Functional Evaluation of Anchored Skin Equivalent Cultured *in Vitro*: Percutaneous Absorption Studies and Lipid Analysis

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INTRODUCTION

In vitro cutaneous models, resulting from recent progress in human cell culture, provide new tools to investigate the development, physiology and pharmacology of the human skin. Indeed, several skin equivalents (SE) have been produced by seeding keratinocytes upon various dermal substrates. One consists of de-epidermized dermis without living fibroblasts but exhibiting an intact basement membrane (1,2). Living dermal models could be obtained *in vitro* by growing dermal fibroblasts on a nylon mesh or in a sponge such that they deposit extracellular matrix (3,4). The reorganization and contraction of collagen matrix by included fibroblasts also results in a tridimensional dermal layer (5). We adapted the latter method to produce an anchored skin equivalent (ASE) combining the following advantages: i) a large number of similar ASE could be generated with cells cultured from a single skin biopsy. Therefore, this obviates the need for a constant supply of human skin. ii) Replicates could be submitted to quality control during their production. iii) This anchorage design impeded collagen gel contraction thus leading to the production of a SE with a large and defined surface area. iv) The absence of any supporting or embedded material into our ASE facilitated its manipulation and prevented any eventual deleterious effect of foreign matter. Furthermore the absence of any extraneous material avoids an eventual adsorption effect (6).

These various SE models shown an organized epidermal layer (1,3,5,6). Further differentiation of the epidermis (with a multilayered stratum corneum), histologically similar to its *in vivo* counterpart, appears when the SE are cultured at the air-liquid interface (1,3,6–8). These SE display barrier function as measured by percutaneous studies absorption (1,3, 6,8). They can discriminate the absorption rate of different substances and rank them in the same order than normal human skin. However, quantitatively, they are more permeable than intact skin (1,3). Their lipid analysis revealed that these SE have the capacity to synthesize all lipid species present in native tissue and that they contain significant amount of ceramides. The latter are characteristic of differentiated cells and thought to be the most important lipid class for the barrier function (2). A lipid structural organization similar to that of native skin appears in SE cultured with appropriate culture conditions (2). Therefore, the cause for the quantitative difference in percutaneous absorption between SE and intact skin is not clear.

This study was undertaken to compare lipid profile, lipid content and percutaneous absorption properties of ASE with those of human and mouse skin. Our results indicate that the greater permeability of ASE reflects their lower content in ceramide per cm^2 of surface area compared to mouse and human skin.

MATERIALS AND METHODS

Chemicals. ^{14}C-Benzoic acid (22 mCi/mmol), ^{14}C-caffeine (59.4 mCi/mmol), ^{3}H-hydrocortisone (54.5 Ci/mmol), ^{14}C-sucrose (4.2 mCi/mmol) and ^{14}C-urea (7.8 mCi/mmol) were purchased from NEN (NEN Dupont, Mississauga, Ontario, Canada). Bovine serum albumin (BSA), phosphatidylcholine, phosphatidylethanolamine, cerebroside II, types III and IV ceramides, oleic acid, cholesterol sulfate and cholesterol were obtained from Sigma (Sigma).

Cell and Skin Equivalent Culture. Human keratinocytes and dermal fibroblasts were obtained from normal adult skin specimens removed during reductive plastic surgery, and used at their 4th passage (9,10). Keratinocytes were seeded at $2 \times 10^5/cm^2$ on an anchored dermal equivalent to produce a SE which was raised at the air-liquid interface 24h after confluence (6). Twelve days later, the samples were used for percutaneous absorption, histological, and lipid analysis (6).

Hairless Mouse and Human Skin. Hairless mouse abdominal skin excised without fat and abdominal human skin obtained from plastic surgery (dermatomed at 750 μm) were frozen at −70°C for 24h, and stored in liquid nitrogen until use.

Permeability Studies. Percutaneous absorption was measured using standard Franz diffusion cell technique (6,11). Hundred μl of the labeled drug diluted in 0.9% NaCl was deposited in the donor compartment. The radioactivity present in the dermal bathing solution (NaCl 0.9%; except for hydrocortisone and sucrose, NaCl 0.9%-BSA 1%) was evaluated at selected intervals (1h, 2h, 4h, 6h, 8h and 24h). The radioactivity present in the epidermis and dermis was determined after digestion with NCS II (Amersham, Ont., Can.) (6).

