Human Keratinocytes Synthesize, Secrete, and Degrade Acetylcholine

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We previously reported that normal human keratinocytes express muscarinic receptors, and that acetylcholine induces attachment of these cells to each other. We have now studied the ability of human keratinocytes to synthesize, secrete, and degrade acetylcholine. To detect and localize the synthesizing enzyme choline acetyltransferase and degrading enzyme acetylcholinesterase, cultured cells and cryostat sections of normal human skin were pre-incubated with specific monoclonal antibodies and stained with an avidin-biotin complex/alkaline phosphatase. The choline acetyltransferase activity was assessed by the conversion of [^{3}H]acetyl CoA to [^{3}H]acetylcholine, and newly synthesized [^{3}H]acetylcholine was detected using thin-layer chromatography. The acetylcholinesterase activity was measured spectrophotometrically. Both cholinergic enzymes were present in cultured keratinocytes, and in basal, spinous and granular epidermal cell layers. Choline acetyltransferase was visualized in the vicinity of cell nuclei, and acetylcholinesterase was observed in or near cell membranes. Newly synthesized acetylcholine was detected in both cell homogenates and culture supernatants. The estimated Vmax of the synthesis of labeled acetylcholine by homogenized keratinocytes was about 20 pmoles acetylcholine produced/mg protein/min at 37°C. A single keratinocyte synthesized a mean of 2×10^{-17} moles, and released 7×10^{-19} moles acetylcholine per minute. Both cell homogenates and culture supernatants exhibited similar acetylcholinesterase activities indicating that human keratinocytes secrete acetylcholinesterase, too. Thus, we have demonstrated that normal human keratinocytes possess choline acetyltransferase and acetylcholinesterase, and synthesize, store, release, and degrade acetylcholine. Because human keratinocytes can also respond to acetylcholine, we believe that keratinocyte acetylcholine works in the epidermis as a local hormone. *J Invest Dermatol 101:32–36, 1993*

We previously reported high-density muscarinic acetylcholine receptors (mAChR) on the cell surfaces of intact human keratinocytes, and the ability of muscarinic ligands to regulate adhesion and motility of these cells [1]. Acetylcholine (ACh) antagonized acantholysis induced by pemphigus antibodies *in vitro,* and restored confluence of keratinocyte monolayers altered by the serine proteinase trypsin, and the calcium chelator ethylenediaminetetraacetic acid. The muscarinic antagonist atropine produced cell-cell detachment (acantholysis) in a dose-dependent manner. Because these atropine-treated acantholytic keratinocytes re-attached to each other spontaneously, we hypothesized that ACh might be synthesized by these cells, and that it is endogenous ACh that eventually reverses the effects of added atropine. We tested this hypothesis and found that human keratinocytes synthesize, store, secrete, and degrade ACh. Herein, we demonstrate localization and activities of keratinocyte cholinergic enzymes choline acetyltransferase (ChAT) that synthesizes ACh, and acetylcholinesterase (AChE) that degrades ACh, as well as the rates of ACh synthesis and release by human keratinocytes in cell culture.

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Abbreviations: AcCoA, acetyl coenzyme A; ACh, acetylcholine; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; KRB, Krebs-Ringer bicarbonate buffer; mAChR, muscarinic acetylcholine receptors.

