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ORIGINAL ARTICLE

CHARACTERIZATION OF HAIR FOLLICLES IN HIROSAKI HAIRLESS RATS WITH DELETION OF BASIC HAIR KERATIN GENES. ENLARGED MEDULLA, LOSS OF CUTICLE AND LONG CATAGEN

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Abstract The Hirosaki hairless rat (HHR) is a mutant strain spontaneously derived from the Sprague-Dawley rat (SDR) and its inheritance is autosomal recessive. Our recent study has revealed that an 80-kb genomic DNA on 7q36 containing basic hair keratin genes, *Kb21*, *Kb23*, *Kb26* and *Krt2-25*, is deleted in HHR. To characterize hair follicles in HHR, progression of hair cycle and expression profiles of basic hair keratins were immunohistochemically studied and compared with those of SDR. The HHR exhibited sparse hairs and their twisted hairs were shorter than SDR hairs. HHR hair follicles entered the catagen phase earlier than SDR and massive destruction of HHR hair follicles and infiltration of inflammatory cells occurred in the catagen phase. In HHR the hair medulla was enlarged and the inner root sheath was thinned while the hair cortex was formed where Kb25 was expressed. In SDR Kb25 was expressed in the hair matrix and medulla. Electron microscopy indicated loss of the cuticle in HHR. These results suggest that hypotrichosis of HHR is due to the deletion of hair keratin genes and expression of a keratin fusion gene. Thus, HHR seems to be a useful model to examine the role of hair keratins in the hair follicle formation.

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Key words: gene deletion; hair cycle; hair follicle; hypotrichosis; keratin.

原　著

塩基性ヘアケラチン遺伝子群を欠失する弘前ヘアレスラットの毛包の性質：毛髄質の拡大と毛小皮の欠損，退行期の延長

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抄録　弘前ヘアレスラット（HHR）はSDラット（SDR）から自然発生した変異ラットで，常染色体劣性の遺伝形式を示す．我々は最近，HHRでは，塩基性ヘアケラチン遺伝子Kb21，Kb23，Kb26とKrt2-25などを含む7番染色体の末端領域7q36が80-kbにわたって欠失することを明らかにした．HHRの毛包の性質を明らかにするため，毛の性状を調べるとともに，毛包の構造と毛周期をHE染色や免疫組織化学により検討し，SDRと比較した．HHRの体毛は屈曲し，SDRの直線状の毛に比べ短く，皮膚の単位面積当りの毛の本数も少なかった．HHRでは，毛包の退行期が早期に始まり，退行期に毛包の構造が完全に破壊され，炎症細胞の浸潤が観察された．HHRでは，毛皮質は形成されたが，髄質が拡大し，内毛根鞘は菲薄化し，毛小皮は欠損した．SDRではヘアケラチンKb25は，マトリックスと髄質に発現したが，HHRではこれらで発現せず，皮質で発現した．HHRの毛の異常は，ヘアケラチン遺伝子の欠失と融合遺伝子の発現によると考えられ，HHRは毛包形成におけるヘアケラチンの役割を解析するモデルとなることが示唆された．

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キーワード：遺伝子欠失；毛周期；毛包；乏毛症；ケラチン．

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INTRODUCTION

The Hirosaki hairless rat (HHR) is a mutant strain spontaneously derived from the Sprague-Dawley rat (SDR), first observed in 1985 in our school, and its inheritance is autosomal recessive[1]. HHRs are nearly bare with some sparse and short hairs, and also distinguishable from normal rats by characteristic bent whiskers [1]. In addition to hair loss, female HHRs show involution of the mammary gland at early stage of lactation, and most newborn rats die within a week because of less feeding[2].

The large keratin multigene family comprises cytokeratins, which are differentially expressed in the various types of epithelia, and hair keratins expressed in hard keratinized structures such as hairs and nails. These keratins can be divided into the acidic type I and the basic-to-neutral type Ⅱ members, which form the 10-nm intermediate filament network through the obligatory association of equimolar amounts of type I and type Ⅱ keratins[3]. Previous studies on the hair keratins of several mammals reveal the presence of 9 type I and 6 type Ⅱ members[3]. In the case of rats, genes encoding the basic members are designated *Kb21-26*. In rats, the type I keratin genes are clustered on the chromosome 10q31 and the type Ⅱ gene cluster on 7q36[4].

