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Expression Pattern of Cyclooxygenase-2 in Normal Rat Epidermis and Pilosebaceous Unit during Hair Cycle

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As an important member of the cyclooxygenase isoenzymes, cyclooxygenase-2 (COX-2) mainly catalyzes the first two steps in prostanoid synthesis. In mammalian animals, although COX-2 was thought to be rarely expressed in most normal tissues and was usually upregulated in a variety of epithelial tumors and inflammatory reactions, recently it was reported that COX-2 could localize in the epidermis as well as the pilosebaceous unit of the normal human and mouse skin. Until now, the function of COX-2 in normal skin has remained unknown. To investigate the possible roles of COX-2 in normal skin by RT-PCR and immunochemistry, we studied the expression pattern of COX-2 in hair cycle of the normal rat skin. The expression of COX-2 mRNA was detected in normal rat skin sample and was related to the hair follicle cycle. When the hair cycle entered catagen and telogen, COX-2 mRNA transcription in skin increased significantly. Furthermore, the location of COX-2 immunoreactivity showed that COX-2 protein is mainly concentrated in the epidermis and pilosebaceous unit. In the stratified epidermis, the strong COX-2 protein expression was detected in the suprabasal layers of epidermis in anagen and declined in catagen and telogen. In hair follicle, COX-2 protein was obviously expressed in the outer root sheath of the anagen hair follicle, and was barely detectable in catagen as well as telogen. In the sebaceous gland, the COX-2 protein expression became more intense in catagen and telogen, with an increase in sebaceous gland size. Our results suggested that COX-2 was not specific to some abnormal tissues and was indeed involved in the normal physiology of rat skin, such as the differentiation of epidermis, the morphogenesis of the hair follicle, the transformation of hair cycle stages, and the lipid production of the sebaceous gland.

Key words: cyclooxygenase-2, pilosebaceous unit, hair cycle, epidermis, sebaceous glands

I. Introduction

As the first line of defense of the mammalian animal body against the environment, the skin epidermis and its appendages provide a protective barrier against ultraviolet light and mechanical, chemical and thermal insults. The epidermis consists of multilayers of squamous epithelium, which are mainly formed by keratinocytes. When these keratinocytes move upwards from basal layer, they differentiate and accumulate abundant keratins in the cytoplasm. Finally, when the keratinocytes of epidermis are filled with keratin filaments, the mature epidermis is formed and becomes the physical barrier of skin [20].

Pilosebaceous units are the appendage of the epidermis. During embryogenesis, a bud from the basal cell layer of epidermis (hair germ) grows downward into the dermis and associates with the underlying condensational mesenchymal cells, the dermal papilla. The crosstalk between the epithelial and mesenchymal cells offers essential signals for fur-

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ther development of hair germ to form pilosebaceous units, consisting of entire hair follicle structures and attached sebaceous glands [3]. The hair follicle is a tubular structure invaginated from the epidermis and provides the growing point of the hair. The lower portion of the hair follicle, which is called a hair bulb, is formed by mitotically dividing cells and shapes the hair shaft and inner root sheath (IRS) through keratinisation. Surrounding the surface of the hair bulb is a stratified epithelium, termed the outer root sheath (ORS), which is located just below the IRS and continuous with the epidermis [8]. Sebaceous glands are usually found to locate in areas with hair and extend from the ORS and consist of sebocytes, which contain large lipids in the cytoplasm and offer lubrication and protection for the skin by releasing the lipid content into the hair follicle.

The structure and function of the epidermis and pilosebaceous units are under the influence of the hair cycle. The hair cycle means the hair follicle grows in repeated cycles and consists of three phases: anagen, catagen and telogen. In the anagen phase, the hair follicle grows and reaches its maximal length, while during the catagen phase, its lower part undergoes apoptosis and the length of hair follicle shrinks. Finally, when the hair follicle enters the telogen phase, the hair follicle is completely at rest and only the upper part of the hair follicle is maintained [2].

