

Cytokeratin Expression in Alopecia Areata Hair Follicles

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Alopecia areata is a human hair disease of unknown etiology. Immunological mechanisms, alterations in the extracellular matrix and follicular growth abnormalities have been suggested as a possible cause. Here we compare the expression of cytokeratins in normal hair follicles to that of alopecia areata using immunohistology with monoclonal antibodies. A number of cytokeratins were specifically expressed in defined anatomical parts of the follicle; however, no gross qualitative or quantitative differences were found between normal and diseased scalp. Interestingly, the expression of cytokeratin 16, which is modulated by conditions that affect the rate of keratinocyte proliferation, was found to be unchanged in the outer root sheath of alopecia areata follicles. This is in contrast with earlier observations of a decrease in the expression of the proliferation-associated, Ki-67 nuclear antigen.

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A number of mechanisms have been suggested to underlie the common hair disease alopecia areata. Immunological abnormalities, including autoantibody formation (1) and aberrant expression of HLA class II antigens, have been found (2, 3). It is, however, not clear whether these factors are causal or consequential. The finding that topical immunotherapy is successful in the treatment of alopecia areata lends further support to the theory of an immune-mediated process (4, 5). Other mechanisms studied in alopecia areata include the expression of extracellular matrix proteins (6, 7) and follicular growth abnormalities (8, 9).

In this study we have investigated whether a disturbance of the differentiation process in the hair follicle keratinocytes is associated with the pathologic changes seen in alopecia areata. As cytokeratins represent the most abundant proteins in the hair follicle and are generally accepted markers for the type and stage of epithelial differentiation (10), these constituents can serve as key molecular markers for differentiation events in the hair follicle. We therefore investigated whether the cytokeratin pattern in alopecia areata is altered, either due to an intrinsic abnormality or as a consequence of the inflammatory process.

MATERIAL AND METHODS

Biopsies

Excisional scalp biopsies were taken from 5 healthy controls and from the margins of active lesions of 5 patients suffering from alopecia

areata. All biopsies were immediately embedded in Tissue-Tek II compound (Miles Inc., Diagnostics Division, Elkhart, Ind., USA), snap-frozen in liquid nitrogen and stored at -80°C until further use. Sections ($5\ \mu\text{m}$) were cut using a 2800 Frigocut-N cryostat (Reichert-Jung, Cambridge Instruments GmbH, Nußloch, Germany) at -28°C (11). The sections were placed on bovine serum albumin-coated slides, air-dried for at least 30 min and then fixed with acetone for 10 min and stored in sealed boxes at -80°C until use.

Immunohistochemistry

Detailed information of the antibodies used for immunohistochemical staining is given in Table I. The immunoperoxidase staining procedure was performed as follows. The acetone-fixed sections were rehydrated with phosphate-buffered saline (PBS) containing 0.01% Tween 80 for 3 min, then incubated in a humidified chamber at room temperature for 30 min with the primary antisera diluted in PBS. After washing in PBS, sections were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) in a dilution 1:50 in PBS with 5% normal human AB-serum for 30 min at room temperature. After washing with PBS, the sections were preincubated with sodium acetate buffer (pH = 4.9) and finally stained in sodium acetate buffer containing 200 mg/l 3-amino-9-ethylcarbazole solution (Sigma Chemical Company, St Louis, Mo., USA) and 0.01% H_2O_2 (prepared freshly) for 10 min at room temperature in the dark. After two washes with demineralized water, the sections were counterstained with Mayer's haematoxylin solution (Sigma) and mounted in glycerin-gelatin. The sections were examined using a Zeiss photomicroscope.

RESULTS

Anagen and telogen hair follicles were studied in biopsies from alopecia areata patients and from normal control subjects in horizontal and vertical sections. The descriptions of the follicular cytokeratin expression patterns apply to anagen of both normal and diseased scalp. The observations are summarized in Fig. 1.

