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INVESTIGATIVE REPORT

# Hsp27 Expression Coincides with Epidermal Stratification during Human Epidermal Morphogenesis

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**Heat shock protein 27 (Hsp27), apart from its protective function in response to stress, is implicated in the regulation of cell growth, differentiation and apoptosis. Data on the expression of Hsp27 in the developing human epidermis are sparse and partially conflicting. Thus, the purpose of the present study was to investigate Hsp27 expression during the morphogenesis of human epidermis. Skin biopsies and dispase-separated epidermal sheets obtained from 7 human embryos (7 and 8 weeks estimated gestational age, EGA), from 79 human fetuses (9–23 weeks EGA) and from 10 healthy adult volunteers were investigated by immunohistochemistry and Western blotting, respectively. The earliest detection of Hsp27 expression was found by immunohistochemistry at the 12th week EGA (basal and intermediate layer) and by Western blotting at the 9th week EGA. From the 16th to the 23rd week EGA immunoreactivity was not detectable in the basal layer, whereas in the overlying layers it revealed a differentiation-related pattern. The simultaneous onset of epidermal stratification and Hsp27 expression (9th week EGA) and the alterations of the latter in the subsequent stages of development, suggest that this stress protein may be involved in the molecular events underlying human epidermal morphogenesis.** ***Key words: skin; stratification; differentiation; stress.***

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Heat shock protein 27 (Hsp27) belongs to a family of abundant and ubiquitous stress proteins, the small heat shock proteins, which are present and can be induced in all eukaryotic and prokaryotic species (1). It is an evolutionary highly conserved low molecular weight protein that is phosphorylated and overexpressed in response to thermal and oxidative stress, cytokines and other mediators of inflammation (2). Since its original identification in the human breast cancer cell line MCF-7, Hsp27 has been detected in the oviduct, uterus, cervix, vagina, breast, placenta, platelets, kidney and in the epidermis and adnexal structures (3, 4). In unstressed cells, Hsp27 exists predominantly as a large oligomeric unit of up to 800 kDa, usually comprising six tetrameric complexes of the protein located in the cytoplasm near the Golgi complex. After stress exposure, Hsp27 is translocated to the nucleus (5), is rapidly phosphorylated and the level of its expression markedly increases (6).

Apart from its protective properties under stress, Hsp27 appears to have a variety of functions as a molecular chaperone and to play an important role in the regulation of differentiation, growth and apoptosis (1). To the best of our knowledge, only two studies have been performed so far on the expression of Hsp27 in the developing human epidermis; however, they were conducted on very small numbers of biopsy samples from developing skin and their results are partially conflicting (7, 8). In the present study we investigated Hsp27 expression during the morphogenesis of human epidermis using Western blotting and immunohistochemistry on a large number of samples.

## MATERIALS AND METHODS

### *Tissue samples*

Biopsy skin specimens obtained from 7 morphologically normal human embryos aged 7 weeks ($n$=3) and 8 weeks ($n$=4) of estimated gestational age (EGA) and from 79 morphologically normal human fetuses of 9 weeks ($n$=4), 10 weeks ($n$=6), 11 weeks ($n$=4), 12 weeks ($n$=8), 13 weeks ($n$=5), 14 weeks ($n$=6), 16 weeks ($n$=7), 17 weeks ($n$=6), 18 weeks ($n$=8), 19 weeks ($n$=9), 20 weeks ($n$=10) and 23 weeks EGA ($n$=6) following legal abortions, were included in the study. The study protocol was approved by the local ethics committee in accordance with the ethical standards laid down in the Helsinki Declaration of 1975, as revised in 1983. The fetuses were confirmed not to have a family history of cutaneous disorders. EGA was determined from maternal histories, fetal measurements (crown, rump and foot length) and comparative histological appearance of the epidermis (9–12). Biopsy specimens obtained from the buttock skin of 10 healthy adult volunteers of both sexes (aged 23–31 years) were also investigated.

### *Western blotting*

Skin specimens were incubated in dispase (250 mg/100 ml dissolved in serum-free DMEM-F12) at 4°C for 18 h. The epidermis was then peeled off the dermis in a continuous sheet

