Cultured Human Sweat Gland Epithelia: Isolation of Glands Using Neutral Red

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INTRODUCTION

Application of current can facilitate the absorption of drugs across the skin. A model of the mechanism of iontophoresis has emerged in which it is assumed that the major pathways for ionic substances to cross the skin are via low resistance skin appendages such as hair follicles and sweat glands (1–3). To our knowledge the only direct evidence in favour of this hypothesis is recent visualization of electrically-activated follicular pores in response to iontophoresis as measured by ion-associated dye formation in hairless mouse skin (4).

The cultured human skin equivalent models consist of multilayers of keratinocytes overlying a layer of collagen type 1 matrix in which fibroblasts are embedded (5,6). Unfortunately, the drawback of such models is that they are oversimplified, as they are devoid of hair follicles, sebaceous glands and sweat glands. Cultured human testskin models are therefore unsuitable for examining the iontophoretic theory of pore pathways through the skin by follicular routes.

The aim of the present study is to improve the cultured human eccrine sweat gland model (7–10) so that adequate tissue can be produced for drug flux studies. Previous work has shown that that such cells can be grown successfully on collagen-coated Millipore filters for Ussing chamber ion transport studies. However, fresh human operation skin of appropriate size is at a premium and it has proved impossible to carry out quantifiable drug flux studies on those few glands that can be isolated and grown using existing methods. In the absence of validated sweat gland epithelial cell lines (11,12), new ways need to be found to optimize gland yield from skin pieces while maintaining cell characteristics.

We present here a novel modified isolation procedure for human sweat glands and show that the resulting higher yield of glands and their derived cells maintain similar growth, electrophysiological and pharmacological properties to normal unstained glands. Further, we report for the first time that cultured human sweat gland epithelia can now be grown successfully on Costar's Transwell-COL inserts. The first drug flux preliminary data across sweat gland epithelial sheets is also presented. The modified non-toxic isolation method described here should allow quantifiable assessment of the contribution, if any, of sweat gland derived-cells to iontophoretic pore pathways for skin permeation.

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MATERIALS AND METHODS

Isolation of Human Sweat Glands

Full thickness non-cauterized skin samples (size 1 cm × 3 cm) were taken from patients undergoing abdominal surgery. Ethical Permission was granted by the Ethics Committee of St. Vincent's Hospital, Dublin. The skin was chopped with a sharp 125 mm scissors in a physiological buffer as previously described (7). The principle is that sweat glands 'pop out' from surrounding collagen and fat due to shearing forces created by the chopping: the finer the suspension, the more the glands become accessible. The resulting suspension was poured into petri-dishes for examination under a light microscope (×20).

The following protocol was employed. Initially, all identifiable sweat glands were picked out of each dish using size 5 fine forceps. Then neutral red in PBS was added to the remaining suspension to a final concentration of 10 μM. Within 2 minutes the remaining smaller shrouded glands became apparent (Fig. 1a): they were highlighted from the background by the appearance of a diffuse red colour in the tubules of the secretory coil and by a thin dark red line in the reabsorptive duct (8,13). Neutral red is actively secreted into the lumen of the duct through organic ion pathways, similar to those originally located in the kidney cortex (14). The stained glands were placed in clear PBS and within 15 min. resumed their colourless appearance. From each subject the stained and control glands were then treated identically for primary culture.

Primary Culture of Whole Glands

Glands were transferred to William's E medium containing collagenase type 2 (2 mg ml^{-1}) and 5% fetal bovine serum. Incubation was with 5% CO_2 in air at 37°C for 30 min. After the collagen was digested the glands were allowed to recover in William's E containing only 5% fetal bovine serum for at least 60 min. Tissue culture flasks ($25cm^2$) containing exactly 1 ml of William's E plus the following additives were prepared as previously described (8,9): l-glutamine (1 mM), penicillin-streptomycin (100 U ml^{-1}, 100 μg ml^{-1}), bovine insulin (10 μg ml^{-1}), hydrocortisone (10 ng ml^{-1}), epidermal growth factor (20 ng ml^{-1}), fetal bovine serum (1%) and trace element mix (0.5%). After 60 min. equilibration the glands were explanted into flasks using A5 insect pins fitted to metal holders. Exactly 1 ml of medium was required to keep the glands moist, yet still attached to the plastic surface. The populations of stained and control glands were explanted into separate flasks and incubated with 5% CO_2 in air at 37°C.

