Original Article

Morphological analysis of patchy thickening and reddish discoloration of active hair growth areas in the skin of New Zealand White rabbits

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Abstract: Patchy thickening and reddish discoloration of active hair growth areas of skin in rabbits are occasionally found, and this gross feature could affect precise evaluation when conducting a dermal irritation test. Since little is known about the mechanism of this phenomenon, we examined the dorsal skin of New Zealand White rabbits morphologically and immunohistochemically in order to identify the possible mechanism responsible for developing these skin changes in relation to the hair cycle. Skin samples from 4 rabbits were divided into three groups (5 samples/group) based on their macroscopic characteristics: a thickened skin, erythematous skin, and smooth skin group. Histomorphological examination revealed that the percentage of hair follicles in the anagen phase, hair follicle length, hair follicle area, and proliferating cell nuclear antigen-positive cells in the hair follicles were greater in the thickened skin and erythematous skin groups than in the smooth skin group. Unlike mice and rats, the dermis was nearly adjacent to the muscular layer with a thin hypodermis, and the whole lengths of hair follicles in the anagen phase were located in the dermis in the rabbit skin. These results suggest that large hair follicles in the anagen phase compressed the surrounding dermis; therefore, the skin was grossly raised and showed thickening. A higher number of CD31-positive blood vessels, suggesting the occurrence of angiogenesis, was observed around the hair follicles in the erythematous skin group, and they seemed to affect the reddish discoloration of skin noted grossly. (DOI: 10.1293/tox.2017-0033; J Toxicol Pathol 2017; 30: 315–322)

Key words: dermal irritation test, rabbit, skin, hair cycle

Introduction

The hair cycle consists of three phases, a growth phase called the anagen phase, regression phase called the catagen phase, and rest phase called the telogen phase. In the anagen phase, hair follicles grow rapidly by cell proliferation and reach their greatest length. After hair follicles undergo involution by apoptosis in the catagen phase, they enter the telogen phase[1, 2].

Rabbit hairs are very dense and arise singly or in multiples from hair follicles. Hair replacement in adult rabbits follows a seasonal pattern, and there are usually two complete coat changes per year. The coat changes usually proceed from the head to the back and then to the abdomen with synchronization of their hair cycles[3, 4]. It has been reported that irregular patches of hair growth appear as raised, thickened islands of skin when compared with the surrounding skin[5, 6]. In New Zealand White (NZW) rabbits, the raised areas are redder than the surrounding skin[3].

Rabbits are widely used to evaluate chemicals and new drugs for dermal irritation in nonclinical safety assessment. The Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals recommend using rabbit skin that is in the telogen phase to perform dermal irritation tests, because the thickening and reddish discoloration of the skin associated with hair growth in the anagen phase may interfere with the evaluation[7, 8]. The skin changes in the anagen phase are thought to be caused by the increasing size of hair follicles and blood vessels due to active hair growth[3, 6], but little is known about the mechanisms that cause these phenomena. In this study, we morphologically and immunohistochemically investigated the changes in hair follicles and surrounding connective tissues in relation to the hair cycle in the skin of NZW rabbits.

Materials and Methods

Animals

Four 25-week-old male NZW rabbits (Kbl:NZW) purchased from Kitayama Labes Co., Ltd. (Ina, Japan), were maintained under the following conditions: temperature 21–23°C, humidity 52–70%, 12-h light/dark cycle, and 5–40 air exchanges/h ventilation frequency. The animals were housed individually in cages and allowed free access to a laboratory pellet diet for rabbits (RC-4, Oriental Yeast Co., Ltd., Tokyo, Japan) and water (automatic water supply system). The animal maintenance and experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals of Taisho Pharmaceutical Co., Ltd.

