Morphological and morphometrical study on the dorsal skin of Wistar and WBN/ILA-*Ht* rats in their developing stage. Evaluation of the proliferation and apoptotic processes

S. Iwamoto, H. Nakayama and K. Doi

Department of Veterinary Pathology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

Summary. The detailed histology of the dorsal skin of Wistar-derived hypotrichotic WBN/ILA-*Ht* rats (HtRs) was examined in their developing stage (at 3 and 7 weeks of age) using Wistar rats (WRs) as controls. As a result, except for the existence of time-lag in the hair follicular cycle and some quantitative differences, there were no essential qualitative differences between the two strains. The follicular epithelial cell number and the amounts of tonofilaments and trichohyalin granules were less in HtRs than in WRs in the same phase of hair follicular cycle. In addition, the diameter of hair shaft was significantly larger in WRs than in HtRs at 7 weeks of age. The number of proliferating cell nuclear antigen (PCNA)-positive epidermal basal cells decreased in HtRs and increased in WRs from 3 to 7 weeks of age, respectively. Apoptosis was sparsely observed in the sebaceous gland epithelial cells, keratinizing portion of hair cuticules and inner root sheath in both strains, but the frequency of apoptosis in the hair follicle was somewhat higher in 7-week-old HtRs. HtRs are considered to be useful experimental animals for dermatotoxicological studies.

Key words: WBN/ILA-Ht rat, dorsal skin, morphology, hypotrichosis, TUNEL, PCNA

Introduction

Hairless or hypotrichotic animals are expected to be suitable experimental animals for dermatology, especially for long-term dermatotoxicological studies, because they do not require hair shaving which probably affects the skin physiology. Among several kinds of hairless experimental animals (Reed and O'Donoghue, 1979; Panepinto and Philips, 1986; Kimura and Doi,

Offprint requests to: Shoko Iwamoto, Department of Veterinary Pathology, Faculty of Agriculture, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

1994), rats seem to be more useful because they have an appropriate body size and there are abundant background data of them used in various toxicity studies including dermatotoxicity.

Many mutant genes which affect the growth of hair follicles have been known among rats (Roberts et al., 1940; Castle et al., 1955; Palm and Ferguson, 1976; Festing et al., 1978; Ohno et al., 1987; Itagaki et al., 1995). However, most of these mutant rats show skin conditions which are widely different from normal ones. For example, hairs are lost during their developing stage, resulting in formation of follicular cysts and/or severe wrinkles (Mann, 1971; Ohno and Yoshida, 1981; Inazu and Sakaguchi, 1984; Hanada et al., 1988; Itagaki et al., 1995; Ishii et al., 1997).

A Wistar-derived inbred strain of hypotrichotic rats named WBN/ILA-*Ht*, which has an autosomal dominant gene (*Ht*: dominant hypotrichosis) responsible for their charasteristics of hypotrichosis, has been developed in Japan (Nishimura and Ishikawa, 1987). WBN/ILA-Ht rats (HtRs) have short downy hairs on the head, dorsum and extremities throughout their life span. Our preliminary studies indicated that, except for hair follicles being less well developed, the skin histology of HtRs is similar to that of haired rats. Therefore, HtRs are considered to be valuable for dermatotoxicological studies (Iwamoto et al., 1997).

The aim of this study is to clarify the details of skin histology of HtRs, compared with Wistar rats (WRs) as controls, during their development.

Materials and methods

Animals

Five each of 3- and 7-week-old male WBN/ILA-*Ht* rats (HtRs) and 5 each of age-matched male Wistar rats (WRs) were purchased from Saitama Experimental Animals Co. Ltd. (Saitama, Japan). They were individually housed using an isolater caging system

(Niki Shoji Co. Ltd., Tokyo, Japan) under standard laboratory conditions (room temperature, 23±2 °C; relative humidity, 55±5%; a 14h/10h light/dark cycle), and were fed basal diet (MF; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. After 3 days of acclimatization, rats were euthanized by exanguination under ether anesthesia and offered to histological examinations.

Histology

The central dorsal skins of each rat were taken and fixed in a commercially available fixative, 18.5% formaldehyde in methanol (Yufix, Sakura Seiki Co., Tokyo, Japan). Paraffin sections of 4 μm were stained with hematoxilin and eosin (HE) and toluidine blue (TB) for histological examinations.

Ultrastructure

For electron microscopic examination, small pieces of the skin were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded in Epok 812 (Oken Shoji Co. Ltd., Tokyo, Japan). Ultrathin sections were double-stained with uranyl acetate and lead citrate and observed under a JEOL-1200EX electron microscope (JEOL Co. Ltd., Tokyo, Japan).

In situ detection of fragmented DNA

DNA fragmentation was examined on the paraffin sections of the skin by the modified TUNEL method first proposed by Gavrieli et al. (1992), using a commercial apoptosis detection kit (ApopTag In situ Apoptosis detection kit; Oncor, Gaitherburg, MD, USA). In brief, the procedure was as follows: multiple fragmented DNA 3'-OH ends on the sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methylgreen.

