

Potential Involvement of the Stem Cell Factor Receptor c-kit in Alopecia Areata and Androgenetic Alopecia: Histopathological, Immunohistochemical, and Semiquantitative Investigations

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Alopecia areata (AAR) and androgenetic alopecia (AGA) are two major forms of alopecia based on altered hair growth condition. In general, the cell cycle is regulated by several mechanisms including the stem cell factor/c-kit signaling. To assess a role for stem cell activity in alopecia, we performed histopathological, immunohistochemical, and semiquantitative analyses of c-kit as well as Ki-67 in scalp biopsy specimens obtained from 14 patients with AAR, 18 patients with AGA, and 6 age-matched control subjects, using the specific antibodies. Formalin-fixed, paraffin-embedded skin sections were examined. Immunoreactivities for Ki-67 and c-kit were localized in keratinocytes and melanocytes in the outermost layer of hair follicles. The mean length of hair follicles was significantly shorter in the AAR and AGA groups than in the control group. The mean number of Ki-67-immunoreactive cells per follicle was significantly reduced in the AAR and AGA groups as compared with the control group. The mean number of c-kit-immunoreactive cells per follicle was significantly increased in the AAR and AGA groups as compared with the control group. Our results indicate that c-kit is upregulated in the hair follicle cells in these forms of alopecia, and suggest that the upregulation reflects a negative feedback mechanism in response to possible down-regulation of the ligand stem cell factor.

Key words: alopecia, c-kit, histopathology, immunohistochemistry, semiquantitation

I. Introduction

Hair has a great social significance for human beings. Human hair growth activity reflects both physiological and pathological conditions. Normal hair is seen in healthy, young individuals, while baldness and white hair increase in an age-dependent manner [2, 32]. Alopecia areata (AAR) and androgenetic alopecia (AGA) are two major common

forms of alopecia that affect young and old people alike [2]. In AAR, the skin lesions are characterized by bald spots, and they usually appear on the scalp and show a local or extensive distribution [2, 23]. In 1–2% of the AAR cases, skin lesions involve the entire scalp (alopecia totalis) or the entire epidermis of the body skin (alopecia universalis) [23]. A growing body of evidence implicates several mechanisms mediated by neuropeptide stimuli, autoimmunity, heredity, stress, and so on in the pathomechanism of AAR [2, 5, 14]. In AGA, skin lesions are characterized by well defined loss of hair that usually involves the marginal zone adjacent to the face and spreads into the bilateral upper sides known as male pattern baldness [2, 11, 23]. Given that male hormones including androgens act as a main regulator of hair growth, the involvement of hormonal imbalance and altered sensitiv-

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ity to androgen in the hair follicle has been discussed; excessive production of androgen and reduced production of estrogen inhibit hair growth [11, 23], and overexpression or variation of the androgen receptor gene promotes male pattern baldness [9, 23].

Recent studies have identified stem cells in various organs and tissues including the epithelium of the hair follicle, skin (epidermis), and intestinal mucosa, as well as the central nervous system and bone marrow [10, 12, 19]. Tissue stem cells are the body's master cells with the ability to differentiate into tissue-specific precursor and specialized cells; the former cells actually proliferate, create tissue bulk, and play a critical role in maintenance of tissue structures and homeostasis [10, 19]. Mounting evidence suggests that altered activities of tissue stem cells have relevance to certain pathological conditions such as tumorigenesis [16] and degenerative disorders [13]. It is known that stromal cells produce and release the growth factor stem cell factor (SCF), which stimulates cells expressing the SCF receptor c-kit [3, 6, 7, 17, 31], also called CD117. This receptor is a transmembrane protein [1] that is localized on the cell surface of hematopoietic stem cells and other cell types and acts as a receptor for SCF [3, 6, 7, 17, 31]. The binding of SCF to c-kit transactivates the receptor tyrosine kinase to activate stem cells, and the SCF/c-kit signaling induces cell survival, differentiation, and proliferation; the latter is evidenced by the cell proliferation marker Ki-67 [8, 18, 30]. In the hair follicle, the cells of the keratinocytic and melanocytic lineage are responsible for hair growth and color, respectively, and express c-kit [4, 25, 26, 29], while stromal cells in the dermis produce SCF [4, 28]. Thus, it is conjectured that the SCF/c-kit signaling is involved in the pathological processes of AAR and AGA. Although this signaling has been well characterized mainly in animals [4, 21, 25, 26], the involvement of c-kit in human alopecias has not yet been fully understood. To address this issue, we performed histopathological, immunohistochemical, and semiquantitative analyses in scalp biopsy specimens, focusing on c-kit.