MATERIALS AND METHODS

Human Keratinocyte Cultures Keratinocytes were obtained from normal human neonatal foreskins. Specimens freed from fat and clotted blood were rinsed in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (Gibco, Grand Island, NY) and placed epidermis up into 35-mm petri dishes (Falcon 3001, Becton Dickinson Labware, Lincoln Park, NJ) containing Dulbecco's modified Eagle's medium (DMEM; Biologos, Inc, Naperville, IL) supplemented to contain 0.125% trypsin, 10 μg/ml gentamicin, and 5 μg/ml fungizone (Gibco). After overnight incubation at 37°C in a humidified atmosphere with 5% CO_2, epidermis was separated from dermis and individual keratinocytes were released by rapid pipetting. The cells were resuspended in DMEM supplemented to contain 20% pooled decomplemented newborn calf serum (Biologos; DMEM-NCS) and centrifuged for 10 min at 200 × *g*. The pellet was resuspended in keratinocyte growth medium (KGM) containing 5 ng/ml recombinant epidermal growth factor and 50 μg/ml bovine pituitary extract (all from Gibco) and grown in 75-cm² Falcon tissue culture flasks (T-flasks) at 37°C in 5% CO_2. The concentration of calcium in KGM was 0.09 mM. The medium was changed every 3 d until keratinocytes covered two thirds of the flask bottom. The cells were released from the T-flasks by a 2-min incubation in trypsin (0.05%)–ethylenediaminetetraacetic acid (0.02%) solution (Biologos), centrifuged for 10 min at 200 × *g* in DMEM-NCS, resuspended in KGM, and inoculated into new T-flasks for further growth. After two passages, the keratinocytes were used in experiments.

The purity of human keratinocyte cultures (greater than 95%) was confirmed immunocytochemically using DAKO-CK monoclonal mouse anti-human cytokeratin antibodies (MNF 116, Dako-

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patts, Carpinteria, CA). The trypan blue dye exclusion test revealed not less than 95% living cells in all keratinocyte cultures.

Immunohistochemical Assays Immunohistochemical procedures were performed in keratinocyte monolayers, and in frozen sections of normal human epidermis obtained from cosmetic surgical procedures. For immunocytochemistry, 2.5 × 7.5 cm specimens were cut out from keratinocyte monolayers (75–80% confluence) growing on the bottom of plastic 75-cm² Falcon tissue-culture flasks. The cutouts were rinsed with 0.05 M Tris-buffered saline (TBS) to remove KGM, incubated for 10 min in 65% acetone to permeabilize cell membranes, placed in TBS for 5 min, incubated for 20 min with normal rabbit serum diluted 1 : 5 in TBS for blocking of non-specific binding, and then incubated for 30 min at room temperature with either anti-ChAT (MAB305) or anti-AChE (MAB303) mouse IgG1 monoclonal antibodies (MoAbs) (Chemicon International, Temecula, CA) diluted in TBS 1 : 50 or 1 : 10, respectively. Following incubation, the specimens were stained with avidin-biotin complex/alkaline phosphatase (DAKO K 376; Dakopatts) [2]. Some specimens were counterstained with Mayer's hematoxylin to highlight morphology. Cryostat sections of normal human skin were fixed in 100% acetone for 10 min, and then treated with MoAbs as above. Negative controls included omitting of the first layer monoclonal antibodies and replacing it with i) normal rabbit serum and ii) the isotype matching anti-CD3 MoAbs (M 756, Dakopatts).

Assays of ChAT Activity Three million viable keratinocytes, released from 75²-cm tissue culture flasks by 2-min incubation in trypsin/ethylenediaminetetraacetic acid, were rinsed in DMEM-NCS and washed in Krebs-Ringer bicarbonate buffer (KRB; Sigma, St. Louis, MO). Cells were resuspended in 3.5 ml KRB containing 145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 2 mM sodium-phosphate/10 mM Tris-HCl buffer, pH 7.4, and 10 mM glucose, put on ice, and disrupted by sonication. The protein concentration in keratinocyte homogenates was determined using the Micro BCA kit (Pierce, Rockford, IL). Experimental vials received 0.5 ml cell homogenate, and control vials received 0.5 ml KRB (blank). To each vial, 0.5 ml incubation buffer, consisting of 50 mM Tris-HCl (pH 8.0) with 1 mM acetyl coenzyme A (AcCoA), 10 mM choline, 1 mM eserine (all from Sigma), and 0.02 mM [³H]AcCoA (0.2 Ci/mmol; NEN, Boston, MA), were added. The assay was performed in the presence of saturating concentrations of non-labeled AcCoA and choline (determined in the standardization of the assay) to estimate the kinetics of [³H]ACh synthesis by putative keratinocyte ChAT. Eserine (i.e., physostigmine; the specific inhibitor of AChE) was included in the incubation buffer to protect newly synthesized [³H]ACh from enzymatic degradation by putative keratinocyte AChE. Vials were capped, vortexed, and incubated in a CO_2 incubator at 37°C for 20 min. After incubation, 5.0 ml of freshly prepared scintillation cocktail, consisting of nine parts INSTA-FLUOR (Packard Instrument Co., Inc, Downers Grove, IL) and one part tetraphenylboron (Sigma) in *n*-butanol, were added to each vial, and the newly synthesized [³H]ACh was extracted from the aqueous phase [3]. To calculate ChAT activity, blank values were subtracted from experimental values, and the obtained differential of disintegrations per minute (dpm) was converted into the amount of ACh produced by mg protein per min.