The homeostasis of the epidermis and the pilosebaceous units in the hair cycle is regulated by many factors via intricate signalling pathways, such as β-catenin, FGF families, noggin, notch, hedgehog and c-myc [10, 15]. Accumulated evidence has shown that prostaglandin could also play key roles in the growth and differentiation control of the epidermis and the pilosebaceous units [1, 4–6, 14]. In prostaglandin biosynthesis, the cyclooxygenases catalyze the key reactions [7]. As an important member of the cyclooxygenase isoenzymes, cyclooxygenase-2 (COX-2) was usually found to be expressed in a variety of epithelial tumors and inflammatory reactions. COX-2 was also localized mainly in suprabasal keratinocytes of normal human skin [18]. Moreover, it was recently reported that in normal mouse skin, COX-2 was expressed in the normal process of morphogenesis of the epidermis and the pilosebaceous units [16, 18]. But until now, the function of COX-2 in normal epidermis as well as the pilosebaceous unit has remained unknown. In this study, we evaluated the expression pattern of COX-2 in the epidermis and the pilosebaceous unit during the hair cycle of normal rat skin, in order to explore the possible roles of COX-2 in the normal structure and function of the epidemis and the pilosebaceous unit.

II. Materials and Methods

Animals

Female SD rats with normal hair follicle cycling behavior were provided by the National Resource Center for Mutant Mice, Nanjing, China. All animals were fed standardized chow in the central animal unit of the Third Military Medical University (Chongqing, China) and demonstrated the normal cycling phenotype. Neonatal (days 7, 18, 26 postpartum) and six-week old adult SD rats were used to obtain a series of skin samples. According to the standard classification of hair follicle cycling [17], days 7, 18 and 26 postpartum respectively correspond to the three typical stages of hair cycle, i.e., anagen, catagen and telogen. For analyzing the characteristics of the pilosebaceous unit, three SD rats were studied at each stage.

RNA isolation and RT-PCR

Total RNA isolation from the dorsal skins was performed as described previously [22]. cDNA synthesis and conventional RT-PCR were performed using the Takara AMV-RT PCR kit (Takara Biomedicals, Otsu, Japan), following the manufacturer's instructions. Primer sequences specific for the rat genes of β-actin (sense: 5'CTG AAC CCT AAG GCC AAC C3', antisense: 5'AAT GTC ACC CAG GAT TTC C3') and cyclooxygenase-2 (sense: 5'ACG CTT CTC GAA ACC TTA C3', antisense: 5'TTA CAG CTC AGT TGA ACG CCT3') were generated from the respective mRNA sequences obtained from the European Molecular Biology Laboratory (EMBL) gene bank. After initial denaturation at 94°C for 2 min, amplification consisting of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 1 min was performed for 25 cycles. The amplified products were identified using electrophoresis on a 2% agarose gel and staining with ethidium bromide.

Histology & immunohistochemistry

Dorsal skins were harvested and embedded in O.C.T compound at –25°C immediately, all samples were sectioned parallel to the vertebral line for the longitudinal hair follicle sections. For histology, sections were fixed with cold acetone and stained with hematoxylin/eosin. For immunohistochemistry, air-dried 8 μm cryosections were collected on poly-L-lysine-coated slides and also fixed in cold acetone for 5 min, then incubated in hydrogen peroxide to block the endogenous peroxidase activity. After they were labelled with cyclooxygenase-2 primary rabbit anti-rat antibodies (Neomarkers, Fremont, CA, USA) followed by sheep anti-rabbit secondary antibodies (Neomarkers, USA), the sections were developed by the avidin-biotin-complex method, using diaminobenzidine as a chromogenic substrate, and counterstained with hematoxylin. As controls, the serum of rabbit (Neomarkers, USA) were used as primary antibody at the same concentration.

