Expression of the High–Affinity Choline Transporter, CHT1, in the Neuronal and Non–neuronal Cholinergic System of Human and Rat Skin

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Choline is an essential component in acetylcholine biosynthesis, and is involved in cell signaling. It is unable to permeate the cell membrane and requires a transporter to enter the cell. Neurons that synthesize acetylcholine take up choline by a recently cloned high-affinity choline transporter (choline transporter 1) that is Na$^+$-dependent and can be blocked by hemicholinium–3. The aim of this study was to determine the expression and to analyze the distribution of choline transporter 1 in human and rat skin. The mRNA for choline transporter 1 was detected in rat and human skin and in the human keratinocyte cell line HaCaT. A polyclonal anti-serum was developed against the N-terminal region of the human and rat protein. In rat and human skin, choline transporter 1 immunoreactivity was present in nerve fibers. In addition, keratinocytes, HaCaT cells and cells of the internal root sheath of the hair follicle contained choline transporter 1 immunoreactivity. The labeling patterns of nonconfluent vs confluent cultured cells and the distribution of choline transporter 1 along the epidermal layer suggest an association of choline transporter 1 with keratinocyte differentiation. In conclusion, this study shows the presence of the high-affinity choline transporter choline transporter 1 in nerve fibers and epithelial cells in the human and rat skin supporting the pivotal role of this transporter in both the neuronal and non-neuronal cholinergic system of the skin. *Key words: HaCaT cells/hair follicle/ keratinocytes/sweat glands/vasculature. J invest Dermatol 119:943–948, 2002*

The skin contains elements of the neuronal and a non–neuronal cholinergic system: eccrine sweat glands receive cholinergic nerve fibers (Landis, 1999), and the keratinocytes of the epidermis synthesize and release acetylcholine (ACh) (Grando *et al*, 1993a, b, 1995; Klapproth *et al*, 1997). Additional non-neuronal sources of ACh are the vascular endothelium and/or leukocytes (Milner *et al*, 1989; Misery 1998; Fujii and Kawashima, 2001; Kirkpatrick *et al*, 2001). The uptake of choline is the rate-limiting step in ACh synthesis (Okuda and Haga, 2000). Choline is an essential component in ACh biosynthesis, and it is also involved in cell signaling. It is unable to permeate the cell membrane and requires a transporter to enter the cell. Neurons that synthesize ACh take up choline by a high-affinity choline transporter (CHT1) that is Na$^+$-dependent and can be blocked by hemicholinium–3 (Swann and Hewitt, 1988; Okuda and Haga, 2000). Recently, the human and rat neuronal CHT1 were cloned (Okuda and Haga, 2000; Okuda *et al*, 2000). It has been proposed that CHT1 is unique to cholinergic neurons as the mRNA for CHT1 was detected by northern blotting and *in-situ* hybridization in different areas of the rat brain but not in non-neuronal tissues (Okuda *et al*, 2000). In this study we aimed to determine the localization of CHT1 in the skin, in particular as to whether non-neuronal cholinergic cells also express the CHT1 protein. To investigate the distribution of CHT1 protein in human and rat skin, a polyclonal anti-serum was developed against the N-terminal region (amino acid residues 29– 40) of the human and rat sequence. In support of the immunohistochemical findings reverse transcription–polymerase chain reaction (reverse transcription–PCR) analysis of skin and the human keratinocyte cell line, HaCaT, was performed.