Immunohistochemical staining for PCNA

To evaluate the cellular proliferative activity in the basal layer of epidermis, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was carried out on the above-mentioned paraffin sections by the avidine-biotin-peroxydase complex method using ABC kit (Vector laboratories, USA). Mouse anti-rat PCNA antibody (clone PC10; Novocastra, Newcastle, UK) was used as the primary antibody. The sections were visualized by peroxidase-diaminobenzidine (DAB) reaction and then counterstained with methyl-green.

Morphometry

The thickness of whole epidermis and dermis (from the epidermal basement membrane to the border between dermis and subcutaneous adipose tissue), the diameter of hair shaft at the level of hair bulge and the numbers of hair follicles and sebaceous glands per unit area were measured on the HE-stained skin sections using computer image analyzing system (image scanner, Power Macintosh 8100/100AV and NIH Image ver.1.56). The number of mastocytes per unit area in the dermis was also measured on the TB-stained skin sections and the number of PCNA-positive cells in the basal layers of the epidermis on the immunostained ones, respectively. Then, PCNA labeling index (LI) was calculated (PCNA-positive cells/total cells in the basal layer x 100).

Statistical analysis

Five areas each of two skin samples from each rat were measured under light microscope (x400) and the mean was calculated for each rat. Then, the mean± standard error (SE) of 5 rats was calculated for each group. Student's t-test or Welch's test were used for statistical analysis.

Results

Histological and morphometrical findings

In both HtRs and WRs, the epidermis consisted of 4 layers, i.e. corneal, granular, spinous and basal. Although not significant, the epidermal thickness tended to increase from 3 to 7 weeks of age mainly due to an increase in number of spinous cells. The epidermis was significantly thicker in HtRs than in WRs at 3 weeks of age, while there was no significant difference between
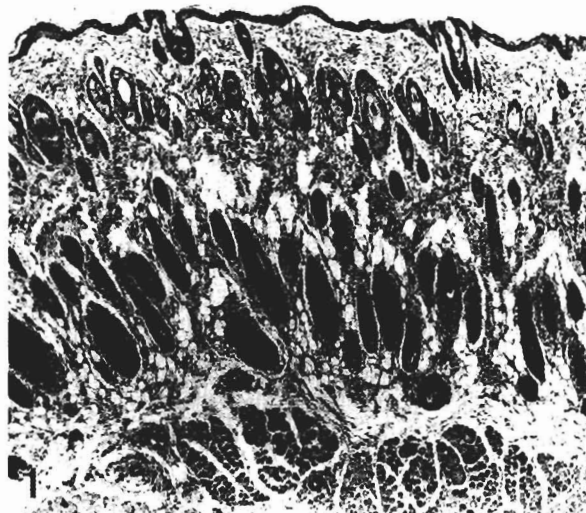

Fig. 1. The skin of HtR at 3 weeks. x 16

both strains at 7 weeks of age. On the other hand, the thickness of the dermis significantly increased from 3 to 7 weeks of age mainly due to an increase in amount of collagen fibers. The dermis of WRs was thicker than that of HtRs at 7 weeks of age (Figs. 1-4).

The number of hair follicles per unit area significantly decreased from 3 to 7 weeks of age in both strains (Fig. 5), and the number of follicles was rather larger in HtRs than in WRs at both ages. The hair follicular cycle at 3 weeks of age was in anagen phase in HtRs, and in telogen phase in WRs, respectively (Figs. 6, 7). At 7 weeks of age, the hair follicular cycle was in catagen phase in HtRs and in anagen phase in WRs, respectively (Figs. 8a,b, 9). To compare the hair structures in the same hair follicular cycle between both

strains, the number of follicullar cells was generally less in HtRs than in WRs, and the whole follicular size was also smaller in HtRs than in WRs (Figs. 6, 9). The diameter of hair shafts was rather larger in HtRs than in WRs at 3 weeks of age, but it showed a prominent increase in WRs while it did not change in HtRs at 7 weeks of age. Therefore, the diameter was significantly larger than in WRs at 7 weeks of age (Fig. 10).

There was no significant difference in the number of sebaceous glands per unit area between both strains and between both ages in HtRs, respectively. The number in
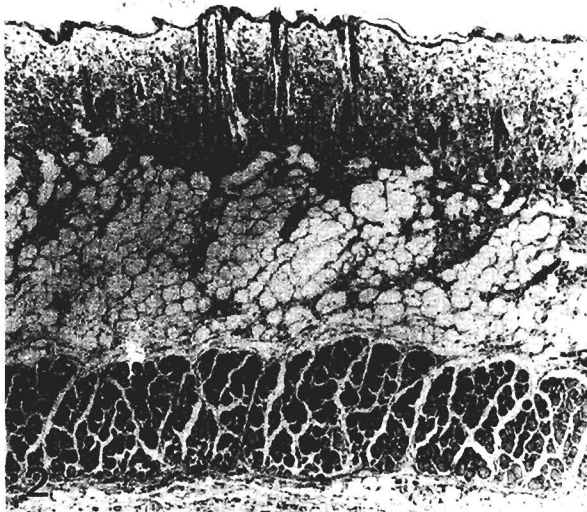