Lipid staining

Dorsal skin cryosections (10 μm) of six-week old SD rats were fixed in 4% paraformaldehyde and washed with distilled water. Sections were incubated in 60% isopropanol for 5 min and then stained with 0.5% oil red solution for 5 min. The stained sections were carefully washed with 60% isopropanol and counterstained with H&E.

Statistical analysis

All data were analyzed using SPSS (Windows version 8.0) software package. Results were expressed as mean±SEM and were representative of at least three separate experiments. $P<0.05$ was used to determine the statistical significance of the data.

III. Results

Expression of COX-2 mRNA in epidermis and pilosebaceous unit

By using RT-PCR, the expression level of COX-2 mRNA in normal epidermis and pilosebaceous unit of normal rat skin was determined. COX-2 mRNA was deteced in rat dorsal skin during the whole phases of hair follicle cycling. Densitometric analyses further showed that compared with anagen, when hair follicle cycle entered catagen as well as telogen, the level of COX-2 mRNA tended to increase remarkably, with the highest expression level appearing at catagen (Fig. 1).

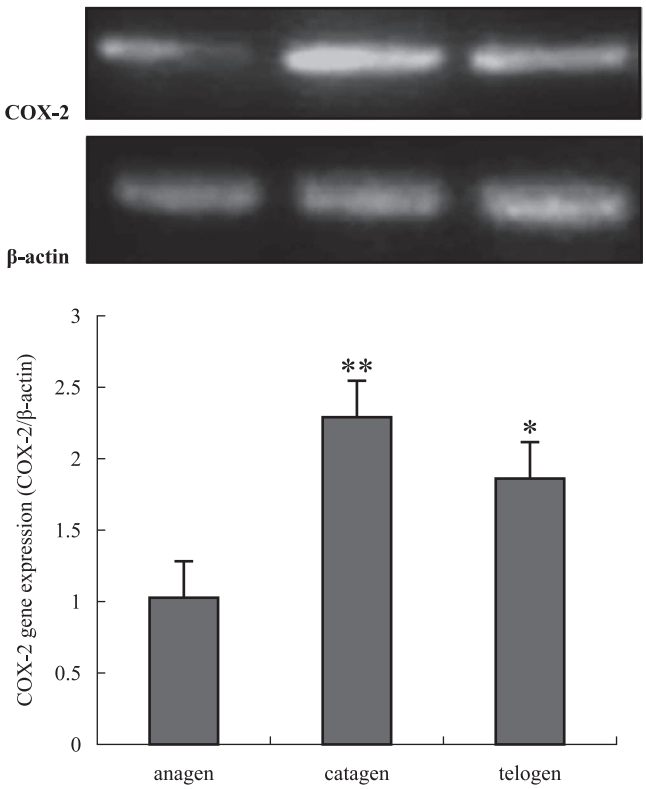

Fig. 1. The mRNA expression of COX-2 in epidermis and pilosebaceous unit of normal rat skin during the phases of hair cycling. Total RNA of rat skin was extracted and mRNA level of COX-2 as well as β-actin were determined by RT-PCR. Relative intensity of COX-2 mRNA was shown by normalization with β-actin mRNA. COX-2 mRNA transcription in pilosebaceous unit increased intensely in catagen and telogen. Upper panel: image of the gel; lower panel: densitometric analyses of COX-2 as ratio to β-actin. Data (mean±S.E.M.) are from four separate experiments. * $P<0.05$, ** $P<0.01$, compared with anagen.

Expression of cyclooxygenase-2 protein in epidermis and pilosebaceous unit

A more detailed analysis of COX-2 expression was carried out using immunohistochemistry. The results showed that in normal rat skin, COX-2 protein was especially distributed in the epidermis and the members of pilosebaceous unit, including the hair follicle and the sebaceous glands. The level of COX-2 protein continually changed along with the progress of the hair cycle.

In the epidermis, compared with the immunohistochemical control (Fig. 2D), the COX-2 protein expression was strongly detected in the suprabasal layers (especially in the lower part, such as the spinous stratum) of the epidermis in anagen (Fig. 2A) and then gradually declined in catagen and telogen (Fig. 2B, C). Meanwhile, the thickness of the epidermis became gradually thinner (Fig. 2A–C).