Hairs are produced in hair follicles, in particular, in a special type of epithelial cells, called the trichocyte. The hair follicle includes not only the medulla and cortex but also the cuticle, inner root sheath (IRS), companion layer, and outer root sheath[5]. Recent studies have revealed that some specific cytokeratins such as K6irs are expressed in the IRS[6] and K6hf in the companion layer and hair medulla[7].

The various types of hairs in mammals undergo a unique cycle throughout life, with phases of growth (anagen), involution (catagen), and rest (telogen)[8]. During the anagen phase, matrix epithelial cells in the prominent hair bulb proliferate, giving rise to progenitors for the layers comprising the hair medulla, cortex, cuticle and IRS[9]. During the catagen phase, the hair bulb migrates to the proximity of the bulge that houses a fraction of skin epithelial stem cells.

The hair follicle morphogenesis and the hair cycle are complex processes dependent on a series of mesenchymal-epithelial interactions in the skin[9]. Numerous growth factors and cytokines such as Wnt[10], transforming growth factor[11], bone morphogenetic proteins[12], fibroblast growth factors[13] and their signal transduction molecules[14], are involved in the processes. Furthermore, loss-of-function mutations of the transcription factor *Foxn*1 are responsible for the mouse nude phenotype[15]. Deletion or overexpression of a member of the highly conserved Hox multigene family, the *Hoxc*13 transcription factor involved in embryonic cell fate determination, result in defective hair formation[16]. However, causative mutations of keratin genes for hair abnormalities are very limited. Mutations of two basic hair keratin genes are noted to lead to monilethrix[17]. Deletion or mutation of genes encoding the specific basic cytokeratins, K6hf or K6irs1, and the acidic cytokeratin K17, result in hair loss in mice[7, 18, 19].

Our recent study has revealed that an 80-kb genomic DNA on 7q36 containing the basic hair keratin gene loci is deleted in HHR[20]. Thus, *Kb21*, *Kb23*, and *Kb26* genes are deleted and the *Kb25* gene is fused to the *Krt2-25* gene. Since the Kb21 and Kb23 are reported to be expressed in the hair cortex[5], the deletion of these genes raised a possibility of aberrant hair follicle formation in HHR. In the present study, the properties of hair follicles were compared between HHR and SDR. Although Kb21 was not expressed in HHR, the hair cortex was formed and Kb25 in place of Kb21 was expressed. The hair medulla was enlarged but the cuticle was devoid in HHR. Furthermore, massive destruction of HHR hair

follicles occurred in the catagen phase.

MATERIALS and METHODS

Rats

Male HHRs maintained in our Department by brother-sister mating of *hhr/+* heterozygous females and *hhr/hhr* homozygous males[20] were used in the present study. SDRs were purchased from Charles-River Japan (Atsugi, Japan). HHRs and SDRs were housed in plastic cages in air-conditioned rooms with a 12 h light/dark cycle in the Institute for Animal Experiments of Hirosaki University Graduate School of Medicine and had free access to water and food. This study was carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

Extraction of rat hair proteins

Proteins were extracted from hairs of SDRs and HHRs, as described by Winter *et al* [17].

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out by the method of Laemmli[21] on 10% w/v polyacrylamide gels. Gels were stained with Coomassie brilliant blue R-250 or electroblotted to Hybond nitrocellulose membranes (GE Healthcare, Tokyo, Japan), according to the method of Towbin *et al* [22].

Production of anti-basic hair keratin antibodies

To produce anti-basic hair keratin Kb25 antibody, the Kb25 protein was isolated from HHR hairs by SDS-PAGE, as described by Nanashima *et al.*[20], and eluted from the gels. The gel portions containing the protein were cut with a razor, homogenized in 1% SDS, 20 mM Tris-HCl (pH 8.0), and then rotated at 25℃ overnight. The supernatant fraction after centrifugation at 15,000 x *g* for 10 min was dialyzed against phosphate-buffered saline. The protein (200 μg protein/1.5 ml) was emulsified with an equal volume of Freund's complete adjuvant (Iatron Laboratories, Tokyo, Japan), and then injected subcutaneously 4 times into an albino rabbit at 1-week intervals. The rabbit was bled on the seventh day after the last injection, and antiserum was purified by ammonium sulfate fractionation. To produce anti-Kb21 antibody, the C-terminal peptide of Kb21 (^{473}SAVSCGRKC^{481}) was synthesized by a peptide synthesizer (model 432A, Applied Biosystems, Foster City, CA, USA). The peptide was coupled to keyhole limpet hemocyanine with *m*-maleimidobenzoyl N-hydroxysuccinimide ester[23]. The peptide-hemocyanine complex was injected into a rabbit, as described above. Specificities of the respective antibodies were confirmed by immunoblotting[20].

