Alteration of Cytokeratin Expression Following Transdermal Lidocaine Hydrochloride Iontophoresis

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INTRODUCTION

Successful transdermal drug delivery systems are those which achieve therapeutically efficacious concentrations into the dermal vasculature thereby avoiding gastrointestinal digestion as well as first-pass metabolism in the liver encountered by oral doses. These systems provide potentially pain-free access to the circulation in contrast to the parenteral route. However, many compounds which might prove to be efficacious cannot traverse the lipophilic skin barrier because they are large, polar peptide molecules. Electrically driven transdermal drug delivery has been applied for some time in attempts to deliver such compounds across the skin (1). The premise of such systems is the use of an electromotive force to drive polar molecules into the underlying dermal vasculature, allowing vital access to the systemic circulation. Laser scanning confocal microscopic results have implicated the transappendageal (hair follicles) route as a major source of iontophoretic transport despite the relatively small proportion of the skin surface area that hair follicle comprise (2). Furthermore, the pathway of iontophoretic current flow has been shown to follow the transappendageal route as evidenced by vibrating probe electrode results (3). However, several studies have provided evidence of an intercellular pathway of charged molecules following both *in vitro* and *in vivo* iontophoresis (4,5,6). Iontophoresis clearly provides a potential mechanism for the successful transdermal delivery of pharmaceutical compounds. However, during its continued development and refinement, concern must be given to ultrastructural and biochemical alterations which might occur in the skin as a result of the application of an electric current across the skin surface, and these alterations may provide insight into the mechanism of delivery.

Lidocaine iontophoresis has been shown to induce epidermal changes in both *in vivo* and *in vitro* pig skin (7,8). These findings indicated an alteration of the tonofilaments in the epidermis, representing the first evidence of damage following iontophoresis. The same alteration has been demonstrated in the isolated perfused porcine skin flap (IPPSF) thus eliminating the possibility of an immunological etiology. Furthermore, in both *in vivo* and *in vitro* systems, the severity of damaged correlated strongly with increased current intensity and duration.

Keratinocytes compose approximately 80% of the cells in the epidermis, and 85% of the total protein content of keratinocytes is keratin, the most diverse of the cytoskeletal proteins. Each keratinocyte contains bundles of intermediate filaments that are 8–10 nm which contact neighboring cells at junctional complexes, desmosomes and hemidesmosomes (9). This intercellular continuity suggests an important role in the maintenance of keratinocyte structural integrity by the intermediate filaments. Keratin expression and keratin filament integrity are major factors affecting the defensive barrier created by the epidermis. Keratins are selectively expressed in the different layers of the epidermis and represent a functional progression rather than merely being stratified histologically (10). Suprabasal keratinocytes primarily contain keratins K1 (56.5 kDa) and K10 (67 kDa). These high molecular weight keratins are produced only in the sufficiently-differentiated cells found in layers superficial to the stratum basale. Keratins K6 (48 kDa) and K16 (56 kDa) are expressed in suprabasal cells only during a repressive state of K1 and K10 expression (i.e., following injury or hyperproliferative disorders). While they are synthesized in the stratum basale (composed of cells which are least-differentiated in the epidermis), the low molecular weight keratins K5 and K14 are also expressed throughout the suprabasal layers (11).

Clearly, keratin plays an essential role in keratinocyte cytoskeletal architecture. This study was performed to evaluate the effects of iontophoresis on keratin expression in the skin. Adversely affecting the expression of keratin polypeptides or the integrity of the intermediate filaments has been shown to effect the functional properties of the skin and ultimately lead to various dermatological disorders (10). Therefore, concern should be given to iontophoretic effects on keratin.