The monoclonal antibody (MoAb) RCK102, directed against cytokeratins 5 and 8, showed staining restricted to the outer root sheath (ORS) (Fig. 2) and the centre of the medulla of the hair (Fig. 3). When the ORS reached the level of the infundibulum only the basal layer showed a positive staining, which continued in the basal layer of the epidermis. In the telogen germinal unit, the centre showed a positive staining.

A quite different pattern was found with MoAb M20, which is a cytokeratin 8 specific antibody. M20 reactivity was found focally in the ORS, showing positive cells in the innermost part of the ORS (Figs. 4, 5). Also the cortex of the hair was stained (Fig. 5).

Expression of cytokeratin 10 (Moab RKSE60) was limited to the suprabasal cells of the ORS, which showed positivity at the level of the drainage of the sebaceous glands and extended to the epidermis, where it continued as a suprabasal staining pattern (not shown).

Table I. Monoclonal antibodies used in this study

MoAb	Cytokeratin specificity	Source/References
RCK 102	Cytokeratins 5, 8	Euro-Diagnostics BV (Apeldoorn, The Netherlands) ¹²
M20	Cytokeratin 8	G. N. P. v Muijen ¹³
RKSE60	Cytokeratin 10	Euro-Diagnostics BV (Apeldoorn, The Netherlands) ¹⁴
2D7	Cytokeratin 13	Euro-Diagnostics BV (Apeldoorn, The Netherlands)
K _s 8.12	Cytokeratins 13, 16	Sigma, St. Louis, USA
K14/CKB1	Cytokeratin 14	Sigma, St. Louis, USA
E3	Cytokeratin 17	S. M. Trojanovsky (Moscow, Russia) ¹⁵
M9	Cytokeratin 18	G. N. P. van Muijen ¹⁶
LP2K/RPN-1165	Cytokeratin 19	Amersham International PLC, Aylesbury, Buckinghamshire, UK ¹⁷

Cytokeratin 13 (MoAb 2D7) showed a positive staining at the dermal level in the most exterior part of the ORS (Fig. 6), which was absent at the epidermal level (not shown) and also in the outer cell layer of the hair cuticula (Fig. 7).

K_s 8.12, which has specificity for the cytokeratins 13 and 16 (and possibly 14), showed positive staining of the not yet differentiated ORS (not shown). At the dermal level, the ORS showed a positive staining of the most exterior part, which continued in the epidermis as a positive basal cell layer (Fig. 8). When a complete hair, composed of medulla, cortex and cuticula, had developed, the layer of Huxley showed some staining, and the layer of Henle was invariably stained.

Cytokeratin 14 (MoAb K14) was expressed in the medulla above the top of the dermal papilla, although less pronounced as compared to the ORS (Fig. 9). Fig. 10 shows cytokeratin 14

expression in the ORS of an anagen hair follicle, with a minor staining of the matrix at the top of the dermal papilla. At the bulbar level the ORS was constituted of one cell layer of positive cells; upwards the number of cell layers in the ORS increased and the total ORS remained positive. At the epidermal level, cytokeratin 14 expression was only found in the basal layer (not shown).

Cytokeratin 17 (MoAb E3) showed weak staining of the ORS with exception of the basal part (Fig. 11). In horizontal sectioning the medulla and cortex occasionally showed a positive staining.

Cytokeratin 18 expression (MoAb M9) in the ORS was found sporadically, in the most internal part, showing some scarce positive cell groups (not shown).

Cytokeratin 19 (MoAb LP2K) was not consistently expressed in the lower part of the ORS, consisting of only one cell layer. When the ORS is multi-layered, positive cell clustering can be seen, which disappears at the level of the papillary dermis (not shown).