II. Materials and Methods

Subjects

This study was carried out on scalp skin biopsy specimens obtained from 14 AAR patients [age: 19–61 (40.50±12.93) yr; sex: 5 males and 9 females], 18 AGA patients [age: 21–55 (35.78±9.45) yr; sex: 18 males] and 6 age-matched control subjects with other diseases [age: 21–68 (43.17±18.77) yr; sex: 5 males and 1 female]. There was no significant difference in age at biopsy between the control, AAR and AGA groups ($P>0.05$). The biopsy was achieved under written informed consent from each subject in accordance with the Declaration of Helsinki revised on 2000. Associated disorders of the control subjects were sebaceous cyst (n=1), eccrine spiradenoma (n=1), basal cell epithelioma (n=1), chronic dermatitis (n=1), schwannoma (n=1) and nevocellular nevus (n=1), and normal-appearing regions in these control specimens were examined.

Histopathological and immunohistochemical analyses

Multiple 3- μ m-thick sections were cut from formalin (10%)-fixed, paraffin-embedded scalp materials obtained from each case, and used for hematoxylin-eosin (H&E) staining and immunohistochemical staining. The primary antibodies employed in immunohistochemistry were mouse monoclonal IgG₁ against Ki-67 (Clone MIB-1; diluted 1:100; Dako, Glostrup, Denmark), rabbit polyclonal IgG against c-kit (Cat. No. A4502; diluted 1:1,000; Dako), mouse monoclonal IgG₁ against pancytokeratin (Clone AE1/AE3; diluted 1:100; Dako), and mouse monoclonal IgG₁ against melan-A (Clone A103; diluted 1:50; Dako). The last two antigens were used as markers of keratinocytes and melanocytes, respectively.

Prior to immunostaining for Ki-67 and melan-A, antigen unmasking was performed by microwaving in Tris-buffered ethylenediaminetetraacetic acid (TB-EDTA), pH 9.0, solution (400 W, 95°C, 40 min). Sections were deparaffinized, rehydrated, quenched for 10 min at room temperature with 3% hydrogen peroxide to inhibit endogenous peroxidase activity, rinsed in phosphate-buffered saline, pH 7.6 (PBS), pretreated for 20 min at room temperature with 3% skim milk/PBS solution to inhibit nonspecific antibody binding, and subsequently incubated overnight at 4°C with the primary antibodies. Antibody binding was visualized by the avidin-biotin-immunoperoxidase complex (ABC) method; 3,3'-diaminobenzidine tetrahydrochloride (DAB) was the chromogen, and hematoxylin, the counterstain. Sections processed with omission of the primary antibodies or incubated with 3% nonimmune serum derived from the same animal species as those producing antibodies served as negative reaction controls.

Immunohistochemical localization of Ki-67 and c-kit was verified by comparison with H&E-stained consecutive sections. Additionally, c-kit-immunoreactive cells were strictly identified by the following double immunostaining procedures. In brief, sections were incubated with the anti-c-kit antibody, and immunoreaction was detected by the polymer-immunocomplex (PIC) method using the Envision system (Dako) and DAB as the chromogen. After taking microphotographs of c-kit-immunoreactive cells, sections were processed by eluting the antibodies by microwaving in citrate buffer, pH 6.0, solution (400 W, 95°C, 10 min) for pancytokeratin and by microwaving in TB-EDTA, pH 9.0, solution (400 W, 95°C, 40 min) for melan-A. Sections were then incubated with the antibodies to pancytokeratin and melan-A, and antibody binding was detected by the PIC method using the Envision system (Dako) and NiCl₂/DAB as the chromogen. The c-kit-immunoreactive cells were identified by comparison of the immunohistochemical localization of these cell markers with that of c-kit on the initially taken microphotographs.

Immunoblot analysis

To verify the specificity of the anti-c-kit antibody, we performed an immunoblot analysis using three control skin samples. Archival, freshly frozen skin materials were ho-

mogenized with a 10-fold-volume of radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitor cocktail Complete Mini® (Roche Diagnostics, Mannheim, Germany) and centrifuged for 1 hr at 10,000×g to obtain total protein extracts. Protein concentration in the tissue samples was determined by the Bradford method. After boiling for 10 min with an equal volume of Laemmli's sample buffer, aliquots (30 µg protein/lane) were separated by 8% sodium dodecyl sulfate gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane. Blots were rinsed in Tris-buffered saline, pH 7.5 containing Tween 20 (TBS-T), pretreated for 1 hr at room temperature with 3% bovine serum albumin, and incubated overnight at 4°C with the anti-c-kit antibody at a dilution of 1:200 in Can Get Signal® (ToYoBo, Tokyo, Japan) solution followed by swine anti-rabbit IgG (Dako) at a dilution of 1:25,000 in the Can Get Signal solution. Antibody binding was visualized by the chemiluminescence method using ECL-plus kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instruction. Blots from which the primary antibody was omitted served as negative reaction controls.