After 24–36 hours the flasks were topped up with 4 mls medium. At this stage the glands were firmly attached. Thereafter, the cells were fed twice weekly. After 18 days the monolayers were subcultured onto collagen-coated nitrocellulose Millipore filters as previously described (9,10). In brief, aliquots of 100 μl cell suspension containing 7×10^5

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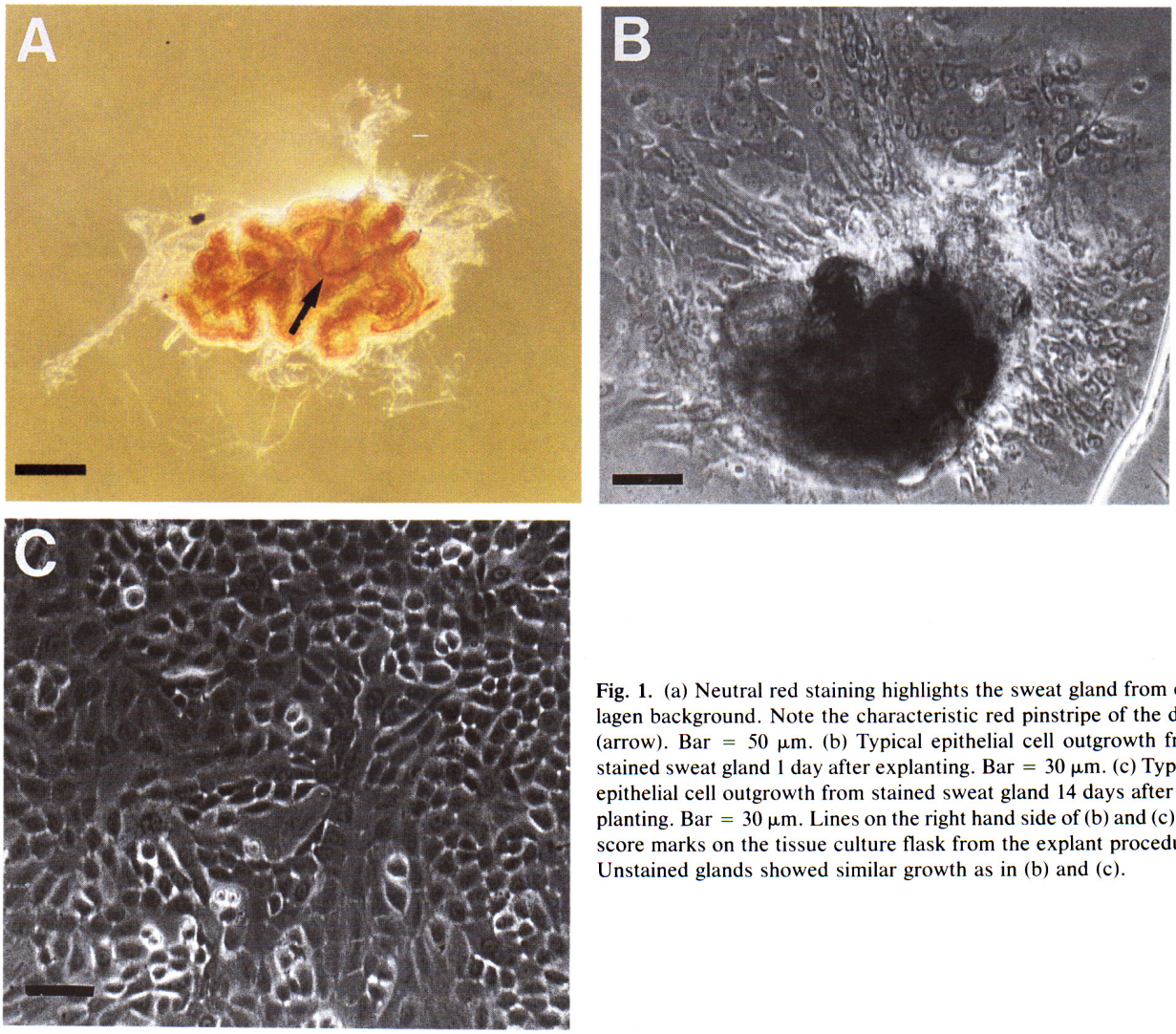

