Agarose Gel Keratinocyte Outgrowth System as a Model of Skin Re-epithelization: Requirement of Endogenous Acetylcholine for Outgrowth Initiation

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To better understand the mechanisms of skin re-epithelization, we developed a simple technique that assays the outgrowth of human keratinocytes. Second-passage foreskin keratinocytes were inoculated at high cell density into 3-mm wells cut from agarose gels in standard 6-well tissue culture dishes. The cells settled on the dish bottom and formed a confluent colony. The cells at the periphery of the colony flattened, spread their cytoplasm, and moved away over the dish surface under the agarose gel. The morphology of migrating keratinocytes was observed microscopically through the transparent agarose, and the migration distance was measured after the gels were removed and after cells were fixed and stained. To determine which cell activities were involved in the outgrowth, the effects of cholinergic compounds on keratinocyte outgrowth were compared with their effects on keratinocyte proliferation, cell-plastic attachment, and spreading measured in separate sets of experiments. Outgrowth was inhibited by the specific inhibitor of acetylcholine synthesis bromoacetylcholine (0.05 mM) and restored by 5 mM exogenous acetylcholine. The irreversible muscarinic antagonist propylbenzilylcholine mustard (0.05 mM) abolished the restorative effects of exogenous acetylcholine and also inhibited outgrowth of intact keratinocytes. In keratinocyte cell cultures, bromoacetylcholine stopped cell division. Propylbenzilylcholine mustard increased cell number, but interfered with cell-plastic attachment and spreading. This suggests that cell-matrix attachment, spreading, and locomotion of human keratinocytes, but not mitosis, mediate the earliest stages of skin re-epithelization, and that endogenous acetylcholine regulates these keratinocyte functions. Specifically, keratinocyte acetylcholine is required to initiate outgrowth. Key words: keratinocyte migration/proliferation/attachment/spreading. *J Invest Dermatol 101:804–810, 1993*

Skin re-epithelization is an important component of wound healing. Growth factors and extracellular matrix proteins facilitate wound healing [1,2], and promote the outgrowth of human keratinocytes *in vitro* [3,4]. Cultured keratinocytes produce growth factors and extracellular matrix proteins [5,6] and respond to them *via* specific receptors expressed on their cell surfaces [7,8]. The activation of growth factor receptors leads to reorganization of the cytoskeleton [9] and stimulates secretion of extracellular matrix proteins by human keratinocytes [10]. This suggests a self-regulatory mechanism that might be involved in re-epithelization of wounds. In addition to growth factors, other recently discovered autocrine- and paracrine-acting factors can regulate the cell cycle of human keratinocytes [11]. We previously reported that human keratinocytes synthesize, store, release, degrade, and respond to acetylcholine (ACh) *via* specific muscarinic acetylcholine receptors, and that ACh regulates keratinocyte functions in an autocrine or paracrine manner [12,13].

Early stages of skin re-epithelization may require keratinocyte mitosis, cell-matrix attachment, spreading, and movement. To clarify the mechanisms mediating early stages of skin re-epithelization, we developed an *in vitro* technique that measures keratinocyte outgrowth under agarose gel, i.e., the agarose gel keratinocyte outgrowth system (AGKOS). This technique combines a previously described high-density cell culture [14], allowing rapid outgrowth of keratinocytes from the culture substrate [15], with the "under-agarose" chemokinesis/chemotaxis assay [16] frequently used to measure leukocyte migration. Because the endogenous synthesis of ACh can be abrogated by bromoacetylcholine (BrACh) [17] and the constant stimulation with endogenous ACh can be interrupted by the irreversible muscarinic antagonist propylbenzilylcholine mustard (PrBCM) [18], we used BrACh and PrBCM to determine which keratinocyte activities initiate outgrowth.

The study showed the efficiency and reliability of the AGKOS technique in assessing keratinocyte outgrowth. We found that endogenous ACh sustains proliferation and viability of cultured human keratinocytes, and controls their cell-matrix attachment, spreading, and locomotion. Apparently, all these cell functions, but not mitosis *per se*, are essential for initiation of keratinocyte outgrowth at an early stage of skin re-epithelization.

MATERIALS AND METHODS

Tissue Culture Media, Plastic Labware, and Reagents The following tissue culture products and reagents were used: the Gibco base keratinocyte growth medium (GM) containing 0.1 mM choline chloride, 6 mM glucose, and 4.5 mM sodium pyruvate (metabolic precursors of ACh), and 0.09 mM Ca^{++}, 1 mM Mg^{++}, 5 ng/ml recombinant epidermal growth factor, and 50

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Abbreviations: ACh, acetylcholine; AGKOS, agarose gel keratinocyte outgrowth system; BrACh, bromoacetylcholine; GM, growth medium; PrBCM, propylbenzilylcholine mustard; SI, spreading index.

