Heat shock protein 25 plays multiple roles during mouse skin development

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Abstract Heat shock protein (Hsp) 25 is a member of the small Hsp family. High levels of Hsp25 can be detected in skin. During adult epidermis differentiation, the concentration of Hsp25 increases as the distance of keratinocytes from the basal layer increases, in parallel with the extent of keratinization. We previously showed that Hsp25, mouse keratin (MK) 5, and MK14 participated in the formation of characteristic ring-shaped aggregates during the differentiation of the PAM212 keratinocyte cell line. We suggested that Hsp25 was involved in the disorganization of the MK5-MK14 keratin network before the establishment of the MK1-MK10 keratin network at the beginning of epidermis stratification. In this study, we have investigated the distribution of Hsp25 and keratins throughout skin development. We demonstrate that the distribution of Hsp25 and MK5 in the epidermis at the beginning of stratification and before keratinization is similar to that observed in PAM212 keratinocytes. These results indicate that there is a strong correlation between the mechanism we described *ex vivo* and the events taking place *in vivo*. Moreover, we show that Hsp25 is produced in different cell types in the epidermis and in the hair follicle at different stages of their development. Thus, our results suggest that Hsp25 is involved in more than one process during skin development.

INTRODUCTION

Heat shock protein (Hsp) 25 (called Hsp27 in human cells) is a member of the small Hsp family, which has 10 members in mammals (Kappe et al 2003). This 25-kDa protein is a molecular chaperone (Jakob et al 1993; Ehrnsperger et al 2000) and can interact with actin (Miron et al 1988; Lavoie et al 1993a, 1993b, 1995; Benndorf et al 1994; Guay et al 1997; Rousseau et al 1997; Schafer et al 1998; Schneider et al 1998) and intermediate filaments (Iwaki et al 1993; Perng et al 1999). Hsp25 has several different roles, including protecting cells against a large range of stresses (Oesterreich et al 1993; Lavoie et al 1995; Mehlen et al 1995; Huot et al 1996; Arrigo et al 2002) and against apoptosis (Garrido et al 1999; Bruey et al 2000; Charette et al 2000). In addition, it is involved in cell migration (Rousseau et al 1997; Piotrowicz et al 1998) and contraction (Muller et al 1999; Meloche et al 2000; Yamboliev et al 2000) and interferes with translation (Cuesta et al 2000). Recent studies have also shown that small Hsps are likely to play a key role in protecting cells against aging (Hsu et al 2003).

The distribution pattern of Hsp25 during mouse embryonic development (Gernold et al 1993; Loones et al 1997; Morange et al 1998; Morange 1999; Davidson et al 2002) suggests that this protein is involved in the differentiation of tissues. Further evidence supporting this hypothesis has been obtained using *ex vivo* approaches (Spector et al 1994; Mehlen et al 1997; Davidson and Morange 2000; Favet et al 2001; Duverger et al 2004). Skin is one of the organs containing high levels of Hsp25, both in the adult and during embryonic development. In the adult, the epidermis consists of several cell layers: basal layer, spinous layer, granular layer, and the stratum corneum. The degree of differentiation increases from the basement membrane to the outside of the body. The pattern of Hsp27 synthesis in mature human skin has been described previously (Gandour-Edwards et al 1994; Trautinger et al 1995) and suggests that this small Hsp is involved in terminal differentiation. Indeed, Hsp27 staining increases with the distance of the keratinocytes from the basal layer in human epidermis. In human hair follicles, Hsp27 is mainly confined to the innermost cell layer of the outer root sheath (ORS). A more recent study suggests that Hsp27 interacts with keratins and proteins of the cornified cell envelope (Jonak et al 2002). The distribution of Hsp25 in mature mouse epidermis is similar to that de-

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scribed for mature human epidermis (Laplanche et al 1998).

During embryonic development, the epidermis is formed from the ectoderm and is initially composed of only one cell layer (Byrne and Hardman 2002). This simple epithelium forms a second layer called the periderm, which is degraded at the end of gestation. Afterwards, an intermediate layer forms between the basal layer and the periderm. From the beginning of keratinization onward, when mouse keratin (MK) 1 and MK10 are produced, the pattern of keratin synthesis is similar to that found in the mature epidermis. MK5 and MK14 are restricted to the cells of the basal layer, the only mitotic cells in the epidermis, whereas MK1 and MK10 are produced in the suprabasal layers where keratinocytes undergo terminal differentiation. The influence of the embryonic origin of the underlying dermal cells results in the kinetics of epidermis stratification and differentiation being region dependent (Byrne et al 1994). In this study, we have focused on the dorsolateral part of the embryo.