In the hair follicle of the pilosebaceous unit, even though the shape of the hair follicle transformed sharply from anagen to telogen during the hair cycle (Fig. 3B–D), compared with the immunohistochemical control (Fig. 2A), the COX-2 immunoreactivity was found primarily in the ORS cell of the anagen hair follicle, and was barely detectable in catagen as well as telogen (Fig. 3).

In the sebaceous glands of the pilosebaceous unit, oil red and HE staining showed that these glands were located in the isthmus of hair follicle and composed of squamous epithelium (Fig. 4A), which have lipid-producing ability (Fig. 4B). When the hair cycle progressed from anagen to telogen, the size of the sebaceous glands obviously increased and meanwhile the COX-2 protein expression also increased intensely and reached the maximum in catagen (Fig. 4C–E), compared with the immunohistochemical control (Fig. 4F).

IV. Discussion

In normal human skin, COX-2 immunostaining was observed not only in the epidermis of skin biopsy sections, but also in the cultured epidermal keratinocytes [13]. In normal mouse skin, COX-2 immunostaining appeared in the epidermis and pilosebaceous unit, such as the hair follicles and the sebaceous glands [16]. In this study, we have firstly showed that COX-2 protein could also be constitutively expressed in the normal skin of rat and are specially localized in the epidermis, hair follicles and sebaceous glands, which confirmed the former findings mentioned above and was not previously found in rat skin.

The localization of COX-2 protein have been detected in the more differentiated, suprabasilar keratinocytes of normal human epidermis [13]. As evidence for the finding in human, in the present study, the COX-2 protein expression was strongly detected in the suprabasal layers of the stratified epidermis during the whole hair cycle, which may indicate the the function of COX-2 in epidermal differentiation. The crucial role of COX-2 in maintaining the normal process of epidermal differentiation has been reported. In K5-COX-2 transgeneic mouse, overexpression of COX-2 was accompanied by the abnormal differentiation of epidermis