Semiquantitative and statistical analyses

Sections stained with H&E and immunostained for Ki-67 and c-kit were scanned and imported into a personal computer. The length of hair follicles (µm) in each case was measured, and the mean length of the follicles was calculated. The number of hair follicle epithelial cells immunoreactive for Ki-67 or c-kit in each case was counted, and the mean number in a follicle was calculated. All of the values in the control, AAR and AGA groups were expressed as mean±standard deviation, and compared between the three groups by non-repeated measures analysis of variance (ANOVA) followed by unpaired Student's t-test. Statistical significance was defined as P value less than 0.05.

III. Results

Histopathological observations

H&E-stained sections demonstrated a variety of histopathological changes between the control, AAR and AGA groups (Figs. 1, 2a, 2d, 2g, 3a, 3d, 3g, 4a, 4d, 4g). In the control group, many of the hair follicles had normal structures characteristic of the anagen phase, and both the hair bulb and the inner and outer root sheaths were clearly identified. In the alopecia groups, the hair follicles tended to show a marked shortening of the longitudinal length as compared with the control group, and these histopathological findings were prominent in the AGA group (Fig. 1). In the AAR group, hair follicles in the anagen phase were reduced in number, while many of the follicles showed dystrophic changes in the hair shafts. Peribulbar lymphocytic infiltrates were, in particular, observed around the catagen and telogen follicles, and perivascular lymphocytic infiltrates were also seen in the dermis and subcutaneous tissue (Figs. 2–4). In the AGA group, the hair follicles in the telogen phase were increased in number and predominant to those in the anagen phase, and displayed miniaturization in the whole structure and hair shaft with variable perivascular and perifollicular inflammation (Figs. 2–4).

Immunohistochemical observations

Immunoreaction product was undetectable in negative reaction control sections without the primary antibodies (data not shown). Ki-67 immunoreactivity was localized to the cell nuclei in the outermost layer of the hair follicles and prominent at the hair bulb, and it was most intense in the control group and the least intense in the AGA group (Figs. 2b, 2e, 2h, 3b, 3e, 3h, 4b, 4e, 4h). By contrast, c-kit immunoreactivity was localized to the cell membrane in the outermost layer of the hair follicles and prominent in the upper half of the hair follicles, and it was least intense in the control group and the most intense in the AGA group (Figs. 2c, 2f, 2i, 3c, 3f, 3i, 4c, 4f, 4i). The Ki-67-immunoreactive cells

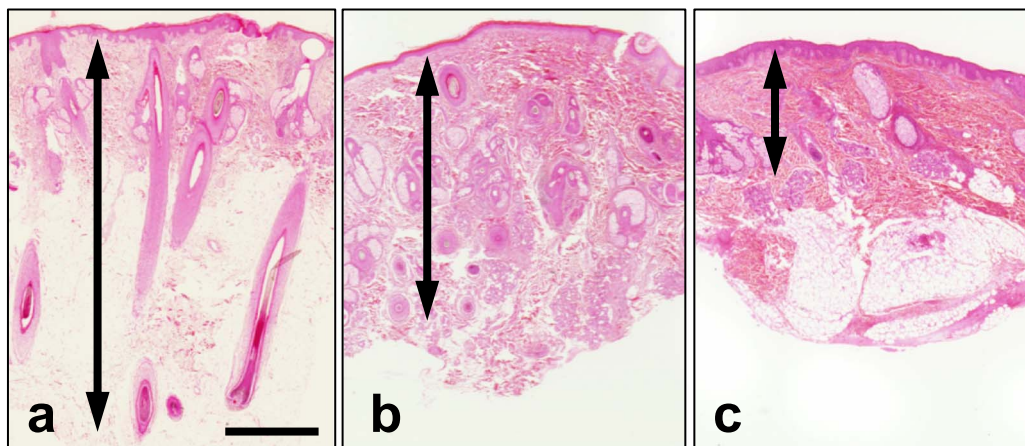


Fig. 1. Semimacroscopic findings of scalp biopsy specimens obtained from control (a), alopecia areata (b) and androgenetic alopecia (c) cases. Hematoxylin-eosin staining. Bar=1 mm (a–c).