Lipid Extraction and Analysis. Lipid were extracted from epidermal layers of human skin, hairless mouse skin and ASE after Ponec *et al* (11). The pellet obtained was weighted for determination of the total lipid content per cm^2 of epidermis, and dissolved in chloroform at a final concentration of 100 mg/ml. A constant amount of total lipids (100 μg) were separated on high performance TLC (HPTLC) with

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the ceramide development system (11). The compounds were identified by their respective Rf value in comparison with lipid standards using an imaging densitometer (Bio-Rad Lab. Ltd, Mississauga) equipped with computer and software for quantitative evaluation of each lipid.

RESULTS

Histological Analysis. Histological analysis of the epidermal layer of the ASE revealed a stratified and differentiated epidermis (Fig. 1).

Permeability Analysis. The absorption profiles obtained using the ASE were similar to those determined in mouse skin but the extent of absorption was always greater with the ASE. Human skin was much less permeable than the two other systems for any of the compounds used (Fig. 2). Considering the limited number of data in the linear portion of the curve, the Kp values were not calculated.

Benzoic acid and caffeine penetrated the ASE without lag-time and the amount absorbed after 4h reached 45% and 80% respectively. After 24h, the two compounds had entirely penetrated through the ASE, whereas 65% of benzoic acid and 90% of caffeine had crossed the mouse skin (Fig. 2a, 2b). A short lag-time period was obtained for the penetration of urea through the ASE, but the compound was completely absorbed after 24h (Fig. 2c). Using mouse skin, the lag-time was longer, and only 60% was absorbed after 24h.

For hydrocortisone and sucrose which are lipophilic and hydrophilic respectively, the rates of absorption were diminished compared to the other compounds. Furthermore, longer lag-times were observed. Only 20% of hydrocortisone had penetrated through the ASE after 4h, and 60 to 70% after 24h. Mouse skin was less permeable, as only about 10% of sucrose and 30% of hydrocortisone had penetrated after 24h (Fig. 2d, 2e). In all cases, the unabsorbed compounds was found mainly in the epidermis.

Lipid Analysis. Human epidermis contained a total lipid concentration of 1 mg/cm^2 which was 3 and 7 times more than hairless mouse (0.3 mg/cm^2) and ASE epidermis (0.15 mg/cm^2) respectively. The analysis of 100 μg of lipids by HPTLC revealed that the ASE epidermis had qualitative lipid profiles similar to human and mouse skin (Fig. 3). In particular, ceramides were largely present in our ASE, as well as cholesterol, cholesterol sulfate and phospholipids (Table I). The relative proportion of phospholipids and cholesterol to total lipids were decreased in ASE compared to normal human skin whereas that of cholesterol sulfate and ceramides were increased. In particular, this augmentation was more than two fold for ceramides III and acylceramides. The proportions in mouse epidermal lipids were more similar to the ASE, except for the cholesterol (low) and the triglycerides (high).

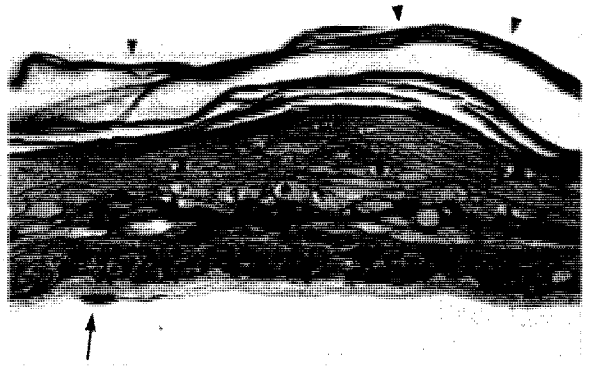

Fig. 1. Histological section of anchored skin equivalent cultured 12 days at the air-liquid interface. In the dermal layer, a fibroblast is longitudinally oriented in relation to the tissue length (arrow). The keratinocytes, cuboidal at the basal layer, differentiate and form the stratum corneum, the uppermost layer of the epidermis (arrow heads). (×250).