The pattern of Hsp27 synthesis in developing human skin has been described previously (Jantschitsch et al 1998). Hsp27 was detected in the human epidermis from week 14 of gestation, when the epithelium is made up of a basal layer, an intermediate layer, and a periderm. At this stage, Hsp27 was confined to the periderm. After the beginning of keratinization, Hsp27 was produced in the suprabasal layers of the epidermis. In the developing hair follicle, Hsp27 was produced in the innermost cells of the ORS and in the bulge. The distribution of Hsp25 in developing mouse skin has not yet been described.

Although it is well established that Hsp25 can interact with intermediate filaments (Iwaki et al 1993; Perng et al 1999), little is known about the interactions between Hsp25 and keratins in the skin. In a recent study, we showed that Hsp25 interacted with MK5 and MK14 in a murine keratinocyte cell line (Duverger et al 2004). During the differentiation of these keratinocytes, Hsp25, MK5, and MK14 participated in the formation of large aggregated structures with a characteristic ringlike shape. Hsp25 seems to be involved in the reorganization of the keratin network by mediating the sequestration of MK5 and MK14 before the establishment of the MK1-MK10 network. However, Hsp25 and MK5 are not produced in the same layers in the mature epidermis. We investigated previously whether these 2 proteins interacted during epidermis development. We found that, at E12.5, both Hsp25 and MK5 were detected in the simple epithelium making up the epidermis before stratification. However, the distribution of Hsp25 and MK5 in these proliferating cells was diffuse. After the beginning of keratinization (E15.5), MK5 was restricted to the basal layer, whereas Hsp25 was produced in the suprabasal layers, where

MK1 and MK10 were also being produced (Duverger et al 2004).

The aim of this study was to define more precisely the dynamics of Hsp25 and keratin distribution during the first steps of mouse epidermis stratification (between E12.5 and E15.5). We investigated the formation of the aggregated structures containing Hsp25 and MK5 to determine whether our previous findings in PAM212 keratinocytes were representative of the process taking place *in vivo*. Because this kind of analysis has never been conducted for developing mouse skin, we extended our study to the later stages of embryonic development and focused on epidermis and hair follicle formation.

It is difficult to establish a correlation between the distribution of a marker in cultured keratinocytes (primary culture or cell line) and the distribution of the same marker in the epidermis. *In situ* analyses of skin are usually performed on tissue sections. However, because keratinocytes are very large and flat, such sections provide only a limited view of the cells. Analyzing the longitudinal plane of the epidermis would give the widest view of the surface of the cells. Such observation would be more similar to that performed on cultured keratinocytes. In this study, we performed 2 kinds of immunohistochemical analyses on developing mouse skin (Fig 1). First, we analyzed paraffin sections to determine the distribution of markers in the different layers of the epidermis and in the different parts of the hair follicle. Second, we performed *in toto* immunohistochemical analyses on skin fragments followed by confocal microscopy observations to analyze the surface of epidermis. This analysis allowed us to characterize more accurately the distribution of the markers in the keratinocytes.

MATERIALS AND METHODS

Animals

OF1 mice used for the experiments were obtained from Charles River-IFFA CREDO, L'Arbresle, France. They were bred according to the French Ethical Committee guidelines (Decree 87-848) and the European Community Directive 86/609/EEC. Embryos were harvested and analyzed at different developmental stages.

Immunohistochemistry on paraffin sections

Embryos were fixed overnight at 4°C in 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS). They were then dehydrated using an ethanol series and embedded in Paraplast plus* (Sherwood Medical, St. Louis, MO, USA). Embryos were then sectioned (thickness: 10 µm) using a Leica microtome. Sections were placed in water on SuperFrost* Plus slides and al-

Fig 1. Immunohistochemical approaches. The distribution of different markers in the developing mouse skin was analyzed by 2 immunohistochemical approaches: immunohistochemistry on paraffin sections (PS-IHC) and in toto immunohistochemistry on skin fragments (IT-IHC).

