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Localization of TIMP in cycling mouse hair

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Summary

TIMP (tissue inhibitor of metalloproteinase) is a glycoprotein inhibitor of metalloproteinases that we hypothesize to be involved in the tissue remodeling that occurs during each hair growth cycle. We examined this hypothesis by studying the expression of TIMP at selected times during a single hair cycle using TIMP–lacZ transgenic mice to localize TIMP gene activity in the hair follicle. TIMP gene induction was visualized by staining mouse back skin for β-galactosidase (β-gal) activity. Paraffin sections were analyzed for the localization of TIMP expression. TIMP gene activation appears in hair follicles only during the mid-anagen (the growing stage of the hair cycle) primarily in Henle's layer of the inner root sheath. Some expression of TIMP is also seen in a few connective tissue cells, in the sebaceous gland and in cells at the proximity of the dermal papilla cells in catagen (regressing) and telogen (resting) follicles. These results are consistent with a role for TIMP in cyclic remodeling of connective tissue in hair follicles.

Key words: TIMP, transgenic mice, β-galactosidase, hair cycle.

Introduction

Tissue inhibitor of metalloproteinase (TIMP) is a M_r 28 000 glycoprotein that blocks the activity of metalloproteinases such as collagenase (Reynolds *et al.* 1981). As such, TIMP may be key in the regulation of connective tissue metabolism and remodeling in many different organ systems including hair.

TIMP plays a key role in helping to maintain a balance between extracellular matrix (ECM) deposition and degradation (Reynolds *et al.* 1981). TIMP expression can be detected in developing osteogenic tissues such as mandible, ribs, calvaria, limbs, tooth buds and vertebrae (Flenniken and Williams, 1990; Nomura *et al.* 1989). Furthermore, Reynolds *et al.* (1981) hypothesized that invading tumor cells may destroy ECM by inducing cells of the invaded tissue to synthesize metalloproteinases by producing factors that alter the normal condition of excess presence of TIMP.

The growth of hair is a result of extensive cyclic remodeling of the follicle involving interactions between dermal and epidermal components of the skin. These interactions that result in the synthesis of hair occur in a well-characterized cycle (Montagna and Parakkal, 1974). Following the anagen growth phase, the follicle regresses to about one-third its growing length and contains a brush-like club hair (catagen phase). This is followed by a resting phase (telogen) and then another anagen phase.

Periods of growth and regression of the hair follicle require extensive remodeling of the epithelial and connective tissue elements involved. Although the exact nature of the controlling mechanisms of the hair growth cycle is unknown, the action of collagenase and other proteinases involved in ECM remodeling is thought to be important in the cyclic remodeling process (Johnson-Wint and Gross, 1984). Furthermore, Parakkal (1969) has shown that the connective tissue sheath around a catagen follicle consists almost entirely of a tightly packed layer of macrophages. He suggests that these macrophages actively engulf and degrade the collagen fibers. Several ECM macromolecules such as fibronectin, laminin, type IV collagen (Couchman and Gibson, 1985; Westgate *et al.* 1984), proteoglycans (Couchman *et al.* 1990; Westgate *et al.* 1989) and growth factors (Pittelkow, 1990; Green, 1989; Green and Couchman, 1984) exhibit spatial and temporal turnover during the hair growth cycle. Thus, inhibitors of ECM proteinases, such as TIMP, may also be important in regulating the degradative properties of proteinases.

This study examines the hypothesis that TIMP is involved in the regulation of hair growth during the hair

cycle. We support this hypothesis by showing that TIMP is produced in the hair follicle at stages critical to the remodeling of the follicle during the hair cycle.

Materials and methods

Transgenic animals

Mice used were TIMP–lacZ transgenic mice generated from a CD-1 parental strain (Flenniken and Williams, 1990). Briefly, transgenic mice were generated by microinjection of the 6 kb TIMP–lacZ fusion gene into fertilized eggs. Transgenic mice were identified at 3 weeks of age by Southern blot analysis of tail DNA and by *in situ* detection of β-gal activity. On the basis of these assays, mice carrying a germ line integration were identified and used to establish homozygous transgenic lines.

