

Abnormal Expression of Ki-67 Antigen in Hair Follicle of Alopecia Areata

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The monoclonal antibody Ki-67 was used to determine the numbers of cycling cells in hair follicles both in alopecia areata and in normal scalp skin. Pronounced nuclear staining was limited to the area below the critical line of Auber and the exterior part of the outer root sheath. In alopecia areata there is reduced nuclear Ki-67 binding in the bulb of anagen hair follicles. These findings indicate that inhibition of keratinocyte proliferation might be a pathogenetic mechanism in alopecia areata.

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The proliferative state of cell populations in the hair follicles is a major factor in the regulation of hair growth. The relative numbers of proliferating cells, i.e. the growth fraction, strongly influences hair growth. In vivo measurement of the growth fraction, e.g., by [³H]thymidine or I-deoxyuridine labelling (1, 2, 3), is difficult for obvious reasons. By using the monoclonal antibody Ki-67, a simple and rapid estimation of the growth fraction of a given human cell subset has become possible (4). If a cell shows nuclear binding of this monoclonal antibody, the cell has passed the G₁-restriction point and is regarded as belonging to the cycling cell population; if a cell shows cytoplasmatic staining without nuclear binding, it is regarded as belonging to the resting (G₀) population (5).

A disturbance in the proliferation rate of follicular stem-cells has been suggested to be of pathogenetic significance in alopecia areata. The purpose of the present study was to compare the growth fraction in keratinocyte populations of normal and alopecia areata hair follicles.

We used both transverse and vertical sections of anagen and telogen hair follicles in alopecia areata lesions and normal scalp, to evaluate the Ki-67 binding.

METHODS

Biopsies

Excisional scalp biopsy samples were taken from healthy individuals free from a history of alopecia areata, and from the margin of active lesions on patients suffering from alopecia areata. All biopsies were immediately embedded in Tissue-Tek II Compound (Miles Inc., Diagnostic Division, Elkhart, Ind., USA), snap-frozen in liquid nitrogen and stored at -80°C until further use. Biopsies were processed as described elsewhere (6). 5-µm sections were cut using a 2800 Frigocut-N cryostat (Reichert-Jung) at -28°C. The cryostat sections were placed on slides coated with bovine serum albumin. The cryostat

sections were air-dried for at least 30 min before fixing in acetone for 10 min and then stored in sealed boxes at -80°C until use.

Histological grading

On biopsies from 5 patients with alopecia areata and 5 normal controls, transverse serial sectioning was performed as described earlier (6). These sections were subdivided into different sample-regions, namely: subcutaneous fat, and transition between subcutaneous fat, non-papillary dermis and the papillary dermis. From each sample region, two sections were investigated. The anagen hair follicle was divided in five levels as shown in Fig. 1. Every hair follicle observed in a transverse section was classified as belonging to one of these five levels. The numbers of positive Ki-67 nuclei of the hair follicle were counted in each section. Vellus hairs were excluded.

Immunohistology

Before use, the sections were placed in acetone-ether (60/40 v/v%) for 10 min. Thereafter the sections were placed in Tween-80 (0.01%) for 3-5 min. Subsequently, the sections were incubated for 30 min at room temperature in a moist chamber with Ki67 (Dakopatts, Copenhagen, Denmark), diluted 1 : 20 in PBS, washed with PBS and subsequently incubated for 30 min with a peroxidase-conjugated rabbit anti-mouse