lowed to dry at 37°C before being stored at 4°C. Paraplast was removed from the sections before immunohistochemical analysis by washing twice for 15 minutes in Histo-clear (National Diagnostics, Atlanta, GA, USA). Sections were then gradually rehydrated using an ethanol series and finally soaked in PBS for 5 minutes. Immunohistochemistry was performed in a humid chamber. Free aldehydes were saturated by incubating the sections with 50 mM NH_4Cl in PBS for 30 minutes. Sections were then permeabilized by incubation for 30 minutes in a blocking solution (3% bovine serum albumin [BSA] in PBS) supplemented with 0.5% Triton X-100. Slides were washed thrice for 10 minutes in 3% BSA and 0.1% Triton X-100 in PBS (dilution buffer) and then incubated for 1 hour with the dilution buffer supplemented with primary antibodies. After washing thrice in dilution buffer, slides were incubated for 30 minutes with the dilution buffer supplemented with secondary antibodies. Sections were washed thrice in PBS and mounted in Mowiol. Slides were examined using a Leica TCS/SP2 confocal microscope. Primary antibodies used were as follows: anti-Hsp25 (Stressgen SPA801, Victoria, BC, Canada), anti-

MK5 (gift from Dr Ouled—IGBMC, Strasbourg, France), anti-MK10 (DAKO M7002, Glostrup, Denmark), and anti-MK1 (Babco PRB-165P, Richmond, CA, USA). Secondary antibodies used were as follows: Cy[®]3-conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson 111-165-003, Bar Harbor, ME, USA) and Alexa Fluor[®] 488-conjugated goat anti-mouse IgG (Molecular Probes, A11001).

In toto immunohistochemistry on skin fragments

Embryos were fixed overnight at 4°C in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS. After fixation, large skin explants were carefully separated from the rest of the embryo and cut into smaller fragments. These skin fragments were prepared for in toto immunohistochemistry as follows. Free aldehydes were saturated by incubation with 50 mM NH_4Cl in PBS for 2 hours. Skin fragments were then permeabilized by incubation for 4 hours in a blocking solution (3% BSA in PBS) supplemented with 0.5% Triton X-100. Fragments were then incubated overnight at 4°C with dilution buffer supplemented with primary antibodies. After washing in dilution buffer thrice for 1 hour, skin fragments were incubated overnight at 4°C with dilution buffer supplemented with secondary antibodies. They were then washed in PBS supplemented with 0.1% Triton X-100 thrice for 1 hour followed by one wash in PBS. Skin fragments were laid flat on glass slides and mounted in Mowiol. They were allowed to flatten between the glass slide and the glass coverslip, allowing a planar imaging of skin surface. Samples were examined on a Leica TCS/SP2 confocal microscope. Primary and secondary antibodies used were as described in the previous section.

RESULTS

Distribution of Hsp25 and MK5 in developing mouse epidermis

We showed previously (Duverger et al 2004) that Hsp25 and MK5 were detected in the same cells of the developing epidermis before the beginning of stratification. In this study, our immunohistochemical analyses carried out on paraffin sections from embryos at E12.5 showed that Hsp25 and MK5 are evenly distributed in the cytoplasm of the cells composing the simple epithelium (Fig 2a–c). At this stage, a small number of cells have already left the simple epithelium and reached the surface of the monolayer. These cells, which are the first cells of the epidermis to become isolated from the basement membrane, also contain Hsp25 and MK5 (Fig 2c). We also performed confocal microscopy on in toto immunohistochemically stained skin fragments to obtain a longitudinal view of the epithelium (Fig 1). The distribution of Hsp25 and

MK5 observed with this approach is consistent with that from the analysis of paraffin sections (Fig 2d–f). However, this approach gave more information on the overall organization of the cell layer. The epithelium was made up of tightly associated and uniformly distributed cells. The proportion of cytoplasm visible with the *in toto* technique was significantly greater than that observed on the paraffin sections, giving more information about the subcellular distribution of the markers. At E12.5, the diameter of the cells was approximately 20 μm .