MATERIALS AND METHODS

Reverse transcription–PCR For reverse transcription–PCR, human and rat skin were quick–frozen in RNazol (WAK-Chemie, Bad–Homburg, Germany), homogenized using a turrax, and the total RNA was isolated using the RNazol reagent technique according to the recommended protocol. The total RNA from HaCaT cells was isolated using the Qiagen-kit (Qiagen, Heiden, Germany) according to the recommended protocol. The contaminating DNA was removed by DNase (1 U per μg total RNA; Gibco-BRL, Life Technologies, Karlsruhe, Germany) in the presence of 20 mM Tris–HCl (pH 8.4), 2 mM MgCl$_2$, and 50 mM KCl for 15 min at 25°C. Equal amounts of the RNA were reverse transcribed in the presence of 3 mM MgCl$_2$, 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 10 mM dithiothreitol, 0.5 mM deoxyribo-nucleoside triphosphate (Gibco-BRL) and 25 μM oligo(dT) (MWG Biotech, Ebersberg, Germany), with 200 University of Superscript RNase H$^-$ Reverse transcriptase (Gibco-BRL) for 50 min at 42°C. For the PCR reaction 5 μl buffer II, 5 μl MgCl$_2$, 1 μl deoxyribonucleoside triphosphate (10 mM each), 0.5 μl (2 U) AmpliTaq Gold polymerase (all reagents from Perkin Elmer) and 1 μl of each primer (20 μM, rat, forward: CAAGACCAAGGAGGAAGCAG reverse: GCAAACATGGAACT-TGCTGA GenBank accession number BAA90484; human, forward: ATCCCAGCCATACTCATTGG reverse: CAGAAACTGCACCA-AGACCA GenBank accession number AB04399, MWG Biotech) were
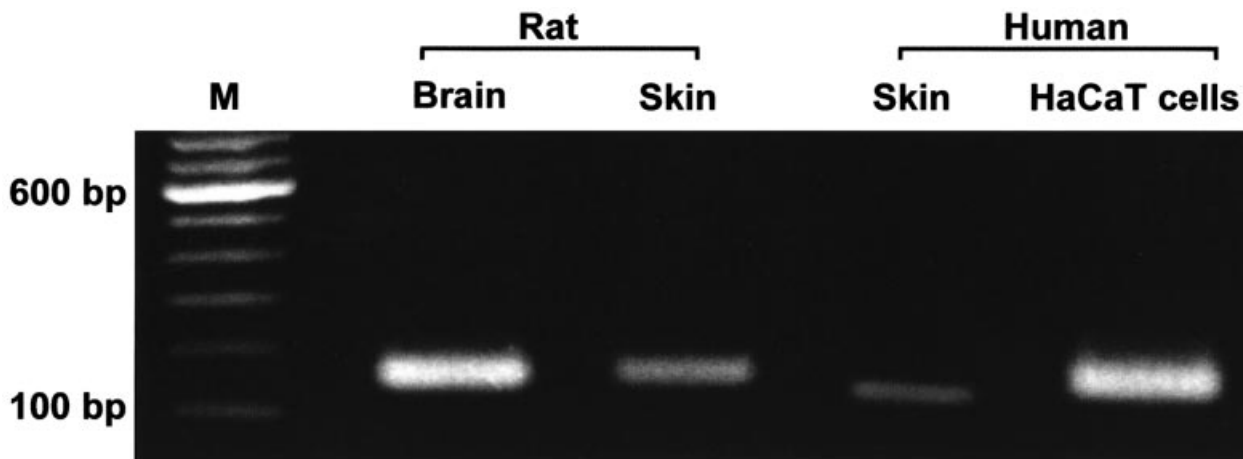
Figure 1. Reverse transcription–PCR detected CHT1-mRNA in rat and human skin. PCR products corresponding to the rat and human CHT1-mRNA could be obtained in rat and human skin and in human HaCaT cells. Rat brain served as a positive control. M = 100 bp marker.

mixed. Cycling conditions for time release hot start PCR were 12 min at 95°C, 45 cycles of 20 s at 94°C, 20 s at 60°C and 20 s at 72°C followed by 7 min at 72°C. Control reactions for reverse transcription–PCR, including the absence of the reverse transcription–reaction before PCR and the absence of template showed no amplification products.

Preparation of the CHT1 anti-sera   Polyclonal guinea-pig and rabbit anti-sera were raised against the synthetic peptide TKNSGNAEERSE that corresponds to the amino acid residues 28–40 of rat CHT1 (GenBank accession number AB030947) and to the AS 29–40 of human CHT1(accession number AAG25940) (Pineda Anti-körper-Service, Berlin, Germany) (Lips *et al*, 2002).