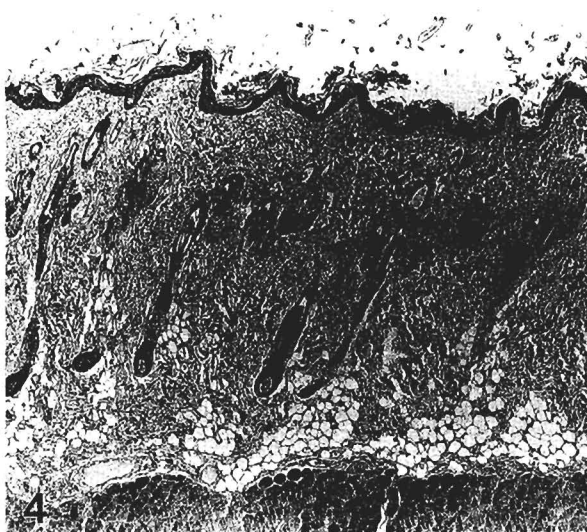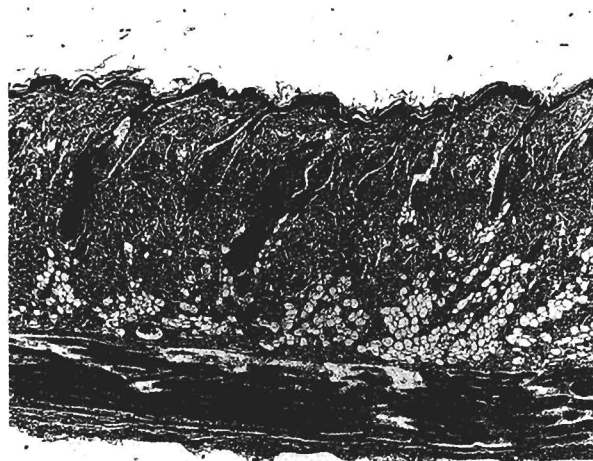**Fig. 2.** The skin of WR at 3 weeks. x 16

Fig. 4. The skin of WR at 7 weeks. x 10

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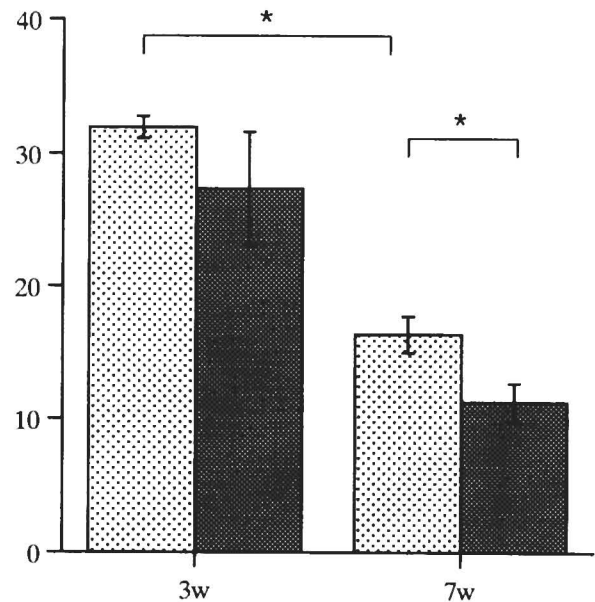**Fig. 3.** The skin of HtR at 7 weeks. x 10

Fig. 5. The number of hair follicules in HtR and WR dermis. Left bars: hairless; right bars: Wistar. *: significantly different from each other (p<0.05)

WRs, however, significantly decreased from 3 to 7 weeks of age (Fig. 11). The size of sebaceous glands was very variable and there was no clear difference between either strains.

On the other hand, the number of mastocytes per unit area in the dermis was significantly decreased from 3 to 7 weeks of age in both strains. At 3 weeks of age, the number was significantly larger in WRs than in HtRs, but it was similar between the two strains at 7 weeks of age (Fig. 12).

Immunohistochemical findings

The PCNA-LI in the basal layer at 3 weeks of age was significantly larger in HtRs than in WRs. The PCNA-LI in HtRs decreased significantly while that in

WRs tended to increase from 3 to 7 weeks of age, respectively. Therefore, there was no significant difference between either strain at 7 weeks of age (Fig. 13).

As to the developement of apoptosis in the skin, apoptosis was sparsely observed in the well-differentiated sebaceous gland epithelial cells, keratinized portion of hair cuticules and inner root sheath in the upper bulb of hair follicle in both strains (Fig. 14a-c). The frequency of apoptosis in the hair follicles was somewhat higher in catagen/telogen phase of 7-week-old HtRs.