Stratification of the epidermis was obvious by E13.5. At this stage, the epidermis was composed of a basal layer plus 1 or 2 additional layers comprising the periderm and an intermediate layer. MK5 was uniformly detected in all layers of the stratifying epithelium. On the other hand, Hsp25 was strongly detected in some cells of the newly formed layers, whereas only a faint signal remained in basal cell (Fig 2g–i). *In toto* observations confirmed that the basal layer produced a high level of MK5, but a low level of Hsp25 (Fig 2j'–l'), and showed that the cells making up this layer were more compact at E13.5 than they were at the previous stage. Indeed, cell density seemed to have increased and mean cell diameter decreased (10–15 μm at E13.5 compared with 20 μm at E12.5; Fig 2k' compared with Fig 2e). Hsp25 and MK5 had very unusual distribution pattern in the newly formed layers. Both proteins were produced in groups of cells in which they were distributed throughout the entire cytoplasm. In the cells surrounding these islets, Hsp25 and MK5 distribution was nondiffuse. These cells contained characteristic ring-shaped aggregates containing both Hsp25 and MK5 (Fig 2j–l). This pattern is very similar to that observed during the differentiation of PAM212 keratinocytes in our previous study. This pattern was also detected at E14.5 (data not shown).

Hsp25 and MK5 were no longer detected in the same epidermal layers from E15.5 onward. Indeed, MK5 was restricted to the basal layer and in some cells of the first suprabasal layer in the epidermis, whereas Hsp25 was exclusively detected in cells of the suprabasal layers, except in those lining the basal layer and producing MK5 (Fig 2m–r). At E15.5 and E16.5, Hsp25 was concentrated in certain regions of the tissue rather than being distributed evenly throughout all the cell layers (Fig 2m). This distribution pattern is described in more detail subsequently. From E17.5 onward, the distribution pattern of the protein was more homogenous on the whole surface of the epidermis. However, the signal was hardly detected in the first suprabasal layers, whereas it was strong in the upper layers (Fig 2p).

Distribution of Hsp25 and MK10 in developing mouse epidermis

MK10 was not detected in the epidermis before E15.5. The presence of MK10 in the epidermis marks the begin-

ning of keratinization. As described above, Hsp25 was detected in the suprabasal layers of the developing epidermis at E15.5. Immunohistochemical analyses on skin sections showed that the protein had a patchy distribution in the suprabasal layers rather than being homogeneously distributed (Fig 3a). MK10 was also produced in the suprabasal layers of the epidermis at this stage of development (Fig 3 b,c). Confocal analysis of *in toto* immunohistochemical preparations clearly showed that Hsp25 was not evenly distributed throughout the entire surface of the tissue but concentrated in groups of cells (Fig 3d). Interestingly, the same groups of cells also produced MK10 at this stage of development, and the intensity distribution of this MK10 signal correlated with that of Hsp25. These results showed that cells producing high levels of Hsp25 also produced high levels of MK10 (Fig 3d–f). The morphology of the cells at E15.5 differed considerably from that observed at earlier stages: cell diameter had reached 40–50 μm and cell shape was more uniform. This change in cell morphology and the characteristic distribution of MK10 and Hsp25 at E15.5 were not apparent from our observations of the paraffin sections.

From E17.5 onward, Hsp25 and MK10 were uniformly detected throughout the surface of the epidermis, and production was no longer restricted to certain parts of the tissue (Fig 3g–i). However, MK10 was produced in all suprabasal layers, whereas Hsp25 was hardly detected in the first suprabasal layer, as described previously for Figure 2r. Our confocal analysis of *in toto* immunohistochemical preparations allowed to detect Hsp25 and MK10 in all the large cells (80–100 μm) covering the surface of the epidermis (Fig 3j–l).

Distribution of Hsp25 and the keratins in developing hair follicles

We also analyzed the pattern of synthesis of Hsp25 and keratins in the developing hair follicles. The stage of hair follicle growth is dependent on the location of the follicle in the body. We did not detect Hsp25 in any part of the developing hair follicles from the dorsolateral part of the embryo before birth, although MK5 was readily detected in the ORS as early as E17.5 (see Fig 2q). Hsp25 was detected in the inner root sheath (IRS) of the hair follicle at birth (P0). At that stage, the hair shaft had not yet emerged, and the IRS formed a column of cells. The stained region spread from the top of the bulb to the surface of the epidermis (Fig 4A; a–f). This column of cells was surrounded by the ORS, which produced MK5 from E17.5 onward (Fig 4B; a–f).