Tissue sampling

Four naïve rabbits were used and anesthetized by inhalation of 4.0% isoflurane when subjected to skin biopsy. Dorsal skin samples (n=5/group) were collected from the rabbits based on their macroscopic characteristics of three dominant features, thickened skin (TS), erythematous skin (ES), and smooth skin (SS), by means of 6-mm biopsy punches (Kai Industries Co., Ltd., Seki, Japan) at 1, 4, and 5 weeks, after shaving their hair. When the dorsal skin sample areas were selected, the macroscopic characteristics were evaluated based on the criteria described as follows. 1) When rabbit skin showed thickened regions accompanied by very slight edema, the region was sampled as a TS group sample. 2) When a skin region appeared to have reddish discoloration around a thickened area, the region was sampled as an ES group sample. 3) Normal skin regions without thickening or reddish discoloration were sampled as SS group samples. The representative gross findings are shown in Fig. 1.
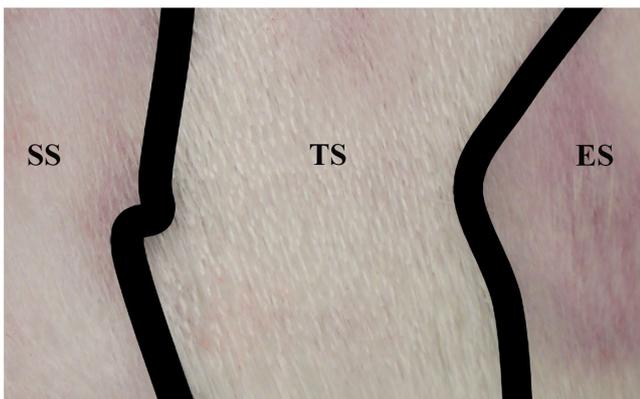
Fig. 1. Rabbit skin was divided into three groups according to the macroscopic characteristics. A thickend region with dense hair growth was observed in the thickened skin (TS) group, and reddish discoloration was observed around thickened areas in the erythematous skin (ES) group. Normal skin regions without thickening or reddish discoloration were sampled for the smooth skin (SS) group.

Histology and immunohistochemistry

All skin samples were fixed with 10% neutral buffered formalin, embedded in paraffin, and cut into 3-μm sections. The sections were stained with hematoxylin and eosin (H&E) and the following immunohistochemical methods. For the detection of cell proliferation, endothelial cells, and collagen type 1 (most prominent structural component of the dermis), monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (M0879, 0.163 μg/ml, 1:2000, Dako, Glostrup, Denmark), monoclonal mouse anti-CD31 antibody (NB600-562, antibody concentration unknown, 1:10, Novus Biologicals., Littleton, CO, USA), and polyclonal goat anti-collagen type 1 antibody (1310-01, 5 μg/ml, 1:80, Southern Biotechnology Associates, Inc., Birmingham, AL, USA) were used[9, 10]. Immunohistochemical staining with anti-PCNA and anti-collagen type 1 was performed using a Ventana HX system Discovery (Roche Diagnostics K.K., Tokyo, Japan). To enhance immunoreactivities for PCNA staining, the sections were heated in CC 1 buffer (950-124, Roche Diagnostics K.K.). After incubation with anti-PCNA or anti-collagen type 1 antibodies, sections were treated with a secondary antibody, biotin-conjugated horse anti-mouse IgG (BA-2001, Vector Laboratories, Burlingame, CA, USA) or biotin-conjugated horse anti-goat IgG (BA-9500, Vector Laboratories). Then the immunoreaction was visualized by peroxidase-diaminobenzidine reaction using an iVIEW DAB research kit (760-093, Roche Diagnostics K.K.). Immunohistochemical staining with anti-CD31 was performed by a manual procedure, and the sections were boiled in EDTA solution (pH 8.0; 00-5500, Invitrogen Corporation, Carlsbad, CA, USA) to enhance immunoreactivities before incubation with the primary antibody. After incubation with the anti-CD31 antibody, the sections were treated with a biotin-conjugated horse anti-mouse IgG secondary antibody (BA-2001, Vector Laboratories). Then immunoreaction was visualized using a Vectastain Elite ABC Kit (PK-6100, Vector Laboratories) and 3,3′-diaminobenzidine (1.02924.0001, Merck Millipore, Billerica, MA, USA).

Morphological analysis

All H&E-stained and immunohistochemically stained specimens were scanned with a ScanScope XT (Leica Biosystems, Nussloch, Germany) to create whole digital slide images. Histological observations and measurements were made with Aperio ImageScope viewing and analysis software (Leica Biosystems)[11].

Hair follicle length and thickness of the dermis were measured with an exclusive ruler tool in the digital images of H&E-stained section. To measure the hair follicle length and thickness of the dermis, three areas per section were analyzed, and mean values were calculated in each sample. The lengths of longitudinally cut hair follicles were measured from the bottom of the hair bulb to the epidermis[12], and these values were used as the hair follicle lengths. The nuclear v9 algorithm was modified and used to count the numbers of PCNA-positive cells in the hair follicles with

hair and the number of PCNA-positive cells in the dermis. The Positive Pixel Count v9 algorithm was modified and used to measure the collagen type 1-positive reaction area and CD31-positive reaction area in the dermis. In addition, the area of hair follicle with hair in H&E-stained sections was measured by the Positive Pixel Count v9 algorithm. Analysis for PCNA-positive cells, collagen type 1-positive reaction area, CD31-positive reaction area, and hair follicle area in each sample were conducted within a width of 3 mm horizontally.