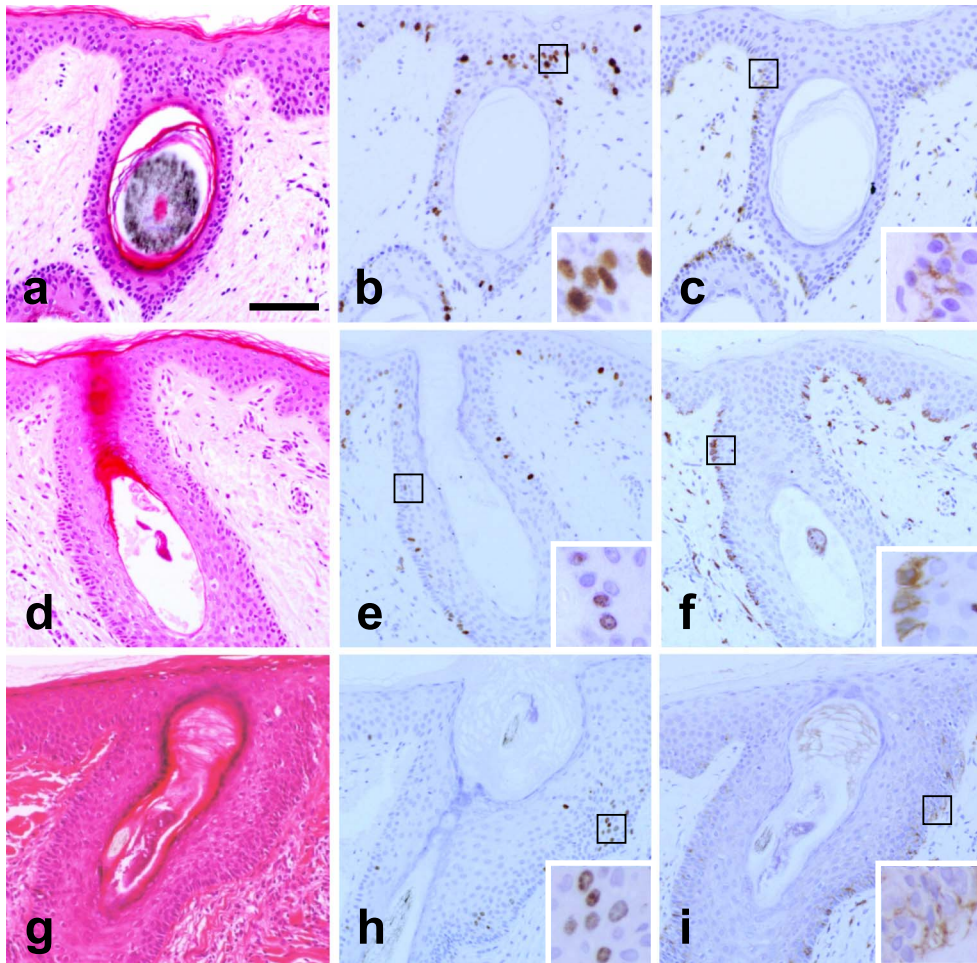


Fig. 2. Photomicrographs of the upper part of hair follicles in control (a–c), alopecia areata (d–f) and androgenetic alopecia (g–i) cases. Hematoxylin-eosin staining (a, d, g) and immunohistochemical staining for Ki-67 (b, e, h) and c-kit (c, f, i). Insets indicate high magnification views of immunoreactive cells in blank squares in the corresponding panels. Bar=200 μ m (a–i).

showed the characteristic morphology of keratinocytes. On the other side, c-kit immunoreactivity was colocalized with pancytokeratin-identified keratinocytes and melan-A-identified melanocytes (Fig. 5). In the examined cases, c-kit immunoreactivity was only very weak or not detectable at all in the hair bulb, where a large number of melanin pigments were accumulated.

Specificity of anti-c-kit antibody

Immunoblot analysis was performed using total protein extracts prepared from skin samples (Fig. 6). Immunoblots disclosed a single band at a 125 kDa mobility. The immunoreactive signals were undetectable on negative reaction control blots.

Changes in length of hair follicles and population of epithelial cells expressing Ki67 and c-kit

The length of hair follicles and population of epithelial cells immunoreactive for Ki-67 and c-kit were statistically compared among the control, AAR, and AGA groups. The

mean length of the hair follicles was 3.73 ± 0.21 mm in the control group, 2.6 ± 0.84 mm in the AAR group, and 2.06 ± 0.46 mm in the AGA group (Fig. 7a). ANOVA disclosed a significant difference in the mean hair follicle length among the three groups, and Student's t-test revealed that the length was significantly shorter in the AAR and AGA groups than in the control group. The mean number of Ki-67-immunoreactive cells per hair follicle was 90.23 ± 31.91 in the control group, 14.19 ± 8.02 in the AAR group, and 3.52 ± 3.91 in the AGA group (Fig. 7b). ANOVA disclosed a significant difference in the mean number of the Ki-67-immunoreactive cells among the three groups, and Student's t-test revealed that the number was significantly reduced in the AAR and AGA groups as compared with the control group and also smaller in the AGA group than in the AAR group. The mean number of c-kit-immunoreactive cells per hair follicle was 3.11 ± 1.58 in the control group, 8.19 ± 2.84 in the AAR group, and 12.89 ± 2.49 in the AGA group (Fig. 7c). ANOVA disclosed a significant difference in the mean number of the c-kit-immunoreactive cells