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that was washed with $Ca^{2+}$- and $Mg^{2+}$-free Hank's buffer saline solution (BSS) and incubated in 0.25 mg/ml trypsin (in 0.01% EDTA) at 4℃ for 10 min. Keratinocytes were obtained by dissociating the epidermal sheet by shaking; subsequent to trypsin neutralization they were centrifuged (1500 rpm) at 4℃ for 5 min. The pellet was resuspended in Hepes-BSS and centrifuged at 1500 rpm at 4℃ for 5 min. The pellet was then resuspended in a buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and after addition of NP-40 (10%) the keratinocytes were homogenized with a motor-driven glass homogenizer and centrifuged (1500 rpm) at 4℃ for 5 min. Subsequent to determination of protein concentration using the Bradford protein assay, the supernatant (total protein extract) was separated on 13% polyacrylamide/SDS gel and electrotransferred to a PVDF membrane (Immobilon-P, Millipore) for 1 h at 50 V. The membrane was initially incubated with a 1/1000 dilution of mouse monoclonal $IgG_1$ antibody against Hsp27 (Biogenex, USA; Clone G3.1) and consequently with a 1/2000 dilution of goat anti-mouse IgG HRP conjugated for 1 h at room temperature. Antigen–antibody complexes were visualized using the Amplified Opti-4CN Substrate and Detection Kit (Bio-Rad) following the manufacturer's instructions.

### *Immunohistochemistry*

The expression of Hsp27 was investigated by immunohistochemistry in formalin-fixed and paraffin-embedded skin biopsy specimens (4-$\mu$m sections) using a primary mouse monoclonal $IgG_1$ antibody against Hsp27 (Biogenex; Clone G3.1; dilution 1:100) and the peroxidase-labelled streptavidin-biotin standard technique (13).

### *Statistical analysis*

All data were considered evaluable and were included in the analysis. The outcome variable was the intensity of Hsp27 immunoreactivity graded on a scale of 0–3: 0=negative stain; 1=weak intensity; 2=moderate intensity and 3=strong intensity. The Jonckheere-Terpstra test (14, 15) was used to detect any possible trends between gestational age and intensity of Hsp27 immunoreactivity of each epidermal layer in all specimens. Furthermore, all pairwise comparisons between specimens of different gestational ages were performed using the Wilcoxon rank sum test (16). The level of significance was fixed at $\alpha=5\%$. A $p$ value of $\leqslant 0.05$ was considered to indicate statistical significance. The statistical analysis was carried out with the software product SAS® (SAS Institute, Cary, NC, USA), version 8.02. Due to the exploratory nature of the study the derived $p$ values were not adjusted.

## RESULTS

### *Western blotting*

The results of Western blotting are summarized in Table I. Expression of Hsp27 was found in all specimens of the adult and in all specimens of fetal epidermis (9th to 23rd week EGA) but was absent in all specimens of embryonic epidermis (7th and 8th week EGA) under the conditions tested (Fig. 1).

### *Immunohistochemistry*

Epidermal Hsp27 immunoreactivity in each age group as found in our study and in the two previous studies (7, 8) is shown in Table I, whereas Hsp27 expression in each epidermal layer of all examined specimens is shown in Table II. The results are discussed separately below.

Table I. *Hsp27 expression in embryonic (7 and 8 weeks EGA), fetal (9–23 weeks EGA) and adult human epidermis detected by immunohistochemistry (IHC) and Western blotting (WB) in the present and previous studies*

| EGA (weeks) | Present study | | Previous studies | |
|---|---|---|---|---|
| | IHC | WB | Ref. 7* IHC | Ref. 8* IHC |
| 7 | − | − | ND | ND |
| 8 | − | − | ND | ND |
| 9 | − | + | ND | ND |
| 10 | − | + | ND | ND |
| 11 | − | + | ND | ND |
| 12 | + | + | − | ND |
| 13 | + | + | ND | − |
| 14 | + | + | ND | + |
| 16 | + | + | ND | + |
| 17 | + | + | ND | + |
| 18 | + | + | ND | + |
| 19 | + | + | ND | + |
| 20 | + | + | − | + |
| 23 | + | + | ND | + |
| Adult | + | + | + | + |

ND, not done; EGA, estimated gestational age.
*Western blotting not performed.

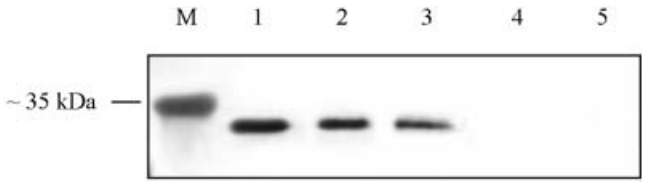


*Fig. 1.* Western immunoblotting of Hsp27 from human epidermis: 2 $\mu$g of total protein extracts from adult (lane 1) and fetal human epidermis at 9 weeks estimated gestational age (EGA) (lanes 2 and 3) and at 8 and 7 weeks EGA (lanes 4 and 5, respectively) were electrophoretically analysed, transferred and blotted with mouse monoclonal anti-Hsp27 antibody. Lane M, protein marker of an apparent molecular weight of 35 KDa.

*Specimens of the 7th to 20th week EGA (Fig. 2A–D).* In all specimens peridermal cells revealed no Hsp27 immunoreactivity.