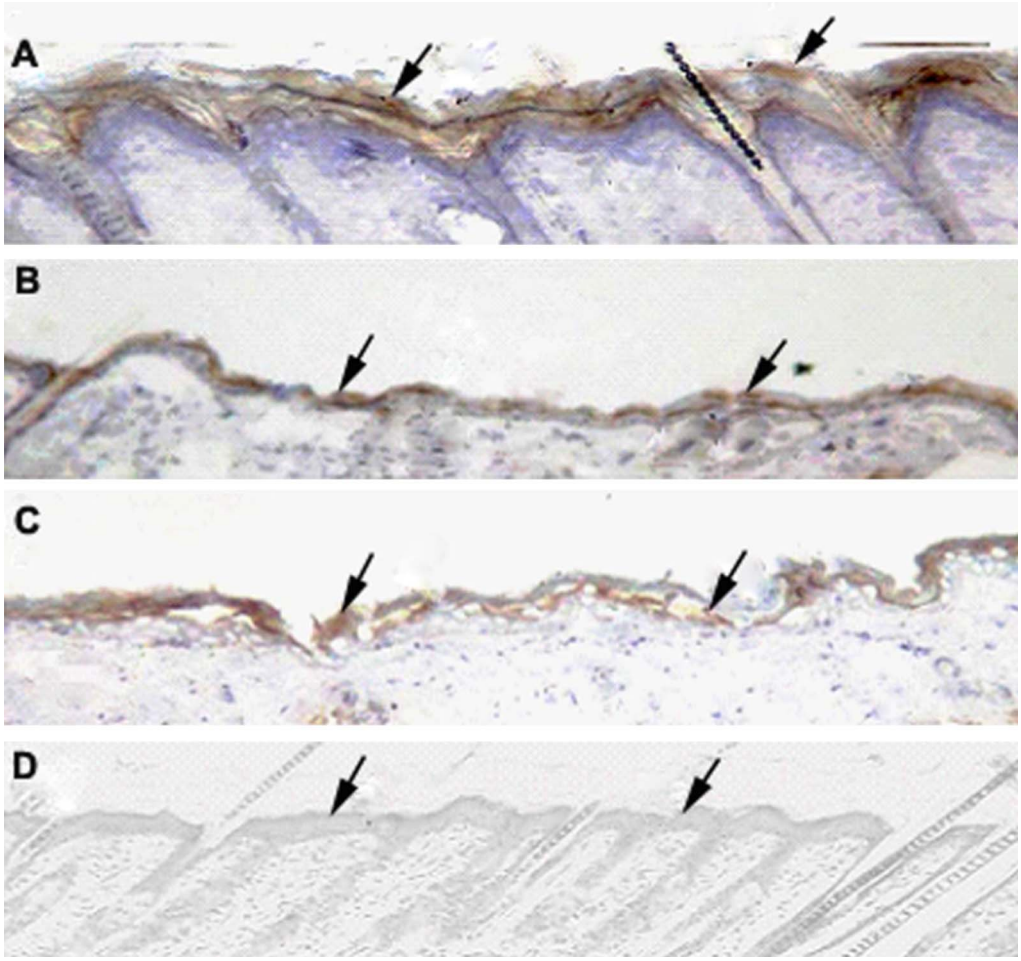

Fig. 2. Immunocytochemistry results of COX-2 in the suprabasal layers of epidermis (arrows) of the pilosebaceous unit during the phases of hair cycling. The stronger COX-2 expression was detected in the suprabasal layers of epidermis of anagen (**A**) (×400) in a manner of relative stabilization in the suprabasal layers of epidermis (arrow) but negative in the basal layer. When the skin became gradually thinner, the COX-2 protein expression gradually declined in catagen (**B**) (×400) and telogen (**C**) (×400), compared with the immuno-histochemical control (**D**).

with decelerated desquamation of cornified cells, and the reduced as well as disturbed expression of many markers of epidermal differentiation were observed [16, 18]. In addition, COX-2 deficient mice displayed the premature onset of terminal differentiation of epidermal keratinocytes. The markers of epidermis early differentiation, K10 and K1, expressed in advance. COX-2 were thus thought to regulate the epidermis differentiation at early stages by controlling the expression of K1 and K10, which coordinated the differentiation of basal cells into spinous cells [21]. In our study, because COX-2 was more concentrated in the lower part of the suprabasal layers of the stratified epidermis, this raises the possibility that the role of COX-2 in epidermis differentiation mentioned above may also be found in the rat normal epidermis herein. Of course, this suggestion needs to be investigated by more detailed works.

It was reported that during the hair cycle of normal mouse skin, the immunostaining of COX-2 firstly appeared in the basal ORS of anagen hair follicles and then gradually became weak in catagen as well as telogen phase, which changed in a hair cycle-dependent manner [16]. This result was in line with our findings in rat skin and proposed the role of COX-2 in hair follicle biology, such as promoting the hair follicle entry from anagen to catagen, which was documented in COX-2 transgenic mice [16, 18]. Recent studies showed prostaglandin action involved in the regulation of hair follicle physiology and cycling, but the mechanism remains unclear [5, 6]. As a key enzyme of the production of prostaglandin [19], COX-2 is thought to be responsible for the action of prostaglandin in the ORS and could initiate the cross talk between the ORS and the dermal papilla cells to regulate the transition from anagen to catagen [12, 16]. In the present study, COX-2 was also found to be mainly expressed in the ORS of anagen hair follicle, thus we invite further studies to ascertain whether the same mechanism could be applied to explain the meaning and the possible role of COX-2 in rat hair follicles shown here.