Immunohistochemistry   Specimens of human skin were obtained from surgery. Hairy skin of the head and glabrous skin of the inguinal region were dissected. Specimens of glabrous and hairy skin of rat hind paws were obtained from Wistar rats of either sex that were killed by chloroform inhalation. The specimens were immersed in Zamboni's fixative (Zamboni and deMartino, 1967) and then washed repeatedly in 0.1 M phosphate buffer, cryoprotected in the same buffer with 18% sucrose added, snap frozen in liquid nitrogen and stored at −20°C until processed for immunostaining. They were sectioned at a thickness of 10 μm with a cryostat (Jung Frigocut 1900 E, Leica, Bensheim, Germany). Sections were covered for 1 h with blocking medium (phosphate-buffered saline containing 10% normal porcine serum, 0.1% bovine serum albumin, and 0.5% Tween 20) followed by incubation with the primary anti-sera (CHT1, guinea-pig anti-serum 1 : 1000; CHT1, rabbit anti-serum, 1 : 1600; VAChT goat anti-serum, 1 : 800, Biotrend, Köln, Germany). Then the sections were washed in phosphate-buffered saline and covered for 1 h with secondary reagents. The sections were covered with either a fluorescein isothiocyanate-conjugated mouse anti-goat IgG (1 : 200, Sigma, Deisenhofen, Germany) or with Texas Red-conjugated donkey anti-guinea pig IgG (1 : 100, Dianova, Hamburg, Germany) or a Cy-3-conjugated donkey anti-rabbit immunoglobulin anti-serum (1 : 1000, Dianova). For double-labeling immunofluorescence the sections were incubated overnight with CHT1 and VAChT anti-sera, washed and covered with fluorescein isothiocyanate-conjugated mouse anti-goat IgG in combination with either Texas Red-conjugated donkey anti-guinea-pig IgG or a Cy-3-conjugated donkey anti-rabbit immunoglobulin anti-serum.

HaCaT cells (Boukamp *et al*, 1988) were cultured to confluence in RPMI 1640 tissue culture medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin/amphotericin at 37°C under normoxic conditions (5% CO_2, 95% air). For the experiments, the cells were detached with 0.1% trypsin/0.05% ethylenediamine tetraacetic acid (1 : 1) and resuspended in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin. Five thousand cells per well grew in eight-well chamber slides for 2 d. After 1 d, 10^{-4} M of the specific CHT1 inhibitor, hemicholinium-3 was added. Control cells grew in absence of hemicholinium-3. After 2 d, cells were fixed in Zamboni's fixative for 20 min followed by washing steps and incubation with primary and secondary anti-sera as described above. After incuba-

tion with the secondary reagents, the slides were washed in phosphate-buffered saline and coverslipped in carbonate-buffered glycerol at pH 8.6. Preabsorption with the corresponding synthetic peptide (20–100 μg antigen per ml diluted anti-serum) abolished immunolabeling. The slides were evaluated by epifluorescence microscopy (Olympus BX 60F, Hamburg, Germany) using appropriate filter combinations for Cy3 (excitation filter 525–560 nm, barrier filter 570–650 nm) and fluorescein isothiocyanate (excitation filter 460–490 nm, barrier filter 515–550 nm).

RESULTS

Reverse transcription–PCR   In samples of human and rat skin and rat spinal cord, PCR products of 150 bp in length could be amplified corresponding to the region 1153–1303 of the rat. A PCR product 169 bp in length, corresponding to the region 1131–1300 of the human CHT1 mRNA could be amplified from human skin and the human keratinocyte cell line, HaCaT (**Fig 1**).
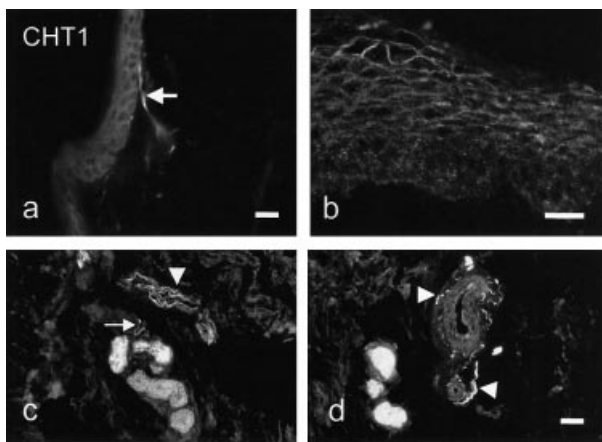
Immunohistochemistry   The rabbit and the guinea-pig CHT1-anti-sera showed similar staining of nerve fibers in rat skin (**Fig 2**). Double-labeling immunofluorescence revealed colocalization of CHT1 immunoreactivity and VAChT immunoreactivity in nerve fibers at sweat glands and in motor endplates (**Fig 2**).