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μg/ml bovine pituitary extract (Gibco, Grand Island, NY); minimal essential medium with Earle's salts, L-glutamine, and sodium bicarbonate (MEM; Celox Corp., Hopkins, MN); heat-inactivated newborn calf serum (NCS; Sigma Chemical Co., St. Louis, MO), 0.05% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA) (Biologos Inc., Naperville, IL); Ca^{++}- Mg^{++}-free phosphate-buffered saline (PBS; Gibco); trypan blue dye (TBD) solution (Sigma); Wright stain (Cat. No. WS16; Sigma); agarose type HSA (Litex, Denmark; distributed by Accurate Chemical & Scientific Corp., Westbury, NY); mouse IgG1 monoclonal antibody (MoAb) against human Ki-67 antigen (M 722; DAKO A/S, Denmark); fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody and mouse IgG1 isotype control (Sigma); 75-cm^2 flasks (T-flasks; Corning Glass Works, Corning, NY); 35-mm Petri dishes (Falcon 3001, Becton Dickinson Labware, Lincoln Park, NJ); 6.5-mm polycarbonate collagen-treated membrane-bottomed (pore size 0.4 μm) cell culture chamber inserts (Transwell-COL; Costar, Cambridge, MA); 6-well tissue culture plates (Falcon 3046); 24- and 96-well tissue culture plates (Corning); [^{3}H]thymidine (6.7 Ci/mmol; NEN Research Products, Boston, MA; NEN); ACh (Sigma); BrACh (Research Biochemical Incorporated, Natick, MA); and PrBCM (NEN). Prior to experiments, PrBCM was cyclized in 10 mM PBS at 30°C for 20 min to activate the aziridinum ions [19].

AGKOS Technique

Preparation of Agarose Gel: Half of a gram of agarose was dissolved in 25 ml of sterile distilled water, and the solution was boiled for 5 min, cooled in a 56°C water bath for 30 min, and then mixed 1:1 with prewarmed (37°C) GM. Four milliliters of the solution were poured into a tissue culture dish (35-mm Petri dish or a well of 6-well tissue culture plate), cooled at room temperature for 10 min, and then placed into a freezer (−25°C) for temporary storage. On the day of experiments, three wells (to allow triplicate measurements) were cut into the solidified gel using a 3-mm in diameter sterile punch with an inside bevel (kindly provided by Dr. R.D. Nelson, Department of Dermatology, University of Minnesota, Minneapolis, MN).

Keratinocyte Migration Under Agarose: Keratinocytes were obtained from trypsin-split epidermal sheets of normal human neonatal foreskins, and cultured in T-flasks in GM at 37°C in humid atmosphere with 5% CO_2 as previously described [12]. Second-passage cells from the same foreskin donor were used in all experiments. Keratinocytes were released from T-flasks by a 2-min incubation in trypsin-EDTA solution at 37°C, washed in MEM supplemented to contain 20% NCS, resuspended in GM, and counted with a hemocytometer. The cells were then concentrated by centrifugation, resuspended in GM, and loaded at high density (i.e., $2.5 \times 10^4/10$ μl) into each well of the agarose gel for a 24-h incubation. The transparency of the agarose gel allowed time-lapse microscopic video-monitoring of the cells moving over the plastic surface of the plates.

Measurement of Keratinocyte Migration Distances: Keratinocyte migration was stopped by flooding the dishes with 0.25% glutaraldehyde and cooling them at 4°C for 24 h to fix the cells. The gels were removed with a spatula, and the fixed cells were stained with Wright stain. The distance of keratinocyte outgrowth was measured in mm using a projection microscope at a magnification of 10 times. The diameter of the projection image of the initial keratinocyte colony (i.e., after the loaded cells settled on the bottom of the cutting wells) was always equal to the actual diameter of cutting wells. The edge of the well was considered to be the starting point of keratinocyte outgrowth. The projected distances between the starting point and the leading edge of the culture after a 24-h incubation were measured on six sides of each colony in mm and divided by 10,000 to obtain the actual distance of keratinocyte outgrowth in μm.

Assay of Keratinocyte Outgrowth Keratinocytes were loaded in the AGKOS plates and incubated for 24 h in GM containing 5 mM ACh, 0.05 mM BrACh, or 0.5 mM PrBCM. Control cells received GM without test compounds. After 2 h of incubation, some ACh-pretreated cells were exposed to PrBCM, and the PrBCM-pretreated cells were exposed to ACh. Some BrACh-pretreated cells were exposed to ACh and PrBCM either alone or together. The plates were returned to a CO_2 incubator for an additional 22 h to allow outgrowth.

Assays of Keratinocyte Proliferation and Viability In AGKOS plates, proliferating keratinocytes were labeled with MoAb M 722, stained by FITC-conjugated secondary anti-mouse IgG antibody, and observed under the Nikon phase-contrast inverted fluorescent microscope. To determine the effects of ACh, BrACh, and PrBCM on cell number and viability, 2.5×10^4 viable keratinocytes were inoculated in each well of standard 96-well plate and incubated for 4, 24, or 48 h at 37°C in a humid atmosphere with 5% CO_2 in the presence (experiment) or absence (control) of test compounds dissolved in GM. The numbers of the TBD-positive and -negative keratinocytes were counted after each incubation period in a hemocytometer.