To determine the release of ACh, 3 × 10⁶ keratinocytes in KGM were inoculated into 75-cm² tissue culture flasks, and the flasks were incubated overnight to assure complete settling of the cells (confirmed by phase-contrast microscopy). Then, the monolayers were washed with prewarmed (37°C) KRB, fed with 7.0 ml incubation buffer/KRB (1 : 1 [v/v]), and incubated for 1 h in a CO_2 incubator, after which 1.0 ml aliquots of the supernatant (chasing medium) were added to scintillation vials to extract [³H]ACh. Blank value vials contained 1.0 ml incubation buffer/KRB instead of chasing medium.

To estimate the rates of ACh synthesis and release, the differential dpm were converted into the mean amount of ACh produced or released by a single cell per min.

Newly synthesized [³H]ACh in keratinocyte homogenates and chasing media was also detected by thin-layer chromatography (TLC) [4] on 10 × 20 cm silica Gel GF plates (Analtech, Inc, Newark, DE) using [³H]AcCoA, and [³H]ACh (90.0 mCi/mmol; NEN) as external standards and butanol : ethanol : acetic acid : water (8 : 2 : 1 : 3) as solvent system. After a 5-h run, the plates were dried, and 1.0 × 0.25 cm pieces of silica were scratched from the plates and transferred into scintillation vials containing 0.7 ml 0.5 M acetic acid to extract radioactivity. After shaking the vials, each of them received 6.0 ml of the scintillation cocktail Aquasol, and the radioactivity was measured in the LKB liquid scintillation counter.

As another control, 10 U AChE (Sigma) were added instead of eserine to some cell homogenate-, and chasing medium–containing samples, and to some samples containing authentic [³H]ACh.

Assay of AChE Activity Pre-confluent keratinocyte cultures were incubated with 5.0 ml KRB overnight at 37°C in a CO_2 incubator. Supernatants were collected and immediately used in the AChE assay. The flasks were washed with fresh KRB, and keratinocytes were scraped from the flasks with a rubber policeman. The cells were resuspended in 5.0 ml ice-cold KRB, put on ice, and disrupted by sonication. The protein concentration was measured using the Micro BCA kit. The AChE activity was immediately assessed spectrophotometrically by measuring the optical density at 412 nm of a solution containing acetylthiocholine iodide as a substrate and dithiobisnitrobenzoic acid as a reagent that yields a yellow color in the presence of thiocholine [5]. The optical density was measured each min at room temperature in both experimental and control samples. Experimental samples contained 0.5 ml keratinocyte homogenate or culture supernatant, 20.0 μl of 0.075 M substrate, and 100.0 μl of 0.01 M reagent in 0.1 M phosphate buffer, pH 8.0 (total volume 3.0 ml). Control samples contained only the substrate, reagent, and buffer in the same total volume. After 15 min, the optical density increment of control samples (non-enzymatic hydrolysis of the substrate) was subtracted from that of experimental samples, and the rates of enzymatic hydrolysis were converted into absolute units. One unit (U) of AChE activity hydrolyzed 1 μmole of substrate to thiocholine and acetate per min at pH 8.0 and 25°C.

The specificity of the assay was confirmed in another set of experiments in which keratinocyte cultures were incubated in the presence of 0.5 mM eserine.

Statistics All experiments were performed in triplicate, or quadruplicate, and the results were expressed as mean ± SD. Significance was determined using the Student t test. Results were deemed significant if the calculated p value was less than 0.05.

