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The distribution of $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins identifies distinct subpopulations of basal keratinocytes in the outer root sheath of the human anagen hair follicle

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Abstract. The human hair follicle is composed of different concentric compartments, which reflect different programmes of differentiation. Using monoclonal antibodies against $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins we demonstrated a shift in their expression, from a basolateral distribution in the basal cells of the lower outer root sheath, to an apicolateral expression in the upper outer root sheath, as in epidermis. This shift takes place in a transition zone, localized to the midpart of the follicle. The distinct basolateral distribution of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in the lower portion of the outer root sheath coincides with the presence of basal cell protrusions and is probably linked to the presence of the vitreous membrane which surrounds the bottom part of the anagen human hair follicle. Moreover, we showed that the expression of $\alpha 6\beta 4$ integrin is discontinuous along the hair follicle and coincides with that of laminin 5. Together these results establish that within a given compartment – namely the outer root sheath – several domains can be clearly identified, which probably reflect the onset of successive differentiation pathways along the hair follicle.

Key words. Outer root sheath; hair follicle; $\alpha 2\beta 1$; $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins; laminin 5.

Hair follicle formation results from precisely timed and located interactions between fetal epidermis and specialized populations of dermal cells [1]. In the human adult scalp, the pilo-sebaceous unit appears as a composite structure comprising an epithelial component, the follicle itself and the sebaceous gland, mesenchymal cells, basement membrane and mesenchyme-derived fibrous sheath, and the dermal papilla. Altogether, more than 20 distinct populations of cells coexist and interact within the pilo-sebaceous unit [2]. These subpopulations have been identified and characterized by specific histological and immunological markers [3, 4] including proteoglycans [5], keratins [6–8], cadherins [9], various glycoprotein antigens [10, 11], trichohyalin [12], transglutaminase I [13], metallothionein [14], and calcyclin [15].

The outer root sheath (ORS) has attracted a sustained interest since it was shown that pluripotent keratinocytes reside in this follicle compartment [16] and that stem cells could be located in the bulge area of mouse hair follicle [17, 18] or in the midportion of human hair follicle [19, 20], rather than in the follicle end bulb region [21]. Specialized structures such as knob-like or villous projections have also been seen in the bulge area by scanning electron microscopy [21]. The outer root sheath thus appears as a functionally compartmentalized structure. Since $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins are involved in signal transduction for extracellular matrix [22, 23] cell-cell/cell-substrate adhesion of human keratinocytes [24–26] and cell fate [27, 28], we decided to investigate the distribution of these integrins along the ORS of human hair follicle, in parallel to extracellular matrix components. Two types of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin distribution patterns were observed, while $\alpha 6\beta 4$ expression was discontinuous along the follicle. The results are discussed in terms of both the differentiation programme and the proliferative activity of the ORS keratinocytes.

Materials and methods

Tissue preparation. Samples of human scalp skin were obtained from plastic surgery. A total of 14 biopsies were studied, obtained from seven informed and consenting donors. Samples were cut into small pieces containing about six intact follicles. Unfixed tissue samples were then embedded in Tissue-Tek OCT compound (Miles, Naperville, IL, U.S.A.), quick frozen over dry ice (−80 °C), and stored at −80 °C until used for immunohistochemistry.

Immunohistochemistry. Longitudinal frozen sections (5 μm) of hair follicles were prepared on a cryostat HM 500 M (Microm, Francheville, France) whose chamber temperature was set at −40 °C. Sections were then air dried and stored at +4 °C overnight before being processed. Immunolabelling was performed as follows: sections were fixed in acetone at −20 °C for 10 min followed by several washes in phosphate buffered saline (PBS), and endogenous peroxidase activity was quenched by immersing the slides in 0.1% hydrogen