Assay of Keratinocyte Cell-plastic Attachment Cholinergic effects on keratinocyte cell-plastic attachment were measured by a previously described microassay [20]. Briefly, viable keratinocytes released from T-flasks were loaded in 96-well tissue culture plates at a concentration of 2.5×10^4/well, incubated for 2 h in a humid 5% CO_2 incubator at 37°C, stained with crystal violet solution, gently washed with tap water (to remove non-attached cells), and decolorized with sodium dodecyl sulfate. The optical density (OD) of the crystal violet dye retained by the cells attached to the plastic surface of the wells was measured *in situ* at 590 nm by a computer-linked enzyme-linked immunosorbent (ELISA)-plate reader. The more cell attached to plastic, the higher the OD values obtained.

Assay of Keratinocyte Spreading To assess spreading of keratinocyte cytoplasm (i.e., cell flattening) shortly after inoculation of suspended cells, we modified a two-chamber assay of keratinocyte monolayer permeability described elsewhere [13]. One hundred microliters of GM containing 5×10^4 viable keratinocytes were added to the upper chambers, i.e., the Transwell-COL chambers, inserted into the lower chambers, i.e., the wells of 24-well tissue culture plates, containing 1 ml GM. Experimental cells were loaded in GM containing test compounds, and control cells were loaded in GM without test compounds. After a 2-h incubation, the spreading of the cytoplasm of keratinocytes attached to the dish bottom was quantified by measuring the amount of [^{3}H]thymidine that moved between the cells and across a porous membrane. The results were expressed as a spreading index (SI) calculated as follows:

$$\mathrm{SI} = 100 - \left(\frac{\text{c.p.m. in experimental cultures}}{\text{c.p.m. in control cultures}}\right) \times 100.$$

The more keratinocytes spread their cytoplasm, the less tracer passed through the membrane and the higher the SI values obtained.

RESULTS

Keratinocyte Outgrowth Under Agarose The 2-h incubation of keratinocytes loaded into wells cut into agarose gel allowed all cells to settle on the bottom of the wells and to form a culture substrate, i.e., a round megacolony of tightly packed cells (Fig. 1*A*). At this time point, actual diameters of the colonies were always equal to the diameters of the wells, i.e., 3000 μm. The cells at the edges of the culture substrate had flattened and extended fan-like cytoplasmic aprons toward the peripheral empty space. Optically dense stress fibers formed a framework for the cytoplasmic aprons (Fig. 1*B*). As most of the frontal keratinocytes had not moved yet and remained attached to the culture substrate, the migration distance at this time point was zero.

By 6 h, the size of the colonies had slightly increased (Fig. 1*C*). This increase was caused by advancement of the leading edge under agarose gel out of the well up to 100–200 μm (migration distance). Keratinocytes moving individually exhibited typical migrating patterns, i.e., they elongated and acquired a bipolar shape. Their front ends formed filopodia, whereas their back ends developed tail-like cytoplasmic processes, i.e., a ruffle (Fig 1*D*).

By 24 h, the leading edges of the colonies had advanced up to a distance of 400–600 μm (Fig 1*E*). Most keratinocytes migrated as a cellular sheet constituting the leading front (Fig 1*F*). However, some cells detached from the sheet, advanced considerably farther than others, and formed small satellite colonies (Fig 1*E*). Measurements of keratinocyte outgrowth were always made from the edge of the well to the leading edges of the megacolony.

After the cells were fixed and stained, a rim of intense cell proliferation was visible between the leading fronts and more internal parts of the megacolony. Optically dense black spots, corresponding to most rapidly proliferating keratinocytes, were seen in more central parts of the megacolony (Fig 1*E*). The presence of proliferating cells in these areas was confirmed immunocytochemically. As seen in Fig 2, the cells located in or inside a rim of intense