Rat skin   A subpopulation of nerve fibers in the rat skin exhibited CHT1 immunoreactivity. Sweat glands of hairy and glabrous skin received a dense CHT1/VAChT immunoreactivity innervation (**Fig 2**). The underlying plantar skeletal muscles showed intense CHT1/VAChT immunoreactivity in motor endplates and nerve fibers (**Fig 2**). CHT1 immunoreactivity fibers were present in small numbers in the plexus at the epidermal–dermal junction and run parallel to the basal lamina but immunoreactive axons could not be observed in the epidermal layer (**Fig 3**). Keratinocytes of hairy and glabrous skin showed CHT1 immunoreactivity (**Fig 3**). CHT1 immunoreactivity nerve fibers were found in nerve fiber bundles and at larger blood vessels in the subcutis (**Fig 3**). CHT1 immunoreactivity nerve fibers occurred at a nerve plexus adjacent to the inner epithelial layer of the hair follicles (**Fig 4**). Cells of the internal root sheath of the hair follicle were strongly CHT1 immunoreactivity (**Fig 5**). The skin area used contained no piloarrector muscles. Sebaceous glands were devoid of an innervation by CHT1 immunoreactivity axons (not shown).

Human skin   Nerve fiber bundles in the dermis and subcutis showed intense CHT1 immunoreactivity, whereas the nerve fiber plexus at the epidermis–dermis junction was devoid of CHT1 immunoreactivity axons. In addition, single CHT1 immunoreactivity nerve fibers were found at the hair shaft, in arrector pili muscles, and around eccrine sweat glands and blood vessels (**Fig 6**). CHT1 immunoreactivity nerve fibers innervating the sweat glands
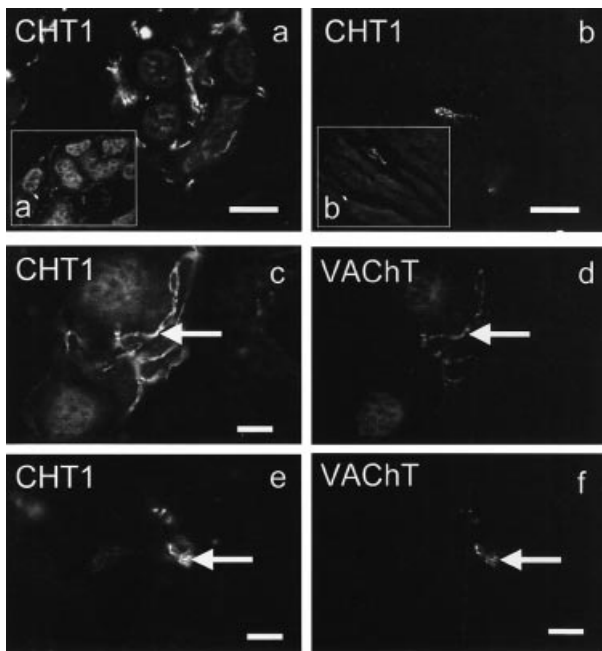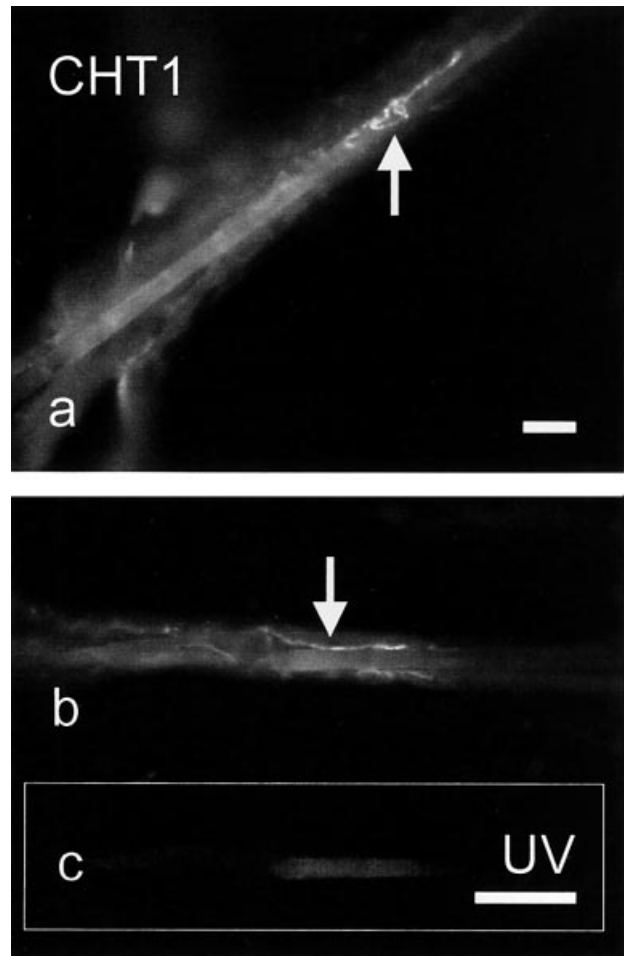
Figure 2. Indirect immunofluorescence of CHT1 in the rat sweat gland and striated muscle. The guinea-pig anti–CHT1 (*a,b*) and the rabbit anti–CHT1 (*a′,b′*) anti-serum labeled nerve fibers at eccrine sweat glands (*a,a′*) and motor endplates in plantar striated muscles (*b,b′*). Both,the fibers at eccrine glands (*c,d*) and the motor endplates (*e,f*) showed immunoreactivity for CHT1 and VAChT (*arrows c–f*). *Scale bars* = 50 μm.