The pattern of Hsp25 production in the hair follicle dramatically changed 4 days after birth (P4), when the hair shaft had emerged. At that stage, the IRS no longer formed a column of cells but a real sheath surrounding

Fig 2. Patterns of heat shock protein (Hsp) 25 and MK5 synthesis in developing epidermis. Embryonic mouse skin was collected at E12.5 (a–f), E13.5 (g–l, j'–l'), E15.5 (m–o), and E17.5 (p–r) and fixed in 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde. Hsp25 and MK5 were detected by immunohistochemistry using secondary antibodies conjugated to cy3 (red labeling; a, d, g, j, j', m, and p) and Alexa 488 (green labeling; b, e, h, k, k', n, and q), respectively. The merged images are shown in c, f, i, l, l', o, and r. Immunohistochemical analyses were performed on paraffin sections (PS-IHC; a–c, g–i, and m–r), and in toto (IT-IHC; d–f, j–l, and k'). Tissue fragments and sections were analyzed by confocal microscopy (single scans). At E13.5, in toto preparations were analyzed on 2 different focal planes: at the level of the basal layer (z1, j'–l') and at the level of suprabasal layers (z2, j–l). The white arrow (c): cell separated from the basement membrane. Scale bar = 40 μ m. Inserts: 4-fold enlargements.

the hair shaft and surrounded by the ORS. Hsp25 was not detected in the cells of the IRS but was found in the ORS and in the inner part of the bulb (Fig 4A; g–i). Hsp25 and MK5 were both detected in the ORS, whereas the matrix of the bulb produced only Hsp25 (Fig 4B; g–i). Thus, the distribution pattern of Hsp25 in the part of the hair follicle close to the epidermis differed from that in the deepest part of the skin appendage.

These patterns of synthesis were investigated further by immunohistochemical analyses of perpendicular sections of the entire length of the hair follicle. These sections were used to investigate the distribution of Hsp25 and MK5 in the different parts of the hair follicle between the epidermis and the smooth muscles (Fig 4C). Close to the epidermis, Hsp25 and MK5 were detected throughout the

Fig 3. Patterns of heat shock protein (Hsp) 25 and MK10 synthesis in developing epidermis. Embryonic mouse skin was collected at E15.5 (a–f) and E17.5 (g–l) and fixed in 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde. Hsp25 and MK10 were detected by immunohistochemistry using secondary antibodies conjugated to cy3 (red labeling; a, d, g, and j) and Alexa 488 (green labeling; b, e, h, and k), respectively. The merged images are shown in c, f, i, and l. Immunohistochemical analyses were performed on paraffin sections (PS-IHC; a–c and g–i), and in toto (IT-IHC; d–f, and j–l). Tissue fragments and sections were analyzed by confocal microscopy (single scans). The dotted lines (c and i): basal lamina. Scale bars = 40 μ m.

entire ORS, made up of about three layers of cells (Fig 4C; a, g, and m). We found that the distribution pattern of MK5 production remained constant as we examined sections from regions farther away from the epidermis (Fig 4C; g–j); however, the pattern of Hsp25 production changed considerably. In sections from within the dermis, Hsp25 was restricted to the innermost cells of the ORS (Fig 4C; b, h, and n). In subsequent sections, Hsp25 was detected in the innermost cells of the ORS and in the cortex (distal part) of the growing hair shaft (Fig 4C; c, i, and o), separated by the IRS, which is not stained. Hsp25 was also present in the less compact proximal part of the cortex (Fig 4C; d, j, and p). In sections from regions approaching the smooth muscles, we found that Hsp25 was produced in the matrix of the hair follicle (appears on the section as a crown of cells), whereas we no longer detected MK5 at this level (Fig 4C; e, k, and q). Finally, we detected neither Hsp25 nor MK5 in the deepest part of the hair follicle adjacent to the smooth muscles (Fig 4C; f, l, and r).

