Colocalization of Cystatin M/E and its Target Proteases Suggests a Role in Terminal Differentiation of Human Hair Follicle and Nail

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The cysteine protease inhibitor cystatin M/E is a key regulator of a biochemical pathway that leads to epidermal terminal differentiation by inhibition of its target proteases cathepsin L, cathepsin V, and legumain. Inhibition of cathepsin L is important in the cornification process of the skin, as we have recently demonstrated that cathepsin L is the elusive processing and activating protease for transglutaminase 3, an enzyme that is responsible for crosslinking of structural proteins in cornified envelope formation. Here, we study the localization of all players of this pathway in the human hair follicle and nail unit in order to elucidate their possible role in the biology of these epidermal appendages. We found that cathepsin L and transglutaminase 3 specifically colocalize in the hair bulb and the nail matrix, the regions that provide cells that terminally differentiate to the hair fiber and the nail plate, respectively. Furthermore, transglutaminase 3 also colocalizes with the structural proteins loricrin and involucrin, which are established transglutaminase substrates. These findings suggest that cathepsin L and transglutaminase 3 could be involved in the pathway that leads to terminal differentiation, not only in the epidermis but also in the human hair follicle and nail unit.

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INTRODUCTION

Recent work of our group has revealed a previously unreported biochemical pathway that controls skin barrier formation by regulation of both crosslinking and desquamation of the stratum corneum. The cysteine protease inhibitor cystatin M/E, which is a key molecule in this pathway, has two important regulatory functions in epidermal differentiation and hair follicle morphogenesis. First, cystatin M/E regulates crosslinking of structural proteins by transglutaminase 3 (TGase 3) in the cornification process of the epidermis and the hair follicle by controlling cathepsin L (CTSL) and legumain activities (Zeeuwen *et al.*, 2004; Cheng *et al.*, 2006). We provide evidence for this assumption when we demonstrated that human CTSL is the elusive enzyme that is able to process and activate human TGase 3, an epidermis-specific enzyme responsible for the crosslinking of loricrin and small proline-rich proteins (SPRs) in the cornification process of the skin (Cheng *et al.*, 2006). During keratinocyte differentiation, TGase 3 is activated by limited proteolysis (Hitomi *et al.*, 1999; Ahvazi *et al.*, 2002), a process that is apparently under control of cystatin M/E, at least during skin morphogenesis in the neonatal phase (Zeeuwen *et al.*, 2004). We suppose that processing and activation of TGase 3 in terminally differentiating keratinocytes is regulated by the inhibitory activity of cytoplasmic cystatin M/E against CTSL. Legumain inhibition by cystatin M/E could have a regulatory role in CTSL activity as legumain is involved in CTSL processing (Maehr *et al.*, 2005). As a second regulatory function, cystatin M/E could have a function in desquamation by the regulation of cathepsin V (CTSV) protease activity, which is involved in the degradation of corneodesmosomal components. We recently showed that cystatin M/E and CTSV are separately transported within lamellar granules and that both proteins are secreted in the extracellular space of the stratum corneum where they associate with corneodesmosomes as determined at the ultrastructural level (Zeeuwen *et al.*, 2007).

Misregulation of this pathway by unrestrained protease activity, as seen in cystatin M/E-deficient mice, leads to abnormal stratum corneum and hair follicle formation, disturbance of skin barrier function, and neonatal death (Sundberg *et al.*, 1997; Zeeuwen *et al.*, 2002, 2004). The importance of regulated proteolysis in epithelia is well demonstrated by the recent identification of the *SPINK5*

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Abbreviations: CTSL, cathepsin L; CTSV, cathepsin V; IRS, inner root sheath; ORS, outer root sheath; TGase, transglutaminase; THH, trichohyalin

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serine protease inhibitor as the defective gene in the Netherton syndrome, cathepsin C mutations in the Papillon-Lefevre syndrome, ichthyosis because of matriptase deficiency, and targeted ablation of CTSL and cathepsin D in mice (for a comprehensive review see Zeeuwen, 2004).

The hair follicle and nail unit are epidermal appendages that also show processes of terminal differentiation. Hair follicles undergo cycles of growth, regression, and rest, which are, respectively, named anagen, catagen, and telogen phase (Paus and Foitzik, 2004; Alonso and Fuchs, 2006). During the anagen phase the entire hair from tip to root is produced when matrix cells in the hair bulb proliferate and differentiate into the several layers of the hair follicle (Figure 1): the medulla, cortex, and cuticle layer of the hair shaft (the actual hair fiber); the Huxley's and Henle's layers, and the cuticle of the inner root sheath (IRS), and the outer root sheath (ORS) (Niemann and Watt, 2002; Fuchs, 2007). Keratinization of hair follicles occurs in specific compartments, that is the hair shaft, cuticle, and IRS, through crosslinking of structural precursor proteins by TGases (Commo and Bernard, 1997; Thibaut *et al.*, 2005). The degeneration of the IRS in the infundibulum of the hair follicle near the opening of the sebaceous duct requires desquamation, a process in which proteolytic enzymes are involved (Ekholm and Egelrud, 1998).