The hair follicles with dermal papillae in the entire area of H&E-stained specimens were classified into three phases of the hair cycle (the anagen, catagen, and the telogen phases) according to the method of Müller-Röver *et al.*[2]. The percentage of hair follicles in each phase in each sample was calculated.

Statistical analysis

The mean values and standard deviation of the numerical data in each group were calculated. A multiple comparison test was performed to analyze the differences among the TS group, ES group, and SS group. First, the homogeneity of variance was analyzed by Bartlett's test followed by a one-way analysis of variance when the variance was homogeneous. If a significant difference was found among the groups, Tukey's test (parametric) was performed to test the differences among the mean values in the three groups. When the variance was heterogeneous, the Kruskal-Wallis *H*-test was performed, and if a significant difference was found among the groups, the Steel-Dwass test (nonparametric) was performed to test the differences among the mean values in the three groups. The significance level for the Bartlett's test, one-way analysis of variance, and the Kruskal-Wallis *H*-test was set at 5% (two-sided), and the significance levels for the other tests were set at 0.1%, 1%, and 5% (two-sided). The statistical analysis was performed using the Exsus, ver. 7.7, software (CAC Corporation, Tokyo, Japan).

Results

Histomorphological observations and classification of hair cycle phase

In the skin of the SS group, small hair follicles with ball-shaped hair bulbs classified into the telogen phase were located in the shallow part of the dermis (Fig. 2A). The percentage of fair follicles in the telogen phase was predominant (80.44 ± 18.64%), whereas the percentage of those in the catagen phase was low (19.56 ± 18.64%) (Fig. 3). In the ES group, hair follicles classified into the early and late anagen phases grew down into the dermis, and the hair bulb surrounding the dermal papilla and inner root sheath could be identified (Fig. 2B). Various lengths of hair follicles were observed: the hair follicles in the early anagen phase were slightly longer than those in the telogen phase, whereas hair follicles in the late anagen phase were long, and their hair bulbs resided deep in the dermis. The percentage of hair fol-

licles in the anagen phase was predominant (76.65 ± 9.12%), whereas the percentage of those in the telogen and catagen phases were low (20.60 ± 7.53% and 2.75 ± 4.57%, respec-
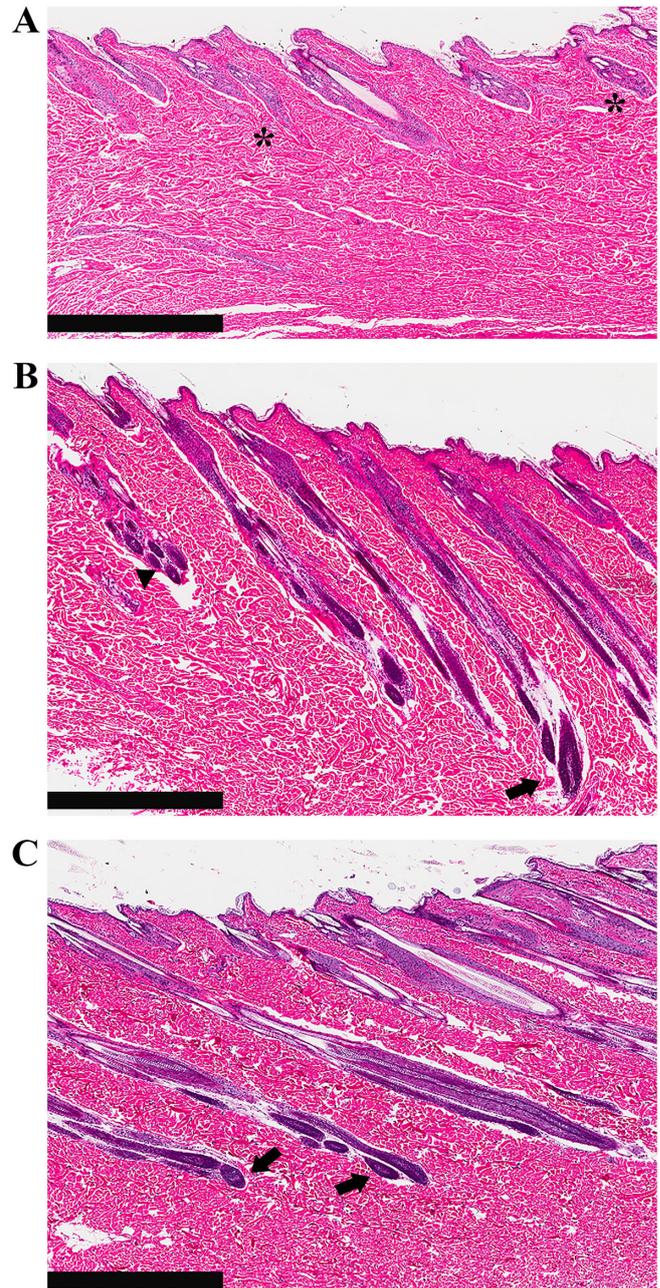
Fig. 2. H&E-stained sections of rabbit skin. In the SS (smooth skin) group (A), small hair follicles in the telogen phase (asterisk) were predominant. In the ES (erythematous skin) group (B) and TS (thickened skin) group (C), hair follicles in the anagen phase were predominant. Various length of hair follicles in the early anagen phase (arrow head) or late anagen phase (arrow) were observed in the ES group, whereas almost all hair follicles were classified into the late anagen phase (arrow) in the TS group. The whole lengths of hair follicles including their hair bulbs were located in the dermis in all three groups. Bar = 800 μm.