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peroxide for 10 min. To block nonspecific binding sites and minimize background, the sections were covered with 1% no-fat milk (Régilait, Saint-Martin-Belle-Roche, France) in PBS for 15 min. Primary antibodies (see table 1) were diluted in PBS-Tween 0.05%, containing 10% of normal serum corresponding to the conjugated secondary antibody species. Primary antibody was applied for 30 min at room temperature, followed by three washes with PBS-Tween. Biotinylated or FITC-conjugated species-specific secondary antibody was applied for 30 min at room temperature, followed by three washes with PBS-Tween. Avidin-biotin peroxidase complex (ABC) (Dako, Trappes, France) was prepared as indicated by the manufacturer, and sections were covered with the ABC solution for 15 min at room temperature once or twice for light or strong amplification staining, followed by extensive washes with PBS-Tween. Substrate solution (AEC/Sigma) was prepared and applied for 6 min as indicated by the manufacturer, the colour reaction being stopped by washing with double distilled water. The slides were finally mounted under cover glass using glycerol gelatin (Sigma, Saint Quentin Fallavier, France). A Zeiss Axioscop microscope (Carl Zeiss, Oberkochen, Germany) was used for microscope observations and documentation. Negative controls were performed by replacing the primary antibody with irrelevant monoclonal antibodies to *Aspergillus niger* glucose oxidase, all controls yielding the expected negative results.

Results

The use of longitudinal sections from dermal papilla to epidermis allows the direct analysis and comparison of antibody staining in the different compartments of anagen hair follicle at a sensitive level.

Heterogeneity of the anagen hair follicle extracellular matrix

It is known that the vitreous membrane surrounding the human hair follicle can be shown with standard histochemical technics using periodic acid-Schiff (PAS) staining [2] (fig. 1a–e). The PAS staining gives an intense fuschia dye showing the basement membrane. This staining is positive all along the hair follicle and the basement membrane zone of the epidermis. Immediately adjacent to the basement membrane of the hair follicle, a faintly coloured vitreous surrounding, or hyalin, membrane is detected, separating the basement membrane from the connective tissue sheath. This vitreous membrane (VM) is clearly evident from the end bulb area to the middle part of the follicle (fig. 1b arrow, fig. 1c) where it becomes thinner and thinner. On the other hand, VM cannot be observed either in the upper part of the hair follicle (fig. 1d) or at the dermal-epidermal junction of epidermis (fig. 1e). These results underline a heterogeneity in the extracellular matrix composition and/or structure along the human hair follicle. Since integrins were originally identified as adhesion receptors utilized by cells for attachment to the extracellular matrix, the presence of this heterogeneity prompted us to investigate the distribution of $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, which are receptors for matrix proteins known to be present in epithelial basement membranes [30].

Table 1. List of antibodies used.

Antibody	Specificity	Dilution	Origin
MoAb P1E6	integrin $\alpha 2$	1/100	Oncogene science
MoAb AK7	integrin $\alpha 2$	1/100	Cymbus
MoAb P1B5	integrin $\alpha 3$	1/100	Oncogene science
MoAb 11G5	integrin $\alpha 3$	1/100	Cymbus
MoAb 1972	integrin $\alpha 6$	1/10	Chemicon
MoAb P3E4	Laminin 5	1/100	Chemicon
MoAb MM1	Ag Ki67	1/100	Novocastra

Heterogeneity of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin subunit distribution in the anagen hair follicle

Serial dilutions of $\alpha 2$ and $\alpha 3$ integrin antibodies were used, in order to detect not only qualitative but also quantitative variations in integrin expression. Since $\beta 1$ integrin associates with too many α subunits [31], $\beta 1$ integrin antibodies were not used. Moreover, to limit artefacts, antibodies of two different origins (see table 1) were used for both α integrin types and gave similar results.