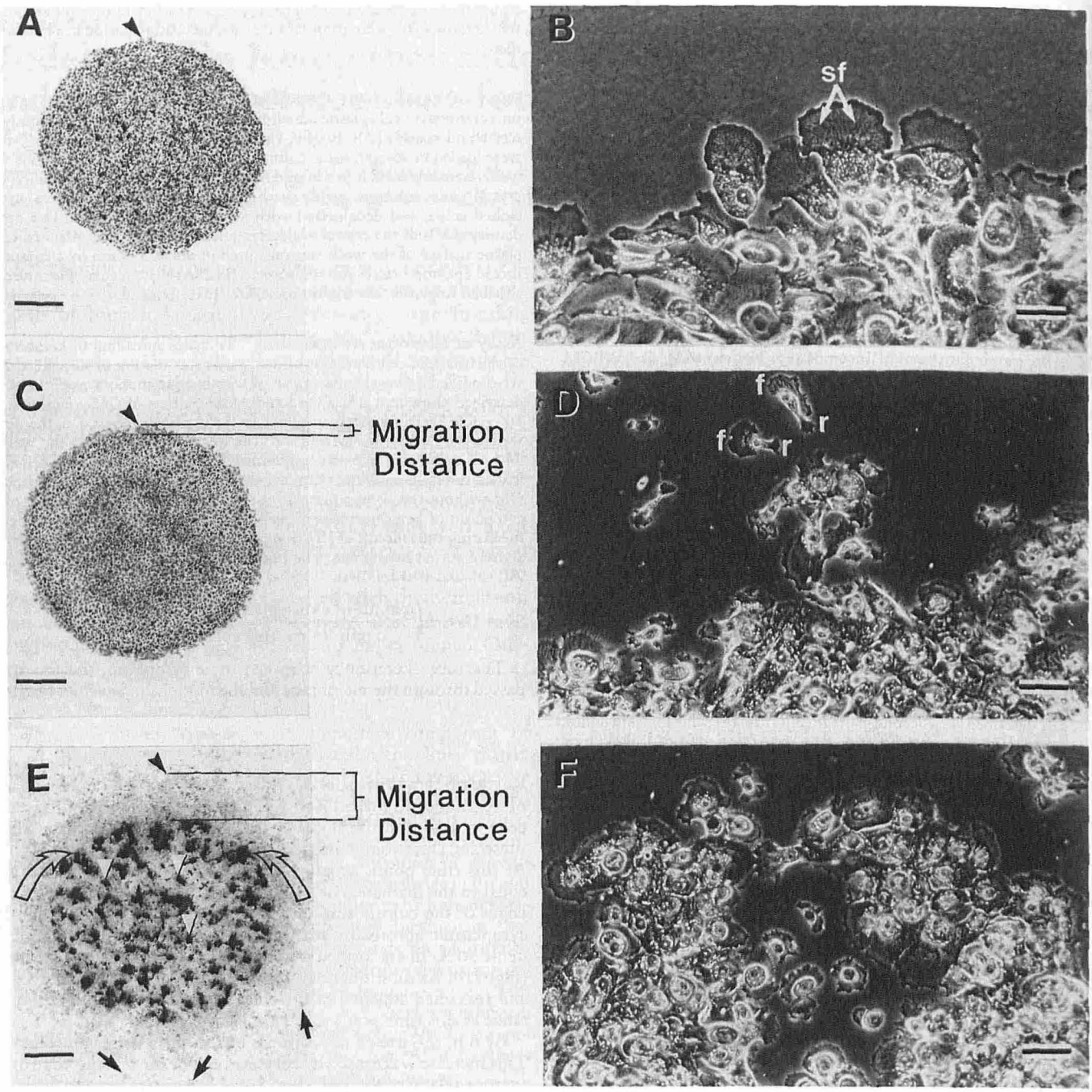

Figure 1. Keratinocyte morphology in the AGKOS plates. *A,B)* By 2 h, human keratinocytes loaded at a cell density of 2.5×10^4/well of agarose gel in 35-mm Petri dish and incubated in a 5% CO_2 incubator had formed a megacolony of tightly packed cells. The outermost cells *(black arrowhead)* flattened and spread their cytoplasmic aprons containing numerous stress fibers (sf). *C,D)* By 6 h, the leading fronts became visible at the periphery of the megacolony *(black arrowhead)*. The cells moving individually exhibited typical migrating morphology: they acquired a bipolar shape, extended filopodia (f) in their direction of movement, and formed a ruffle (r) behind. *E,F)* By 24 h, the megacolony had considerably enlarged in size. Keratinocytes that migrated as a cellular sheet formed the leading front of the megacolony, and the cells migrating individually formed satellite colonies *(arrows)* outside the leading edge of the megacolony. The most rapidly proliferating cells formed a black rim *(outlined arrows)* separating the leading front from the more central parts of the colony, where mitotic figures were seen as black spots *(white arrowheads)*. The measurements of migration distances were made by the leading edges of the megacolony *(black arrowhead)*. Light *(A,C,E)* and phase-contrast *(B,D,F)* microscopy of fixed and stained cells. *A,C,E) Bar,* 638 μm; *B) Bar,* 57 μm; *D,F) Bar,* 128 μm.

proliferation expressed Ki-67 antigen, whereas the cells located in the leading front did not.

Alterations in ACh Metabolism Interfere with Keratinocyte Outgrowth under Agarose

Abrogation of Outgrowth: Exogenous ACh caused a moderate ($p > 0.05$) increase in keratinocyte outgrowth compared to control (Fig 3). The exposure of untreated or ACh-pretreated cells to PrBCM significantly ($p < 0.05$) decreased outgrowth. The migration distance of the cells exposed to BrACh was less than half that of control cells. Exogenous ACh restored keratinocyte outgrowth in the BrACh-pretreated cultures but failed to do so in the PrBCM-pretreated cultures. When the BrACh-pretreated cells were exposed simultaneously to both ACh and PrBCM the restorative ef-