Tissue sampling

Mice were killed by cervical dislocation at selected times to follow the expression of TIMP through one hair growth cycle. Skin samples were taken with a 6 mm biopsy punch from the area on the back at the midline between the scapula and between the pelvis at 3, 6, 12, 17, 25 and 31 days of age.

Representative sections indicating the stage of the hair cycle are shown in the results.

Tissue staining

The skin samples were cut into 1 mm strips before fixing for 15 min in 0.5 % glutaraldehyde in PBS at room temperature. Following the fixation period, the tissue was washed three times for 5 min each in PBS+2 mM $MgCl_2$. After rinsing, the tissue was permeabilized in PBS+2 mM $MgCl_2$+0.01 % sodium desoxycholate+0.02 % NP40 for 10 min. The tissue blocks were then stained in X-gal solution (Dannenberg and Suga, 1981) overnight at 37°C.

After staining, the tissue was rinsed in PBS, dehydrated in a graded alcohol series, cleared in xylene and embedded in paraffin. Sections were cut on a rotary microtome at 6 μm and counterstained with eosin. As controls, non-transgenic mouse tissue were examined and found to be routinely negative for β-gal activity.

Results

TIMP gene expression was judged by staining for TIMP promotor regulated β-gal activity. Clear cycle-dependent changes in the level of TIMP gene expression were observed in the inner root sheath of hair follicles. We found that hair follicles were in anagen through day 12, catagen at day 17, telogen at day 25 and well into the second hair cycle by day 31. These times are in approximate agreement with the observations of Dry (1926).

Early anagen follicles (3-day-old mouse) indicated TIMP activity in the inner root sheath of the follicle (Fig. 1A). TIMP expression has also been detected by northern blot hybridization in RNA extracted from isolated whisker follicles of a 3-day neonate mouse (data not shown). By mid-anagen (6 days old), the expression of TIMP in the follicles was striking (Fig. 1B). The inner root sheath was intensely positive for the transgenic β-gal activity while other areas of the hair and surrounding dermal connective tissue were relatively negative. The follicle bulb and matrix cells were conspicuously negative. By day 12 (late anagen), almost no TIMP gene expression was detected (Fig. 1C). Some TIMP gene expression was present near sebaceous glands below the surface of the skin.

The follicle remained negative for TIMP gene expression during catagen (17 days old) and telogen (25 days old) except for residual expression in the sebaceous gland and in the deepest portion of the follicle bulb in the proximity of dermal papilla cells (Figs 1D,E, 2A). With the initiation of the second anagen cycle (31 days old) of the hair follicle, TIMP gene expression was once again clearly detectable in the inner root sheath but negative in the follicle bulb (Fig. 1F).

TIMP gene expression was limited to Henle's layer of the inner root sheath. Longitudinal sections of an anagen follicle demonstrated that the outer root sheath was β-gal negative (Fig. 2B) while a cross-sectional cut of an anagen follicle indicated that the expression of TIMP was limited to Henle's layer of the inner root sheath (Fig. 2C).

Discussion

Other tissues in which TIMP gene expression has been studied suggest an important role for this proteinase inhibitor in ECM remodeling. Nomura *et al.* (1989) and Flenniken and Williams (1990) have shown that TIMP expression occurs in the developing ossification centers of calvaria, limbs, ribs, mandible, tooth buds and vertebrae. Additionally, Nomura *et al.* (1989) found a high level of TIMP expressed in the corpora lutea of the ovary suggesting that TIMP may prevent premature destruction of the corpora lutea and therefore play a role in the maintenance of pregnancy.