While the matrix cells of hair bulb display a weak pericellular labelling for both $\alpha 2$ and $\alpha 3$ subunits, this staining decreases rapidly and becomes negative in the different structures of the inner root sheath (IRS) (result not shown). In the lower third of the ORS (L-ORS) the labelling is restricted to the basal cells (figs 1f and j), with a strong basolateral distribution for both $\alpha 2$ and $\alpha 3$ integrins. It is noteworthy that positively labelled cellular protrusions extend from the basal cells deep into the vitreous membrane. In the midpart of the ORS (M-ORS), modifications in $\alpha 2$ integrin expression occur (fig. 1g). Still confined to the basal cells, a discontinuous pattern of weak and intense apicolateral labelling is observed. Moreover, the location of $\alpha 2$ integrin shifts from a basolateral to an apicolateral distribution. Although the distribution of $\alpha 3$ integrin undergoes nearly the same modifications (fig. 1k), it appears less intensively expressed, with a discontinuous pattern of faintly and clearly labelled cells. This transition area in the distribution of both $\alpha 2$ and $\alpha 3$ integrins (fig. 1g, k) is coincident with the disappearance of both the vitreous membrane (fig. 1b, c) and basal cell protrusions (fig. 1f, g and j, k). In the upper third of the ORS (U-ORS),

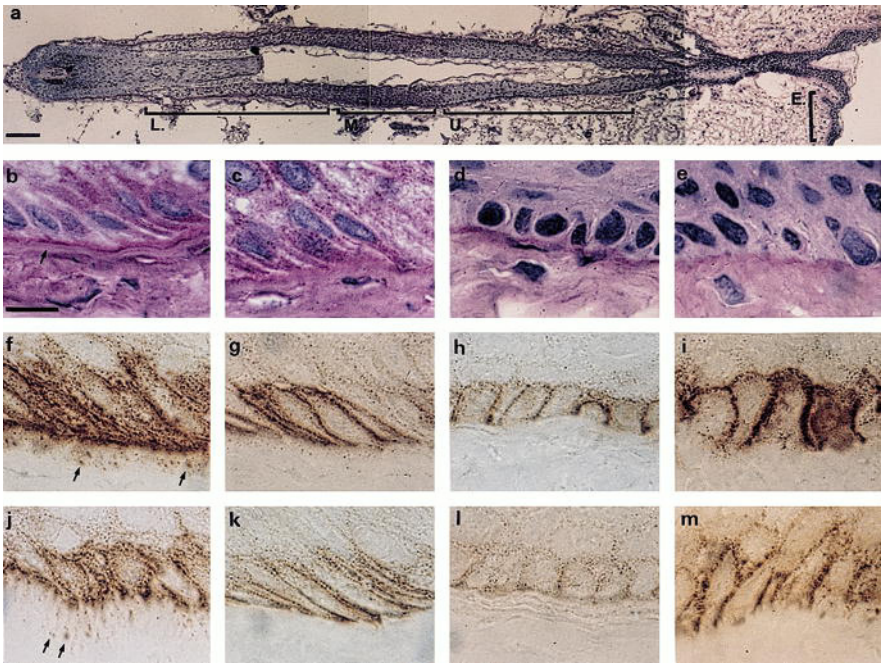

Figure 1. Localization of vitreous membrane, $\alpha 2\beta 1$ integrin and $\alpha 3\beta 1$ integrin in longitudinal frozen sections of anagen human hair follicle. Cryostat sections (5 μm) of fresh human scalp skin samples were stained with PAS (*a–e*), or reacted with anti-$\alpha 2\beta 1$ integrin P1E6 (*f–i*) and with anti-$\alpha 3\beta 1$ integrin P1B5 (*j–m*) monoclonal antibody followed by reaction with biotinylated secondary antibody and avidin-biotin peroxidase complex, as described in Materials and methods. (*a*) General view of a hair follicle (L, M, U and E refer to lower-, mid-, upper ORS and epidermis respectively). (*b*, *f*, *j*) Close-up of L-ORS. (*c*, *g*, *k*) Close up of M-ORS. (*d*, *h*, *l*) Close-up of U-ORS. (*e*, *i*, *m*) Close-up of epidermis. Along the hair follicle, $\alpha 2\beta 1$ integrin (*f–h*) and $\alpha 3\beta 1$ integrin (*j–l*) undergo a progressive redistribution from a basolateral to an apicolateral location, this latter being similar to that found in epidermis (*i*, *m*). A transition zone is found in the midpart of the follicle, which colocalizes with the disappearance of the vitreous membrane (*b–d*). Note the cellular protrusions extending from the basal pole of the basal cells, through the vitreous membrane (arrows in inserts *f* and *j*). Bar represents 100 μm (*a*) or 10 μm (*b–m*).