RESULTS

Immunolocalization of ChAT and AChE in Human Keratinocytes Both keratinocyte cultures and frozen sections of human epidermis contained ChAT. In the monolayers comprised of non-differentiated basal cells, ChAT immunoreactivity was confined to the perinuclear area of the cell cytoplasm (Fig 1*a*). In epidermis, cells in the basal, spinous, and granular layers stained, but cells in the horny layer did not (Fig 1*b*).

In addition to ChAT, keratinocytes in both tissue culture and skin sections contained AChE. AChE immunoreactivity was predominantly observed in the vicinity of cell membranes. Anti-AChE antibodies also stained intercellular filament bundles (Fig 1*c*). Among the AChE-positive epidermal basal, spinous, and granular cell layers, the former stained most intensively (Fig 1*d*).

The anti-AChE MoAb did not stain a keratin epitope, as judged by the absence of staining in western blots utilizing a panel of human epidermal keratins (Sigma; catalogue number K-0253; lot number 41H0308) as an antigenic substrate, and mouse anti-cytokeratin AE1/AE3 MoAbs (BioGenex, San Ramon, CA) as positive controls. The immunoblotting experiments showed that the anti-

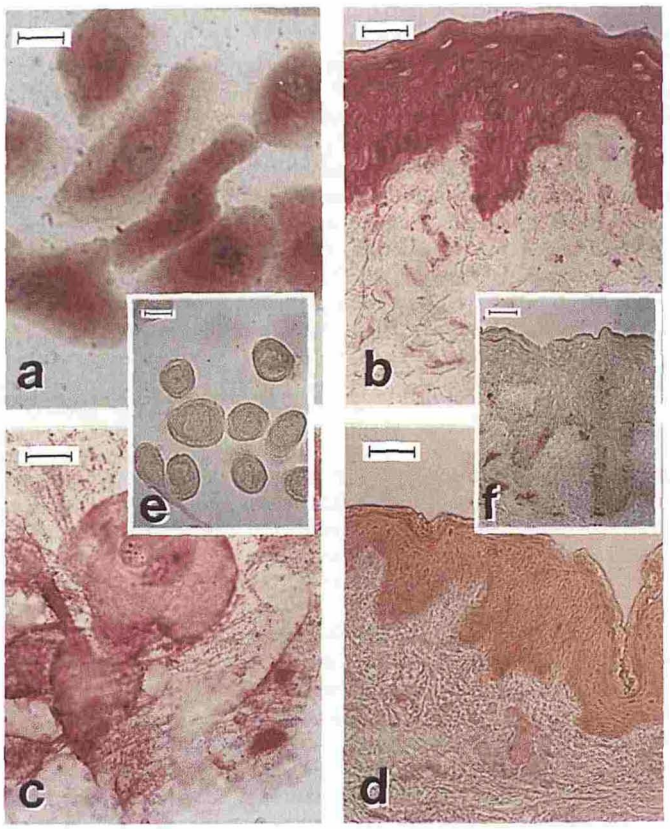

Figure 1. *Immunohistochemical visualization of keratinocyte cholinergic enzymes. The ChAT immunoreactivity is seen in the cytoplasm of cultured human keratinocytes (a) and throughout normal human epidermis (b). Tissue culture slides were counterstained with Mayer's hematoxylin to highlight cell morphology. The AChE immunoreactivity is localized on or near the cell membrane of cultured keratinocytes (c), and in normal human epidermis (d). Negative controls included replacing the first-layer anti-ChAT or anti-AChE monoclonal antibodies with i) normal rabbit serum (not shown) and ii) isotype (mouse IgG1) matching anti-CD3 monoclonal antibodies (e,f). a,b,e, bar, 20 μm; b, bar, 65 μm; d,f, bar, 50 μm.*

AChE MoAb does recognize a membrane-bound form of human AChE (type XIII; Sigma; catalogue number C-5400; lot number 81H9333) (not shown).

The specificity of keratinocyte labeling with ChAT and AChE was also confirmed by the absence of staining in cell culture and skin specimens treated with the isotype, i.e., IgG1, matching mouse anti-CD3 antibodies (Fig 1 *e,f*), and in specimens treated with rabbit serum instead of the first layer MoAb (not shown).