tively) (Fig. 3). In the TS group, hair follicles classified into the late anagen phase resided deep in the dermis, and the dermis was slightly thickened (Fig. 2C). The percentage of hair follicles in the anagen phase was significantly high (90.02 ± 8.40%), whereas the percentage of those in the telogen phase was low (9.98 ± 8.40%) (Fig. 3).

Histological examination of H&E-stained sections revealed that the whole lengths of hair follicles including the hair bulbs were located in the dermis in all three groups. The hypodermis, which lies between the dermis and the muscular layer, was thin, and the dermis was almost directly adjacent to muscular layer. The predominant hair cycle phase in the ES group and TS group was the anagen phase, but it was the telogen phase in the SS group. There was no obvious melanogenesis, which is promoted in pigmented animals during hair growth[13], in the hair follicles in any of the groups.
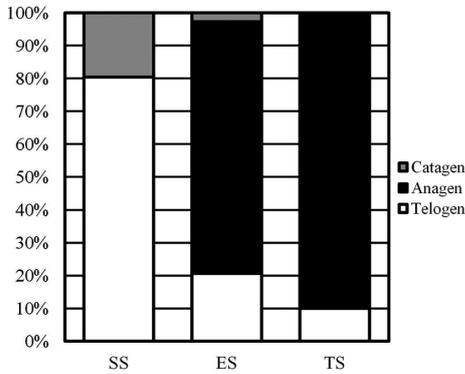
Fig. 3. Percentage of hair follicles in each phase in each group (n=5 samples/group). The predominant hair cycle phase in the ES (erythematous skin) group and TS (thickened skin) group was the anagen phase, whereas it was the telogen phase in the SS (smooth skin) group.

Measurements of hair follicle length, thickness of the dermis, and hair follicle area

The results of measurements for hair follicle length, thickness of the dermis, and hair follicle area in each group are shown in Fig. 4A–4C. Hair follicle length was 0.63 ± 0.12 mm in the SS group, 1.71 ± 0.32 mm in the ES group, and 2.81 ± 0.10 mm in the TS group; thickness of the dermis was 1.49 ± 0.12 mm in the SS group, 1.78 ± 0.12 mm in the ES group, and 2.19 ± 0.24 mm in the TS group; and hair follicle area was 0.24 ± 0.04 mm^2 in the SS group, 0.76 ± 0.17 mm^2 in the ES group, and 1.47 ± 0.38 mm^2 in the TS group. All of the measurement data were highest in the TS group and were higher in the ES group than in the SS group.

Histomorphological observation and analysis of the hair follicles and dermis using immunostaining for PCNA, collagen type 1, and CD31

The PCNA-positive reaction was prominent in the lower part of the hair follicle, especially in the hair bulb in the ES and TS groups, whereas it was not obvious in the hair bulb in the SS group (Fig. 5). The number of PCNA-positive cells in the hair follicles was statistically higher in the ES group (4185.2 ± 839.6) and TS group (4005.0 ± 1599.9) than in the SS group (585.4 ± 255.5) (Fig. 6A). Although the number of PCNA-positive cells in the dermis tended to be higher in the ES group (839.2 ± 277.2) and TS group (867.8 ± 408.5) than in the SS group (498.0 ± 200.2) (Fig. 6A), the difference was not statistically significant. The collagen type 1-positive reaction was observed in almost all areas of the dermis in all three groups. There were no significant differences among the three groups in collagen type 1-positive reaction area in the dermis (Fig. 6B).