*Intermediate layer.* In the specimens of the 7th to the 11th week EGA, the intermediate layer demonstrated a negative reaction to Hsp27. A moderate to strong expression of Hsp27 was found in the cells of the intermediate layer in the specimens of the 12th to 17th week EGA and mostly strong in the specimens of the 18th to 20th week EGA. In the specimens of the 16th to 20th week EGA Hsp27 immunoreactivity was confined to the upper intermediate layer (Jonckheere-Terpstra test: $p<0.0001$). Application of Wilcoxon rank sum test revealed statistically significant differences between fetuses of gestational age $\leqslant 11$ weeks and $\geqslant 12$ weeks ($p<0.05$).

Table II. *Expression of Hsp27 in the epidermal layers (EL) of embryonic (7 and 8 weeks EGA), fetal (9–23 weeks EGA) and adult skin: immunohistochemical results scored*

| | Estimated gestational age (weeks) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 (*n*=3) | | | | 8 (*n*=4) | | | | 9 (*n*=4) | | | | 10 (*n*=6) | | | | 11 (*n*=4) | | | | 12 (*n*=8) | | | | 13 (*n*=5) | | | | 14 (*n*=6) | | | | 16 (*n*=7) | | | | 17 (*n*=6) | | | | 18 (*n*=8) | | | | 19 (*n*=9) | | | | 20 (*n*=10) | | | |
| EL | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| PD | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| IL | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 2 | 0 | 4 | 2 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 3* | 4* | 0 | 0 | 3* | 3* | 0 | 0 | 3* | 5* | 0 | 0 | 3* | 6* | 0 | 0 | 3* | 7* |
| BL | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 6 | 2 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 3 | 3 | 7 | 0 | 0 | 0 | 7 | 6 | 0 | 0 | 8 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |

| | 23 (*n*=6) | | | | Adult (*n*=10) | | | |
|---|---|---|---|---|---|---|---|---|
| EL | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| HL | 6 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| GL | 0 | 0 | 2 | 4 | 0 | 0 | 1 | 9 |
| USL | 0 | 1 | 5 | 0 | 0 | 0 | 7 | 3 |
| LSL | 4 | 2 | 0 | 0 | 2 | 8 | 0 | 0 |
| BL | 4 | 2 | 0 | 0 | 6 | 4 | 0 | 0 |

PD, periderm; IL, intermediate layer; BL, basal layer; HL, horny layer; GL, granular layer; USL, upper spinous layer; LSL, lower spinous layer.
*Only in the upper immediate layer.




*Fig. 2.* Immunohistochemistry (×400) of Hsp27 in human epidermis from fetuses of various ages and adult skin. (A) No immunoreactivity at 9 weeks of estimated gestational age (EGA). (B) Hsp27 expression in fetal human epidermis is detectable for the first time in the basal layer at 12 weeks EGA. (C) At 16 weeks EGA, fetal epidermis reveals a moderate to strong Hsp27 immunoreactivity only in the upper intermediate layer. (D) At 20 weeks EGA, the basal layer is devoid of any immunoreactivity. The intensity of Hsp27 expression in the overlying layers increases with their distance from the basement membrane, whereas periderm demonstrates a negative reaction. (E) In the adult epidermis the horny layer reveals no Hsp27 immunoreactivity. The intensity of Hsp27 expression in the underlying layers shows a differentiation-related pattern.

*Basal layer.* In the specimens of the 7th to 11th week EGA and in those of the 16th to 20th week EGA, no Hsp27 immunoreactivity was found in the basal cells. A moderate to strong expression of Hsp27 was found in the basal layer of the specimens of the 12th to 14th week EGA. Application of Wilcoxon rank sum test in all pairwise comparisons confirmed that the detected differences were statistically significant ($p<0.005$).

*Specimens of the 23rd week EGA and adult epidermis (Fig. 2E).* No immunoreactivity to Hsp27 was found in the horny layer (Wilcoxon rank sum test: $p=0.5179$).

*Granular layer.* A moderate to strong expression of Hsp27 was found in the granular layer of the fetal epidermis at the 23rd week EGA and in the adult epidermis.

*Upper spinous layer.* In most (5/6) specimens of fetal epidermis (23rd week EGA) and of adult epidermis (7/10) there was a moderate expression of Hsp27. The difference was not statistically significant (Wilcoxon rank sum test: $p=0.1264$).
*Lower spinous layer.* In most specimens of the 23rd week EGA (4/6) there was no Hsp27 immunoreactivity. A weak expression of Hsp27 could be observed in only two specimens. In the adult, 8 of 10 specimens showed a weak expression of Hsp27 (Wilcoxon rank sum test: $p=0.1181$).
*Basal layer.* Most specimens of the 23rd week EGA (4/6) and adult epidermis (6/10), revealed a negative Hsp27 immunoreactivity (Fig. 2E). In the remaining specimens there was a weak expression of Hsp27. The difference was not statistically significant.