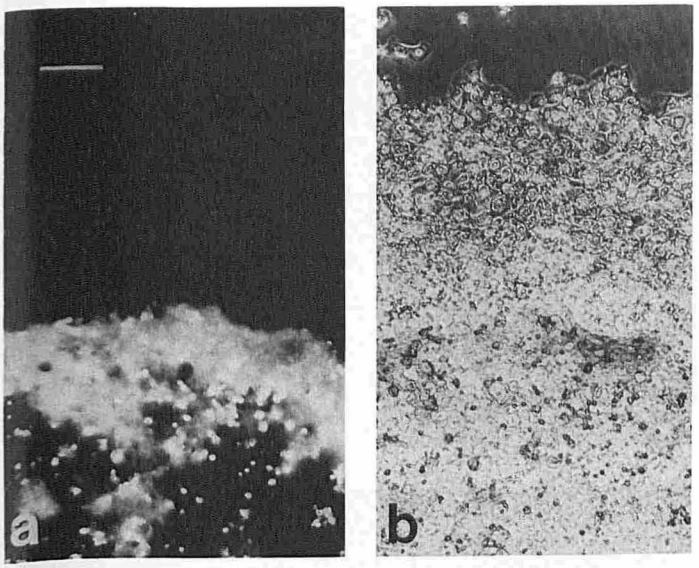

Figure 2. Localization of proliferating keratinocytes in AGKOS plates. *a)* After a 24-h incubation, the megacolony of human keratinocytes in AGKOS plate was treated with MoAb M 722, and keratinocytes expressing Ki-67 antigen were visualized using FITC-conjugated goat anti-mouse IgG antibody. Negative controls omitted the first layer MoAb or replaced it with the mouse isotype control IgG1 (not shown). Note: MoAb M 722 stained nuclei in keratinocytes localized in the rim of intense proliferation and those irregularly scattered in the more central areas of the megacolony, but did not react with cells in the leading front. *b)* The phase-contrast image of the same microscopic field. *Bar,* 319 μm.

fect of exogenous ACh on the outgrowth of these cells was attenuated. As expected, the addition of PrBCM to the BrACh-pretreated cells did not overcome the inhibitor effects of BrACh (Fig 3).

Changes in Morphology of Frontal Keratinocytes: Cell morphology of the outermost keratinocytes in the cultures that received exogenous ACh did not differ from that observed in control, non-treated cultures. In marked contrast, most of the cells exposed to PrBCM did not flatten but remained round-shaped. The leading fronts of these colonies contained only a few keratinocytes with migrating patterns but a large number of cells with typical mitotic patterns, i.e., small,

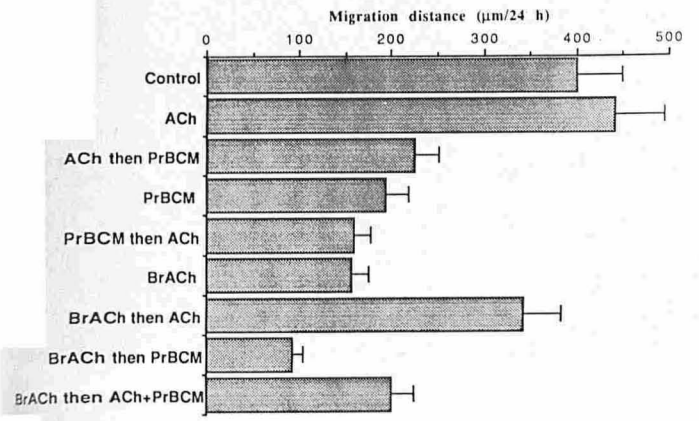

Figure 3. Cholinergic effects on keratinocyte outgrowth. Experimental keratinocytes were loaded on AGKOS plates in GM containing 5 mM ACh, 0.05 mM BrACh, or 0.5 mM PrBCM and incubated for 24 h after which migration distances were measured and compared with the migration distances of control keratinocytes incubated in GM without test compounds. After a 2-h incubation, some cultures received additional treatments as indicated. *Error bars,* mean ± SD of three independent experiments.

optically dense, ball-like clustered cells (Fig 4*A*). The addition of ACh to these cultures did not change this morphologic picture (not shown). In contrast, the majority of keratinocytes that received PrBCM after preincubation with ACh were flattened and elongated, but neither acquired the migrating pattern nor moved away from the culture substrate (Fig 4*B*).

The morphologic appearance of keratinocytes exposed to BrACh was different (Fig 4*C*). Although these cells flattened, their cytoplasmic aprons were smooth and transparent; they never acquired a bipolar shape nor enriched their cytoplasmic aprons with visible stress fibers characteristic of migrating cells. Most of these keratinocytes did not move out of the culture substrate. When PrBCM was added to the BrACh-pretreated cultures, the cells contracted their cytoplasm and rounded up but did not move (Fig 4*D*). The addition of exogenous ACh to the BrACh-pretreated cultures overcame the effects of BrACh; most of the cells acquired the migrating pattern and moved under agarose (Fig 4*E*). When these cells moved, they left empty spaces in the more central parts of the megacolonies (Fig 4*F*).

Still another morphologic pattern was exhibited by keratinocytes that were pretreated with BrACh and then received GM containing both ACh and PrBCM. Although these cells flattened, elongated, produced filopodia, and moved out from the culture substrate, most of them remained "anchored" to neighboring cells by long cytoplasmic processes that "stretched" during their migration (Fig 4*G*) instead of detaching and retracting into a trailing uropod. Proliferating cells were often seen near the leading edges of these cultures.

Cholinergic Effects on Keratinocyte Proliferation, Viability, Cell-plastic Attachment, and Spreading

Proliferation: Control keratinocytes, plated at a concentration of 2.5×10^4 on 96-well tissue culture plates, gradually increased in number during 48 h of incubation (Table I). The presence of 5 mM exogenous ACh did not change keratinocyte growth rates compared to controls ($p > 0.05$).