Histologic analysis and immunohistochemistry

Skin tissues from the anterior dorsal regions of rats were fixed in 10% formaldehyde and embedded in paraffin. Tissue sections (4 μm thick) were routinely passed through xylene and a graded alcohol series and stained with hematoxylin and eosin (HE). Immunohistochemical staining for signal transducer and activator of transcription (STAT) 3 or basic hair keratins Kb25 or Kb21 was performed by the avidin-biotin-peroxidase complex (ABC) method[24] with the respective antibodies. Anti-STAT3 antibody (sc-8019) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The biotinylated anti-rabbit antibody and Vectastain ABC kit were obtained from Vector Laboratories (Burlingame, CA, USA). The specific binding was visualized with a 3,3'-diaminobenzidine tetrahydrochloride solution. Sections were then lightly counterstained with hematoxylin for microscopic examination. The specimens were examined and photographed using a microscope (COOLSCOPE, Nikon, Tokyo, Japan) interfaced with a computer.

Tdt-mediated dUTP nick end labelling (TUNEL) assay

Cell death was located in tissue sections by the TUNEL assay[25]. This was performed using a commercial kit, following the manufacturer's instructions (in situ apoptosis detection kit, TAKARA, Shiga, Japan). The TUNEL labels were visualized with 3,3'-diaminobenzidine as

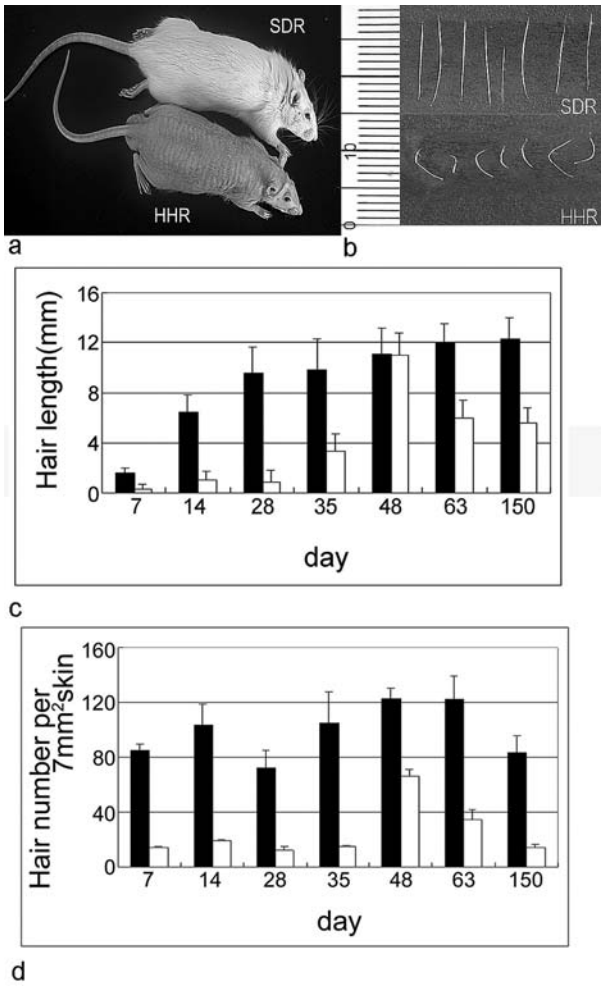

Fig. 1 Gross appearance and hair features of the Hirosaki hairless rat. (a) Five months-old rats. An SDR (upper) has a normal pelage, but an HHR (lower) shows short and sparse pelage. (b) Close-up view of hairs from 14 days-old rats. The hairs of SDR (upper) are straight and longer than those of HHR (lower). HHR hairs are twisted. A rule is in mm. (c) The hair length of SDR (closed bars) and HHR (open bars) at different postnatal days. The length of twisted HHR hairs was expressed by the summation of straight portions. Lines indicate the values of standard deviation. (d) The hair densities of SDR (closed bars) and HHR (open bars), expressed as hair number per 7 mm^2 skin, at different postnatal days. Lines denote the values of standard deviation.

a peroxidase substrate. Postweaning mammary tissue was included as a positive control.