Figure 4. Indirect immunolabeling of rat hairy skin demonstrated CHT1 in nerve fibers. CHT1 immunoreactivity nerve fibers could be demonstrated in nerve fibers adjacent to the inner epithelial layer of the hair follicles (*arrows in a,b*). Slight autofluorescence of the hair is demonstrated in *c* under ultraviolet illumination. *Scale bars* = 20 μm.
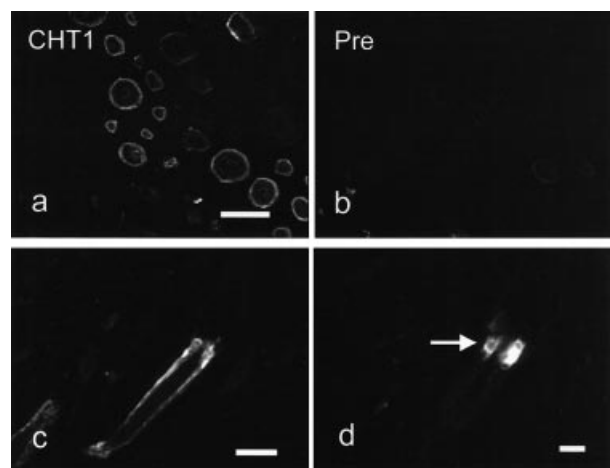

Figure 3. Indirect immunofluorescence of rat glabrous skin showed CHT1 in nerve fibers and keratinocytes. In the rat glabrous skin CHT1 immunoreactivity nerve fibers (*arrow in a*) occurred at the epidermis–dermis border (*a*). In the deeper dermis, CHT1 immuno-reactivity was present nerve fiber bundles (*arrowhead in c*) and in perivascular nerve fibers (*arrowhead in d*). Note the nerve fiber near the eccrine gland duct (*arrow in c*). The keratinocytes of the epidermis showed staining of the membrane (*b*). Epithelial cells of the eccrine ducts exhibited strong fluorescence that could not be preabsorbed (*c,d*). *Scale bar* = 20 μm.

Figure 5. Indirect immunofluorescence of rat hairy skin revealed the presence of CHT1 in cells of the internal root sheath of the hair follicle. Immunoreactivity for CHT1 was found in hair follicles in the outer epithelial layer (*a,c,d*). Cells of the internal root sheath (*c,d*) showed strong CHT1 immunoreactivity. The CHT1 immunoreactivity was strongly reduced after preabsorption (Pre) with the corresponding antigen (*b*). *Scale bars* = 20 μm.

were found in close apposition to acini and ducts. (**Fig 6**). Larger vessels of the deeper dermis received a perivascular innervation by CHT1 immunoreactivity nerve fibers (**Fig 6**). The vessels in the superficial layer at the epidermis–dermis junction showed no innervation by CHT1 immunoreactivity axons. Immunoreactivity for CHT1 was found in the anagen hair follicle. Cells of the internal root sheath showed strong CHT1 immunoreactivity