$\alpha 2$ integrin still exhibits a strong apicolateral distribution (fig. 1h) while $\alpha 3$ integrin is weakly expressed on the apicolateral sides of the basal cells (fig. 1l). In the same section, the epidermis gives an intense and homogeneous apicolateral labelling of the basal keratinocytes for both $\alpha 2$ (fig. 1i) and $\alpha 3$ (fig. 1m) integrins, suggesting that these subunits are closely associated in these cells.

The above observations strongly suggest that the basal keratinocytes of the ORS have a specific distribution for $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins compared to the epidermis. Three different areas clearly emerge. First, the L-ORS where the basal keratinocytes strongly express both $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins with a basolateral distribution, markedly distinct from the apicolateral distribution observed in the epidermal basal cells. This lower part of the follicle is also characterized by the presence of basal protrusions positively labelled with both antibodies, which might represent unique and specialized cell-substrate adhesion structures. Then, in the midfollicular portion, a transition area appears. In this region, a fraction of the basal keratinocytes still strongly expresses $\alpha 3\beta 1$ and $\alpha 2\beta 1$ integrins, while some have already stopped expressing one of them. In addition, a shift from a basolateral to an apicolateral distribution occurs, confirming that modifications in the expression and the location of the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins exist. Finally in the U-ORS, $\alpha 2\beta 1$ integrin is expressed in a manner similar to that in epidermis, while $\alpha 3\beta 1$ integrin expression is weak compared to epidermis.

Discontinuous expression of $\alpha 6$ integrin and laminin 5 in the anagen hair follicle

The $\alpha 6$ integrin subunit is expressed on the basal pole of the matrix cells surrounding the dermal papilla (DP), while at the bulb tip, $\alpha 6$ subunit is not detectable on the basal cells (fig. 2a). Above the Auber line, moderately positive structures extending through the vitreous membrane are observed. The $\alpha 6$ subunit is then progressively detected on the basal pole of the basal L-ORS keratinocytes, with positive cellular protrusions still detectable through the vitreous membrane. In the M-ORS and the U-ORS $\alpha 6$ integrin subunit is strongly expressed and restricted to the basal pole of basal keratinocytes, in a linear fashion, as observed in epidermis. Since laminin 5 (Ln5) has been reported to colocalize with $\alpha 6$ integrin [32], its distribution along the hair follicle has also been investigated. Ln5 is observed in the basement membrane between DP and matrix cells. It is noteworthy that the basement membrane is nega-