It was reported that during the first hair cycle of the normal rat skin, the sebaceous glands continually developed in the anagen and attained its fully development at catagen

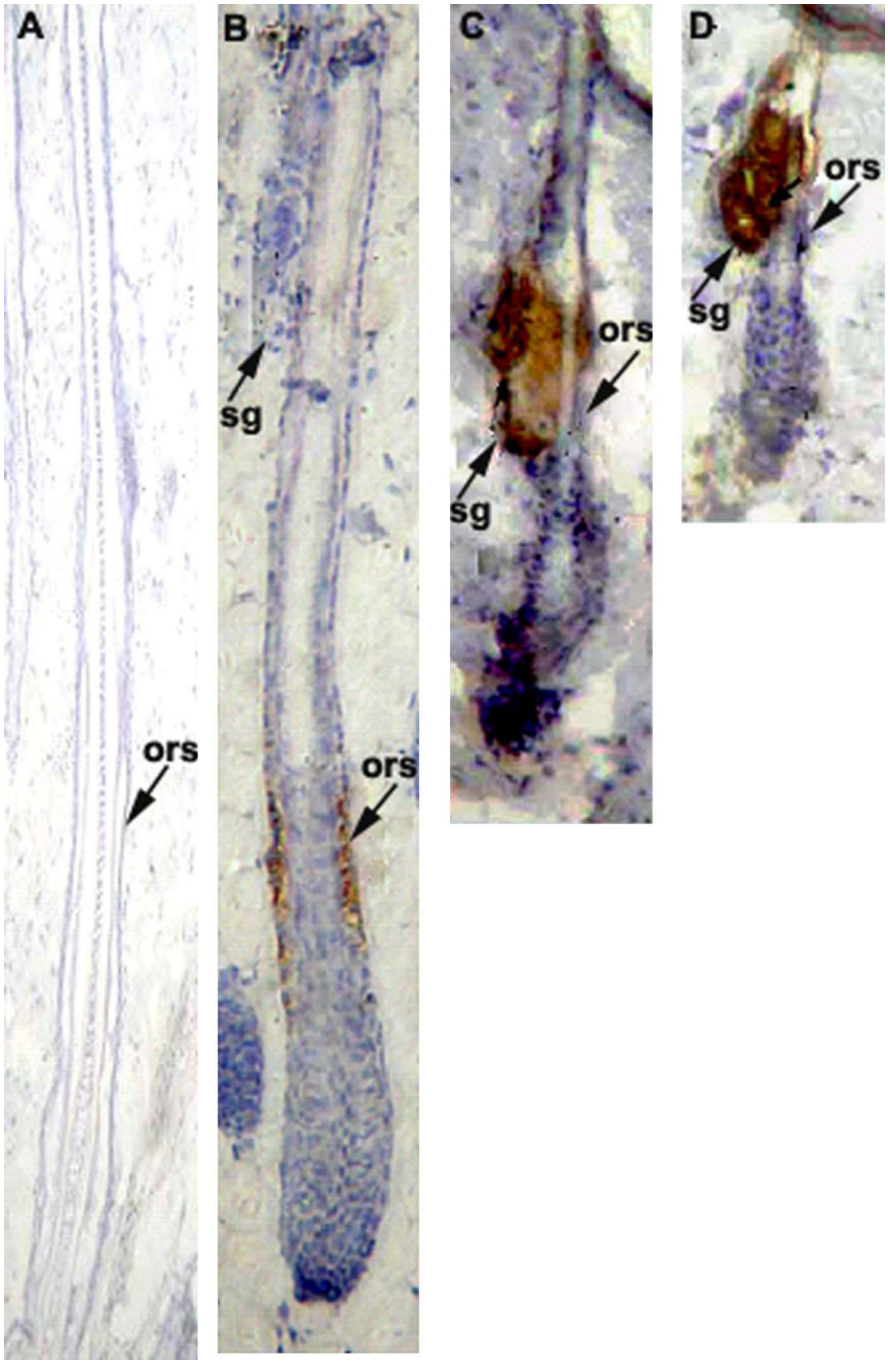

Fig. 3. Immunocytochemistry results of COX-2 in hair follicle during the phases of hair cycling. COX-2 immunoreactivity was obviously found in the outer root sheath (*arrow ors*) of the anagen hair follicle (**A**) (×400), and was barely detectable in catagen (**B**) (×400) and telogen (**C**) (×400) hair follicle as well as the immuno-histochemical control (**D**). The *arrows sg* indicate the sebaceous glands.