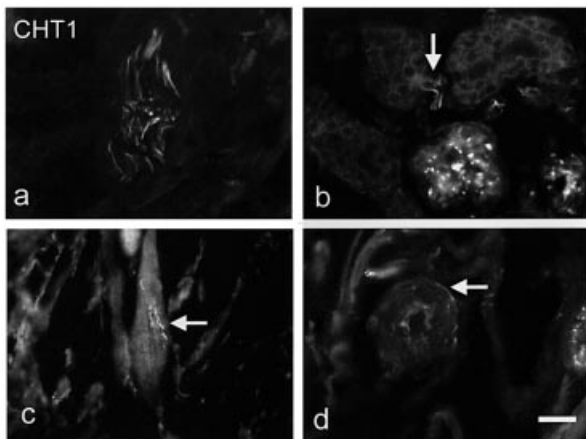(**Fig 7**). Staining for CHT1 was also observed in keratinocytes of the epidermal layer (**Fig 8**).

HaCaT cells   After 2 d of culture, about two-thirds of the chamber area were covered by a confluent monolayer. The keratinocytes showed variations in size and shape. CHT1 immunolabeling of the HaCaT cells was more intense using the rabbit anti-serum. CHT1 immunoreactivity was observed in two different patterns. Most cells grown in a monolayer expressed strong immunoreactivity at cell–cell contacts and a slight cytosolic CHT1 immunoreactivity. The membranous CHT1 immunoreactivity was present only within monolayers, whereas intense cytosolic CHT1 immunoreactivity was found in border regions of the monolayer and in cells islets after 1 d in culture (**Fig 8**). The immunoreactivity of the cell membrane increased after exposure to hemicholinium-3 for 1 d, whereas the cytosolic CHT1 immunoreactivity decreased (**Fig 8**).
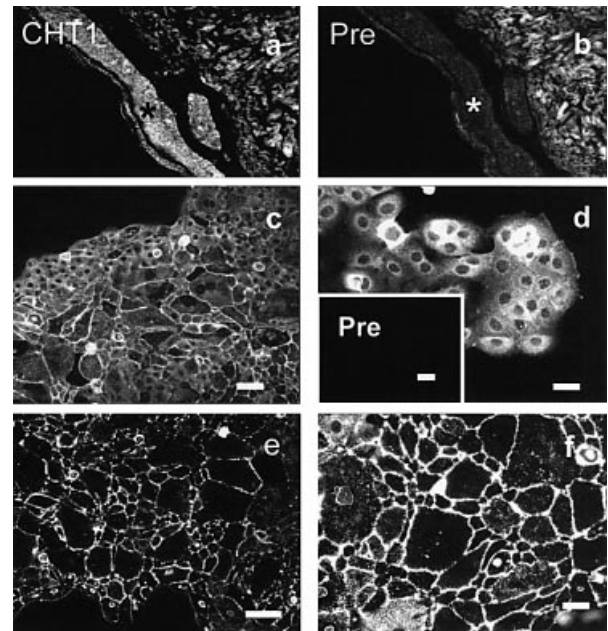
DISCUSSION