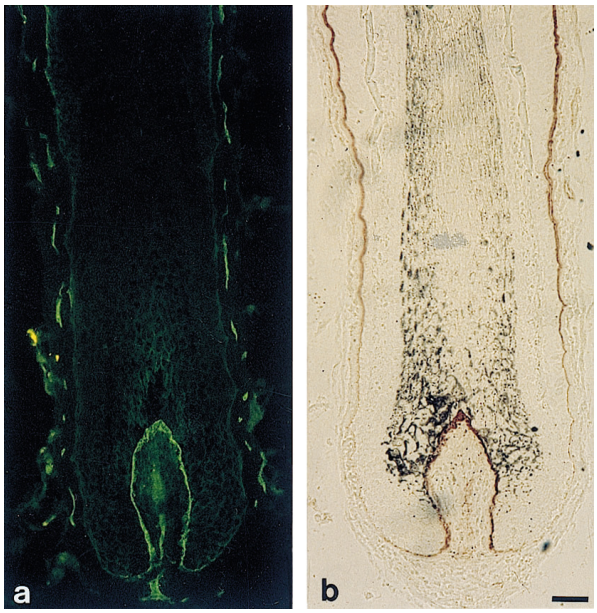

Figure 2. Localization of $\alpha 6\beta 4$ integrin (*a*) and laminin 5 (*b*) in longitudinal frozen sections of anagen human hair follicle. Cryostat sections (5 µm) of fresh human scalp skin samples were stained with anti-$\alpha 6\beta 4$ integrin 1972 followed by reaction with FITC-conjugated goat anti-rat antibody (*a*) and anti-laminin 5 P3E4 monoclonal antibody followed by biotinylated secondary antibody and avidin-biotin peroxidase complex (*b*) as described in Materials and methods. Bar represents 40 µm. Note the perfect match of laminin 5 expression in the basement membrane with $\alpha 6$ integrin subunit distribution at the basal pole of basal keratinocytes. Specifically, note the discontinous expression of both $\alpha 6\beta 4$ integrin and laminin 5 at the tip of the hair bulb.

tive for Ln5 labelling at the bulb tip but becomes positive above the Auber line (fig. 2b). At the L-ORS level the Ln5 distribution is weak, and looks diffuse in the vitreous membrane, with stronger positive structures crossing it. Its expression is more detectable from the M-ORS and in the U-ORS. At these levels, Ln5 expression is progressively restricted to a thin layer corresponding to the basement membrane. The epidermal-dermal junction is continuously positive for Ln5 labelling. It is noteworthy that Ln5 expression in the basement membrane perfectly matches $\alpha 6$ integrin subunit distribution at the basal pole of the basal ORS keratinocytes and basal matrix cells.

Evidence for distinct proliferative compartments in the hair follicle

Since the expression level of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins correlates with the probability of colony-forming ability and self-renewal of human epidermal keratinocytes [28, 29], proliferative compartments of the hair follicle were further studied. Ki67 nuclear antigen is expressed during late G1, S, M, and G2 phases of the cell cycle [33], and is thus detected in the nucleus of actively dividing cells. As expected, MM1-MAb strongly labels the matrix cells of hair bulb under the Auber line (fig. 3a, b). In the L-ORS a moderate portion of the basal and suprabasal cells gives an intense staining (fig. 3c). Further up towards the epidermis, the labelling is progressively confined to the basal cells of the ORS and becomes almost negative in the U-ORS (fig. 3d). In comparison a high proportion of the epidermal basal cells is positively stained with MM1-MAb (result not shown).

These observations support the concept that matrix cells are probably responsible for IRS formation and hair shaft elongation [34], and suggest that the L-ORS might be involved in the sustained renewal of ORS during the anagen phase. Interestingly, this lower part of the follicle corresponds to a basal keratinocyte population which expresses $\alpha 2$ and $\alpha 3$ integrins in a basolateral fashion and at the highest level.

Discussion

Previous work has clearly established that the human anagen hair follicle is composed of different concentric compartments [2], which reflect different pathways of differentiation. The corresponding subpopulations have been identified and characterized by specific histological and immunological markers [3–15], and also by the formation of boundaries and communication compartments [35]. With respect to the ORS, both functional [19, 20] and immunocytochemical [9–11] data had indicated some heterogeneity in this compartment, suggesting that distinct differentiation programmes occurred in the U-ORS and L-ORS [6].

In the present work, we further extended this notion and demonstrated the existence of two distinct basal cell populations in the ORS, separated by a transition zone. Interestingly, this transition zone corresponds to a modification of the surrounding extracellular matrix of hair follicle, i.e. the disappearance of the vitreous membrane. The L-ORS basal keratinocyte is characterized by a basolateral distribution of both $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins, while the U-ORS basal keratinocyte exhibits an apicolateral distribution of these integrins, similar to epidermis. Moreover, the intensity of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin expression is much higher in L-ORS than in U-ORS (see fig. 1). The biological significance of this integrin redistribution is not clear. It has to be noted, however, that the basal expression of these integrins coincide with the presence of cellular protrusions, reminiscent of those previously observed when germinative cells were cultured in the presence of DP cells [21]. Under these conditions, germinative cells indeed synthesize an extracellular structure identical to the vitreous membrane. Fingerlike extensions from basal germinative cells were then clearly observed, traversing the basement membrane and the vitreous membrane. These cellular protrusions might thus be a hallmark of a specific differentiation programme, directly linked to the presence of the vitreous membrane. Since $\alpha 2\beta 1$