[9]. This development pattern of sebaceous glands of rat skin was repeated in our study, with the sizes of the sebaceous glands obviously increasing in the transition from anagen to catagen and holding steady in catagen and telogen. Our study also revealed that accompanying the development of sebaceous glands, the expression of COX-2 protein increased intensely and the maximum of expression appeared in the catagen, which indicated the possible role of COX-2 in the sebaceous glands development. COX-2 played an important role in the production of prostaglandin [19], which involved in sebocyte lipogenesis and augment the formation of lipid droplets [11, 23]. We thus propose that, in rat normal skin, the continuous activation of COX-2 protein in the shift from anagen to catagen may be beneficial for the development of the sebaceous glands, through promoting lipid production as well as sebocytes differentiation.

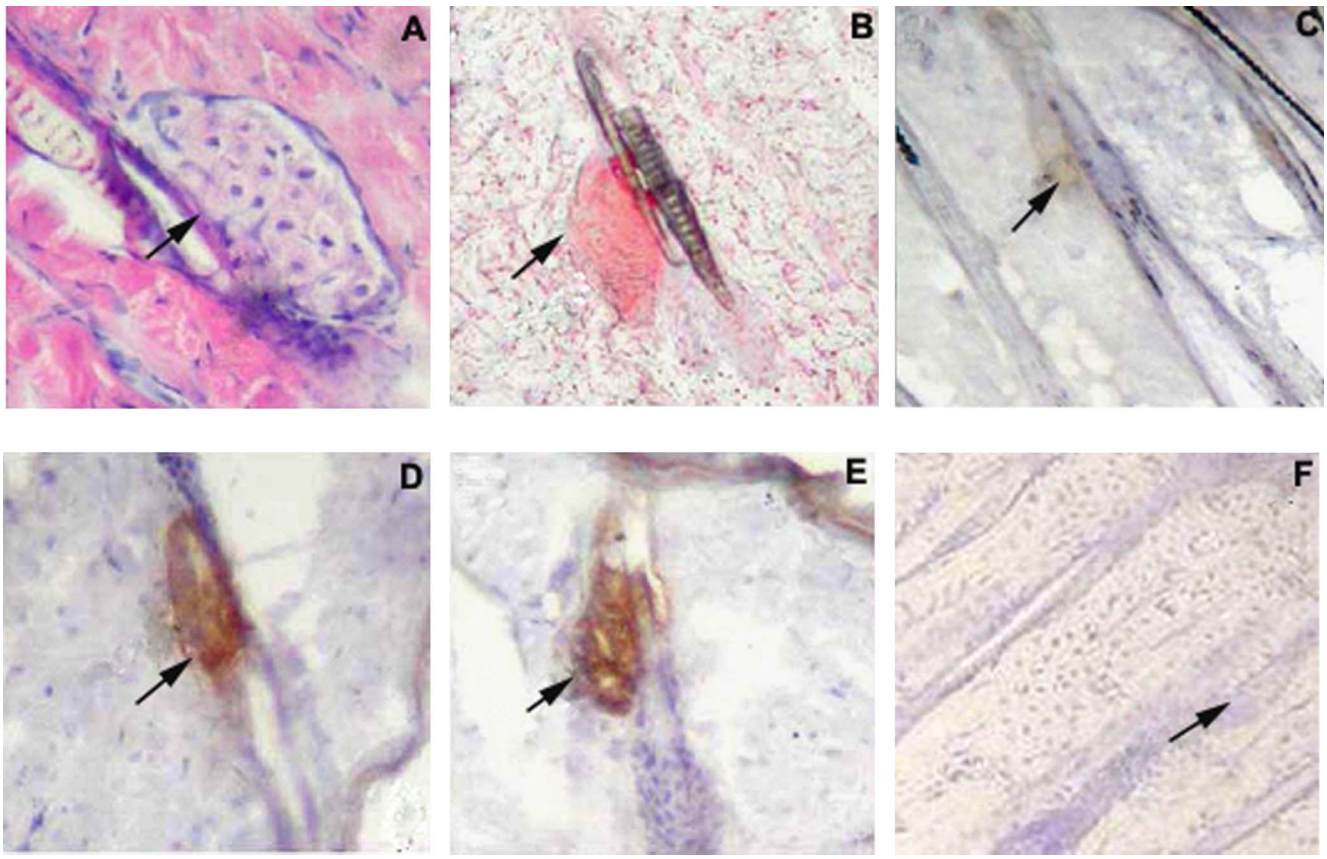

Fig. 4. Histological changes of sebaceous gland and the immunohistochemical reaction of COX-2 in sebaceous gland at different phases: (**A**) the sebaceous glands during catagen in H/E staining (arrow) (×400); (**B**) the sebaceous glands during catagen in oil red staining (arrow) (×200), and COX-2 expression in the whole sebaceous gland (arrows) during anagen (**C**) (×200), catagen (**D**) (×200), and telogen (**E**) (×200). Compared with the immuno-histochemical control (**F**), the intensity of positive reaction was very weak in the anagen; it was, however, very strong in catagen and telogen phases. The size of sebaceous gland also became larger following the process of hair cycle entering from anagen to catagen.

Although previous studies have shown that, in sebaceous glands, sebocytes could constitutively produce cyclooxygenase 2 (COX-2) *in vivo* and *in vitro* [11], until now there is still very little information about the expression pattern of COX-2 protein in sebaceous glands during the hair cycle. In mouse skin, COX-2 protein was detected in the sebaceous glands only at anagen [16]. Extending the findings to mouse, in our present study, COX-2 protein was detected in all three stages of hair cycle, with its strongest expression appearing in catagen. While