METHODS AND MATERIALS

Seven female Yorkshire pigs were sedated with xylazine/ketamine. All pigs were clipped (Oster electric clippers, size 40 blade) 24 hr prior to treatment. Active electrodes, saturated in 4% lidocaine hydrochloride by immersion for two minutes, were placed on either side of the ventral surface of the pig, just caudal to the umbilicus in the inguinal area. Indifferent electrodes, saturated with phosphate-buffered physiological saline, were attached caudal to the active electrodes on either side. Current was delivered to the electrode at varying current intensity and duration (1.0 mA = 100 $\mu A/cm^2$ and 3.0 mA = 300 $\mu A/cm^2$ for 10, 45, and 100 min). Passive control electrodes consisted of no applied current. Two 6 mm punch biopsies were extracted from an area under the active electrode site and placed in half-strength Karnovsky's fixative. Tissue samples were then processed through a graded ethanol/xylene series and embedded in paraffin. Paraffin embedded tissues were sectioned at 6–8 μm on a rotary microtome, mounted on positively-charged microscope slides, and stained with hematoxylin and eosin (H&E). Archival, paraffin-embedded samples from an identical iontophoretic protocol for lidocaine delivery in the isolated perfused porcine skin flap (IPPSF) (7) were used to evaluate the same endpoints in a closed system. To compare lidocaine

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iontophoresis with that of another charged compound, paraffin-embedded samples from a previous study on *in vivo* iontophoresis of luteinizing releasing hormone (LHRH) (1mg/ml) were used (12). For cytokeratin localization, immunohistochemical protocols using monoclonal antibodies directed against 3 individual cytokeratins 10, 14, and 16 (Biomeda), and one pan-cytokeratin (K1, 5, 10, 14, Enzo Diagnostics) were used. Briefly, 6 μm paraffin sections were rehydrated and antigen unmasking was performed by incubation in 0.1% trypsin. Endogenous peroxidases were quenched using 3% hydrogen peroxide. After blocking nonspecific binding sites with normal horse serum, primary antibodies were added and incubated at 37°C (pan cytokeratin) or at room temperature (all others) in a humid chamber as per manufacturer specifications. Indirect immunoperoxidase staining was performed using biotinylated horse antibodies against mouse immunoglobulins (Vector Labs) as secondary antibodies. An avidin-peroxidase coupled tertiary antibody (Chemicon) was then added and visualization was achieved colorimetrically by incubation in Stable DAB solution (Research Genectics). Staining intensity was recorded for each sample and photographed using an Olympus BH-2 light microscope equipped with a PH-10ADS automatic photomicrographic system.

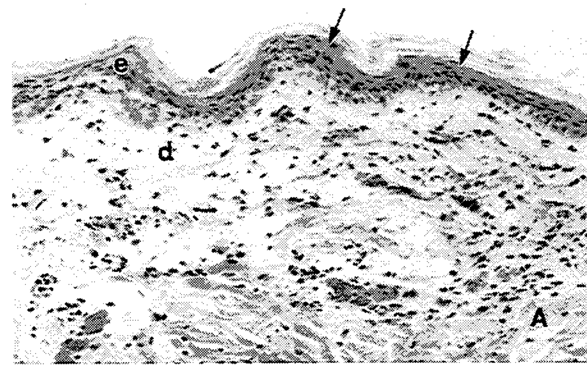
In conducting research using animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publication 85-23, revised 1985).

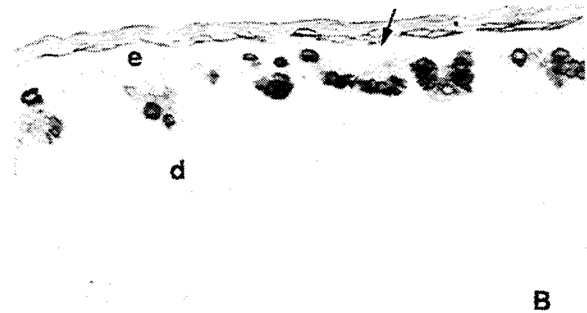
RESULTS

As previously reported (7), focal microscopic alterations characterized by dark basophilic staining nuclei and eosinophilic staining of the cytoplasmic layer was seen only at epidermal sites directly underneath the active lidocaine electrode (Fig. 1A). Severity of damage correlated to increasing current intensity and duration. Similar alterations were seen in the LHRH treated animals, as well as in the lidocaine treated IPPSFs.