Electron microsopy

Hairs from the anterior dorsal regions of HHRs and SDRs were plucked, mounted on cylindrical metal scanning electron microscopy stubs, and coated with gold. Specimens were loaded, examined and photographed using a scanning electron microscope (model 840A, JOEL, Tokyo, Japan).

RESULTS

Properties of HHR hairs

The HHR exhibited sparse hair cover over the whole body (Fig. 1a, lower), and their twisted hairs with a single bend (Fig. 1b, lower) were shorter than the straight hairs of SDR. The HHR also showed twisted vibrissae. The data of hair length and hair density of HHR (open bars) and SDR (closed bars) over 7—150 postnatal days are summarized in Fig. 1c and d, respectively. During these periods the hair

length of HHR was 2—10-fold shorter than that of SDR except at the day 48. The hair density of HHR was 3—6-fold lower than that of SDR.

Early entry of HHR follicles into the catagen/telogen phases

Hair follicles undergo a cyclic process of anagen, catagen, and telogen. The progression of hair cycle was evaluated on HE-stained tissue sections and compared between SDR and HHR during 1-48 postnatal days. Both SDR and HHR hair follicles were in the first anagen phase and exhibited similar morphologies during 1 to 14 days (Fig. 2a-c, h-j). The density of hair bulbs was not different between SDR and HHR. SDR hair follicles were in the catagen phase on the day 21 (Fig. 2d) and entered the second anagen on the day 28, with new hair plugs at the base of old follicles (Fig. 2e). On the other hand, in HHR, the structures of hair follicles, including the infundibulum near the epidermis, were destroyed and cysts were formed on the day 21 (Fig. 2k). Cyst formation as well as leukocyte infiltration was continued and hair follicles in the anagen were hardly observed on the day 28 (Fig. 2l). On the day 35, both SDR and HHR hair follicles showed the features of the full anagen phase (Fig. 2f and 2m), and hair bulbs were located near the muscle layer. On the day 48, SDR hair follicles were in the catagen phase (Fig. 2g), and HHR ones were also in the catagen phase but accompanied cystic lesions (Fig. 2n). The time course of catagen and the second anagen in SDR was similar to that reported for Wistar rats[26].

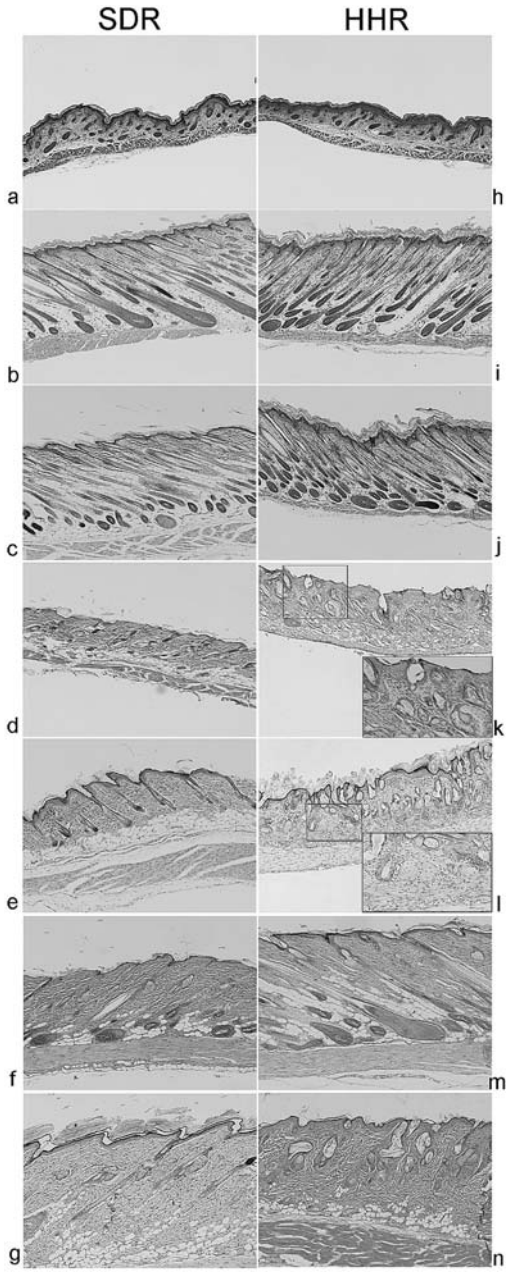

Fig. 2 Comparison in hair cycle progression between SDR and HHR. SDR and HHR skin sections of postnatal day 1 (a and h), day 7 (b and i), day 14 (c and j), day 21 (d and k), day 28 (e and l), day 35 (f and m) and day 48 (g and n) were stained with HE to evaluate hair cycle progression. Magnification, x 50. Images of inserts in k and l, x 200 magnification.