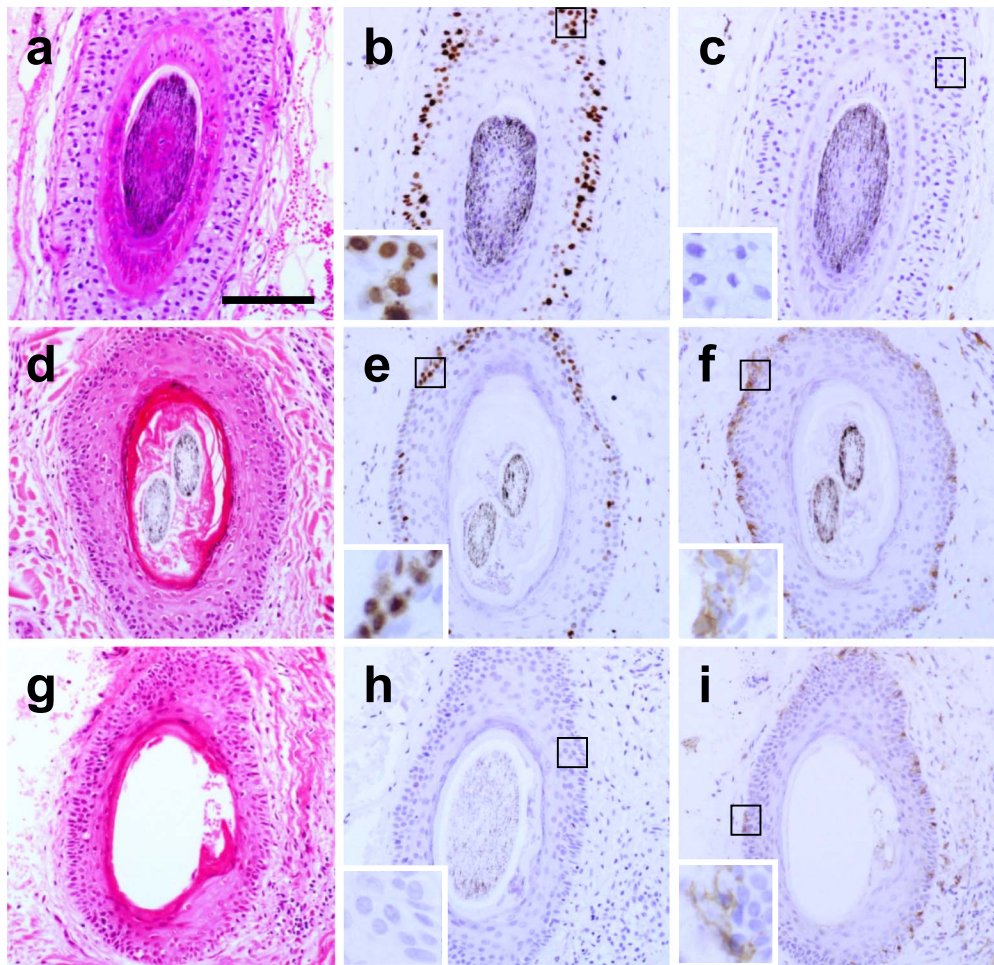


Fig. 3. Photomicrographs of the middle part of hair follicles in control (a–c), alopecia areata (d–f) and androgenetic alopecia (g–i) cases. Hematoxylin-eosin staining (a, d, g) and immunohistochemical staining for Ki-67 (b, e, h) and c-kit (c, f, i). Insets show high magnification views of immunoreactive cells in blank squares in the corresponding panels. Bar=200 μ m (a–i).

among the three groups, and Student's t-test revealed that the number was significantly increased in the AAR and AGA groups as compared with the control group and also increased in the AGA group as compared with the AAR group.

IV. Discussion

The structure of hair consists of the hair bulb and shaft, which exist in the deep dermis and the superficial layer of subcutaneous tissue, and the hair shaft grows upward through the epidermis to protrude outside [15, 20, 22, 24, 29]. The hair follicle can be divided into the following three parts: (1) the infundibulum, (2) the isthmus as the entrance of the sebaceous gland to the attachment site of the arrector pili muscle, and (3) the inferior segment. Hair growth depends on the activity of the highly proliferative hair matrix cells in the hair bulb of the inferior segment. The hair matrix cells are supplied by slow-cycling and multipotent stem cells existing in the bulge near the insertion of the arrector pili muscle [22, 24, 29]. The hair growth cycle consists of the

anagen, catagen, and telogen phases [15, 20, 27, 29]. The anagen phase is a growth condition of the hair follicle and normally lasts for several years. The catagen phase is an involuting or regressive condition that follows the anagen phase and lasts for two or three weeks. The telogen phase is a resting condition that lasts for approximately three months. The hair growth cycle varies in length on different parts of the body [15, 27], and the mean hair growth rate over the body is 0.3–0.4 mm/day [20, 27]. Thus, our histopathological finding of reduced anagen follicles and increased catagen and telogen follicles in the AAR and AGA groups suggests a slowed hair growth cycle in alopecia. This is closely relevant to another fact that the mean length of the hair follicle length was significantly shorter in alopecia. However, it should be noted that the longitudinal length depends on the cell proliferating activity in the inferior portion of the hair follicle.