This study is one of the first to demonstrate the localization and distribution of the high-affinity choline transporter, CHT1, in the skin of humans and rat. In addition to nerve fibers, CHT1 is present in non-neuronal structures, i.e., keratinocytes of the epidermis and hair shafts.
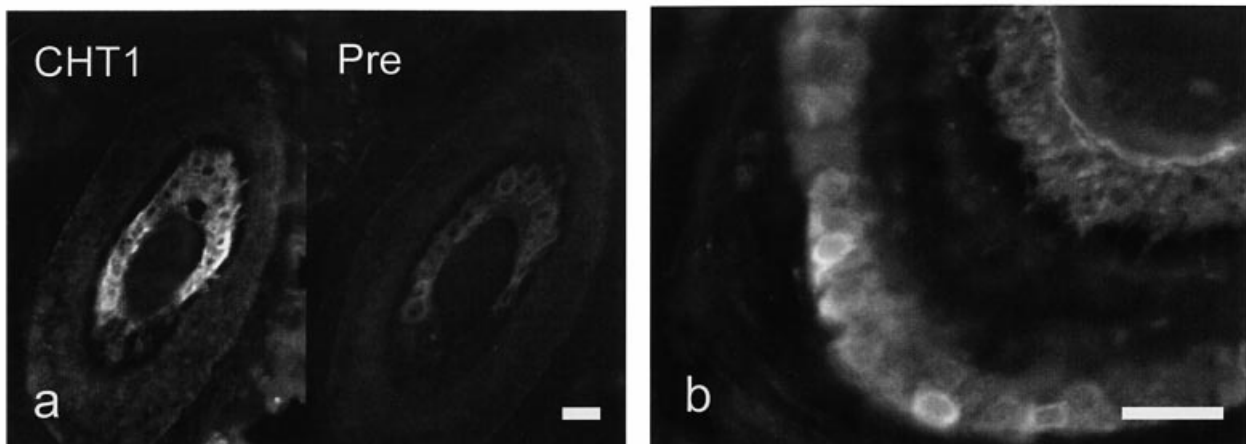
CHT1 in nerve fibers   In the CNS, CHT1 is present in cholinergic neurons as demonstrated by detection of its mRNA by northern blotting and *in-situ* hybridization in different areas of the rat brain, and by localizing the CHT1 protein in spinal motoneurons and in motor endplates by immunohistochemistry (Okuda *et al*, 2000; Kobayashi *et al*, 2002; Lips *et al*, 2002). In the skin, one well known cholinergically innervated tissue are eccrine sweat glands (Ernsberger and Rohrer, 1999). Sweat glands are

Figure 8. CHT1 immunolabeling of human epidermis and cultured HaCaT cells. The epidermis of human skin (asterisk) revealed CHT1 immunoreactivity (*a*) that was absent after preabsorption (*b*). In monolayers of HaCaT cells strong CHT1 immunoreactivity is present at the membrane of cell–cell contacts (*c,e,f*) and/or in the cytosol (*c,d*). Preabsorption (Pre) with the corresponding antigen abolished immunolabeling (*d*, inset). After treatment with hemicholinium-3, the CHT1 immunoreactivity of the membrane increased (*e,f*). Note the strong CHT1 immunoreactivity in the membrane of cell–cell contacts (*f*). *Scale bars* = 50 μm.

Figure 6. Indirect immunofluorescence of human skin showed CHT1 immunoreactivity nerve fibers. In the human skin CHT1 immunoreactivity nerve fibers (*arrows* in *b,c,d*) were present in fiber bundles (*a*), in eccrine glands (*b*), at piloarrector muscles (*c*), and at vessels in the deeper dermis (*d*). *Scale bar* = 20 μm.

Figure 7. CHT1 immunolabeling of human hair follicles. Immunoreactivity for CHT1 was found in hair follicles (*a,b*). Cells of Huxley's layer (*b*) showed strong CHT1 immunoreactivity. The immunolabeling was strongly reduced after preabsorption (Pre) with the corresponding antigen (*a*). *Scale bars* = 20 μm.

innervated by sympathetic neurons that undergo a change in transmitter phenotype from noradrenergic to cholinergic during development (Landis *et al*, 1988; Guidry and Landis, 1998; Fernandez-Fernandez *et al*, 1999). Evidence for the presence of a high-affinity choline uptake in eccrine glands has been provided by the inhibition of the choline-mediated glandular stimulation by hemicholinium-3, a specific blocker of CHT1 (Lamb *et al*, 1983). Here, we could show the presence of CHT1 immunoreactivity in nerve fibers innervating the glands in the subcutis. The fibers exhibited colocalization of CHT1 immunoreactivity and immunoreactivity for VAChT. This corroborates previous immunohistochemical studies that showed immunoreactivity for VAChT and the ACh synthesizing enzyme choline acetyltransferase in nerve fiber bundles at or surrounding the glands (Guidry and Landis, 1998; Zancanaro *et al*, 1999). Thus, the CHT1 immunoreactivity axons surrounding the glandular acini in the rat paw represent the sympathetic cholinergic sweat gland innervation.

In addition to the eccrine sweat glands, the vasculature in the deeper dermis is densely surrounded by CHT1 immunoreactivity nerve fibers, whereas the vessels at the epidermis–dermis border received no CHT1 immunoreactivity fibers. It is not clear if these fibers simply accompany or really innervate the vessels. To clarify if CHT1 immunoreactivity fibers indeed innervate deeper vessels, the vasculature has to be examined at the ultrastructural level. Functional studies on rat and human skin indicate the contribution of cholinergic fibers in the control of the skin vasculature (Low and Westerman, 1989; Muck-Weymann *et al*, 1996).