In areas of severe iontophoretic epidermal damage (i.e., extending to the stratum basale) which was indicated by H&E staining, intense cytokeratin staining (pan keratin, K10 and K14) was present at the stratum granulosum. Cell layers below the stratum granulosum at these focal regions of alterations either remained unstained or lightly stained (Fig. 1B and 1C). In addition, underneath the active electrodes at non-specific areas of the epidermis, "hotspots" of intense staining were seen in both the stratum basale and the stratum spinosum layers (Fig. 1B). These cell layers corresponded to areas with extensive intracellular epidermal edema as observed by H&E light microscopic sections. Similar anti-pan-cytokeratin staining patterns were seen in the lidocaine treated IPPSF samples (Fig. 1D), and in the LHRH treated animals (Fig. 1E).

Figure 2A represents an area of normal pig skin showing no damage to the epidermal cells. Homogeneous cytokeratin staining of the epidermal layers occurred only at these areas which were devoid of the iontophoretic alteration. Anti-pan-cytokeratin staining was seen throughout all cell layers of the epidermis (Fig. 2B). Although staining intensity was reduced, anti-cytokeratin 14 staining was seen throughout the stratum basale cell layer. Anti-cytokeratin 10 staining was seen throughout all suprabasal cell layers (Fig. 2C). No staining for cytokeratin 16 was observed in any of the epidermal cell layers.





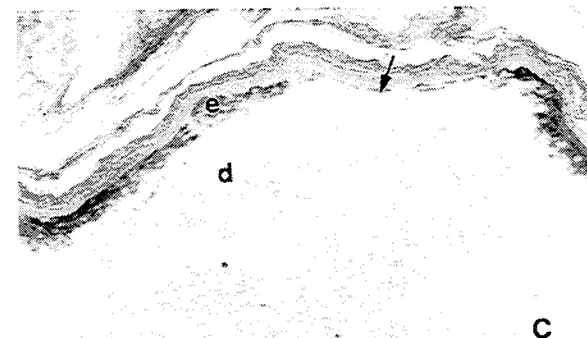


Fig. 1. Staining pattern of a focal epidermal alteration (arrows) produced by lidocaine hydrochloride iontophoresis. (A) H&E. (B) anti-pan-cytokeratin staining within the epidermis corresponding to a focal epidermal alteration. (C) anti-cytokeratin 10 staining within the epidermis corresponding to a focal epidermal alteration. e, epidermis; d, dermis. 495X.

CONCLUSIONS

Structural integrity for the cell is provided by a converging of two filament systems, the nuclear envelope proteins (e.g., laminins) and cytoplasmic intermediate filament proteins (e.g., cytokeratins), on the nuclear pore (13). Therefore, keratins play an important role in the maintenance of mechanical integrity