A large number of CD31-positive blood vessels containing large blood vessels were detected around the transient region of hair follicles in the anagen phase in the ES and TS groups, whereas a few small CD31-positive blood
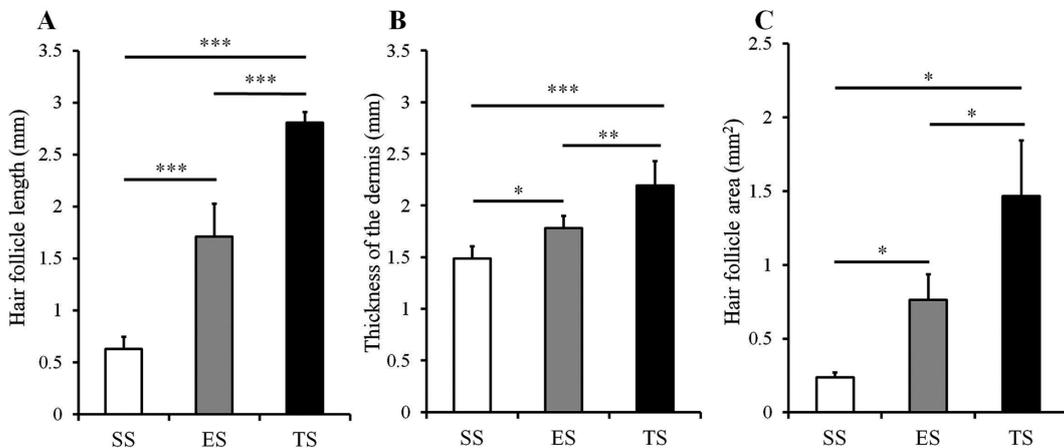
Fig. 4. Hair follicle length (A), thickness of the dermis (B), and hair follicle area (C). Values are shown as the mean ± SD (n=5 samples/group). *p<0.05; **p<0.01; ***p<0.001 for comparisons between two groups (A and B, Tukey's test; C, Steel-Dwass test). All measurement data were highest in the TS (thickened skin) group, followed by the ES (erythematous skin) group and then the SS (smooth skin) group.

vessels were detected around the hair follicles in the telogen phase in the SS group (Fig. 7). The areas of CD31-positive blood vessels in the ES group (0.0189 ± 0.0046 mm^2) and TS group (0.0262 ± 0.0078 mm^2) were greater than that in the SS group (0.0080 ± 0.0024 mm^2) (Fig. 8).