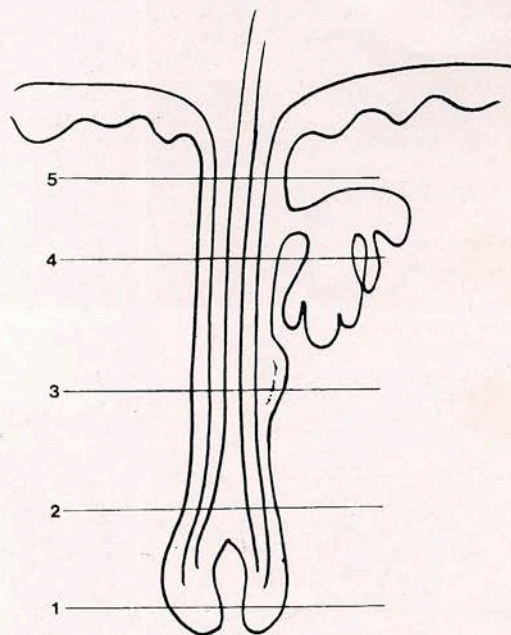


Fig. 1. Schematic representation of a hair follicle.
level 1: Hair bulb at the level of the centre of the dermal papilla.
level 2: The suprabulbar area.
level 3: Level below the sebaceous gland, in which the hair shaft is surrounded by partially keratinized inner root sheath, enclosed by the outer root sheath.
level 4: Level below the sebaceous gland, showing a fully keratinized inner root sheath.
level 5: Infundibulum.

Table I. Ki-67-positive nuclei per transverse section of the hair follicle in different stages; all values are presented as means \pm SEM; n = number of follicles studied.

Level	n	Normal scalp	n	Alopecia areata
1	14	120 \pm 14	13	82 \pm 8
2	56	20 \pm 3	23	11 \pm 3
3	45	27 \pm 1	15	12 \pm 3
4	229	15 \pm 1	123	11 \pm 1
5	10	7 \pm 1	63	21 \pm 2

antibody (Dakopatts) at a dilution of 1 : 25 in PBS containing 5% human AB serum. After being washed with PBS, the bound peroxidase was developed with the 3-amino-9-ethylcarbazole/hydrogen peroxide reaction for 10 min. Finally the sections were rinsed in distilled water and counterstained with haematoxylin, and mounted in glycerin gelatin. The number of positive nuclei was counted per hair follicle at different stages, as observed in transverse sections.

Statistics

All variance analysis was done with the GLM and NPARIWAY procedure of the S.A.S. software statistical package.

RESULTS

The results are summarized in Table I and in Fig. 2A-E.

The Ki-67 positivity is significantly influenced by the level in the hair follicle ($p < 0.001$, Kruskal-Wallis test), though this is largely due to level 1, whereas the other levels showed no marked differences (Waller-Duncan grouping, $\alpha = 0.05$). Ki-67 positivity in alopecia areata is not significantly different from that of normal scalp, if we ignore the level of the follicle (pooled data from all levels (Mann-Whitney test, $p > 0.1$)). However, when the background level is taken into account, alopecia areata shows a dramatic decrease in Ki-67 positivity at levels 1-4 (analysis of variance, $p < 0.001$). Interestingly, at level 5 (the subepidermal level of the follicle) the number of Ki-67-positive nuclei in alopecia areata is significantly greater than in normal scalp skin.