Activity of Keratinocyte ChAT The enzymatic activity of keratinocyte ChAT was detected by the conversion of [^{3}H]AcCoA into [^{3}H]ACh. The maximum rate (Vmax) of the synthesis of labeled ACh in disrupted keratinocytes at 37°C was about 20 pmoles ACh produced/mg protein/min, and the calculated Michaelis-Menten constant (Km) was about 9 μM (Fig 2*a*). Newly synthesized [^{3}H]ACh was detected both in cell homogenates and in the chasing media from keratinocyte monolayers fed with [^{3}H]AcCoA (Fig 2*b*). The mean rates of ACh synthesis and release by a single cell were estimated to be 2×10^{-17} mol/min and 7×10^{-19} mol/min, respectively. The presence of newly synthesized [^{3}H]ACh in both cell homogenates and culture supernatants was confirmed by TLC using purchased [^{3}H]ACh and [^{3}H]AcCoA as external standards (Fig 2*c*). As seen in the graph, the TLC profile of radioactivity in the cell homogenate and chasing medium samples corresponded to that of the standard [^{3}H]ACh preparation, whereas the radioactivity peak of incubation buffer corresponded to [^{3}H]AcCoA.

AChE decreased the radioactivity of cell homogenates and chasing media to the blank levels in the radioenzymatic assay (Fig 2*b*), and eliminated the radioactivity peaks of authentic and endogenously synthesized [^{3}H]ACh in the TLC assay (Fig 2*c*).

Activity of Keratinocyte AChE AChE activity was detected in both cell homogenates and culture supernatants. The levels of AChE were similar, i.e., 1.13 ± 0.15 U/mg protein in cell homogenates and 1.24 ± 0.2 U/mg protein in culture supernatants ($p > 0.05$). If the cultures were incubated in the presence of 0.5 mM eserine, the AChE activity disappeared.

DISCUSSION

ACh is synthesized from AcCoA and choline by the specific cholinergic enzyme ChAT. To inactivate ACh, another enzyme, AChE, hydrolyzes it to acetate and choline. In addition to its role as a mediator of the parasympathetic neurotransmission, ACh is involved in memory, skeletal and smooth muscle contraction/relaxation, heart activity, glandular secretion, and cell division, adherence and motility [6–10]. In the skin, ACh regulates eccrine sweat gland secretion [11], pigmentation [12], blood flow [13], vascular permeability [14], and mast cell activity [15]. The presence of extraneural ACh in human blood vessel endothelium [16], parotid gland epithelium [17], small intestine mucosa [18], sperm [19], and now human keratinocytes suggests that it may have a biologic role. A cholinergic, non-synaptic system for chemical transmission has been recently demonstrated in the skin of the squid *Lolliguncula brevis* [20], and, earlier, in the tube feet of starfish [21]. Although the source and metabolic pathways of the squid skin ACh remained unspecified, it was demonstrated that ACh regulates the optical properties of active iridophores in the dermis [22].

In humans, ACh acts on target cells via specific nicotinic and muscarinic receptors. Nicotinic ACh receptors are expressed in neuro-muscular junctions. mAChR are expressed on the cell surfaces of a variety of non-neuronal cell types. Some of them, e.g., fibroblasts, express mAChR in high density (i.e., 2×10^5 receptors/cell) [23], whereas others, e.g., lymphocytes, express only 6×10^3 receptors per cell [24]. Upon activation by ACh, mAChR regulate the metabolism of cyclic AMP, cyclic GMP, phosphatidyl inositol, calcium and eicosanoids, and ion (potassium, calcium, chloride) channel functions [25,26]. These same messenger molecules regulate keratinocyte proliferation, differentiation, adherence, and migration [27–30]. Different mAChR subtypes are coupled by different G proteins. These either inhibit adenylyl cyclase (i.e., Gi proteins) or activate phospholipase C (i.e., Gp proteins). There are at least five molecular and three pharmacologic subtypes of muscarinic receptors [31].