Electron microscopic findings

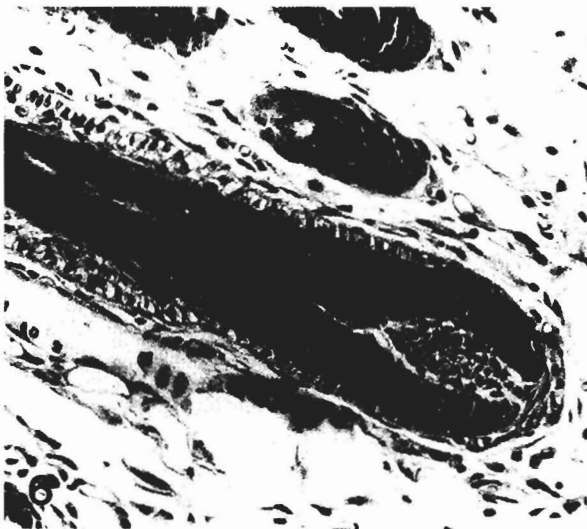There was no essential difference in the ultra-

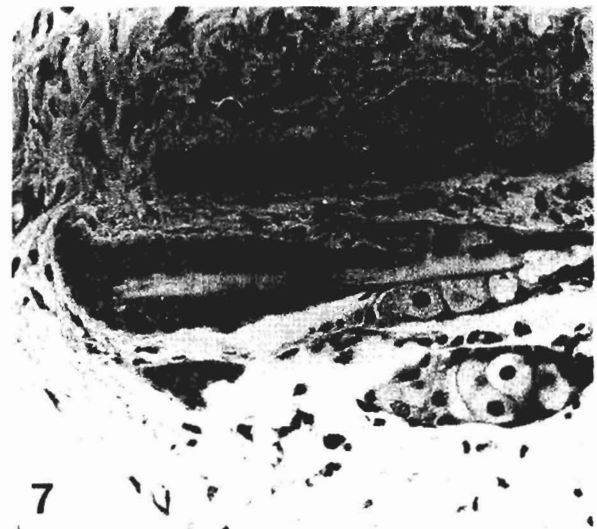**Fig. 6.** The hair follicles of HtR at 3 weeks (anagen phase). x 100

Fig. 7. The hair follicles of WR at 3 weeks (telogen phase). x 100
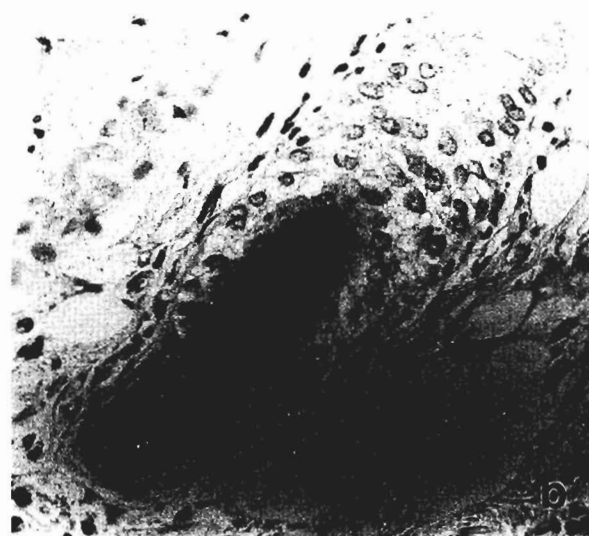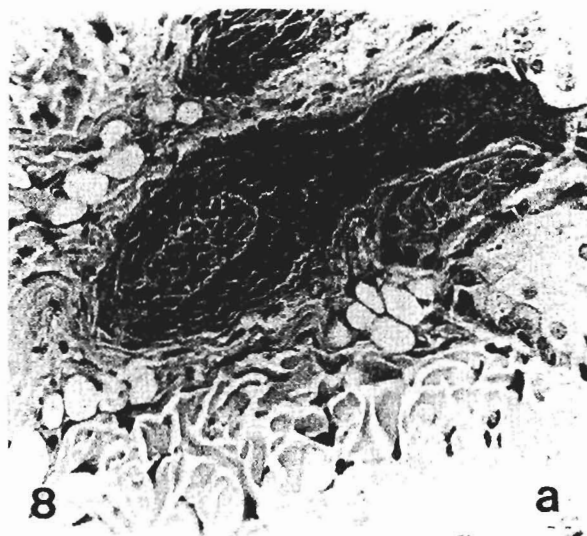

Fig. 8. The hair follicles of HtR at 7 weeks (catagen phase). x 100

structure of epidermal cells between either strain. Langerhans' cells containing racket-shaped Birbeck granules in their cytoplasm were sparsely observed in the epidermal basal layer and in the outer root sheath beneath the basal membrane (Fig. 15), but melanocytes were not observed at all. In addition, there was also no essential difference in component cells and in cellular differentiation process in hair follicles between HtRs and WRs. However, between HtRs and WRs with the same hair follicular phase, the cell number was less in each layer of hair follicles in HtRs than in WRs. In addition, the amounts of tonofilaments and trichohyalin granules

observed during the keratinization process were also less in HtRs than in WRs (Figs. 16, 17).