Discussion

In this study, we examined morphological changes of rabbit skin in relation to the hair cycle using skin samples showing three characteristic gross appearances: thickened skin, erythematous skin, and smooth skin. In the SS group, small hair follicles showing low cellular proliferating activity in the telogen phase resided in the shallow part of the
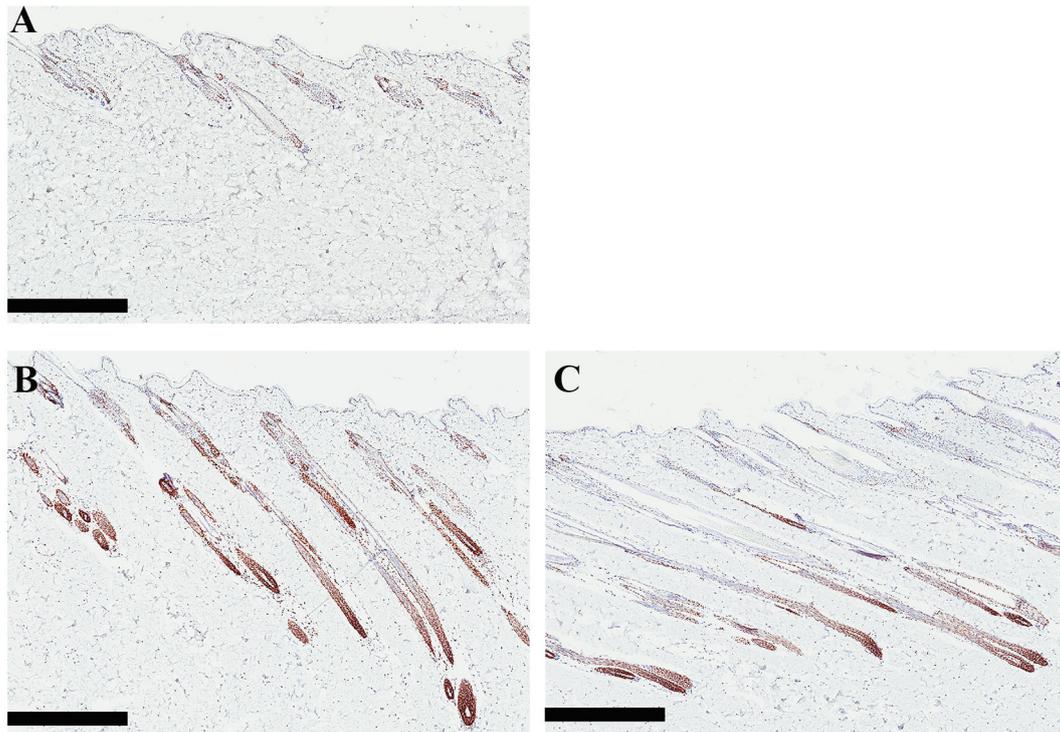
Fig. 5. Immunostaining for PCNA in rabbit skin. A prominent PCNA-positive reaction was observed in the lower part of hair follicle, especially in the hair bulb in the erythematous skin group (B) and thickened skin group (C). A: smooth skin group. Bar = 600 μm.
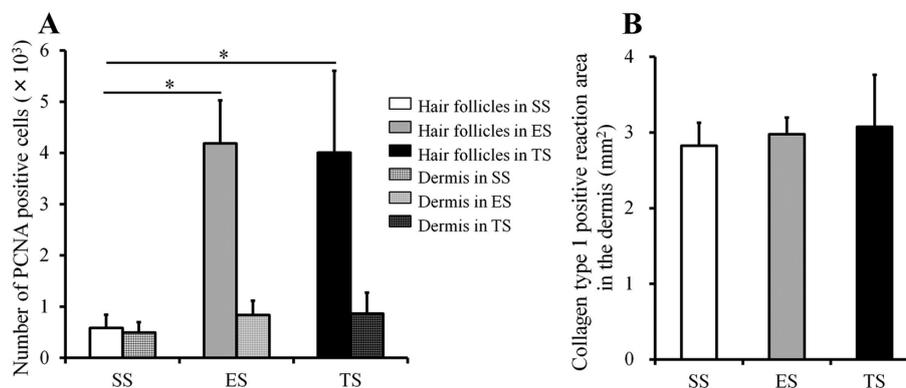
Fig. 6. Number of PCNA-positive cells in the hair follicles and dermis (A) and collagen type 1-positive reaction areas in the dermis (B). Values are shown as the mean ± SD (n=5 samples/group). *$p<0.05$ for comparisons between two groups (Steel-Dwass test). The number of PCNA-positive cells in the hair follicles was statistically higher in the ES (erythematous skin) and TS (thickened skin) groups than in the SS (smooth skin) group (A). The number of PCNA-positive cells in the dermis tended to be higher in the ES and TS group than in the SS group (A), but there were no significant differences among the three groups in collagen type 1-positive reaction area in the dermis (B).

dermis, and hair follicles were covered with a few small blood vessels. On the other hand, hair follicles showing active cell proliferation in the ES and TS groups grew down into the deep dermis, and the hair follicles became long and matured as the hair follicles transferred from the early anagen phase to the late anagen phase. Increases of blood vessels around hair follicles were also observed in the ES and TS groups. Thus, we considered that the various gross appearances of rabbit skin such as thickening and reddish discoloration were characterized by histological variation of the hair cycle of NZW rabbits.

The skin consists of three main layers, the epidermis, the dermis, and the hypodermis, and the hypodermis is in contact with skeletal muscle[14]. In mice and rats, the whole lengths of hair follicles are located in the dermis in the telogen phase; however, during the anagen phase, the hair follicles grow, and their hair bulbs penetrate into the hypodermis, which consists chiefly of adipose tissue[2, 15]. On the other hand, the rabbit hair bulbs in the present study were consistently located in the dermis in all phases of the hair cycle and did not penetrated into the hypodermis. In addition, the rabbit hypodermis was very thin, and the dermis was almost directly adjacent to the muscle layer. The gross feature of thickened skin in rabbits might be observed because hair follicles in the anagen phase compressed the surrounding dermis, and this pressure was not absorbed by the thin hypodermis (Fig. 9).

In the ES and TS groups, the number of PCNA-positive cells in the dermis was increased but not statistically significantly, and there were no differences in the collagen type 1-positive reaction area among the three groups. Therefore, the thickening of the skin was affected mostly by the increased area of grown hair follicles in the anagen phase rather than the minimal change of the cellular components in the dermis.