To confirm the catagen phase, tissue sections of the days 14 and 21 were examined by the TUNEL assay. TUNEL-positive cells were not observed in SDR hair follicles on the day 14 (Fig. 3a) but observed on the day 21 (Fig. 3c and e). On the other hand, many positive cells were detected in the hair cortex and medulla of HHR on the day 14 (Fig. 3b). Positive cells were also scattered in hair follicles in the catagen phase on the day 21 (Fig. 3d and f), but not detected in the areas of cystic lesions. These results indicated that HHR hair follicles entered the catagen phase earlier and stayed in it for longer period than those of SDR, and massive destruction of hair

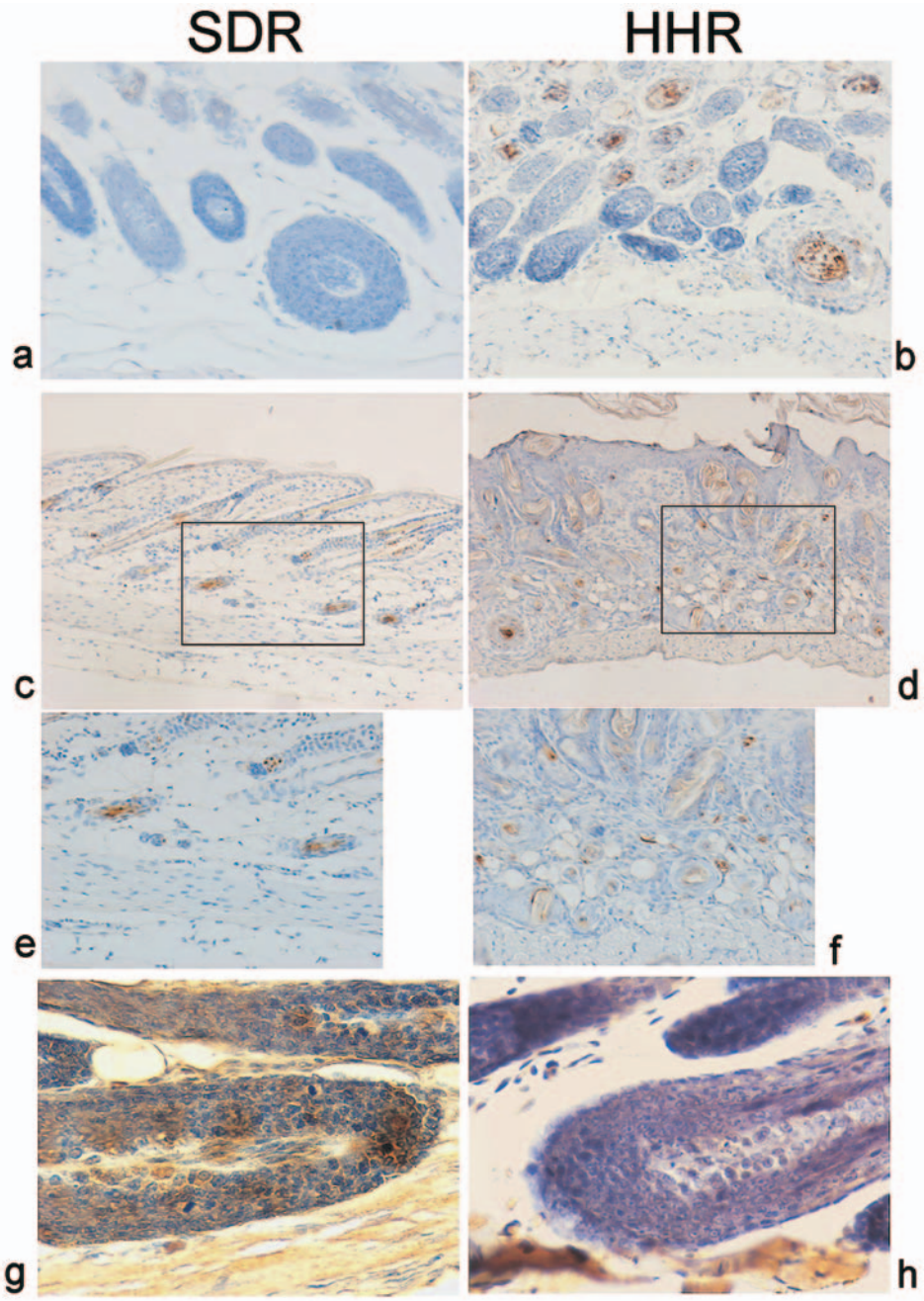

Fig. 3 Immunohistochemical analysis of TUNEL-positive cells and STAT3 expression in SDR and HHR hair follicles. SDR and HHR skin sections of postnatal day 14 (a and b) and day 21 (c, d, e and f) were examined by the TUNEL assay. SDR and HHR skin sections of day 14 (g and h) were stained with anti-STAT3 antibody. Images of a, b, e, f, g and h, 200 x magnification; those of c and d, 100 x. Images of e and f are high magnification of the areas in rectangles of c and d, respectively.

follicles occurred in the catagen phase in HHR. To investigate factors involved in such destructive processes in HHR, expression of STAT3, a protein required for anagen progression in the hair cycle[27], was examined by immunohistochemistry in samples from 14-day-old rats. This showed positive staining in the SDR hair cortex, medulla and matrix (Fig. 3g), whereas HHR hair follicles were negative (Fig. 3h).