No quantitative data on telogen follicles and vertical sec-

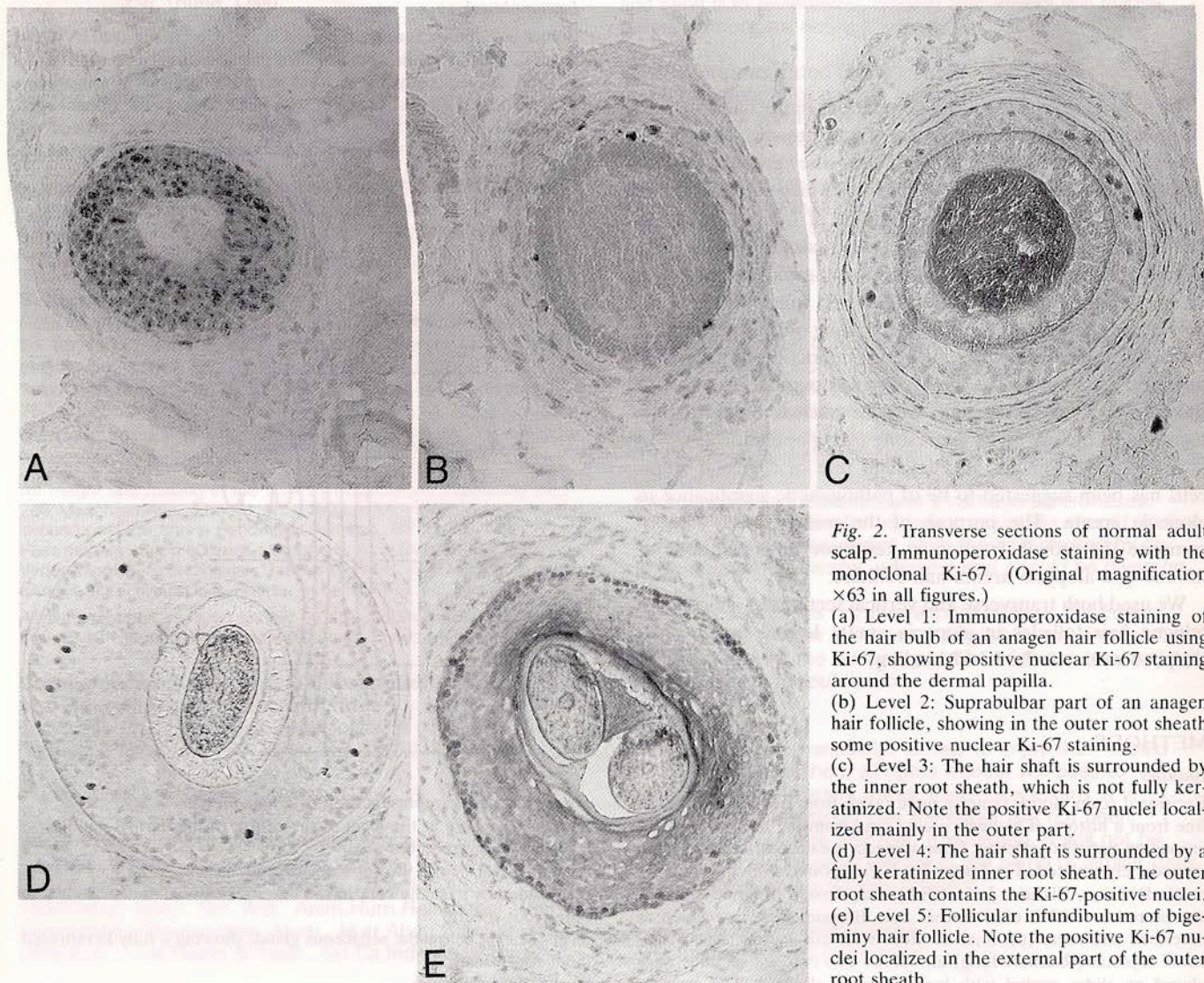


Fig. 2. Transverse sections of normal adult scalp. Immunoperoxidase staining with the monoclonal Ki-67. (Original magnification $\times 63$ in all figures.)

(a) Level 1: Immunoperoxidase staining of the hair bulb of an anagen hair follicle using Ki-67, showing positive nuclear Ki-67 staining around the dermal papilla.

(b) Level 2: Suprabulbar part of an anagen hair follicle, showing in the outer root sheath some positive nuclear Ki-67 staining.

(c) Level 3: The hair shaft is surrounded by the inner root sheath, which is not fully keratinized. Note the positive Ki-67 nuclei localized mainly in the outer part.

(d) Level 4: The hair shaft is surrounded by a fully keratinized inner root sheath. The outer root sheath contains the Ki-67-positive nuclei.

(e) Level 5: Follicular infundibulum of bigeminy hair follicle. Note the positive Ki-67 nuclei localized in the external part of the outer root sheath.



Fig. 3. Vertical section of an early anagen hair bulb, showing most of the Ki-67-positive nuclei localized beneath the line of Auber (arrows). Positive nuclei can likewise be seen in the outer root sheath (arrow-head) ($\times 63$).

tions of anagen hairs are given because only a limited number of the follicles per biopsy can be examined in a vertical section.

In addition, the pattern of Ki-67 is shown in a vertical section of an anagen hair follicle (Fig. 3). Most of the positive nuclei are located below the critical line of Auber (i.e., the widest point of the dermal papilla, approximately at its midpoint) (7).