Differences in the distribution of CHT1 immunoreactivity fibers between rat and humans were present in the nerve plexus at the epidermis–dermis border. This plexus presumably consists of sensory fibers (Dux *et al*, 1999). So far neither choline acetyltransferase nor VAChT protein have been described in cutaneous sensory nerve terminals. Nonetheless, the presence of cholinergic fibers in this plexus cannot be excluded as Sann *et al* (1995) showed choline acetyltransferase immunoreactivity in sensory neurons of small diameter in rat lumbar dorsal root ganglia. Therefore, subpopulations of sensory neurons might well be capable of ACh synthesis and, hence, may require a high-affinity choline uptake. Similarly, CHT1 immunoreactivity nerve fibers occurred also in the nerve plexus termed "follicular network A" adjacent to the inner epithelial layer of the hair follicles, which also primarily contains sensory fibers (Botchkarev *et al*, 1998; Peters *et al*, 2001).

The arrector pili muscle is innervated by nerve fibers that originate from sympathetic ganglia. In the guinea-pig and the rat, practically all of these fibers are immunoreactive for the rate-limiting enzyme in norepinephrine synthesis, tyrosine hydroxylase, and therefore these axons are noradrenergic (Gibbins, 1991; Roth and Kummer, 1994; Bergner *et al*, 2000); however, fibers innervating the human piloarrector muscle showed CHT1 immunoreactivity that has been demonstrated in cholinergic fibers (Okuda *et al*, 2000; Lips *et al*, 2002).

CHT1 in non-neuronal cells The mRNA for CHT1 was detected in human and rat skin specimens and in HaCaT cells. In the skin neurons do not account for the content of CHT1-mRNA, as mRNA is generally restricted to neuronal perikarya and proximal dendrites, but are excluded from axonal processes. The perikarya of neurons innervating the skin are located in sympathetic chain and sensory ganglia at the vertebral column or the base of the skull. Therefore, the detection of CHT1-mRNA in the skin is due to the expression of CHT1 in non-neuronal cells. There are several lines of evidence that these non-neuronal cells are represented by the keratinocytes. Both CHT1-mRNA and CHT1-protein were identified in the keratinocyte cell line, HaCaT, by reverse transcription–PCR and immunohistochemistry, respectively, and CHT1 immunoreactivity was also observed in keratinocytes *in situ*. Thus, the present data are in favor of a choline recycling pathway in keratinocytes, as it has previously been shown that they synthesize, release, and cleave ACh (Grando *et al*, 1993a, b; Grando, 1997;

Ndoye *et al*, 1998; Nguyen *et al*, 2001). The increase of CHT1 immunoreactivity observed in the membrane of HaCaT cells treated with hemicholinium-3 suggests the potential of a dynamic regulation of this recycling pathway similar to that observed in cholinergic neurons where hemicholinium-3 increased membrane-bound CHT1 in synaptosomal preparations (Rylett *et al*, 1993).

A close association of membrane-bound CHT1 immunoreactivity and cellular differentiation was noted both in keratinocytes and HaCaT cells. HaCaT cells preserved the capacity to reconstitute an epidermal tissue when grown as surface transplants on nude mice (Breitkreutz *et al*, 1998; Schoop *et al*, 1999) and undergo the process of differentiation also in culture (Fusenig and Boukamp, 1998). After 1 d in culture CHT1 immunoreactivity was localized intracellularly in cell islets while it shifted to a membrane localization with the onset of the formation of a monolayer after 2 d of culture. Accordingly, membranous CHT1 immunolabeling increased with differentiation of keratinocytes *in situ* along the epidermal layers reaching a maximum before transition into dead corneocytes. This process includes apoptotic secretion that is regulated by cholinergic signaling (Nguyen *et al*, 2001). Similarly, CHT1 immunoreactivity was prominent in a ring in the internal root sheath, which corresponds in location to the so-called "upper ring" where the cells switch to terminal differentiation followed by exfoliation in the pilary canal (Barajon *et al*, 2001). Collectively, these data are in favor of a crucial involvement of CHT1 in the cholinergic signaling pathways regulating keratinocyte differentiation processes.

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