Characterization of HHR hair follicles

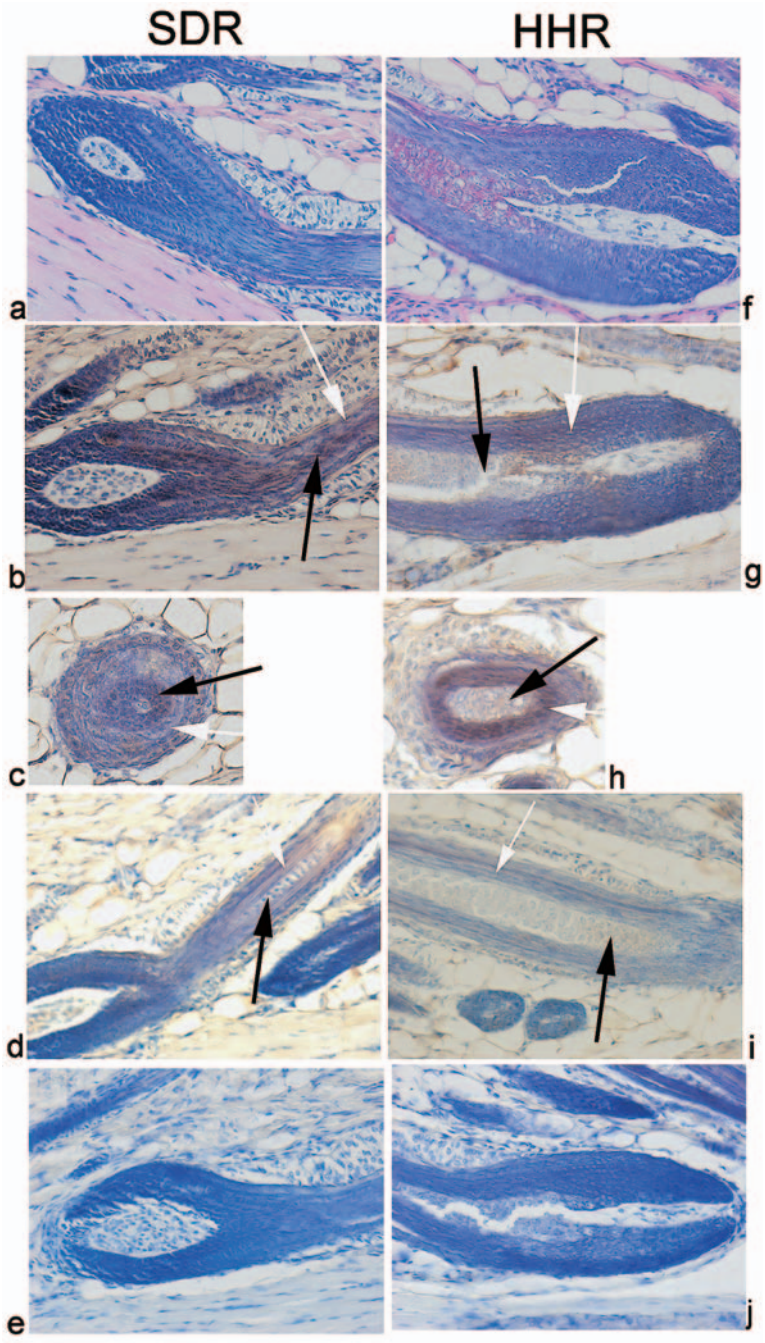

Fig. 4 Immunohistochemical analysis of basic hair keratins Kb25 and Kb21 expression in SDR and HHR hair follicles. SDR and HHR skin sections of postnatal day 35 were stained with HE (a and f), anti-Kb25 antibody (b, c, g and h), anti-Kb21 antibody (d and i), and non-immune control γ-globulin (e and j). The black and white arrows in b, c, d, g, h and i denote the hair medulla and hair cortex, respectively. Magnification, x 200.

Detailed structures of HHR hair follicles were further examined on HE-stained sections from 35-day-old rats. This time point was used because both HHR and SDR were in the full anagen phase (Fig. 2f and m). In HHR, hair matrix formation was not evident and the hair medulla was enlarged (Fig. 4f), as compared with those in SDR (Fig. 4a). The HHR hair medulla near the dermal papilla was eosinophilic. Immunohistochemistry with antibody against

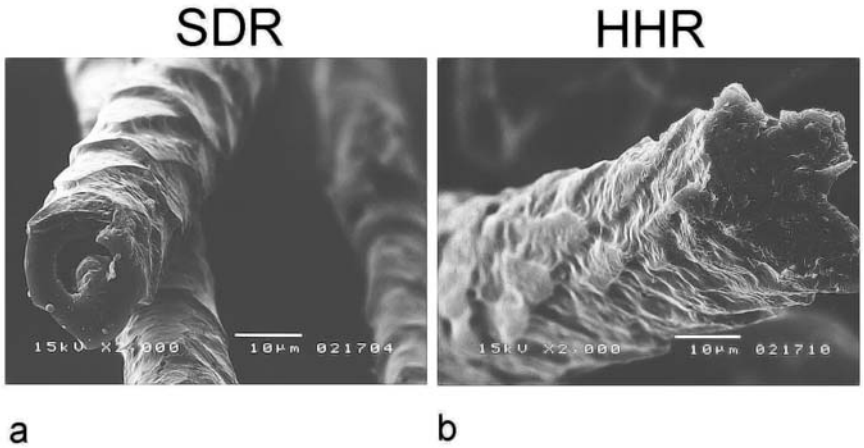

Fig. 5 Scanning electron micrographs of the hairs of 5 months-old SDR (a) and HHR (b). Magnification, x 2,000. Bar, 10 μm.

Kb25, a basic hair keratin expressed in the matrix and medulla[4], was performed to clarify the structures of HHR hair follicles. With this antibody, the medulla as well as matrix was stained in SDR (black arrows in Fig. 4b and c) whereas the hair cortex (white