Late anagen hairs show hardly any Ki-67 positive nuclei (Fig. 4) at the matrix level. In the telogen hair follicles (Fig. 5) there was no positive staining at any level.

DISCUSSION

The monoclonal antibody Ki-67 reacts with nuclei of actively cycling cells in the proliferating phase (G_1 , S, G_2 , M) of the cell cycle (4). We used the immunohistochemical assessment of Ki-67-binding to determine the number of cycling cells in anagen hairs of alopecia areata as compared with normal scalp skin.

The presence of Ki-67-positive nuclei was most pronounced

in the area below the critical line of Auber and the exterior part of the outer root sheath. Notably, positive nuclei of the outer root sheath were located in the outermost part, indicating that relatively rapid cell proliferation is also an essential part of the outer root sheath of the anagen hair follicle (8). It is known from autoradiography, that the cells of the outer root sheath include DNA-synthesizing cells (3). This study shows that also in hair follicles, there is an association between the expression of Ki-67 antigen and cell proliferation.

Recently Ki-67 was studied in human axillary hairs (9). The authors found nuclear staining in a small number of matrix cells and in some outer root sheath cells. They found two different patterns of cytoplasmic staining in the outer root sheath: an intense staining of the innermost cells and a weaker staining of the outer root sheath cells in the isthmus. In scalp hair follicles, no cytoplasmic staining of Ki-67 was found. Whether or not this discrepancy with our data is due to differences in localization remains to be investigated.

Interestingly, the number of Ki-67-positive nuclei at level 1 in alopecia areata showed a significant decrease when com-



Fig. 4. Vertical section of a late anagen hair bulb, showing absence of positive Ki-67 nuclei in the matrix. In the outer root sheath, positive nuclei can be seen in the outer root sheath (arrows) (original magnification: $\times 63$).

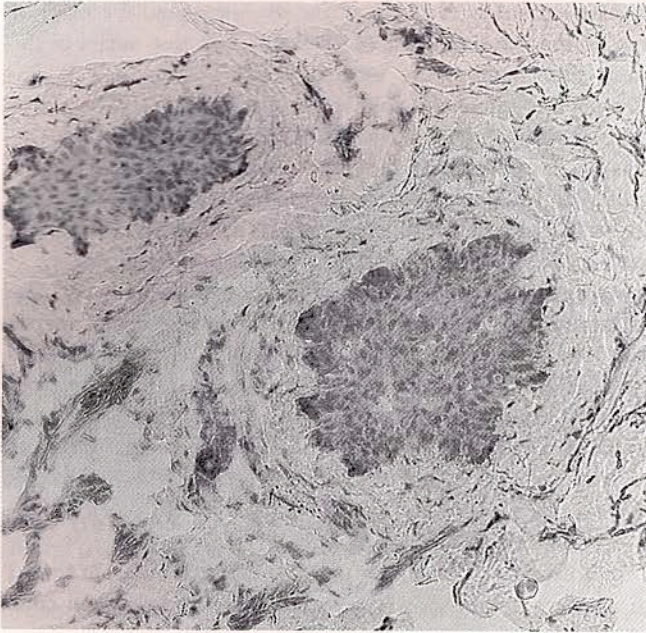


Fig. 5. Transverse section of two telogen germinal units, containing no positive Ki-67 nuclei (original magnification: $\times 63$).

pared with the corresponding level in normal skin. This tallies with earlier studies (10) which showed by direct counting a proportionate reduction in mitotic cells in the matrix. It is known that in alopecia areata the hair bulbs are blocked in anagen stage IV (11) and that the DNA synthesis is restrained (12). We therefore can conclude that Ki-67 reactivity is correlated with findings on DNA labelling as reported in the literature.

The present study demonstrates that an immunohistochemical method using the monoclonal Ki-67, which binds to nuclei of cells in the proliferative phases of the cell cycle, is a useful tool when studying the number of proliferative cells in hair follicles. These findings confirm the results previously obtained by labelling with thymidine and bromodeoxyuridine. The decrease of nuclear Ki-67-binding observed in alopecia areata suggests that abnormal growth of cells in the hair bulbs is of pathogenetic significance.

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