Our data indicate that TIMP expression is regulated and its level of activity changes with the hair cycle. TIMP is primarily expressed in the hair follicle during mid-anagen (growth phase of the hair cycle) and not present during the other stages. This suggests that metalloproteinases are active within the follicle during early growth periods (telogen to early anagen) and during follicle regression (catagen) but are inhibited during the mid-growth phase (mid-anagen) of the follicle. Weinberg *et al.* (1990) have shown that cultured pelage follicles from newborn mice have the ability to lyse the underlying collagen. They presume that this is caused by an elaboration of a type I collagenase. Furthermore, Goodman and Ledbetter (1990) have shown that cultured dermal papilla cells from whisker follicles produce stromelysin, a metalloproteinase known to degrade several ECM macromolecules (Matrisian, 1990).

Several ECM macromolecules are temporally expressed in the hair follicle during the hair growth cycle. Those studied are type IV collagen, laminin, fibronectin, chondroitin sulfate, unsulfated chondroitin, bullous pemphigoid antigen and dermatan sulfate (Couchman

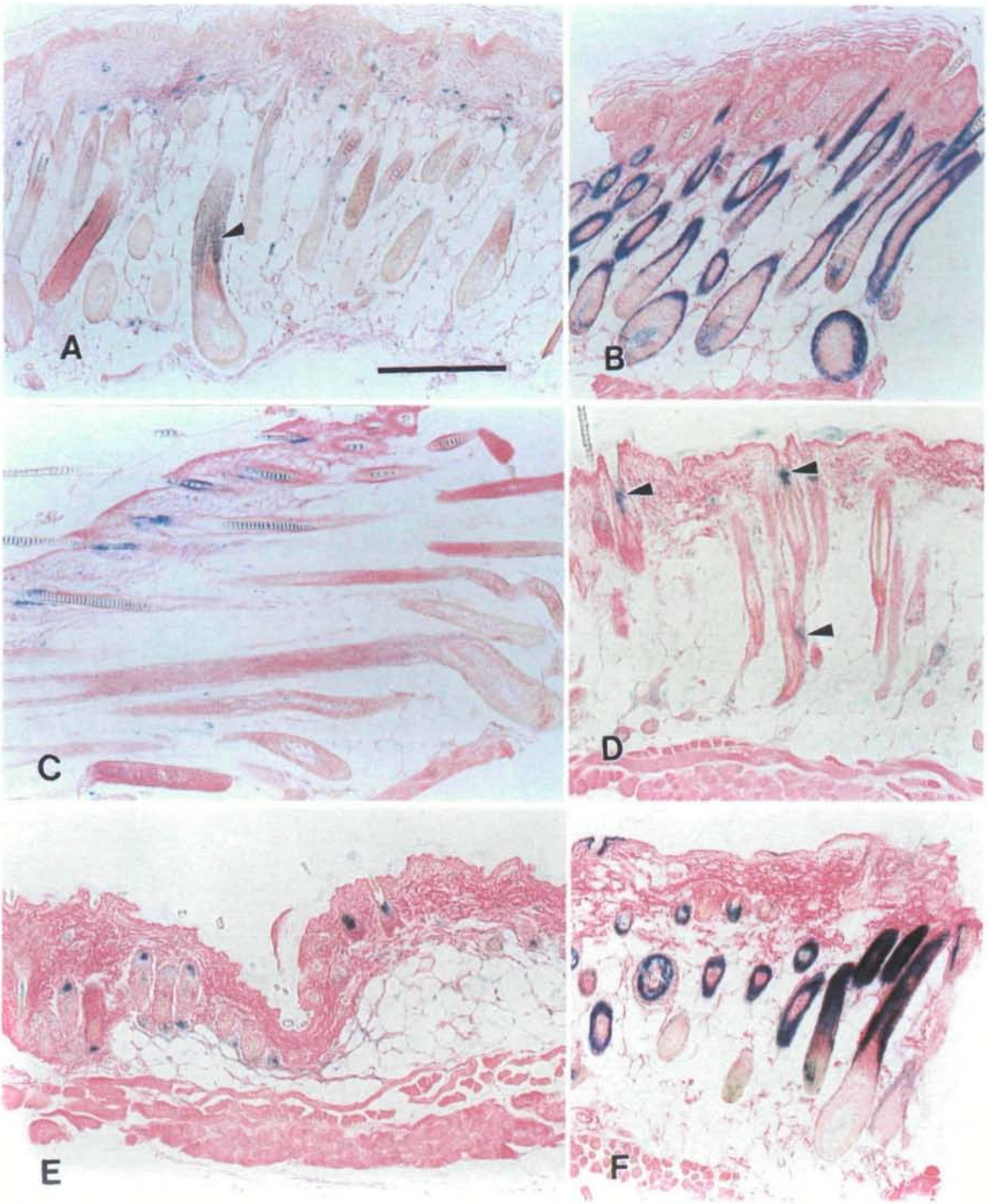