Fig. 1. (a) Neutral red staining highlights the sweat gland from collagen background. Note the characteristic red pinstripe of the duct (arrow). Bar = 50 μm. (b) Typical epithelial cell outgrowth from stained sweat gland 1 day after explanting. Bar = 30 μm. (c) Typical epithelial cell outgrowth from stained sweat gland 14 days after explanting. Bar = 30 μm. Lines on the right hand side of (b) and (c) are score marks on the tissue culture flask from the explant procedure. Unstained glands showed similar growth as in (b) and (c).

cells at a viability of 95% (Trypan Blue exclusion test) were pipetted into each filter well of area size 0.2 cm^2, enclosed by a home-made glued sylgard washer. In addition, aliquots were added to 6.5 mm Transwell-COL polycarbonate filter cup inserts (Costar Cat. No. 3427). The Costar filters have an area of 0.33 cm^2 and the collagen is a mix of type 1 and type 3. Fetal bovine serum concentration in the medium was increased to 5% for 24 hours in order to promote attachment to the filters. All experiments were carried out at day 5–8 after subculturing onto filters.

Short-circuit Current Recording

After 5 days growth on both the Millipore filters and the Transwell-COL inserts, the epithelial sheets were clamped between the two halves of a side-by-side diffusion chamber (15). The volume on each side was 9 mls. Edge damage was avoided because the epithelial sheet was placed in the centre of the chamber window (area 0.63 cm^2) surrounded by a silicone washer. Epithelial sheets grown on Transwell-COL filters were placed in culture cup inserts (area 0.63 cm^2) and mounted in the same size diffusion chambers. The tissues were voltage clamped using a W.P.I. dual voltage clamp (Model DVC 1000) according to previous descriptions (16).

Nicotine Absorption Fluxes

Drug absorption studies were carried out using nicotine (0.1–10 mM) added to the donor apical side. Samples (200 μL) were withdrawn from both the apical and basolateral side every 30 min for 4 hours. Nicotine was measured by HPLC analysis with u.v. detection using a μBondapak C18 column (Millipore): the wavelength was 254 nm and the mobile phase was a mixture of acetonitrile (conc.)/sodium n-decylsulfonate (0.2 M)/sodium acetate (1 M)/water (200/40/75/685 (v/v/v/v)) at a flow rate of 1.5 ml/min. The apparent permeability coefficient (P App) was calculated from the following equation (17).

$$\log (1 - 2C_r/C_0D) = -2A\,(\text{P App})\,T/2.303\,V \quad (1)$$

where C_r = concentration of nicotine (mM) in the receptor compartment at time t, C_0D = concentration of nicotine (mM) in the donor compartment at time zero, A = effective area of the Transwell-COL (cm^2), T = time (seconds), V =

volume in either the donor or receptor compartment (cm^3). The slope of the plot of log ($1 - 2C_r/C_0D$) against T was used in the equation to calculate the P App.

Chemicals and Solutions

Krebs-Henseleit solution was used for Ussing chamber ion transport and drug permeation studies. It consisted of (mM): NaCl 117; KCl 4·7; $NaHCO_3$ 25; $MgSO_4$ 1·2; $CaCl_2$ 2·5; and glucose, 11·1. The pH was 7·4 when gassed with carbogen. All tissue culture reagents were obtained from Gibco (Scotland). Amiloride, carbachol and sodium acetate were obtained from Sigma (UK). Acetonitrile (HPLC grade) was supplied by Labscan (Ireland).