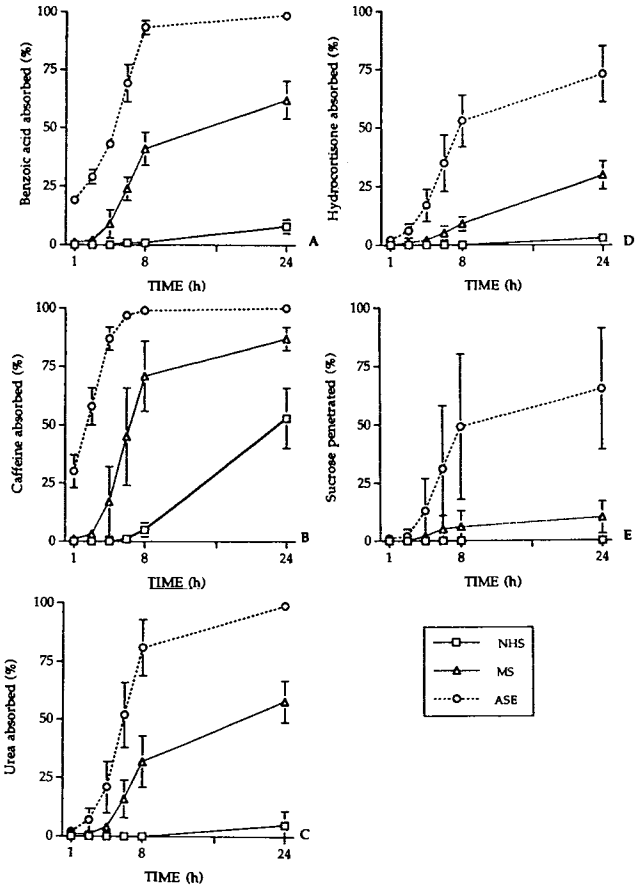

Fig. 2. Percutaneous absorption studies of benzoic acid (a), caffeine (b), urea (c), hydrocortisone (d) and sucrose (e) through normal human skin (NHS, n = 4), hairless mouse skin (MS, n = 6) and anchored skin equivalent (ASE, n = 8). Values represent means ± SD.

DISCUSSION

The results of the present study show that ASE has a significant barrier function although it is more permeable than mouse and human skin. The expected penetration rank order of the 5 substances through human and mouse skin was also found in ASE. It was benzoic acid > caffeine > urea >hydrocortisone > sucrose. Benzoic acid and caffeine penetrated very rapidly without lag-time because the horny layer is relatively highly permeable to substances with high octanol/water partition coefficient (12) and amphiphilic properties (13). On the other hand, the diffusion of a small molecule, urea, through the epidermis was hindered because