We have previously found mAChR in human keratinocytes, and shown that these receptors mediate specific effects of ACh on keratinocyte cell-cell adherence. The mean number of mAChR expressed by a single keratinocyte is 2.5×10^5, as judged by Scatchard analysis of specific binding of the non-selective high-affinity muscarinic ligand [^{3}H]atropine to intact cultured human keratinocytes at 0°C [1]. The molecular and pharmacologic subtypes of mAChR expressed by keratinocytes remain to be determined. The present study describes the other functional elements of the keratinocyte cholinergic system. Human keratinocytes appeared to contain ChAT to synthesize ACh, and AChE to degrade it.

ChAT was found in both human keratinocyte cultures and normal human epidermis. Its immunoreactivity was confined to the perinuclear area of keratinocyte cytoplasm. Keratinocyte ChAT synthesizes about 20 pmol ACh/mg protein/min at 37°C. The localization and kinetic parameters of keratinocyte ChAT are consistent with those reported for this enzyme from other cell systems. For example, ChAT is localized in the vicinity of cell nucleus of endothelial cell, apparently in the mitochondria [32]. The activity of ChAT in retinas ranges from 8 to 330 pmoles ACh produced/mg protein/min, and the reported Km values for AcCoA for the enzyme obtained from neural and non-neural tissues vary from 6 to 72 μM [33–35].