RESULTS AND DISCUSSION

Neutral red staining allowed on average 52% more sweat glands to be isolated from the chopped suspension of human skin. In each of 4 separate skin samples the staining protocol improved the total yield of glands obtained (Fig. 2a). In short, even if the skin is not chopped as fine as possible, the stain will pick up and identify the remaining glands. This is particularly useful in the case of small and fatty glands which would normally be impossible to visualize. Moreover, the stain allows less experienced researchers to be certain of gland identification, while at the same time it reduces the time required for this laborious procedure.

The neutral red-stained glands grew in culture just as successfully as controls. On average 77% of the explanted control glands produced a monolayer culture while the value was 70% for stained glands. There was no statistical difference between the two groups. The data in Figure 2b shows

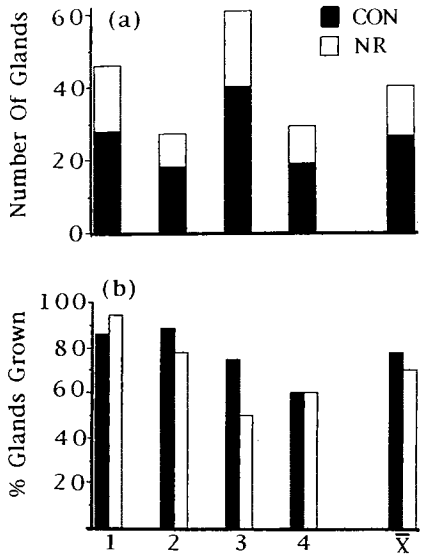

Fig. 2. Neutral red increased the yield of sweat glands isolated from skin and had no toxic effect regarding the number of glands that grew. (a) In each of 4 subjects the unstained glands were first picked out from chopped skin debris. The remainder were then visualized after staining and were picked out. Mean number isolated from the 4 subjects under each condition is given on the right. CON (Control); NR (neutral red). (b) The percentage number of explanted glands that grew in culture in each of 4 subjects is illustrated. Mean percentage grown is given on the right.

that in one subject the stained glands grew better while in another there was no difference in growth. An important point is that the stain was used to find glands after the healthiest and largest unstained ones had been removed: even when there was a bias in favour of the unstained glands, the stained glands grew as well as controls. In Fig. 1b and 1c the epithelial cell growth from neutral red-stained glands at day 1 and day 18 is shown. There was no feature of their growth that was different from controls. The cellular differentiation from both groups was identical in degree and rate. The concentration of 10 μM for neutral red was selected because it is similar to that used for 24 hour cytotoxicity testing in cultured rabbit skin (18).

Previously, it was shown that cultured epithelial sheets from human sweat glands exhibited the sweat duct ion transport property of electrogenic sodium absorption (9,10). The characteristic signature of this process is the inhibition of short-circuit current (Isc) by addition of low concentrations of the epithelial sodium channel inhibitor amiloride to the apical side of the preparation. Further, it was previously shown that the cholinomimetic agent, carbachol, stimulated amiloride-sensitive Isc in cultured human sweat gland epithelia. In both control and neutral-red derived epithelial sheets carbachol stimulated Isc and amiloride inhibited the most of the basal and the stimulated current. A sample tracing from each group of 6 epithelial sheets is shown (Fig. 3a). On average the basal electrophysiological parameters of each group were similar: the basal Isc was close to zero, the mean transepithelial resistance was approximately 70Ω cm^2 and the mean potential difference was approximately 0.5 mV, apical side negative. These values are in the range of those previously reported (9,10). Note that these results include the pooled data from sweat gland cells grown on both collagen-coated Millipore filters and from the Transwell-COL inserts. Neutral red therefore does not alter the electrophysiological characteristics of the derived cultures.

The final criterion was to show that both groups had a similar permeation to nicotine. The well studied nicotine was chosen as a permeant because it seemed premature to approach the complicated variables associated with peptide fluxes (19) in such an experimental system. In 8 epithelial sheets from the skins of 4 subjects examined for each group the P App for nicotine was not different between controls and test epithelial sheets (Fig. 3b). Again, the results include pooled data from both type of filter system. The P App for nicotine from cultured human sweat glands is approximately 10 fold higher than that of in vivo human skin (20). Other cultured human skin models also give higher drug permeability constants than human skin (21), even though rank order relationships are comparable.