arrows) was hardly stained. The IRS was lightly stained in SDR. In HHR, the hair medulla or matrix was not stained (black arrows in Fig. 4g and h) but the hair cortex was homogenously stained (white arrows). Loss of Kb25 in the hair matrix suggested defective hair matrix formation in HHR (Fig. 4g). Although the individual components of SDR hair follicles were arranged in concentric circles (Fig. 4c), the HHR hair medulla and cortex exhibited elliptic shapes and the IRS was relatively thin (Fig. 4h). With antibody against Kb21, a basic hair keratin form expressed in the cortex[4], the hair cortex was stained in SDR (Fig. 4d) but none of the follicle was stained in HHR (Fig. 4i). The loss of Kb21 expression is consistent with the deletion of an 80-kb DNA containing the *Kb21*, *Kb23*, and *Kb26* genes in HHR[20]. These results indicated that the structures of hair follicles and the expression patterns of Kb25 were different between SDR and HHR. Although Kb21 was not expressed in HHR, the hair cortex was formed and Kb25 in place of Kb21 was expressed. The hair medulla was enlarged while the IRS was thinned in HHR. Scanning electron microscopic analysis of hairs revealed scale formation in SDRs, but not in HHRs (Fig. 5). The surface of HHR hairs was rough and the contours of its cross-section exhibited irregular shapes. These results indicated loss of the cuticle in HHR.