## DISCUSSION

In recent years, accumulating evidence indicates that Hsp27, in addition to its protective role during heat or oxidative shock and other forms of environmental and pathophysiological stress, reveals a wide spectrum of functions including regulation of cell growth and differentiation (17) and of cytoskeletal dynamics (18), signal transduction (19) and protection against apoptosis induced by different agents (20).

Gandour-Edwards et al. (21) were the first to investigate the occurrence of Hsp27 in normal human adult epidermis by immunohistochemistry. They reported that this protein is constitutively expressed in a consistent suprabasal location exhibiting an increase in intensity from the spinous through the granular cell layer, whereas the basal and the horny layer are completely devoid of Hsp27 immunoreactivity. The findings of these authors were confirmed by Trautinger et al. (7), who additionally investigated three specimens of developing human epidermis of 5th, 12th and 20th gestational week, respectively. As they were unable to detect any immunoreactivity in the epidermal cells, Trautinger and co-workers suggested that in human fetal epidermis Hsp27 is not expressed in amounts detectable by immunohistochemistry until the 20th week of gestation.

More recently, Jantschitsch et al. (8) performed an immunohistochemical investigation on epidermal Hsp27 in 34 human fetuses of a wide spectrum of EGA groups ranging between 13 and 30 weeks EGA and found that the expression of Hsp27 is detectable for the first time at the 14th week EGA and is confined to the periderm and the intermediate layer. They reported that with advancing EGA the expression of Hsp27 accumulates in the periderm and the upper layers. However, they failed to detect any immunoreactivity in the horny layer in fetuses over 20 weeks EGA.

The results of the present study clearly show that, in contrast to the findings of Trautinger et al. (7) and Jantschitsch et al. (8), the earliest detection of Hsp27 expression in the human developing epidermis is found by immunohistochemistry (basal and intermediate layer) at the 12th week EGA and by Western blotting at the 9th week EGA. This is due to the fact that in immunohistochemistry there is a diffusion of the signal; the target protein can form complexes with other peptides resulting in masking of epitopes. In Western blotting the protein is denatured and confined to a single zone, whereas the epitopes are unmasked. To the best of our knowledge, this is the first report on the expression of Hsp27 in a human fetal tissue as early as the 9th week EGA. However, we were unable to detect any Hsp27 immunoreactivity of the periderm in all gestational age groups.

The distinct alterations in the pattern of Hsp27 expression occurring in the human fetal epidermis with advancing gestational age are reported here for the first time; from the 13th to the 14th week EGA, immunoreactivity is confined to the basal and the intermediate layer. Surprisingly, at the 16th week EGA, only the upper intermediate cells still express moderate to high levels of Hsp27, whereas the basal layer becomes completely devoid of any immunoreactivity and remains negative in all gestational age groups until the 23rd week EGA. In the specimens of the 17th to 20th week EGA, the suprabasal layers also show a negative reaction and Hsp27 immunoreactivity is found only at the overlying epidermal layers and reveals a differentiation-related pattern. In the specimens of the 23rd week EGA the pattern of Hsp27 expression is almost identical to that of the adult human epidermis.

It is well known that the end of the 8th week EGA marks the completion of the embryonic and the onset of the fetal period of human epidermal morphogenesis (22, 23). The first expression of Hsp27 in the human fetal epidermis at the 9th week EGA, as found in the present study by Western blotting, coincides with the beginning of epidermal stratification. It seems reasonable, therefore, to suggest that Hsp27 may be involved in the molecular events underlying this developmental transition.

Jantschitsch et al. (8) reported that the intensity of Hsp27 expression in the developing human epidermis increases with the distance of the keratinocytes from the basal cell layer and concluded that it is closely related to the state of keratinocyte differentiation. This finding was confirmed in the present study. However, in both studies the horny layer, which represents the product of the terminal epidermal differentiation, was found to lack any Hsp27 immunoreactivity, a feature also observed in the adult human epidermis (21, 24). As recently suggested by Jonak et al. (24), the possibility that Hsp27 may occur also in the horny layer in complexes with other proteins which are inaccessible for the monoclonal anti-Hsp27 antibody, cannot be definitely ruled out. Alternatively, it is possible that Hsp27 is

absent in human fetal corneocytes due to its degradation by the proteolytic enzymes occurring in the upper granular layer.

An interesting feature of Hsp27 in the developing human epidermis, reported for the first time in the present study, is its transient expression (until the 16th week EGA) in the basal cells and its 'switch off' until the 23rd week EGA. The significance of this feature for the epidermal morphogenesis and particularly its possible role at the level of the pivotal control between proliferation, apoptosis and differentiation of keratinocytes remain to be elucidated in further experiments currently being carried out in our laboratory.

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