Discussion

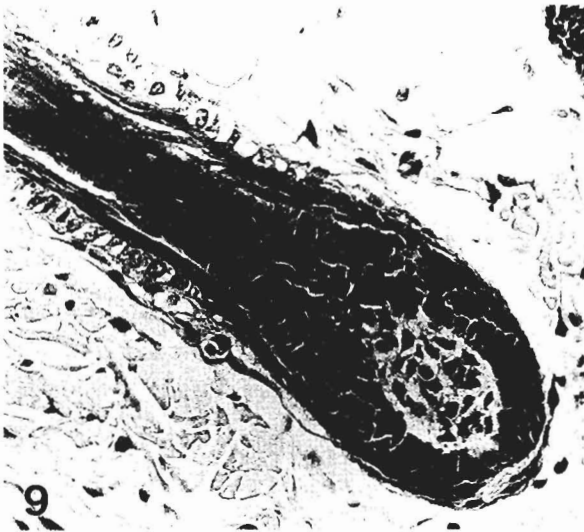Detailed skin morphology was investigated in HtRs during their developing stage and compared with WRs

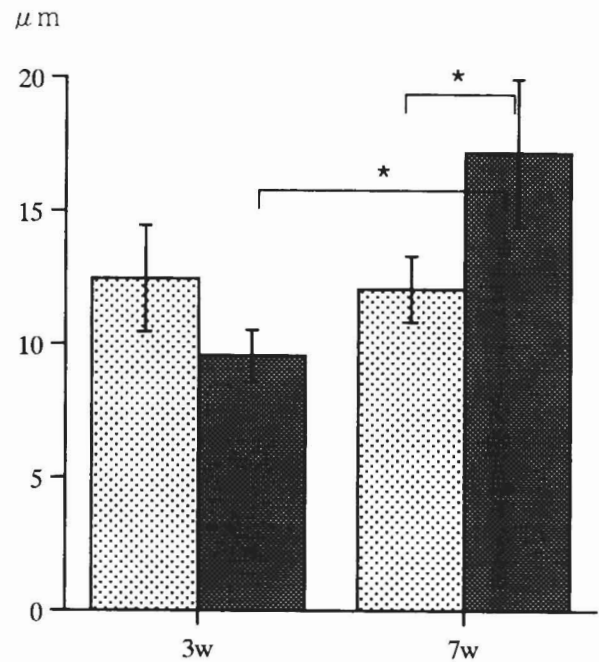**Fig. 9.** The hair follicles of WR at 7 weeks (anagen phase). x 100

Fig. 10. The diameter of hair in HtR and WR dermis. Left bars: hairless; right bars: Wistar. *: significantly different from each other (p<0.05)

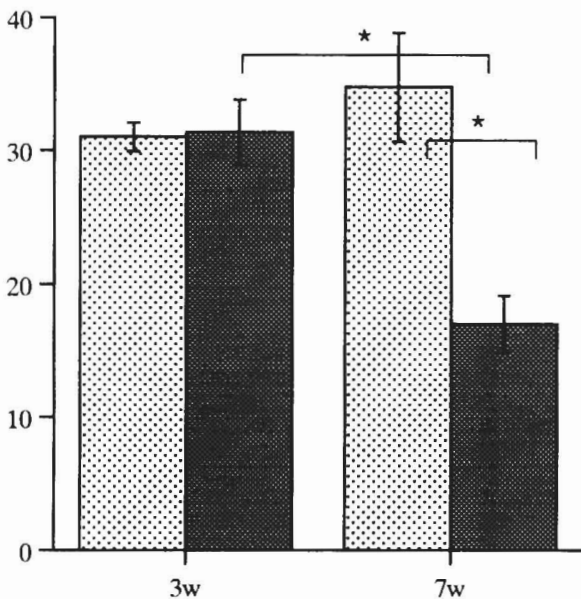**Fig. 11.** The number of sebaceous glands in HtR and WR skin. Left bars: hairless; right bars: wistar. *: significantly different from each other (p<0.05)
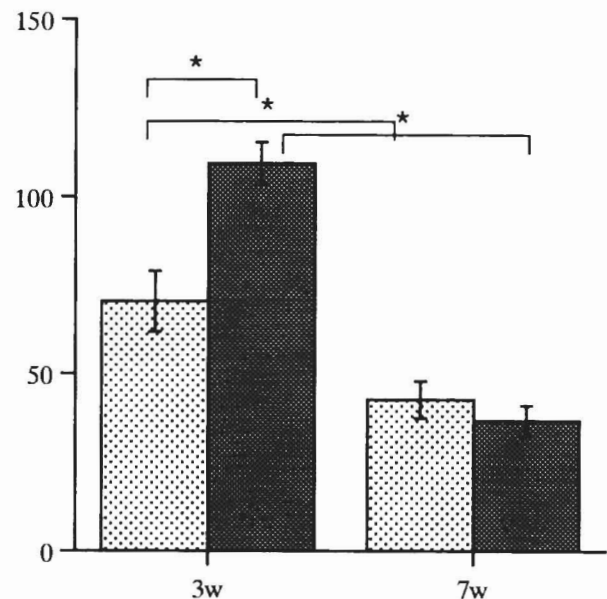