MK1 and MK10 are the 2 main keratins produced by differentiated keratinocytes. We detected both proteins in the suprabasal layers of the epidermis from the beginning of keratinization onward. We detected the MK1 protein in the cortex and matrix of the hair follicle at P4 (Fig 4 D,E). The distribution pattern was similar to that de-

Fig 4. The patterns of heat shock protein (Hsp) 25 and keratins synthesis in developing hair follicles. Mouse skin was collected at birth (P0: A.a–f and B.a–f) and 4 days after birth (P4: A.g–i, B.g–i, C, and D) and fixed in 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde. Paraffin sections were prepared and used for immunohistochemical analyses. (A) Differential interference contrast imaging (a, d, and g) and Hsp25 staining (b, e, and h). The merged images are shown in c, f, and i. Sections were analyzed by confocal microscopy and the results shown are the average of a series of 12 scans collected at 0.2 μ m intervals. The black arrowhead (c): the inner root sheath (IRS) of a developing hair follicle. The black asterisk (a) and (d): the bulb of a developing hair follicle. The white arrow (h): inside of the bulb. The white arrowhead (h): outer root sheath (ORS). (B) Hsp25 and MK5 were detected by immunohistochemistry using secondary antibodies conjugated to cy3 (red labeling; a, d, and g) and Alexa 488 (green labeling; b, e, and h), respectively. Merged images are shown in c, f, and i. Sections were analyzed as described in A, except for d–f, which are single scan images. The white arrow (a) and (d): IRS. (C) Hsp25 and MK5 were detected by immunohistochemistry using secondary antibodies conjugated to cy3 (red labeling; a–f) and Alexa 488 (green labeling; g–l), respectively. Merged images are shown in m–r. The images shown are of sections from 6 different positions between the epidermis and the smooth muscles. Images were taken using a confocal microscope (single scans). The bidirectional arrow (i): thickness of the ORS. The white arrow (o): IRS. The white arrowhead (c): cortex. The white ring (q): matrix. (D) MK1 and MK10 were detected by immunocytochemistry using secondary antibodies conjugated to cy3 (red labeling, a) and Alexa 488 (green labeling, b), respectively. The merged image is shown in c. Sections were analyzed using a confocal microscope (single scans). The white arrow (c): the cortex of the hair follicle. (E) The distribution pattern of MK1 (cy3-labeled: red, a–c) and Hsp25 (cy3-labeled: red, d–f) in the cortex of the hair follicle at the distal (a and d) and proximal (b and e) positions, and in the matrix (c and f). Ep, epidermis; SM, smooth muscles. Scale bar = 50 μ m.

scribed for Hsp25 in the proximal and distal parts of the cortex and in the matrix of the hair follicle (Fig 4E). However, MK10 was not detected in these structures.

DISCUSSION

Comparison of ex vivo and in situ mechanisms

The aim of this study was to validate the mechanisms we had identified previously using a keratinocyte cell line. In

our previous study, we analyzed the distribution of Hsp25 and keratins throughout terminal differentiation of PAM212 keratinocytes (Duverger et al 2004). We showed that Hsp25 and MK5 underwent sequential coaggregation and eventually formed characteristic ring-shaped structures. We assumed that in vivo this phenomenon took place at the beginning of epidermis formation because Hsp25 and MK5 are not produced in the same layers in the adult epidermis. This hypothesis was supported by

our findings that Hsp25 and MK5 were both produced in the single-layered epidermis before stratification, whereas they were produced in different cell layers after the beginning of keratinization: MK5 in the basal layer and Hsp25 in suprabasal layers (Duverger et al 2004). In this study, we showed that the structures formed in the developing epidermis were similar to those observed in the PAM212 cells used in our previous study. We found that at the beginning of stratification and before keratinization, Hsp25 and MK5 formed ring-shaped structures in a large proportion of the cells making up the newly formed layers of the epidermis.

We obtained valuable information concerning the structures formed by Hsp25 and MK5 by *in toto* immunohistochemical analysis of skin fragments. The distribution of Hsp25 and MK5 determined using this technique could not have been deduced by the analysis of the skin sections alone. Similarly, the correlation between the pattern of Hsp25 production and that of MK10 at the beginning of keratinization (E15.5) would not have been detected from analysis of the stained sections. Our results demonstrate that the *in toto* immunohistochemical approach allows larger regions of the cells to be analyzed and facilitates comparisons between tissue and cultured cells. In addition, this technique requires only minimal sample treatment: similar to immunocytochemical analysis on cultured cells, *in toto* immunohistochemical analysis does not require dehydration, inclusion, and sectioning of the sample.

The pattern of Hsp25 synthesis in developing mouse skin compared with that in human skin

It is interesting to compare the dynamics of Hsp25 synthesis during mouse skin development with that described previously for human skin.