Fig. 1. All sections are counterstained with eosin only. The TIMP expression is indicated by the blue staining. All figures are at the same magnification. Bar 200 μm. (A) Skin section of a 3-day-old mouse. Hair follicles are in the first anagen cycle. Some TIMP expression is seen in one of the follicles (arrow). Scattered connective tissue cells also appear to express TIMP. (B) Mid-anagen hair follicles from a 6-day-old mouse. Distinct TIMP expression is seen in all the follicles. The follicle bulb is negative. (C) Late-anagen hair follicles from a 12-day-old mouse. Note the degree of elongation of the follicles and thinning of the epidermis. No TIMP expression appears in the follicles. Some expression appears in the sebaceous gland just below the surface of the skin. (D) Section from a 17-day-old mouse showing catagen hair follicles. No TIMP expression is seen in the hair shaft. Some expression is observed in the sebaceous gland and in the bottom portion of two follicles (arrows). (E) Telogen hair follicles from a 25-day-old mouse. TIMP expression is only seen in the sebaceous gland near the surface of the skin and in the bottom of the follicle. (F) Mid-anagen hair follicles of the second hair cycle from a 31-day-old mouse. Expression of TIMP is identical to that seen in the mid-anagen of the first hair cycle. The follicle bulb remains conspicuously negative.