Fig. 12. The number of mastocytes in HtR and WR dermis. Left bars: hairless; right bars: Wistar. *: significantly different from each other (p<0.05).

as control. As to the epidermis, except for the epidermal thickness, there was no essential morphological difference, irrespective of rat strain and age. In both strains, the thickness of epidermis tended to increase from 3 to 7 weeks of age, though not significant. This increase in epidermal thickness was due to an increase in number of spinous cells. In addition, the epidermis of HtRs was significantly thicker than that of WRs at 3 weeks of age. This seems to have a relation to the fact that PCNA-LI in the basal layer was significantly larger in HtRs than in WRs at 3 weeks of age.

Except for the number of sebaceous glands in HtRs, the numbers of hair follicles, sebaceous glands and mastocytes per unit area decreased significantly from 3 to 7 weeks of age in both strains. In addition, the numbers per unit area at 7 weeks of age were significantly or somewhat larger in HtRs than in WRs. This seems to be related to the fact that the rate of increase in the dermal thickness due to increase in collagen fibers from 3 to 7 weeks of age was prominently larger in WRs than in HtRs. In other words, the density of hair follicles, sebaceous glands and mastocytes became apparently less in WRs than in HtRs at 7 weeks of age, and this may reflect the difference in the numbers per unit area.

It is said that the hair follicular cycle of rats and mice shows the same phase within definite skin area and that there is little individual difference in hair follicular cycle among animals of the same age (Butcher, 1934; Eaton, 1976; Mori and Ueno, 1990; Tezuka, 1990). In addition, it is well known that the sequence of hair follicular cycle after birth is almost similar among mice and rats (Mori et al., 1994). Namely, in WRs, the first anagen phase begins within the first 14 days of life, and catagen/telogen phase starts at about 21 days of age. Thereafter, second anagen phase starts at around 4 weeks of age, and the second telogen phase begins at about 10 weeks of age (Ishii et al., 1997). In the present study, the

hair follicular cycle at 3 and 7 weeks of age in WRs was consistent with these reports. On the other hand, the hair follicular cycle of HtRs differed from that of WRs. The cause of this abberation is obscure at present.

There were no qualitative differences in the morphology of hair follicles in the same hair follicular cycle in either strain. However, there were some quantitative differences between both strains. For example, in the hair follicles in anagen phase, the cell numbers of each follicular layer and the amounts of
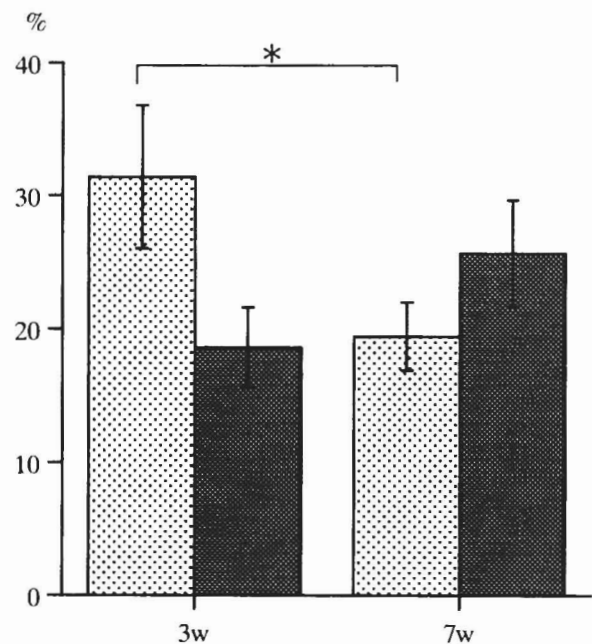