There are three horizontally arranged plexuses of arteries and veins in the skin[1, 14]. The hair follicle is covered by a basket-like network of small blood vessels from these vascular plexuses[16, 17]. In this study, the CD31-positive blood vessels were localized around the hair follicles, and an increase in CD31-positive area in the ES and TS groups that suggested the occurrence of angiogenesis[18] was observed as the hair cycle proceeded from the telogen phase to the anagen phase. The number of perifollicular blood vessels in the skin of pigmented C57BL/6 mice increases when the telogen phase hair follicles enter the anagen phase[18], and their skin color is known to become darker grossly because of active melanogenesis in the hair[19]. Since we used albino rabbits in this study, the reddish discoloration of skin in the ES group appeared to have been intensified by the increase
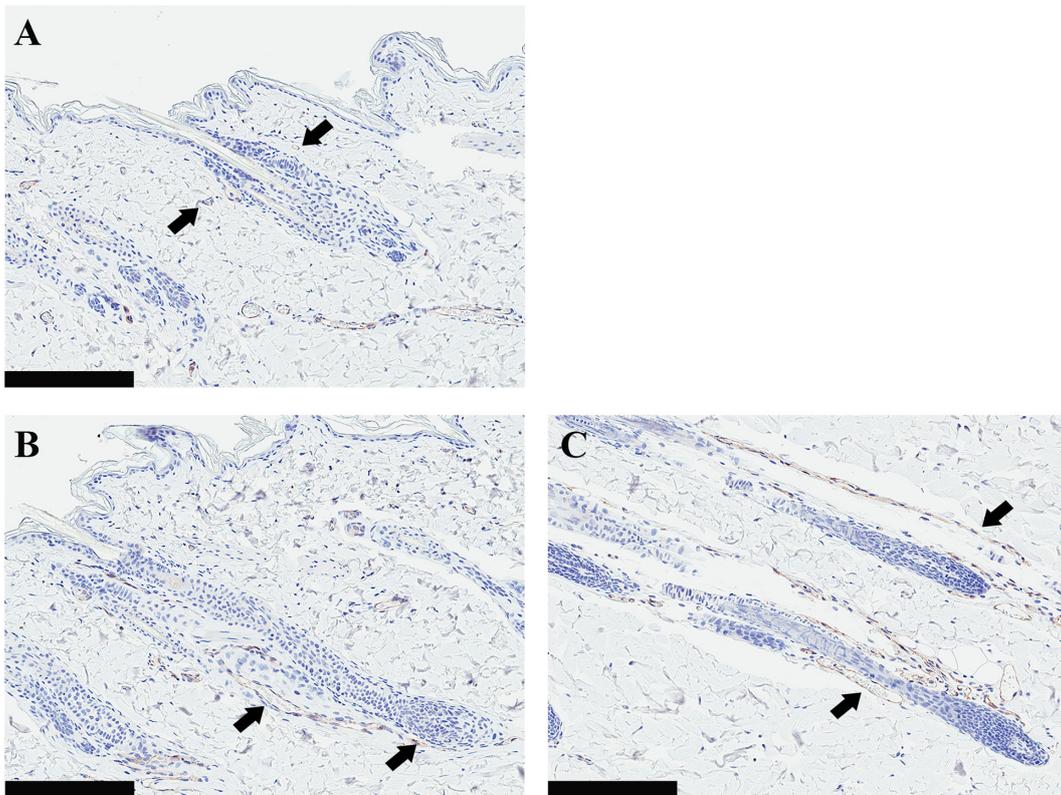
Fig. 7. Immunostaining for CD31 in rabbit skin. CD31-positive reactions (arrows) were detected in the dermis around hair follicles. In the smooth skin group, a few small CD31-positive blood vessels were detected around hair follicles in the telogen phase (A). A large number of CD31-positive blood vessels containing large blood vessels were detected around the transient region of hair follicles in the anagen phase in the erythematous skin group (B) and thickened skin group (C). Bar = 200 μm.

in the number of blood vessels around the hair follicle in the anagen phase in the absence of melanogenesis.