Keratinocyte numbers in cultures exposed to 0.5 mM PrBCM alone or to a combination of PrBCM with ACh significantly ($p < 0.05$) exceeded control numbers after the 24- and 48-h incubation periods. BrACh stopped keratinocyte division, and the presence of ACh and/or PrBCM moderated this effect of BrACh (Table I).

Viability: Neither ACh nor PrBCM, given alone or in a combination with each other, affected keratinocyte viability, whereas BrACh caused death of one-third of the cells after 24 h incubation and almost all cells after 48 h (Table I).

The presence of either ACh or PrBCM or a mixture of ACh and PrBCM in the BrACh-containing GM supported keratinocyte viability.

Cell-plastic Attachment: The OD values of crystal violet retained by keratinocytes attached to plastic surfaces of 96-well tissue culture plates after a 2-h incubation with test compounds are shown in Fig 5. As seen in the graph, among the compounds tested only PrBCM altered cell-plastic attachment. If PrBCM was added together with ACh, the effect of the former was attenuated.

Spreading: The addition of ACh increased the SI values from zero (i.e., control) to 18.5. BrACh caused only small changes in the SI values. The presence of PrBCM significantly ($p < 0.05$) inhibited spreading of keratinocyte cytoplasm (Fig 6).

DISCUSSION

We developed an experimental model for studying the mechanisms of skin re-epithelization *in vitro,* and showed that the initiation of keratinocyte outgrowth requires endogenous ACh.

Keratinocyte outgrowth can be studied in human skin explants as an *in vitro* model for wound healing [21]. However, measurable outgrowth in skin organ cultures can be achieved only after relatively long incubation periods (i.e., 3–4 d) [21–23]. By this time, the earliest and most critical events of re-epithelization may have already occurred [24]. Furthermore, keratinocytes in explant sys-