Fig. 13. PCNA-Labelling Index in basal cell layer in HtR and WR epidermis. Left bars: hairless; right bars: Wistar. *: significantly different from each other (p<0.05)
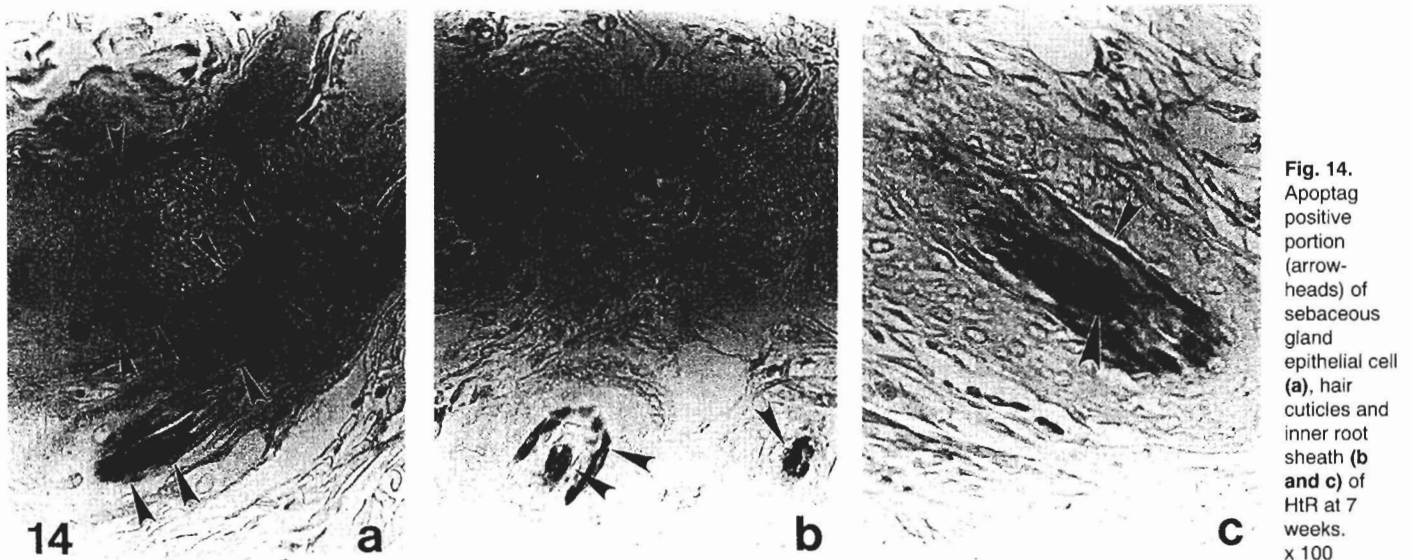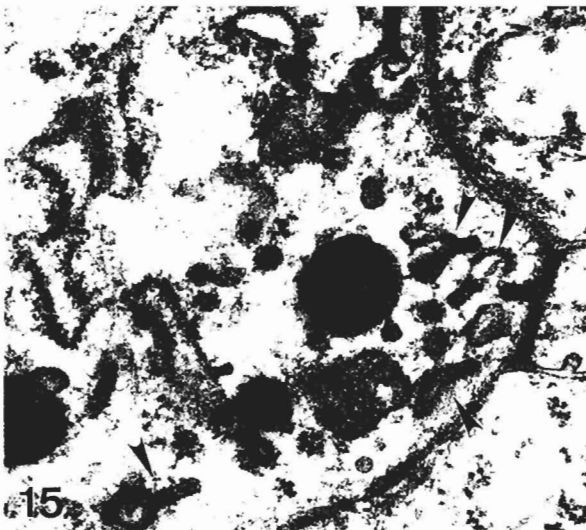

Fig. 14. Apoptag positive portion (arrowheads) of sebaceous gland epithelial cell **(a)**, hair cuticles and inner root sheath **(b and c)** of HtR at 7 weeks. x 100

tonofilaments and trichohyalin granules were less in HtRs than in WRs, and this resulted in smaller size of hair follicles in HtRs. On the other hand, the diameter of the hair shaft at the level of the hair bulge was somewhat larger in HtRs than in WRs at 3 weeks of age while it was significantly larger in WRs than in HtRs at 7 weeks of age. The hair diameter in WRs prominently increased from 3 to 7 weeks of age while that in HtRs did not change. This, in turn, coincided with gross skin characteristics of HtRs in which short, downy hairs are seen throughout their lifespan.

Apoptosis was sparsely seen in well differentiated sebaceous gland epithelial cells, and in hair cuticules and inner root sheaths at upper bulbs of hair follicles in both strains, and the frequency of apoptosis was somewhat