Hsp27 is detected for the first time in developing human skin in the already stratified epidermis. The protein has been detected in the periderm but not in the basal and intermediate layers (Jantschitsch et al 1998). In developing mouse skin, we found that Hsp25 was produced in the single-layered ectoderm before stratification. Thus, studies of Hsp27 distribution in developing human skin did not indicate that the small Hsp and MK5 could be produced in the same cells. At the first steps of the stratification of the mouse epidermis, we found that Hsp25 and MK5 were both produced in the newly formed layers (the periderm and the intermediate layer), whereas only MK5 was present in the basal layer. This distribution of MK5 at the beginning of stratification is consistent with that described previously (Byrne et al 1994).

After the beginning of keratinization, the distribution of Hsp25 in developing mouse epidermis is consistent with that observed previously in developing human skin:

the small Hsp is restricted to the suprabasal layers of the epidermis (Jantschitsch et al 1998). However, Hsp27 is distributed evenly throughout the cytoplasm of the suprabasal cells in human epidermis. In contrast, we found that in mouse epidermis, the protein is initially concentrated in certain parts of the tissue before being synthesized in cells positioned throughout the suprabasal layers.

In developing human hair follicles, Hsp27 was detected mainly in the innermost cells of the ORS and in the bulge. Jantschitsch et al (1998) also detected this protein in the basal cells of the ORS in some sections. However, Hsp27 has never been detected either in the papilla or in the matrix of the hair follicle. We found that in mouse skin, when the hair shaft of mouse hair follicle has already emerged, the pattern of Hsp25 synthesis in the different parts of the appendage varied according to distance from the epidermis. Close to the epidermis, the protein was produced throughout the entire ORS. However, moving away from the epidermis and toward the smooth muscle, we found that Hsp25 synthesis became concentrated in the innermost cells of the ORS and was subsequently only detected in the cortex and matrix of the developing hair follicle. This is the first time that such a distribution has been described for this protein. Moreover, we showed for the first time that Hsp25 was produced in the hair follicle before the hair shaft emerges. We detected Hsp25 throughout the IRS (from the top of the bulb to the surface of the epidermis) at P0.

Our observations on mouse skin, performed at each day of embryo development, between E12.5 and birth, demonstrate that the distribution pattern of Hsp25 can change dramatically within hours, the most rapid changes occurring between E14.5 and E15.5. In studies on human skin development, stages are separated by long periods of time. Thus, many critical steps may have been missed in these studies.

Mainly, 3 of our observations were not described in studies performed on developing human skin: the presence of Hsp25 in the single-layered epidermis, in the IRS before the hair shaft emerges, and in the matrix and the cortex of the hair shaft at the beginning of hair growth. As for MK5, the induction of Hsp25 in the single-layered epidermis occurs a short time before stratification. The presence of Hsp25 in the cortex of the hair follicle is probably a transient phenomenon because it is no longer observed in mature hair follicle. We believe that such steps of skin development can have been missed in the study on human skin. For instance, the distribution of Hsp27 in developing human hair follicle, before the hair shaft emerges, was not analyzed in these studies.

Hsp25 and keratins

Several studies have reported interactions between small Hsps and intermediate filaments. Most of these interac-

tions involve α B-crystallin (Iwaki et al 1993; Nicholl and Quinlan 1994). Hsp27 has been shown to interact with glial fibrillary acidic protein (GFAP) in astrocytes and with keratin 18 in epithelial cells (Perng et al 1999). In both these cell types, Hsp27 colocalizes with the intact intermediate filament network; however, the physiological significance of these interactions has not yet been elucidated. Only 1 immunohistochemical study suggesting that Hsp27 interacts with keratins in the subcorneal layer of the epidermis has been performed thus far on adult human skin (Jonak et al 2002).

In this study, we described in detail the dynamics of the distribution of Hsp25 and keratins in the basal and suprabasal layers throughout mouse epidermis development.

We found that in a large proportion of the cells making up the newly formed layers of the epidermis, Hsp25 and MK5 colocalized in ring-shaped structures at the beginning of stratification and before keratinization. This finding and those of our previous study on PAM212 keratinocytes suggest that these interactions between Hsp25 and basal keratins play an essential role in epidermis differentiation. The results of our previous study suggested that Hsp25 was involved in the disorganization of the MK5-MK14 keratin network before the establishment of the MK1-MK10 network (Duverger et al 2004). Interestingly, this function of Hsp25 seems to be no longer required later, in mature epidermis, when the stem cells of the epidermis quit the basal layer to enter terminal differentiation and switch from a MK5-MK14 network to a MK1-MK10 network. Thus, the process involving Hsp25 that takes place when the cells switch from the basal to the suprabasal keratin network is specific to the beginning of epidermis development. Other mechanisms must be involved later when epidermis differentiation is based on continuous renewal of the epidermal cells.