We also found AChE in keratinocyte cultures and normal epider-

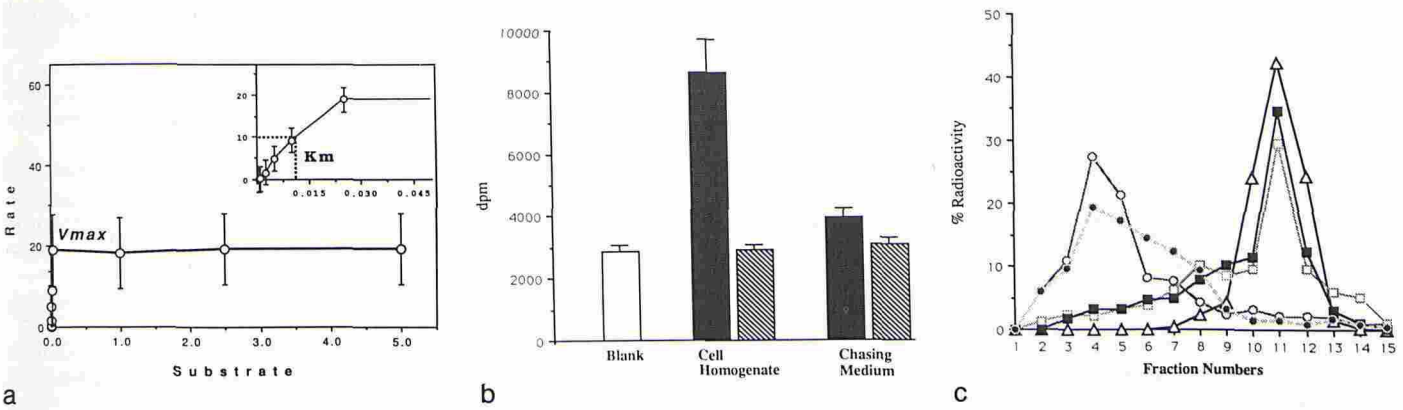

Figure 2. ACh synthesis and release by cultured human keratinocytes. (*a*) The rate of ACh synthesis by keratinocyte ChAT. The results from a representative experiment utilized homogenized 3×10^6 second-passage keratinocytes as the source of the enzyme. Abscissa: AcCoA concentrations in mM (with [^{3}H]AcCoA present as 1/50 of the total AcCoA added). Ordinate: rate of reaction, i.e., pmol ACh produced/mg protein/min. The calculated maximum rate (Vmax) was about 20 pmol ACh produced/mg protein/min, and the Michaelis-Menten constant (Km) occurred at a concentration of 9.1 μM *(inset)*. *Error bars,* SD of the data. (*b*) The amounts of newly synthesized [^{3}H]ACh in cell homogenates and chasing media. The amounts of radioactivity extracted from keratinocyte homogenates or the chasing media covering keratinocyte monolayers in the presence of eserine (■) significantly ($p < 0.05$) exceed the amount of radioactivity extracted from control samples containing incubation buffer/KRB (□). The differences in measured radioactivity represent newly synthesized [^{3}H]ACh. This is proved by a decrease of the radioactivity in samples containing AChE instead of eserine (▨) to the blank levels. Newly synthesized [^{3}H]ACh was degraded by AChE to the non-extractable catabolites. *Error bars,* SD of three independent experiments. (*c*) TLC with authentic [^{3}H]ACh and [^{3}H]AcCoA as external standards. The radioactivity peaks in experimental samples containing cell homogenate (□) or chasing medium (■) correspond to [^{3}H]ACh (△), whereas the radioactivity peak of control sample containing incubation buffer (●) corresponds to [^{3}H]AcCoA (○). Each fraction represents the radioactivity of a single piece of silica gel removed from the TLC plates starting at the load point. If the samples were pre-incubated with AChE, most of the radioactivity of authentic [^{3}H]ACh, cell homogenate, and chasing medium moved to fraction 8, which apparently represents the location of [^{3}H]acetyl covalently bound to AChE, the intermediate product of the reaction of enzymatic degradation of [^{3}H]ACh by AChE. Up to 20% total radioactivity, apparently carried by subsequent products of the reaction, moved to the last 38–56 fractions (not shown).

mis. In cell cultures, AChE was localized in or near cell membranes, and appeared to be associated with intercellular filament bundles. AChE in other types of non-neuronal cells, such as myocytes, hepatocytes, and a variety of hemopoietic cells, is also associated with the cell surfaces [36,37]. In epidermis, all living layers exhibited AChE immunoreactivity, but the basal cell layer stained more intensively. AChE is present in melanocytes as well [12]. Anti-AChE antibodies did not stain dermis. Although cholinesterase activity has been previously reported in human dermis [38], that study measured the activity of pseudocholinesterase but not true (i.e., AChE) cholinesterase.

Because keratinocyte AChE activity was detected not only in cell homogenates, but also in culture supernatants, keratinocytes apparently secrete AChE. The ability of neuronal and non-neuronal cells to secrete AChE has been demonstrated previously [39]. It was suggested that, by controlling ACh in non-neural tissues, AChE modulates cell-to-cell communication by mechanisms similar to neurotransmission [40].

Because ACh is usually associated with neural tissues, the presence of ACh in keratinocytes suggests a possible relationship with neuronal cells, which, like keratinocytes, are of ectodermal origin. On the one hand, keratinocytes produce nerve-growth factor [41] responsible for the maintenance, development, and differentiation of cholinergic neurons, including their abilities to synthesize ACh in culture [42]. On the other hand, proliferation of cultured keratinocytes can be stimulated by a product of neuronal cells, vasoactive intestinal polypeptide [43], that co-exists with ACh in parasympathetic nerves [44], and by a product of neuroendocrine cells in bovine pituitary extract [45]. Both keratinocytes and neuronal cells metabolize the same mediators, such as ACh, epinephrine [46], and enkephalins [47].

Bovine pituitary extract may conceivably contain "neural" substances such as ACh and cholinergic enzymes. Its composition is unknown. We used bovine pituitary extract to grow keratinocytes, but it did not affect the results of this study because 1) keratinocyte ChAT and AChE were visualized in frozen sections of human epidermis and 2) the activities of these enzymes were measured using KRB, which did not contain hormones, growth factors, and bovine pituitary extract.

Thus, this study provides evidence that human keratinocytes contain ChAT and AChE, and that these cells can synthesize, store, secrete, and degrade ACh. Because we have previously demonstrated that keratinocytes express specific receptors for ACh and that ACh can affect cell-cell attachment [1], we suggest that ACh acts as a regulatory autocrine/paracrine mediator. Because changes in ACh metabolism have been found in skin lesions of various dermatologic patients [12,48–51], it is conceivable that perturbations of the keratinocyte cholinergic system may be involved in skin diseases, and drugs influencing this system may be useful in treating them.

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