It has been known for some time that neutral red can be used to highlight features of the human sweat gland. Kealey and colleagues used the stain to help prove that microdissected secretory coil and absorptive duct were isolated cleanly from the transition region of the whole gland (8). The present study provides convincing evidence that use of low concentrations of the dye for a short period can improve upon the previous isolation procedure for whole sweat glands (7). The healthiness of the stained glands is further indicated by their ability to pump away the dye from the ductal lumen when placed in fresh buffer (not shown).

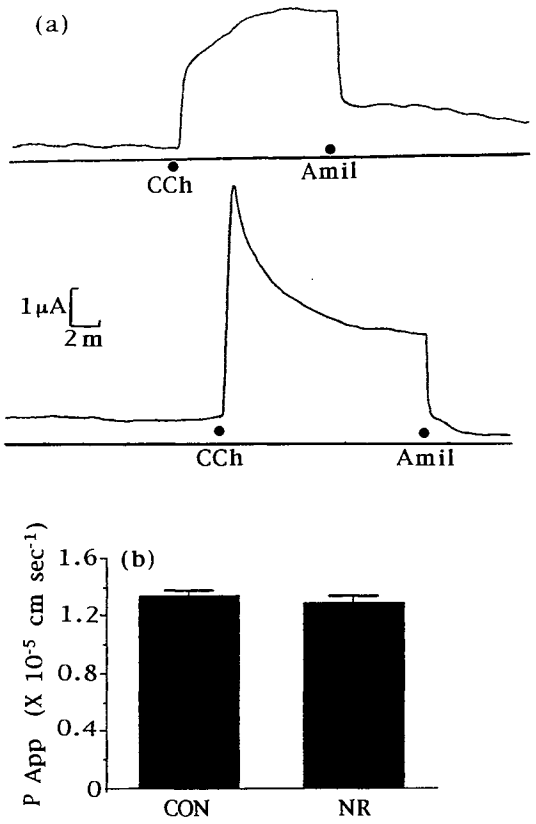

Fig. 3. Neutral red-derived cultured sweat gland epithelial cells express similar electrophysiology and drug permeation properties as controls. In (a) carbachol (CCh, 10 μM) was added to the basolateral side of an 8 day old epithelial sheet derived from unstained (upper trace) and stained (lower trace) glands. Subsequent to the stimulation of an inward current, amiloride (Amil, 10 μM) was added to the apical side of both preparations and inhibited Isc. Baselines denote zero μA. Both recordings were from cultures grown on 6.5 mm Transwell-COL inserts. Responses are typical of each of 6 cultures from both conditions. In (b) the absorption of nicotine (0.1–10 mM) was tested in both types of preparation (n = 8, both groups) after addition to the apical side. CON, control epithelial sheets; NR, neutral red. There was no difference between the two groups (ANOVA).

Others isolate sweat ducts from skin by overnight incubation of skin with collagenase followed by epidermal stripping (22,23). These methods are unsuitable for whole gland isolation and, moreover, do not produce many cells in culture by comparison with the present method. Furthermore, the requirement to microdissect eccrine sweat glands into coil and duct is questionable in view of previous work showing that cultured coil, duct and whole gland express at least one common electrophysiological phenotype, namely that of ductal electrogenic sodium absorption (9).

The electrophysiological and drug flux results from all sweat gland epithelial sheets grown on Millipore filters or Transwell-COL inserts were similar, allowing data pooling. For the first time sweat gland epithelia have been grown on commercially available inserts, which in turn are coated uniformally allowing porosity to be retained throughout. The area of 0.33 cm^2 is the largest growth area yet reported for sweat gland cells grown on supports: areas of 0.07 cm^2 are more typical, but so small that accurate resistance measurements are difficult to record (24).

In summary, the neutral red method has been shown to be non-toxic under the criteria tested. Using this optimized procedure, we are now ready to answer questions of whether the permeation properties of sweat gland epithelia in culture, tested with a range of drugs and electrical parameters, are relevant to either the route of penetration or the barrier properties of human skin in vivo. Other applications of the method could include the screening of antiperspirants for toxicity when these agents are applied as a pad on filter-grown sweat gland cells.

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