Contrary to hairs, nails show continuous growth of the hard nail plate out of the nail matrix, sliding over the underlying nail bed (Dawber *et al.*, 2001; Baran *et al.*, 2005). Keratinization of the nail starts in the nail matrix, but the relative contribution of TGases to the formation of nail corneocytes which end up in the nail plate is not exactly known. Nevertheless, it is known that lack of TGase 1 in lamellar ichthyosis patients could be reflected in structural nail alterations (Rice *et al.*, 2003).

In the present study we sought to address the possible role of cystatin M/E and its physiological target proteases in the biology of the human nail unit and hair follicle. We have examined the localization of cystatin M/E, CTSV, CTSL, TGase 3, and legumain in (longitudinal and/or transversal) sections of the human hair follicle and nail unit by immunofluoresence microscopy. We also examined the localization of known TGase substrates like the structural proteins involucrin, loricrin, and trichohyalin (THH). We found colocalization of CTSL, TGase 3, involucrin, and loricrin in the hair bulb and the nail matrix, suggesting that these proteins are key players in the pathway that initiate biochemical changes in the terminal differentiation of these epidermal appendages.

RESULTS

Hair follicle

Immunofluorescence microscopy was performed on both longitudinal and transversal sections of human hair follicle in order to examine the localization of cystatin M/E and its physiological target proteases in the different layers of the hair follicle. For a comprehensive biochemical and kinetic analysis of the interaction between cystatin M/E and its target proteases (see Cheng *et al.*, 2006). Double staining was performed for cystatin M/E with its target proteases, for TGase 3 with its activating protease CTSL, and for TGase 3 with its presumed substrates (Figures 2–4).

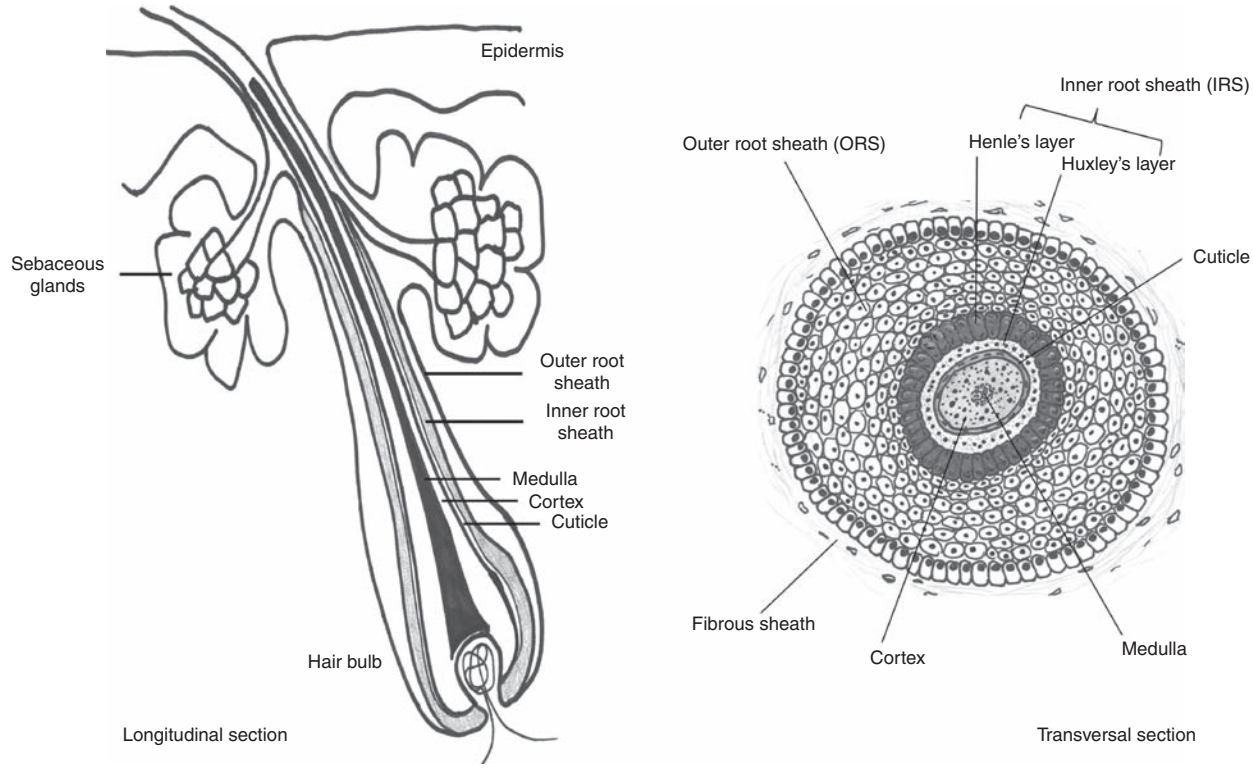

Figure 1. Schematic overview of longitudinal and transversal sections demonstrating the various components of the hair follicle.