Ki-67 is a nuclear protein that is strictly expressed only in actively proliferating cells and thus undetectable in resting cells as a result of which this protein has been used as an

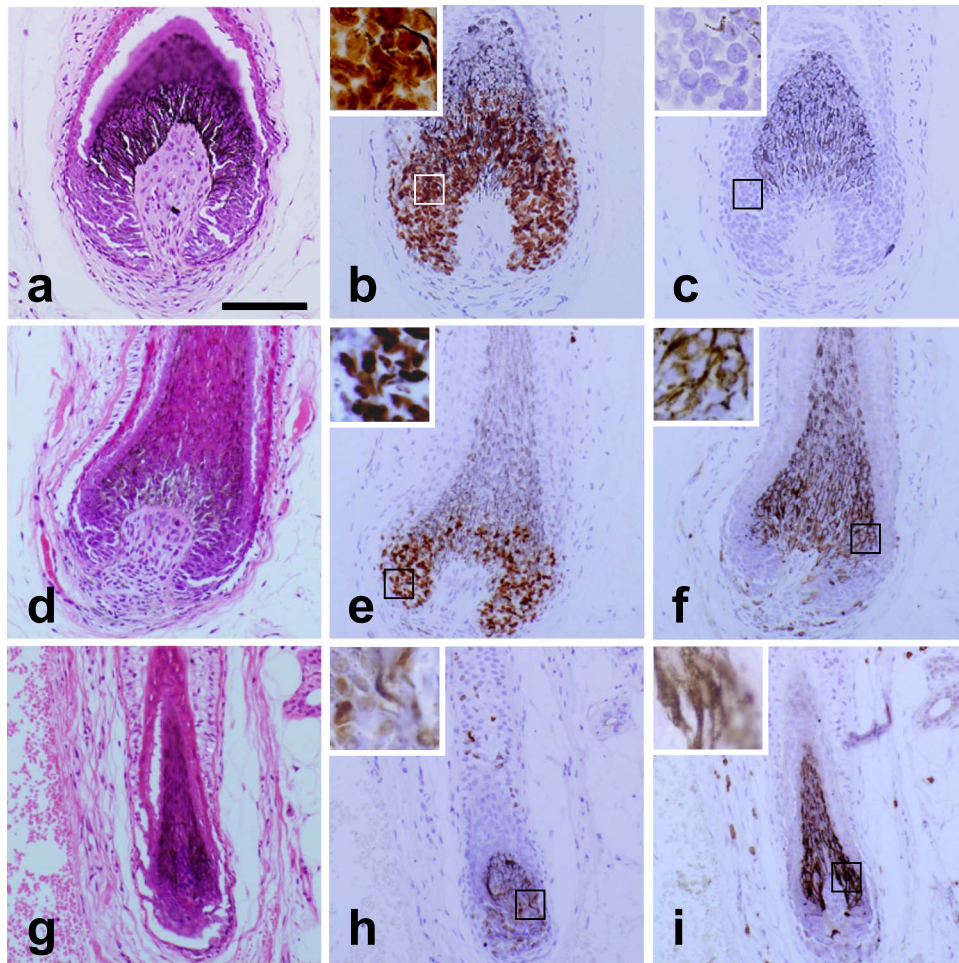


Fig. 4. Photomicrographs of the lower part of hair follicles in control (a–c), alopecia areata (d–f) and androgenetic alopecia (g–i) cases. Hematoxylin-eosin staining (a, d, g) and immunohistochemical staining for Ki-67 (b, e, h) and c-kit (c, f, i). Insets indicate high magnification views of immunoreactive cells in blank squares in the corresponding panels. Bars=200 μm (a–i).