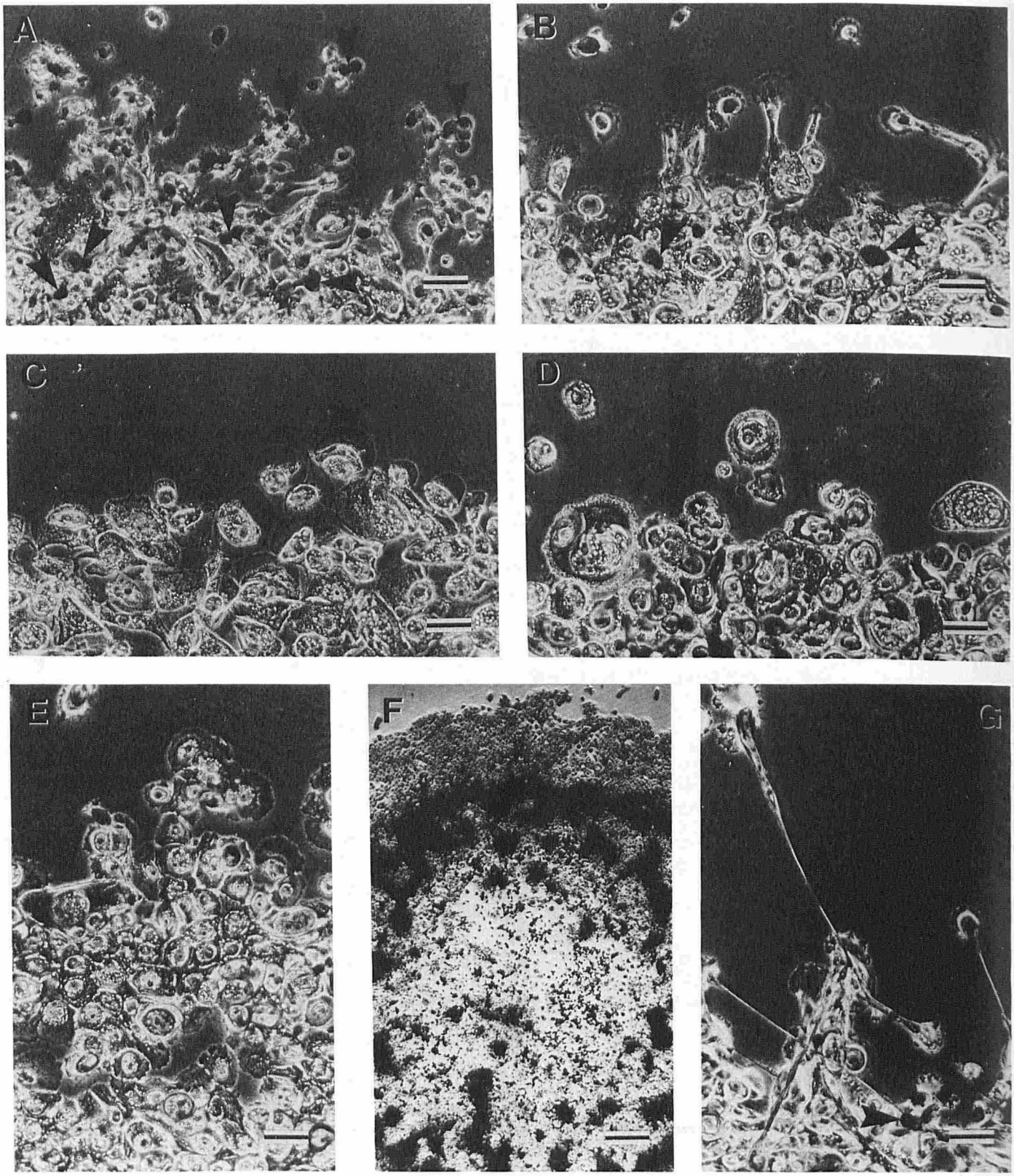

Figure 4. Cholinergic effects on keratinocyte morphology in the AGKOS plates. *A)* Incubation with PrBCM. Note round shaped, clustered, and dividing *(arrowheads)* cells. *B)* Preincubation with ACh followed by addition of PrBCM. Note elongated, bipolar cells, and several mitosis figures *(arrowheads)*. *C)* Incubation with BrACh. Note flattened but immotile cells. *D)* Preincubation with BrACh followed by addition of PrBCM. Note spread, round-shaped, and immotile cells. *E,F)* Preincubation with BrACh followed by addition of ACh. Note: the migration morphology of keratinocytes was restored (see Fig 1*F* for comparison). As cells moved away, empty spaces appeared in the more central parts of the megacolony (see Fig 1*E* for comparison). *G)* Preincubation with BrACh followed by addition of ACh together with PrBCM. Note: Although keratinocytes elongated, acquired a bipolar shape, extended filopodia, and moved forward, they remained "anchored" to the culture substrate by long cytoplasmic processes. Mitotic figures *(arrowhead)* appeared near the edge of the megacolony. Phase-contrast microscopy of fixed and stained cells. *A,B,E) Bar,* 128 μm; *C,D,G) Bar,* 63 μm; *F) Bar,* 319 μm.

Table I. Total Cell Number and Percentage of Trypan Blue Dye–Positive Cells in Keratinocyte Cultures Incubated with Cholinergic Compounds for 4, 24, and 48 h[a]

	4 h		24 h		48 h	
	Total[b]	TBD-Positive	Total	TBD-Positive	Total	TBD-Positive
Controls (no test compounds)	25.9 ± 0.8	13.7 ± 10.0	42.3 ± 2.5	8.8 ± 1.9	53.1 ± 2.7	9.8 ± 2.8
ACh	29.2 ± 1.6	10.4 ± 3.0	45.8 ± 1.4	9.3 ± 0.3	54.8 ± 2.5	10.5 ± 0.2
BrACh	25.2 ± 1.2	8.0 ± 0.4	26.7 ± 1.6[c]	34.2 ± 1.3[c]	29.5 ± 0.9[c]	96.4 ± 1.2[c]
PrBCM	24.8 ± 3.2	7.3 ± 2.3	52.8 ± 2.5[c]	8.3 ± 0.5	70.7 ± 4.5[c]	8.0 ± 0.2
ACh + BrACh	26.4 ± 2.3	8.0 ± 1.3	36.4 ± 1.6	21.6 ± 0.6[c]	42.2 ± 1.9[c]	48.9 ± 0.7[c]
ACh + PrBCM	27.5 ± 1.4	6.0 ± 0.9	53.6 ± 1.8[c]	8.6 ± 0.7	77.0 ± 1.4[c]	8.5 ± 1.1
BrACh + PrBCM	25.2 ± 0.7	6.9 ± 1.5	29.6 ± 0.8[c]	23.8 ± 1.4[c]	33.8 ± 1.4[c]	54.1 ± 5.2[c]
ACh + BrACh + PrBCM	29.7 ± 0.9	14.0 ± 1.1	29.6 ± 4.6	19.6 ± 1.8[c]	35.0 ± 0.7[c]	27.2 ± 0.5[c]

[a] Mean ± SD.
[b] Cells × 10^3/well.
[c] $p < 0.05$ compared to control; other $p > 0.05$.

tems may be influenced by cytokines and growth factors released by cells present in dermis, such as fibroblasts [25] and leukocytes [26], and by biologically active catabolites of basement membrane zone components degraded by dermal cells [27] or keratinocytes themselves [28]. Therefore, cell culture seems preferable as an isolated model to study self-regulation of keratinocyte outgrowth.

The AGKOS proved to be a reliable and highly specific *in vitro* technique for studying keratinocyte activities mediating an early stage of skin re-epithelization. It allows study of the initiation of keratinocyte outgrowth based on cell-matrix attachment, spreading, and locomotion. Both the cell population response and individual cell behavior can be studied simultaneously in one and the same assay. *In vivo,* in wounded skin, keratinocytes migrate both individually and as a cellular sheet [29,30]. *In vitro,* in our AGKOS plates, they also exhibited these two distinct migration patterns. The speed of locomotion of keratinocyte migrating as a sheet was about 0.4 μm/min, and that of individually moving cells was up to 4.5 μm/min.