Catagen and telogen hairs are structurally different from anagen hair follicles in that most of the structures and cell layers that characterize anagen hair follicles, like the matrix, inner and outer root sheath and the hair cuticula, are lacking. When we examine the cytokeratin expression in catagen and telogen hairs, it is found for Moab RCK102 and K_s 8.12 that the whole centre of the telogen germinal unit is strongly positive, this in contrast with anagen hair follicles in which RCK102 and K_s 8.12 staining is restricted to the ORS. The other MoAbs tested showed similar staining patterns, depending on the level in the dermis.

In alopecia areata, the biopsies examined were from margins of active lesions, in the so-called acute progressive phase, characterized by infiltrates around the growing anagen hair follicles and different grades of hair matrix destruction. The heavily infiltrated follicles showed a disturbance of the follicular architecture. However, the cytokeratin expression pattern in the hair follicles of alopecia areata compared with normal scalp showed no gross differences.

DISCUSSION

Expression of cytokeratins in normal human hair follicles has been documented by several authors, using either biochemical or immunohistochemical methods. It was shown by 2-dimensional gel electrophoresis that keratins 5, 6, 14, 16 and 17 were

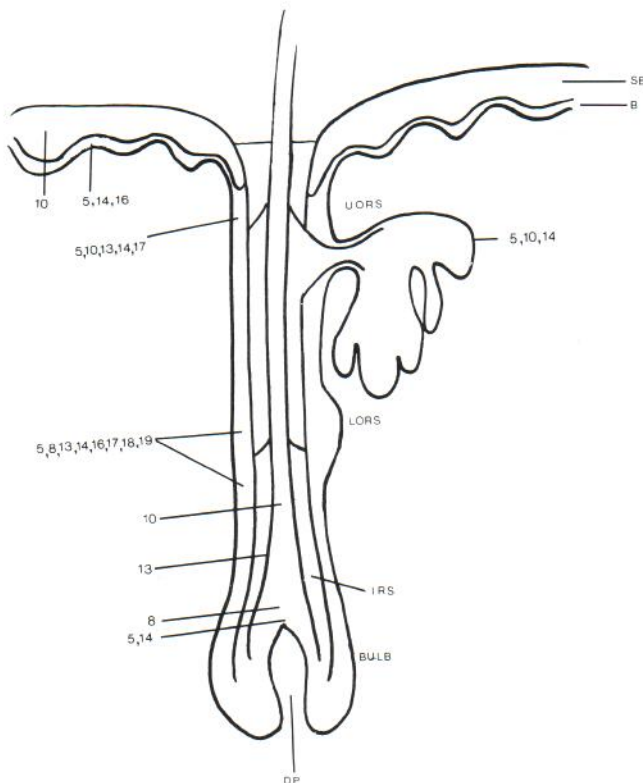


Fig. 1. Schematic representation of the expression of cytokeratins in an anagen hair follicle. UORS, upper outer root sheath; LORS, lower outer root sheath; IRS, inner root sheath; DP, dermal papilla; SB, suprabasal; B, basal.