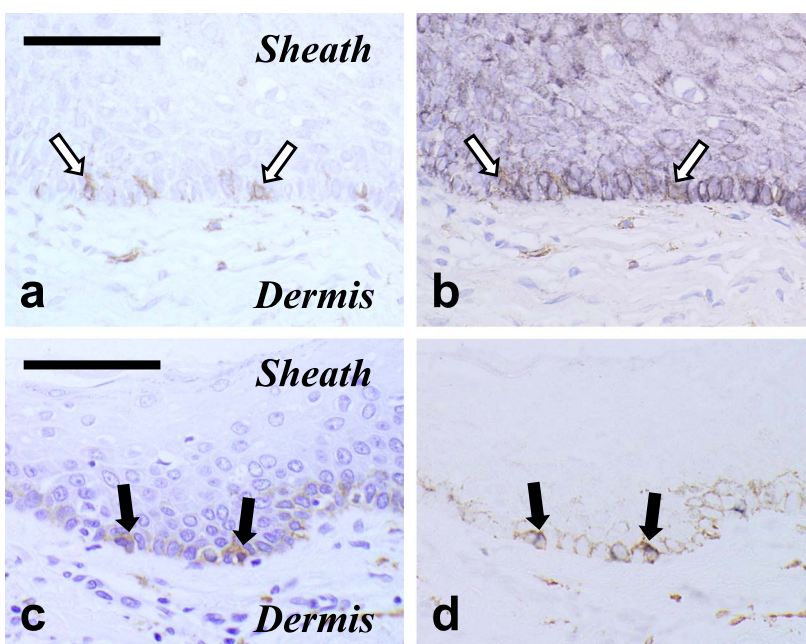


Fig. 5. Identification of c-kit-immunoreactive cells in the hair follicles of AGA patients by double immunolabeling. Panels (a) and (b) show the same region on a section immunostained with the anti-c-kit antibody (brown) and double immunostained with the anti-pancytokeratin antibody (blue), respectively. Panels (c) and (d) show the same region on a section immunostained with the anti-c-kit antibody (brown) and the anti-melan-A antibody (blue), respectively. Blank and solid arrows indicate keratinocytes and melanocytes, respectively. Polymer-immunocomplex method using 3,3-diaminobenzidine tetrahydrochloride (DAB) (a, c) and NiCl_2/DAB (b, d). Bar=20 μm (a–d).

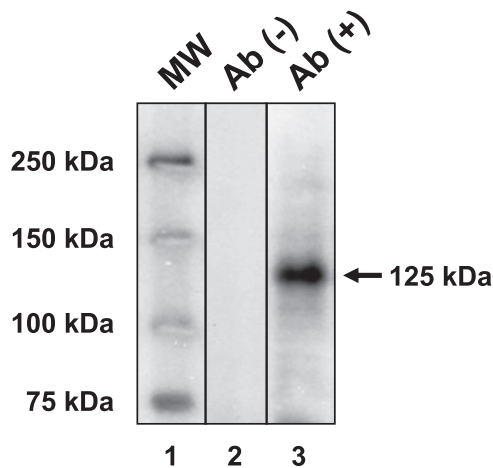


Fig. 6. Immunoblots of total protein extracts from skin samples using the anti-c-kit antibody. Lanes 1, 2 and 3 indicate molecular weight markers, negative reaction control, and c-kit-immunoreactive signals at a 125 kDa mobility, respectively. Chemiluminescence method. Abbreviation: Ab, antibody; MW, molecular weight.

immunohistochemical marker of cell proliferating activity [8, 18, 30]. In the present study, the hair follicles in alopecia showed a shorter length and a reduced number of Ki-67-immunoreactive cells. These observations indicate hair growth suppression and the resultant hair follicle atrophy. It has been shown that Ki-67-immunoreactive cells are mainly localized in the hair bulb [18], and that a reduced number of Ki-67-immunoreactive cells is found in the hair bulb of AAR patients [33]. Thus, our finding of a reduced number of the immunoreactive cells may result from regression of the hair follicles. Based on evidence for the mechanisms by which male hormones regulate the proliferative activity of hair follicle cells, it has been suggested that the altered function of male hormones is involved in AGA [9, 11, 23]. The representative male hormone testosterone is produced in the testes and hematogenously transported into the dermal hair papilla, where 5α -reductase catalyzes the conversion of this hormone to 5α -dihydrotestosterone (5α -DHT), which exerts more powerful effects [11, 27]. These male hormones reduce cell division activity and suppress hair growth activity [11, 27]. Thus, it is conceivable that the increased effects of male hormones in AGA may be responsible for our finding that the lowest hair growth activity, as evidenced by the lack of Ki-67-immunoreactive cells, was in the AGA group.

In the anagen phase, the hair follicle keratinocyte stem cells migrate from the bulge and proliferate to participate in hair bulb formation during hair growth [22, 24, 29]. By contrast, in the catagen and telogen phases, the inferior portion of hair follicles disappears [15, 20, 23, 29]. The slow-cycling stem cells in the hair follicles can follow the two independent stem cell migration pathways. In the bulb-epidermis stem cell pathway, the corresponding cells migrate upward into the epidermis along the basal lamina, proliferate within the stratum basalis, and differentiate verti-