Keratinocytes synthesize ACh from choline and acetyl coenzyme A at a relatively high rate, i.e., 20 pmol ACh produced/mg protein/min [12]. Keratinocytes release ACh, and ACh mediates autocrine or paracrine effects by activating muscarinic acetylcholine receptors expressed by human keratinocytes on their cell surfaces in high density, i.e., 2.5×10^5 receptors/cell [13]. Various human cells that synthesize and secrete ACh use endogenous ACh for self-regulation of mitosis [31], organization of membrane skeleton [32], cell-substrate attachment [33], and movement [34]. We found that human keratinocytes also use their ACh to regulate these cell functions. As shown by the AGKOS technique, both the inhibitor of ACh synthesis BrACh and the irreversible muscarinic antagonist PrBCM considerably diminished keratinocyte outgrowth. Exogenously added ACh restored outgrowth in the BrACh-pretreated cultures, but failed to do so in the presence of PrBCM because keratinocyte muscarinic receptors were blocked.

To clarify how BrACh and PrBCM abolished keratinocyte outgrowth under agarose, we measured the direct effects of these cholinergic compounds on keratinocyte proliferation, viability, cell-matrix (plastic) attachment, and spreading in separate assays. We found that BrACh inhibited keratinocyte proliferation but did not affect cell-plastic attachment and spreading. In marked contrast, PrBCM stimulated proliferation but inhibited cell-plastic attachment and spreading. Because the PrBCM-induced proliferation of cultured keratinocytes did not increase their outgrowth, cell migration rather than mitosis *per se* mediates the initial outgrowth under agarose. The decrease in cell density in central parts of keratinocyte megacolonies in those AGKOS plates to which ACh was added after preincubation of the cells with BrACh (Fig 4*F*) suggests that the BrACh-mediated inhibition of keratinocyte proliferation prevented the replacement by cell division of keratinocytes in areas where other keratinocytes had moved away. As shown by experiments with MoAb M 722, which labels proliferating cells [35], the actively moving keratinocytes do not divide.

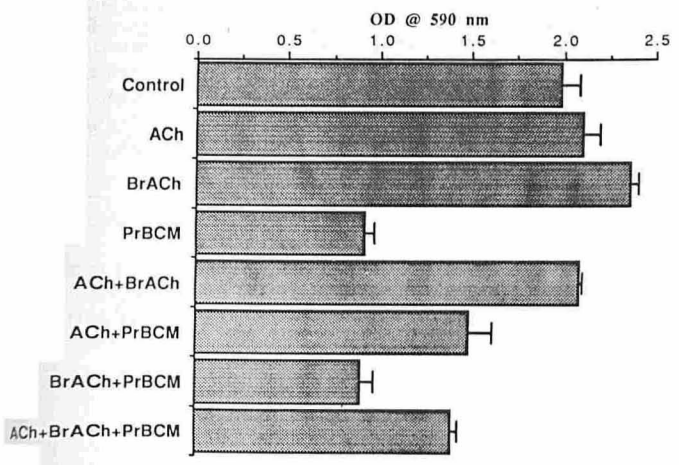

Figure 5. Cholinergic effects on keratinocyte cell-plastic attachment. The OD values were measured after 2-h incubation of keratinocytes in 96-well tissue culture plates in GM containing 5 mM ACh, 0.05 mM BrACh, or 0.5 mM PrBCM given alone or in combinations as indicated. *Error bars,* mean ± SD of three independent experiments.

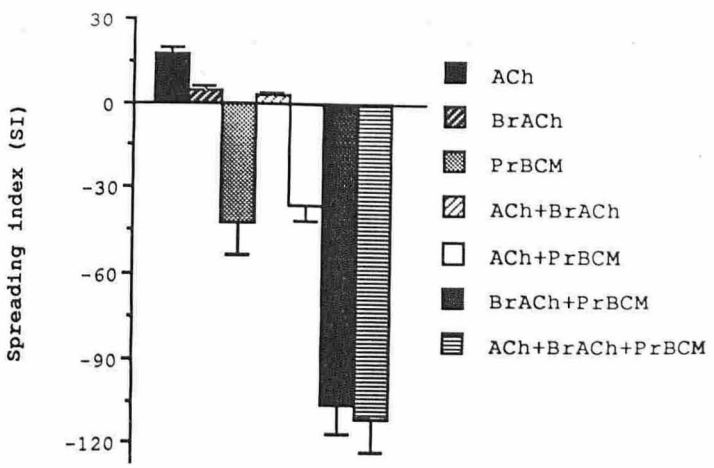

Figure 6. Cholinergic effects on keratinocyte spreading. The SI values were measured 2 h after keratinocytes were loaded in membrane-bottomed chamber inserts being suspended in GM containing 5 mM ACh, 0.05 mM BrACh, or 0.5 mM PrBCM given alone or in combinations as indicated. The SI value in control keratinocyte cultures were always equal to zero (see *Materials and Methods* for formula determining SI). *Error bars,* mean ± SD of three independent experiments.

Thus, we found that keratinocyte outgrowth was inhibited by the effects of PrBCM and BrACh on cell-matrix attachment, spreading, locomotion, and cytoplasm motility, but not by their effects on keratinocyte mitotic rates. Endogenous ACh appeared to be essential for keratinocyte viability and sustained outgrowth. This suggests that cholinomimetic compounds might be useful adjuncts to wound healing. The AGKOS is a simple and reliable technique allowing accurate measurement of keratinocyte outgrowth *in vitro*. It can be used to study the self-regulation of keratinocyte behavior at an early stage of skin re-epithelization. We used the assay to show that initiation of keratinocyte outgrowth requires endogenous ACh.

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