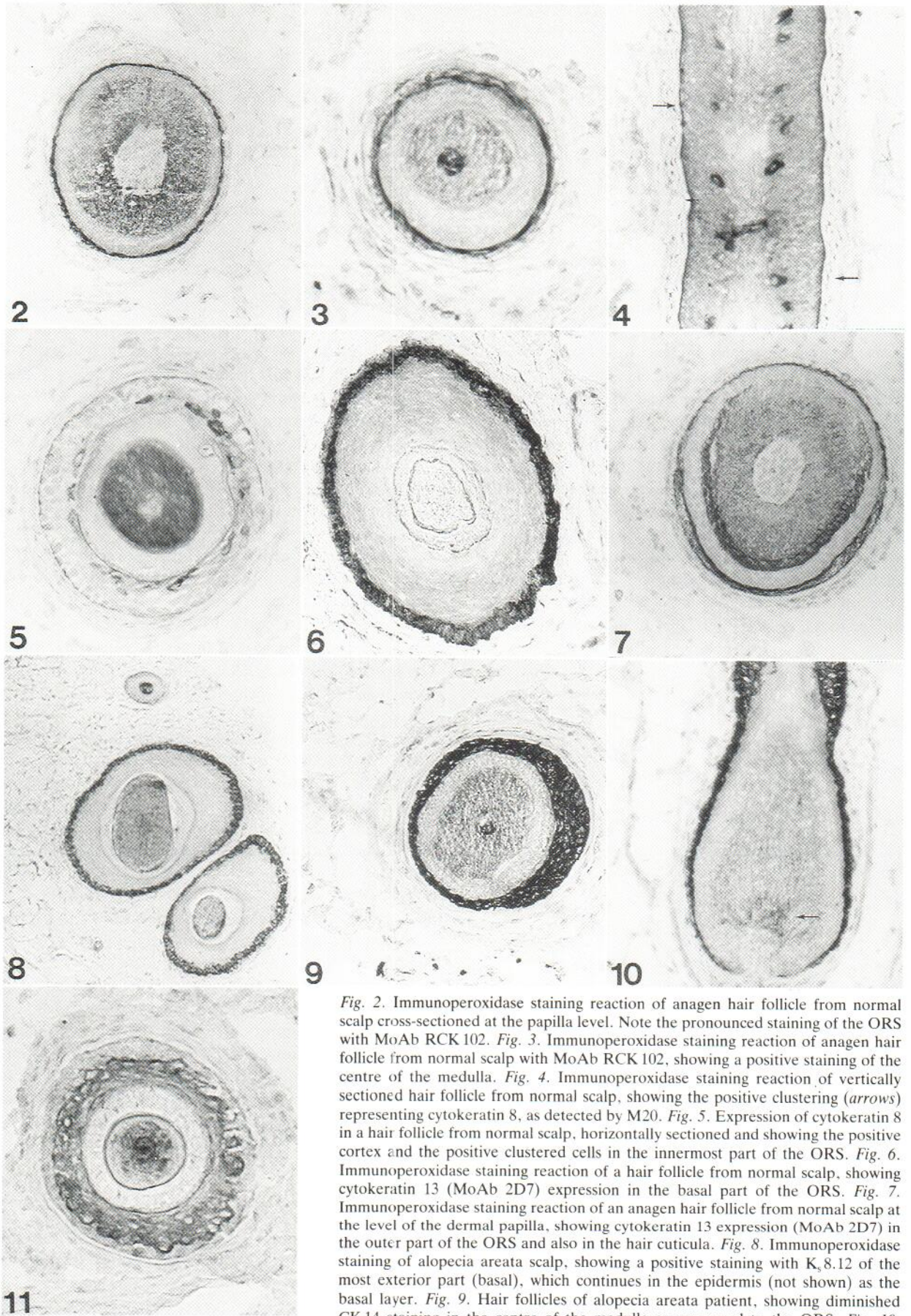


Fig. 2. Immunoperoxidase staining reaction of anagen hair follicle from normal scalp cross-sectioned at the papilla level. Note the pronounced staining of the ORS with MoAb RCK 102. *Fig. 3.* Immunoperoxidase staining reaction of anagen hair follicle from normal scalp with MoAb RCK 102, showing a positive staining of the centre of the medulla. *Fig. 4.* Immunoperoxidase staining reaction of vertically sectioned hair follicle from normal scalp, showing the positive clustering (arrows) representing cyokeratin 8, as detected by M20. *Fig. 5.* Expression of cyokeratin 8 in a hair follicle from normal scalp, horizontally sectioned and showing the positive cortex and the positive clustered cells in the innermost part of the ORS. *Fig. 6.* Immunoperoxidase staining reaction of a hair follicle from normal scalp, showing cyokeratin 13 (MoAb 2D7) expression in the basal part of the ORS. *Fig. 7.* Immunoperoxidase staining reaction of an anagen hair follicle from normal scalp at the level of the dermal papilla, showing cyokeratin 13 expression (MoAb 2D7) in the outer part of the ORS and also in the hair cuticula. *Fig. 8.* Immunoperoxidase staining of alopecia areata scalp, showing a positive staining with K_{8,12} of the most exterior part (basal), which continues in the epidermis (not shown) as the basal layer. *Fig. 9.* Hair follicles of alopecia areata patient, showing diminished CK 14 staining in the centre of the medulla as compared to the ORS. *Fig. 10.* Vertically sectioned anagen hair follicle in normal scalp with a positive cyokeratin 14 staining of the ORS with hardly any staining of the top of the dermal papilla (arrow). At the hair bulb level only a single positive cell layer is shown, which becomes positive in all the layers in the distal direction. *Fig. 11.* Cross sectioning of a hair follicle from normal scalp with mild cyokeratin 17 (MoAb E3) staining of the ORS with exception of the basal cell layer. (Original magnification × 200).