After the beginning of keratinization, Hsp25 is produced in the suprabasal layers of the epidermis, where MK1 and MK10 synthesis has just been induced. Our *in toto* immunocytochemical analysis revealed that Hsp25 and MK10 were produced in the same groups of cells, and the intensity distribution of the MK10 signal correlated with that of Hsp25. This observation suggests that the kinetics of Hsp25 production paralleled those of MK10 production in the suprabasal layers.

Thus, after being produced in cells containing MK5, Hsp25 is present in MK10-positive cells, such as in mature epidermis.

Hsp25 may also be involved in managing keratin networks in the hair follicle. Indeed, the protein was detected in the matrix and the cortex of developing hair follicles, the base of hair shaft formation. Thus, in this part of the hair shaft, Hsp25 may interact with keratins that are involved in the formation of the hair shaft. The small Hsp

has not been detected in the cortex of adult hair follicles. Thus, Hsp25 may be required at the beginning of hair shaft elongation but not during the whole hair follicle development, a situation similar to that described above for the involvement of Hsp25 at the beginning of stratification.

Interestingly, MK1 was also detected in the cortex and matrix of the hair follicle, with a distribution similar to that of Hsp25. The hair shaft is normally composed of specific keratins, called hair keratins (hard keratins), which are involved in the formation of hard keratinized structures such as hair, nails, etc. These proteins constitute a large family of keratins that have different and specific distribution patterns in the adult hair follicle and different biochemical properties from the cytokeratins (soft keratins) (Langbein et al 1999, 2001). The antibody we used to detect MK1 does not cross-react with other cytokeratins (soft keratins). Given the structural and biochemical differences between soft and hard keratins, anti-MK1 is unlikely to cross-react with a member of the hair keratin family. Thus, MK1 could be produced in the cortex of the developing hair shaft, at the beginning of hair shaft formation, before being replaced by hair keratins.

All keratin networks are a result of the association between a type I and a type II keratin. In the epidermis, MK1 is associated with MK10 to build the main keratin filaments accumulating in suprabasal cells. The absence of MK10 in the developing hair shaft implies that MK1 is associated with another type II keratin in these structures.

Hsp25 probably plays multiple roles during skin development

The small Hsp has long been believed to play a role in the differentiation of the epidermis. The differentiation-related production of Hsp27 has been demonstrated both in mature (Edwards et al 1980; Trautinger et al 1995) and in developing human skin (Jantschitsch et al 1998). Our observations confirm this differentiation-related pattern and further demonstrate that Hsp25 is not associated with only terminal differentiation. We found that Hsp25 was produced in the suprabasal layers of the epidermis after the beginning of keratinization and its distribution correlated with that of MK10. These findings support the idea that Hsp25 is involved in terminal differentiation. However, we found that Hsp25 was also produced in the single-layered epidermis and in the proliferating cells making up the epidermis before keratinization, during the first steps of stratification. At these stages, Hsp25 synthesis is not associated with terminal differentiation. In the developing hair follicle, we detected Hsp25 in the innermost cells of the ORS, a region composed of differentiated keratinocytes. This distribution confirms that de-

scribed previously in human skin (Jantschitsch et al 1998) and further supports the suggestion that there is a link between Hsp25 synthesis and terminal differentiation. However, Hsp25 was also detected in the outermost cells of the ORS, in the cortex, and in the matrix at the base of the hair shaft. The presence of Hsp25 in these cells was dependent on the position along the growing hair follicle. These results suggest that Hsp25 is involved in the differentiation of the hair shaft, a mechanism that is distinct from that of keratinocyte terminal differentiation. Moreover, we found that Hsp25 was also produced in the IRS before the hair shaft emerged.

Thus, Hsp25 is synthesized in different skin cell types at various stages of embryonic development. Our results suggest that this protein plays different functions in different cell types during skin development by interacting with different partners.

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