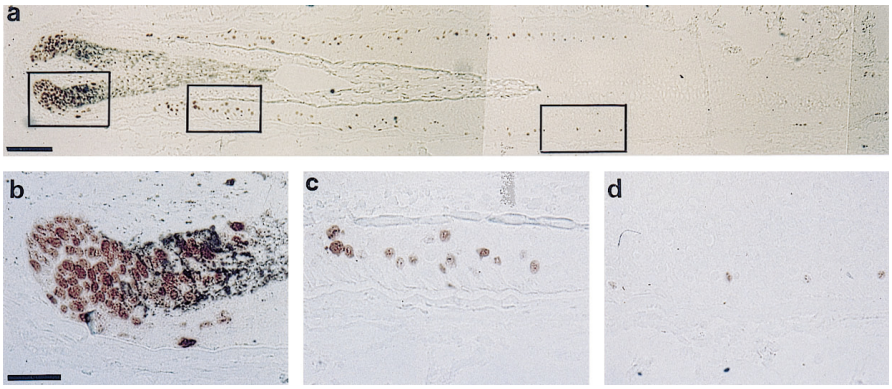

Figure 3. Localization of the proliferative compartments of anagen human hair follicle. Cryostat sections (5 μm) of fresh human scalp skin samples were stained with MM1(Ki67) monoclonal antibody followed by reaction with biotinylated secondary antibody and avidin-biotin peroxidase complex as described in Materials and methods section. Note the intense labelling of the hair bulb (*b*), and the labelling in the L-ORS (*c*). Bar represents 100 μm (*a*) or 40 μm (*b–d*).

integrin mediates interactions between developing embryonic retinal cells and collagen [36] and $\alpha 3\beta 1$ integrin is frequently associated with tumour and transformation progression [37], the basolateral distribution of both integrins might thus be related not only to the specific composition/structure of the vitreous membrane, but also to the high proliferative rate of the L-ORS keratinocytes, as evidenced by Ki67 antigen expression. Moreover, recent experimental data of regrowth of grafted scalp hair after removal of the bulb [38, 39] clearly establish that the lower portion of the ORS and dermal sheath contain pluripotent epithelial, mesenchymal, and melanocyte reservoirs able to regenerate new pigmented hairs.

The present study also shows a discontinuous expression of both $\alpha 6$ integrin subunit distribution at the basal pole of ORS keratinocytes and matrix cells, and Ln5 in the BM. This discontinuous expression of Ln5 in the BM underlines its heterogeneous composition along the hair follicle, thus substantiating structural differences recently observed by electron microscopy [40]. Furthermore, the striking matching between $\alpha 6$ integrin subunit distribution and Ln5 expression strongly suggests that Ln5 is a specific substrate for $\alpha 6$ integrin subunit in the hair follicle. In fact Ln5 can colocalize with integrin $\alpha 6\beta 4$ in hemidesmosome-like stable anchoring contacts (SACs) [41] and generate stable adhesion that retains cells at the BM. So, the absence of $\alpha 6$ integrin subunit and Ln5 at the bulb tip as well as at the L-ORS level confirms that no hemidesmosomes participate in the dermo-epithelial junction in these regions [40]. This can be related to the significant tissue remodelling occurring in these areas. On the other hand, there is evidence for SACs distribution on the matrix cell basal pole which could help to preserve the DP integrity.

Taken together, our results identify a hair compartment localized in the L-ORS and characterized by a unique differentiation programme as revealed by a basolateral distribution of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins, a discontinuous expression of $\alpha 6\beta 4$ integrin correlated with Ln5 expression in the basement membrane, and a high proliferation rate. This compartment may be involved in the ORS sustained renewal during anagen phase and its unique characteristics may be linked to a particularly high regenerative capacity as suggested by recent graft experiments both in humans [38] and nude mouse [39].

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