the reasons for this inconsistency are unknown, one of them may be the species difference in the development diversity of the sebaceous glands between mouse and rat. According to the relationship of COX-2 protein expression with the development of sebaceous glands, we hypothesize that during the shift from anagen to catagen, the sebaceous glands may keep on developing in rat skin, but stop developing in mouse skin, because the sebaceous glands may already have fully matured, hence the promoting role of COX-2 may not be needed.

The pattern of COX-2 mRNA expression in normal mouse skin was reported to be hair cycle-dependent and the expression of COX-2 mRNA was higher in the anagen and decreased in the catagen and telogen [16]. The data here indicated that in normal rat skin, the COX-2 mRNA expression was also in a hair-cycle-dependent manner, but the stronger intensities of COX-2 mRNA expression appeared in catagen and telogen, not in anagen. During catagen and telogen phase of hair follicle, in normal rat skin of the present experiment, COX-2 protein was abundantly detected in the sebaceous glands and so COX-2 mRNA coming from the sebaceous glands should be the main contributor of the total mRNA of skin. But in mouse catagen and telogen skin, the expression level of COX-2 protein was so low that it was scarcely detected not only in epidermis and hair follicles, but also in the sebaceous glands [16]. The persistent expression of COX-2 in sebaceous gland in catagen and telogen thus may be an important candidate for discrepancy between our results and those in mouse skin.

In conclusion, we profiled the expression pattern of COX-2 in normal rat skin during the hair cycle, and suggested the possible functions of COX-2 in epidermis and the two members of the pilosebaceous unit. Further studies are need to address the mechanisms of COX-2 action involved in the

normal physiology and function of epidermis and pilosebaceous unit, such as the differentiation of epidermis, the morphogenesis of the hair follicle, the transformation of hair cycle stages, and the lipid production of the sebaceous gland.

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VI. References

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