Vertically sectioned anagen hair follicle in normal scalp with a positive cyokeratin 14 staining of the ORS with hardly any staining of the top of the dermal papilla (arrow). At the hair bulb level only a single positive cell layer is shown, which becomes positive in all the layers in the distal direction. *Fig. 11.* Cross sectioning of a hair follicle from normal scalp with mild cyokeratin 17 (MoAb E3) staining of the ORS with exception of the basal cell layer. (Original magnification × 200).

expressed in the ORS (10) and keratins 1 and 10 in the inner root sheath and cuticle (18). Some of these cytokeratins could be located at specific anatomical sites within the hair follicle by means of immunohistochemistry (19–21), indicating that also in the hair follicle, cytokeratins can be used as tissue- and differentiation-specific markers.

Here we present a detailed study, using a panel of 9 monoclonal antibodies (most of them monospecific) showing the spatial distribution of a number of cytokeratins in follicles of normal scalp. The findings were compared with scalp tissue from alopecia areata patients. Although not all MoAbs were monospecific, and the specificity of some of them needs further characterization, most of the antibodies used are informative markers for follicular compartments. It has to be noted that the cytokeratins studied here do not include hair-specific "hard" keratins. As shown by others (19–21) and also in this study, cytokeratins typical of basal cells (CK 5, 14 and 17), hyperproliferative keratinocytes (CK 16), suprabasal differentiated keratinocytes (CK 10) and simple epithelia (CK 18 and 19) can be found in hair follicles.

It has been shown in several studies that alterations in the expression of cytokeratins can be found in certain diseases (22–24). Disturbance of intermediate filament formation due to mutation of the CK 14 gene was shown to contribute to the cause of epidermolysis bullosa simplex (25). In other diseases (e.g. psoriasis), cytokeratin 16, which is not normally expressed suprabasally in epidermis, can be found in the suprabasal compartment of the hyperproliferative epidermis (26, 27). In addition, the expression of CK 10 was found to be diminished (28). Here, we compared the cytokeratin expression pattern of alopecia areata with that of normal scalp, in order to investigate abnormalities in follicular differentiation. As we have shown before that alopecia areata hair follicles show a decrease in the expression of the nuclear proliferation-associated Ki-67 antigen (9), we anticipated a possible down-regulation of the expression of the hyperproliferation-associated cytokeratin, concomitantly with effects on CK 10 expression, i.e. the reverse effect of what is found in psoriatic conditions. However, as shown above, no gross quantitative or qualitative differences were found. Whether this means that expression of CK 16 and a hyperproliferative status of the tissue are not coupled per se, or that the decrease of follicular growth rate in alopecia areata is too marginal to exert a measurable effect histologically, is not clear. It was recently shown that keratin 16 antibody LL025 in squamous cell carcinoma of the human lung, stains more cells with increasing degree of differentiation, suggesting that keratin 16 is a marker for (abnormal) squamous differentiation rather than for a hyperproliferative state (29). Future studies will be directed towards the expression of hair-specific keratins in alopecia areata and are needed for further investigation of aberrant differentiation processes in this disease.

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