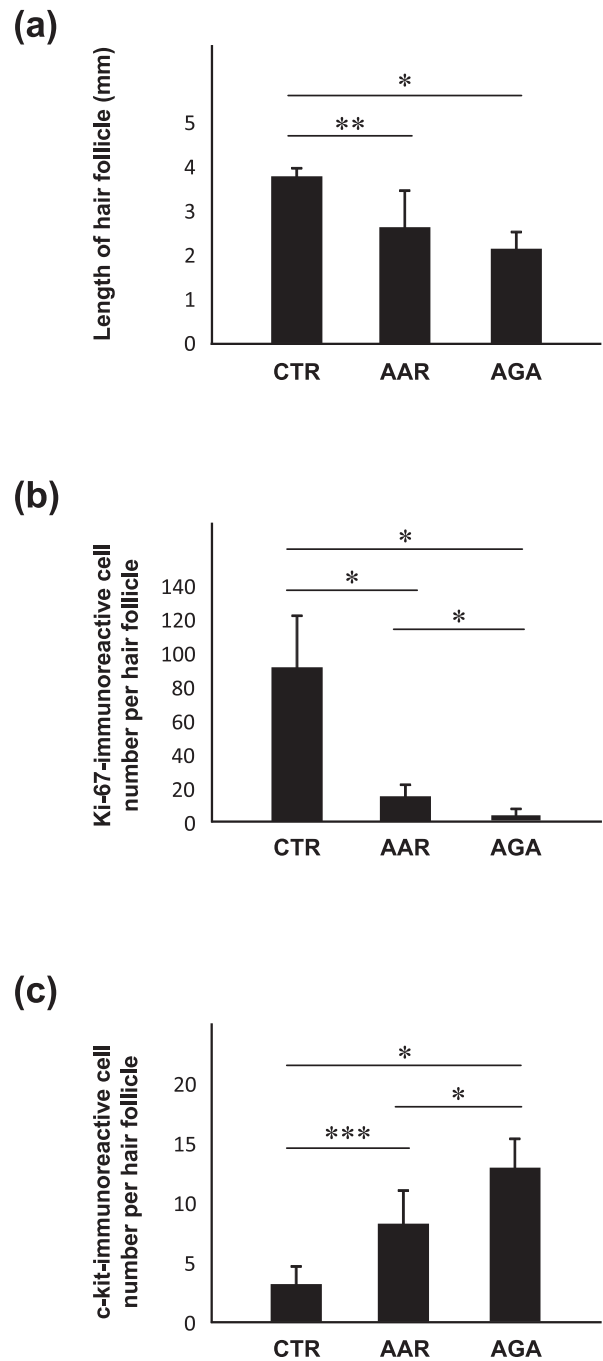


Fig. 7. Statistical comparison of the mean length of hair follicles (a) and mean population of hair follicle epithelial cells immunoreactive for Ki-67 (b) and c-kit (c) between the control, alopecia areata and androgenetic alopecia groups. Asterisks indicate two-tailed P values obtained with unpaired Student's t-test. * $P < 0.001$, ** $P < 0.01$, *** $P < 0.05$.

cally into the keratin-rich cells of the stratum corneum [22]. In the bulb-hair stem cell pathway, the corresponding cells migrate downward and induce stromal cells located at the apex of dermal hair papilla [22, 24, 29]. These cells form the internal root sheath as well as the cortex and medulla of the hair shaft [22, 24, 29].

There is increasing evidence for the presence of SCF and c-kit in the skin, although stem cells in the bulge do not express c-kit [29]. Previous studies documented the expression of c-kit in melanocytic lineage cells [4, 21, 26, 28, 29, 32]. Recent studies demonstrated that c-kit is expressed in keratinocytic lineage cells, destined for hair growth, and the hair matrix of the hair follicles [25], while SCF is expressed in stromal cells of the hair bulb [4, 28]. Thus, c-kit may be responsible for hair growth as well as hair pigmentation. Many of these observations are obtained from animal skins and in keeping with our findings of the presence of c-kit in the outermost layer of the hair follicles, with an upper part-predominant manner, as well as the increased population of c-kit-immunoreactive hair follicle cells in the AAR and AGA groups as compared with the control group. However, our finding of the lack of c-kit in the hair matrix is inconsistent with the results from animal studies. This disparity suggests a difference in the expression pattern of c-kit between humans and animals. On the other side, the present study also demonstrated reduced activity of cell proliferation activity, as evidenced by Ki-67 staining, in hair follicle cells in the AAR and AGA groups. In relation to these observations, it is of interest that both the production and release of the c-kit ligand SCF from stromal cells in dermal hair papilla are impaired in AGA [28]. Thus, our finding of the upregulation of c-kit in the hair follicle cells may reflect a protective mechanism against the potential downregulation of SCF in stromal cells in dermal hair papilla.

Taken together, we demonstrated not only the presence of c-kit and Ki-67 in epithelial cells and melanocytes of the hair follicles in the control and alopecia cases but also the reduced population of Ki-67-expressing cells and increased population of c-kit-expressing cells in the AAR and AGA groups as compared with the control group. The present results suggest a negative feedback mechanism of keratinocyte stem cells in the hair follicles in response to the possible downregulation of SCF in stromal cells in dermal hair papilla. However, both the immunohistochemical localization and expression levels of SCF in alopecia skin tissue remain to be determined. Elucidating these and other issues concerning the crosstalk between SCF and c-kit in the skin will contribute to a better understanding of the complete pathomechanisms of alopecias and establishment of new therapeutic strategies.

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