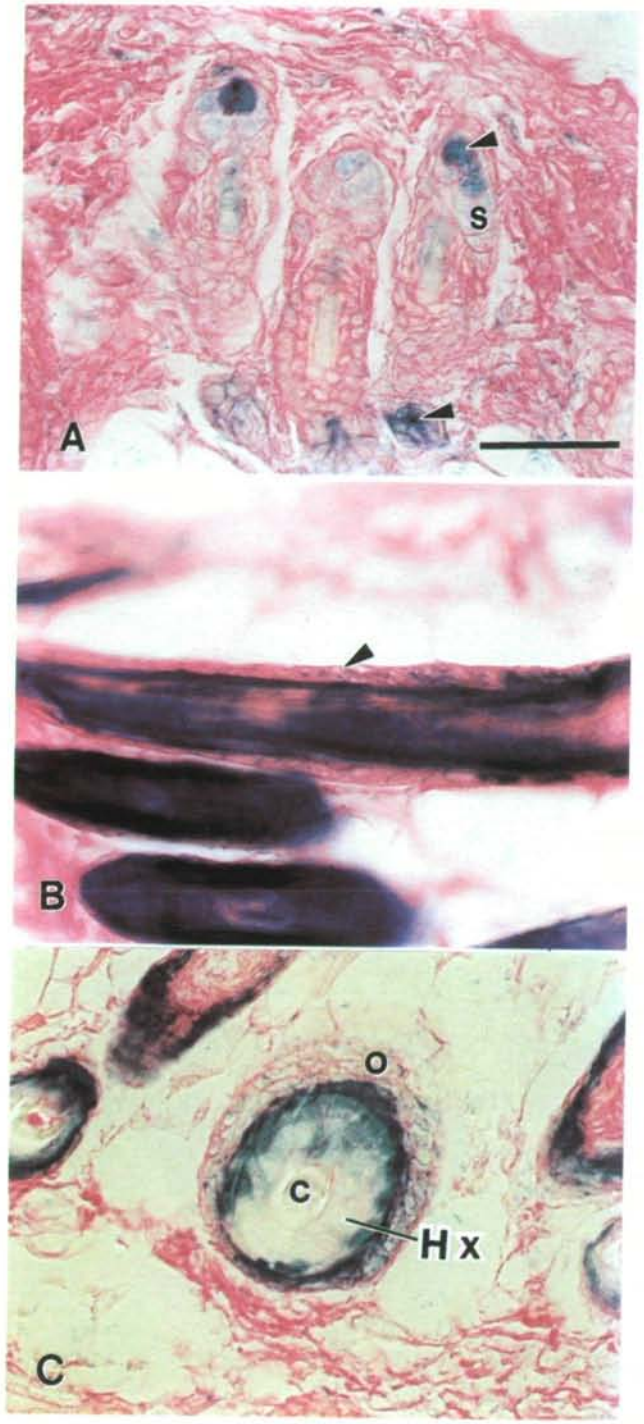

Fig. 2. All sections are counterstained with eosin only. The TIMP expression is indicated by the blue staining. All figures are at the same magnification. Bar, 50 μm.
(A) Telogen follicles showing TIMP expression (arrows) in the sebaceous gland (s) and in the bottom of the hair follicle. The cells stained in the bottom of the hair follicle are in the proximity of the dermal papilla. The exact nature of these cells is unknown. (B) Longitudinal section through a mid-anagen hair follicle. This shows the unstained outer root sheath. Virtually all of the TIMP expression is in the inner root sheath. (C) A cross-section of a mid-anagen hair follicle. The outer root sheath (o) is unstained as is Huxley's layer of the inner root sheath (Hx) and the hair cortex (c). Only Henle's layer of the inner root sheath shows TIMP expression.

et al. 1990; Couchman and Gibson, 1985; Westgate *et al.* 1989; Westgate *et al.* 1984). These are all present during the anagen growth phase and absent or greatly reduced during the regressing catagen and telogen resting phases. The cyclic presence of the ECM macromolecules correlate with the presence of TIMP expression during the hair growth cycle.

EGF and TGF-β_1 are also temporally expressed and present during anagen and absent during catagen and telogen (Green, 1989; Green and Couchman, 1984). Edwards *et al.* (1987) have shown that TGF-β selectively represses the induction of collagenase, but interacts cooperatively with EGF and other growth factors to induce TIMP expression *in vitro*. Thus, the temporal expression of TIMP during the anagen phase of the hair cycle would facilitate tissue remodeling necessary for follicle growth by inhibiting the degradative actions of metalloproteinases.

The appearance of TIMP only during anagen is consistent with the hypothesis that the activity of metalloproteinases in connective tissue remodeling of the dermis during the hair cycle can be spatially and temporally regulated. This localization of TIMP expression, residing only in the inner root sheath and noticeably absent in the follicle bulb and outer root sheath, was both interesting and surprising to us. One may speculate that the presence of TIMP expression in the inner root sheath may protect the newly formed hair shaft from the degradative action of secreted proteinases. Metalloproteinases secreted by the dermal papilla cells (Goodman and Ledbetter, 1990) may facilitate the regrowth at the tip of a hair follicle. This would provide for a uni-directional remodeling of the ECM allowing the follicle bulb to form a pit as the follicle grows deeper into the dermis while repressing proteinase activity distal to the bulb. During catagen, the reduced TIMP activity may facilitate follicle regression because there is no inhibition of ECM proteinases.

Our observations are consistent with the possibility of the involvement of TIMP in the regulation of follicle and connective tissue remodeling during the hair cycle. To our knowledge, this is the first example of cyclical gene activity attributed to the inner root sheath of hair follicles. The temporal expression of TIMP correlates with the concept of its role in controlling connective tissue remodeling during the hair cycle.

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