DISCUSSION

Our previous study has revealed that an 80-kb genomic DNA containing the *Kb21*, *Kb23*, *Kb26*, and the most part of *Krt2-25* is deleted and the last exon of *Kb25* is fused to the remaining portion of *Krt2-25* in HHR[20]. Since fusion occurs between the exons of the two genes with the same sequences, the product of a fusion gene is identical with the wild-type Kb25 protein, and is expressed as the dominant basic hair keratin in HHR hairs[20]. Since the *Kb21* and *Kb23* genes are expressed in the hair cortex[4, 5], deletion of these genes in HHR raises the possibility of defective cortex formation. However, the hair cortex was formed while the medulla was enlarged in HHR. Furthermore, the IRS was thin and the cuticle was not formed in HHR (Figs. 4 and 5). Thus, the structures of HHR hair follicles were different from those of SDR. The Kb25

was expressed in the hair matrix and medulla in SDR while expressed in the hair cortex in HHR (Fig. 4). The altered structure of the *Kb25* gene by fusion to the rest of *Krt2-25* gene[20] may be involved in its aberrant expression profile in HHR. Altered structures of the hair follicle in HHR seemed to be due to the loss of the basic keratins by the deletion of their genes. Thus, the hair matrix may be intact by itself but its differentiation towards the individual components of the hair follicle may be disturbed. On the other hand, loss of Kb25 in the hair matrix suggested defective matrix formation in HHR and this might result in the aberrant structures of the hair follicle.

Hair number per unit skin area was 3—6-fold lower in HHR, as compared with the value in SDR (Fig. 1), whereas the number of hair bulbs on tissue sections was not different between them. Since hair number was counted from the skin surface, a lower value in HHR suggests that most hairs remain under the skin surface. In SDR, 20% of hairs were more than 15 mm in length and 80% were in the range of 7—15 mm on the day 35. Thus, in HHR only hairs corresponding to the long ones in SDR (more than 15 mm) may be counted while the dominant hairs corresponding to the short ones (less than 15 mm) are not. This interpretation, however, may not be applicable to the same length of HHR and SDR hairs observed only at a day 48. The shortness of HHR hairs at most other time points seems to be due to the deletion of the basic hair keratin genes, altered expression regulation of *Kb25* gene, and early entry of HHR hairs into the catagen phase.

In HHR, apoptosis occurred earlier and more massively than in SDR. Destruction of HHR hair follicles and cyst formation were dependent on the hair cycle, and only occurred in the catagen phase. Deletion of the acidic cytokeratin *Krt17* gene in mice develops alopecia due to a premature entry into the catagen phase[19]. K17 interacts with TNF receptor 1-associated death domain protein to suppress the TNFα-signaling pathway. Conditional ablation of *Stat3* gene in keratinocytes resulted in impairment of the second hair cycle, indicating that STAT3 is essential for hair cycle progression[27]. *Stat3*-disrupted mice remained in a telogen phase by the day 35 and displayed the infiltration of inflammatory cells. These findings are analogous to those of HHR. STAT3, an anti-apoptotic transcription factor for hair follicles[27], was not expressed in HHR (Fig. 3h) but expressed in SDR (Fig. 3g). Thus, loss of STAT3 expression may be involved in apoptosis induction in HHR. Although the lack of STAT3 in HHR seems to be linked to the deletion of the basic hair keratin genes, the mechanism leading to the loss of the transcription factor remains to be clarified. Some growth factors are suggested to be responsible for activating STAT3 in the skin.

Destruction of hair follicles accompanied the infiltration of inflammatory cells (Fig. 2k and l). Such cell reaction may be secondary to the destruction of hair follicles. Some lymphocytes, however, are known to possess cytotoxic activities[28], raising another possibility that inflammatory cells in HHR may be a cause of the destruction of hair follicles. In this case, apoptotic cell death may recruit inflammatory cells. HHR is a model of hereditary hypotrichosis but the transient infiltration of inflammatory cells is interesting to consider the pathophysiology of alopecia areata[29].

Besides HHR, several hereditary atrichotic or hypotrichotic rat models have been reported. These include Charles River "hairless", naked, Rowett nude, fuzzy, hairless, shorn, and bald rats[30, 31]. With the exception of the nude mutation[32], responsible genes remain to be clarified. Because hypotrichosis of HHR is due to the deletion of hair keratin genes and expression of a keratin fusion gene, it will provide a useful model to examine the role of hair keratins in the hair follicle formation.

Acknowledgments

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