The dermal irritation score is evaluated based on signs of erythema and edema (observed as a raised region) in the dermal irritation test[7]. Rabbit skin in the telogen phase is used in dermal irritation studies because the thickening and reddish discoloration of the skin associated with hair growth in the anagen phase may interfere with the evaluation[7, 8].
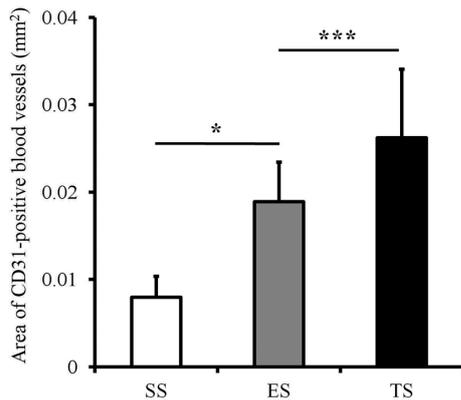
Fig. 8. Area of CD31-positive blood vessels in the dermis. Values are shown as the mean ± SD (n=5 samples/group). *$p<0.05$; ***$p<0.001$ for comparisons between two groups (Tukey's test). The areas of CD31-positive blood vessels in the ES (erythematous skin) group and TS (thickened skin) group were greater than in the SS (smooth skin) group.

However, in cases in which the skin area used in a dermal irritation study shifts from the telogen phase into the anagen phase because of the physiological changes of the hair cycle or the effects of a test article on hair growth during the dosing period, it is difficult to distinguish between changes induced by the irritation of the test article and effect of hair growth based on gross observation. The histopathological features shown in the present study are considered to provide useful information to judge whether the skin reaction found in a dermal irritation study is test article-induced irritation or a hair cycle-related change.

In conclusion, we analyzed the mechanism responsible for the gross skin changes, patchy thickening, and reddish discoloration of active hair growth areas observed in NZW rabbits, in relation to the histological features of the hair cycle. The results of this study suggest that gross patchy thickening and elevation of the rabbit skin is caused by large hair follicles in the anagen phase that seem to compress the surrounding dermis and that the reddish discoloration of the skin is caused by an increase in the number of blood vessels around the hair follicles in the anagen phase in the absence of melanogenesis. Histopathological examination can distinguish whether the gross features of thickening and reddish discoloration of the rabbit skin are most likely due to hair cycle-related changes or chemical irritation when a precise judgment is needed.
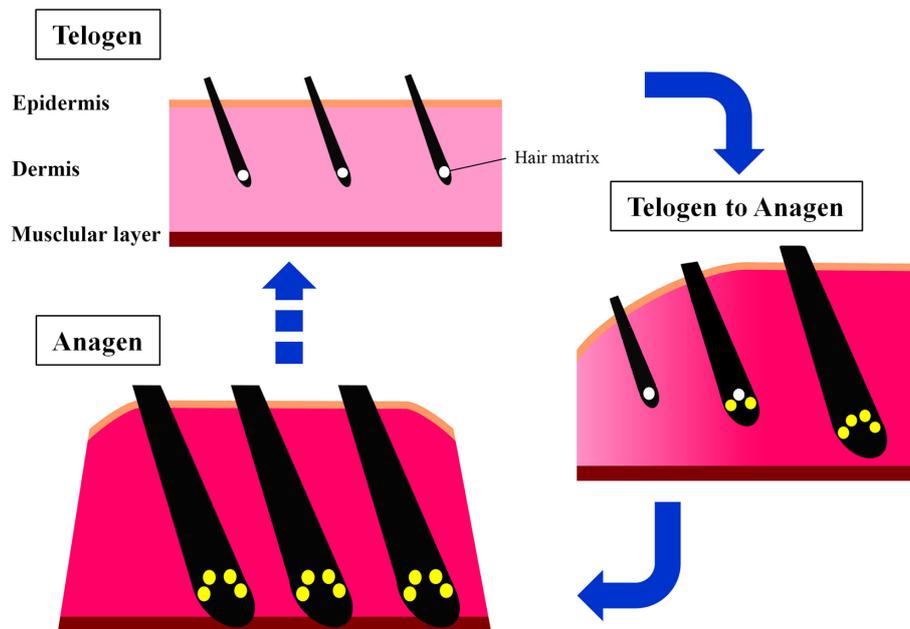
Fig. 9. Schematic drawing of the process leading to thickened skin along with the variation of the hair cycle. Hair follicles in the telogen phase are short and small. When the hair follicles enter the anagen phase, they rapidly increase in size through cell proliferation and reach their greatest length and volume. The gross feature of thickened skin in rabbits might be observed because hair follicles in the anagen phase compressed the surrounding dermis, and this pressure was not absorbed by the thin hypodermis.

Disclosure of Potential Conflicts of Interest: All the authors are employees of Taisho Pharmaceutical Co., Ltd., and there are no known conflicts of interest associated with this publication.

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