Fig. 15. Birbeck granules (arrowheads) in the Langerhans cell of HtR at 3 weeks. x 40,000
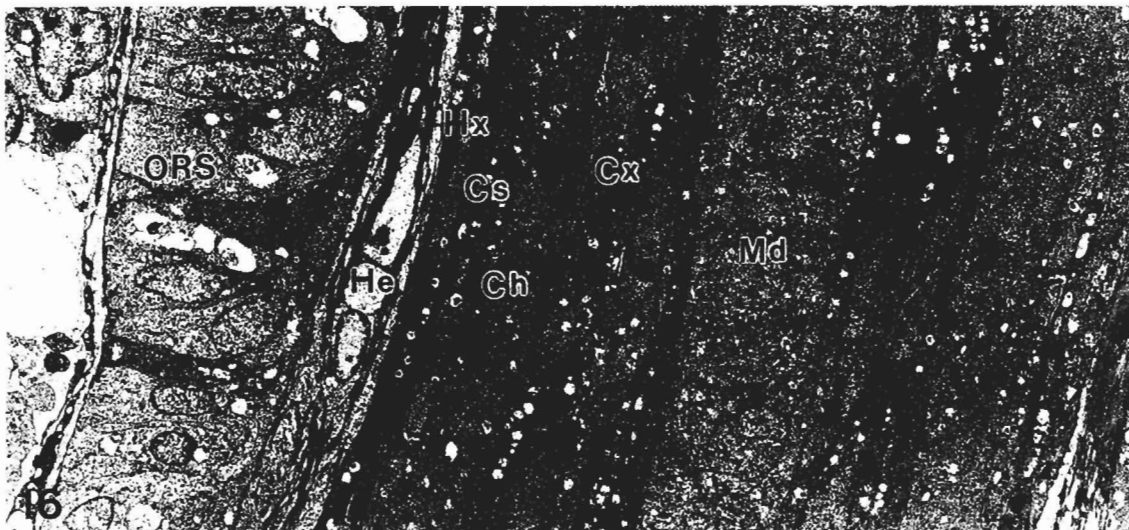

Fig. 16. The haifr follicle of HtR at 3 weeks. ORS: outer root sheath; He: Henle layer; Hx: Huxley layer; Cs: sheath cuticule; Ch: hair cuticule; Cx: hair cortex; Md: medulla. x 2,640
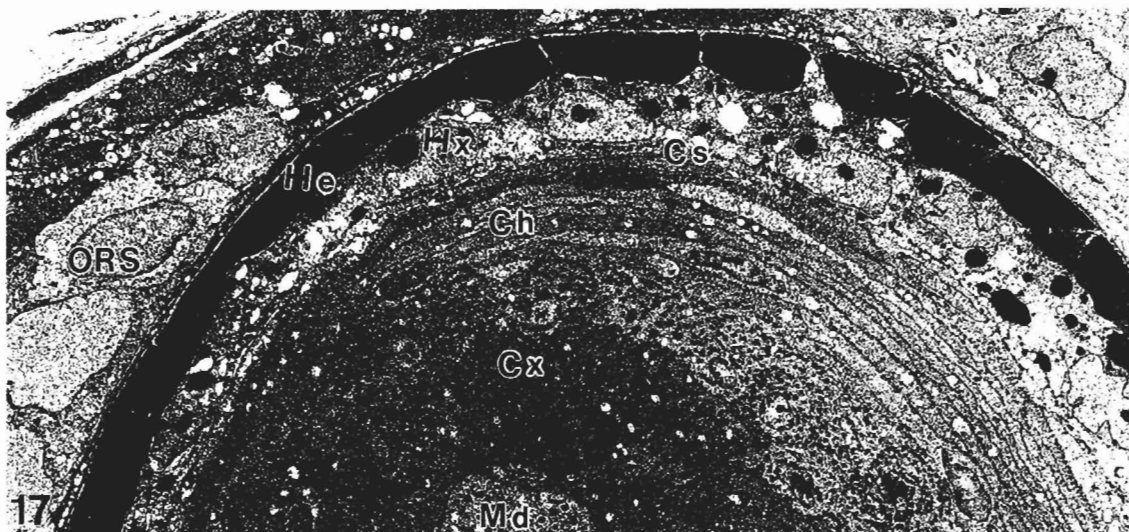

Fig. 17. The hair follicle of WR at 7 weeks. See the footnote of Fig. 16. x 2,640

higher in hair follicles in catagen/telogen phase of HtRs. Apoptosis seen in the sebaceous glands was thought to be correlated with the process of sebaceous gland cell discharge by holocrine (Tamada et al., 1994).

Two kinds of apoptosis have been reported in normal hair follicles. One is observed at the hair cone/hair canal region of developing hair follicles, and this seems to play a role in making the canal through which differentiating hairs go out (Robins and Breathnach, 1969; Polakowska et al., 1994). The other is observed at the lower part of hair follicles, and this seems to be related with retraction of hair follicles in catagen phase (Weedon and Strutton, 1981; Paus et al., 1993). The latter case was also detected in the present study especially in the hair follicles of HtRs at 7 weeks. However, the former case could not be detected in this study. This may be explicable by the fact that apoptosis at the hair cone/hair canal occurs at only a certain short time during the anagen phase.

As stated above, except for the existence of time-lag in the hair follicular cycle and some quantitative differences, there were no essential qualitative differences in the skin morphology between developing HtRs and WRs. In addition, such abnormal findings as formation of follicular cysts and wrinkles seen in other strains showing atrichous or hypotrichous characters from the youth were never found in HtRs (Ishii et al., 1997). On the other hand, it has also been reported that Cryptothrix mouse has normal hair follicle but the top of hair becomes folded and the hair fails to come out of the skin surface (Mori et al., 1994). Such lesion was also not seen in HtRs. Therefore, we consider that HtRs are useful experimental animals for dermatotoxicological studies. Now, detailed studies on the skin morphology of HtRs through their lifespan are in progress.

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