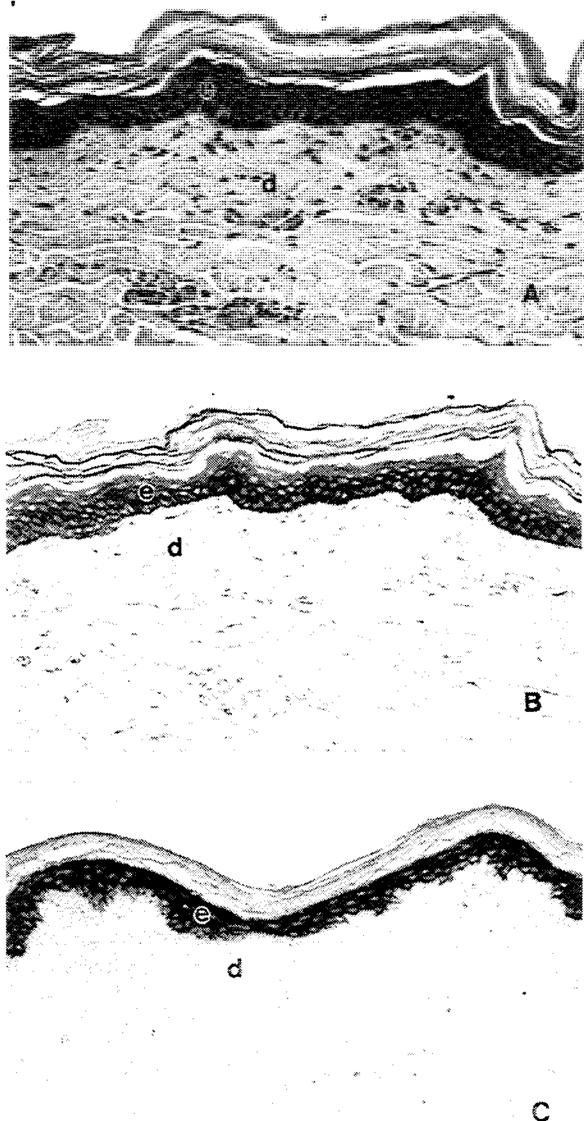


Fig. 2. Epidermal staining pattern in normal pig skin: (A) H&E. (B) Homogeneous anti-pan-cytokeratin (K1/5/10/14) staining. (C) Homogeneous anti-cytokeratin 10 staining. e, epidermis; d, dermis. 495X.

in many epithelial tissues. Fuchs *et al.* (14) have used transgenic mice models to further endorse this function in mouse skin. Keratinocytes compose approximately 80% of the cells in the epidermis and keratinocytes contain bundles of intermediate filaments that are 8–10 nm in diameter. These filaments help play a major role in the forming the cytoskeletal architecture and thus contribute to the barrier function of the skin. Approximately 85% of the total protein content of keratinocytes is keratin, the most diverse of the cytoskeletal proteins. These immunohistochemical investigations on porcine skin that has been subjected to iontophoresis indicate that there is a correlation between the iontophoretic alterations in the skin and cytokeratin expression. Monoclonal antibodies to K1, 5, 10, 14 and 16 have been utilized. The absence of staining with anti-cytokeratin 16 could indicate that hyperplasia is not induced. Alternatively, this could also reflect poor interspecies cross-reactivity to the anti-human antibody.

These results provide several possible interpretations regarding the alterations created by iontophoresis observed by both light and transmission electron microscopy (7). These cytokeratins may be altered in such a way that the antibody is either no longer recognizing them (no stain), or is more effectively recognizing them (intensely-stained hotspots). The expression of these proteins may be enhanced (intensely-stained hotspots) or inhibited (no stain). These cytokeratins could be modified by iontophoresis such that induction of chronic effects on the keratinization process occurs.

Previous studies using saline iontophoresis have predicted that electrical current alone does not appear to alter the biochemical homeostasis of the skin (15). However, an electrical etiology has been proposed for these cytoskeletal alterations based on several conclusions. By performing the same immunohistochemical stains on IPPSF samples that were subjected to an identical iontophoretic treatment protocol, an immunological etiology was eliminated as an identical staining pattern to the *in vivo* samples was seen. A lidocaine specific etiology was ruled out by staining samples iontophoresed with luteinizing releasing hormone (instead of lidocaine), which had similar morphological alterations, with anti-pan keratin antibodies creating an identical staining pattern.

This immunohistochemical data represents modification in protein expression, but does not elucidate the biochemical mechanism of the modification. Keratin plays such a vital role in epidermal integrity that these results warrant further investigation. The possibility of transcriptional modifications caused by iontophoresis needs to be addressed in future studies by investigating changes in mRNA levels. Finally, potential damage to the dynamics of cytokeratin filament assembly must also be explored by examining the chronic effects of such alterations to determine if cytokeratin expression is restored.

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