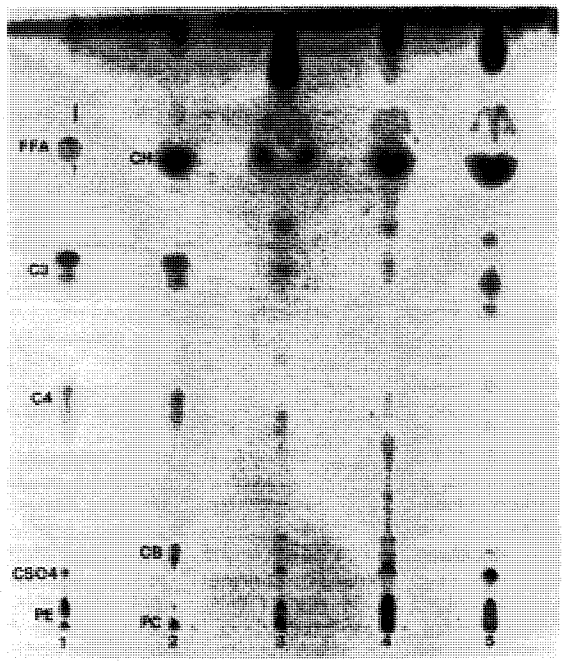

Fig. 3. Total lipid content of epidermal layers of human skin, hairless mouse skin and ASE, analyzed by HPTLC using the ceramide development system. Track 1: standard lipids: free fatty acids (FFA: oleic acid, 1 μg), ceramides types III and IV (C3, C4, 10 μg), phosphatidylethanolamine (PE, 10 μg), and cholesterol sulfate (CSO_4, 10 μg). Track 2: standard lipids: cholesterol (CH), cerebroside II (CB, 2 μg), ceramides types III and IV (C3, C4), and phosphatidylcholine (PC, 10 μg). Track 3 to 5: lipids (100 μg) extracted from epidermis of normal human skin, hairless mouse skin and ASE, respectively.

it takes the intercellular pathway, which is longer than the transcellular route (14). Hydrocortisone had a slower rate of absorption, in the three systems used, due to its high lipid solubility. Finally sucrose has many polar groups and consequently had a slow rate of penetration. Therefore, our work confirms and extends previous percutaneous absorption studies performed with other SE models (1,7,15). *In vitro* cultured ASE and other SE exhibits significant barrier functions and are able to differentiate between low and high permeability compounds. Furthermore SE could rank them in the order previously determined by *in vitro* and *in vivo* studies with intact animal or human skin (16,17, 18).

Although ASE, normal human and mouse skin exhibits similar qualitative percutaneous absorption properties, SE are quantitatively more permeable. The importance of a stratum corneum rich in nonpolar lipids is required for intact barrier function (19). Therefore, we compared the epidermal lipid amount and composition of human skin, mouse skin and ASE. Our results show that the lipid profile of ASE was comparable to those of mouse and human skin. However, the characteristic lipids from the stratum corneum (ceramides and acylceramides corresponding to ceramide I (2)) were proportionally enhanced. This can be related with the histological feature which demonstrated an increased relative proportion of the stratum corneum to the Malpighi layer in the ASE. These results, as well as other variations in lipids between normal human skin and SE were in accord with those previously described (2). In contrast, quantitation of total lipid content per unit surface of ASE revealed a lower value for ASE compared to animal and human skin. Indeed, absolute data demonstrated 4–5 times or 2.5 times more ceramides per cm^2 in normal human skin compared to ASE or mouse skin respectively. It is likely that the greater permeability of cultured ASE reflects their lower ceramide density. Therefore, the comparable qualitative results reflects the similar types of lipids present in the three systems and the quantitative differences observed in the rate of absorption of organic compounds correlate with the lower amount of lipids, especially of ceramides.

In conclusion, we propose an original type of SE that can be easily prepared and manipulated for permeability studies. We demonstrated a relationship between lipid type, content and percutaneous absorption rate in the ASE. Because of similar lipid profiles, the qualitative percutaneous absorption properties were comparable to normal human and mouse skin. However, the lower ceramide density in ASE epidermis likely explained its higher permeability. Cell culture conditions could be further investigated to increase lipid amount in order to approach intact skin barrier properties.

Table I. Lipid Composition of Normal Human Skin, Mouse Skin and Anchored Skin Equivalent Epidermis

Components	Rf	NHS (n = 2)	MS (n = 2)	ASE (n = 4)
Phospholipids	0.03	25.6 ± 0.1[a]	17.1 ± 3.5	10.9 ± 2.0
Cholesterol sulfate	0.03	2.7 ± 0.3	1.7 ± 0.2	4.3 ± 1.7
Cerebrosides	0.09	2.2 ± 0.3	2.2 ± 0.4	0.9 ± 0.3
Ceramides		11.7 ± 0.1	13.9 ± 1.5	17.1 ± 4.0
AGC[b]		1.9 ± 0.5	0.7 ± 0.3	0.2 ± 0.1
C IV	0.38	3.9 ± 0.4	2.9 ± 0.5	3.7 ± 0.6
C III	0.58	2.7 ± 0.8	2.1 ± 1.5	6.5 ± 0.9
AC[c]		2.0 ± 0.6	5.5 ± 0.3	4.3 ± 2.2
Cholesterol	0.73	17.6 ± 1.4	3.7 ± 0.8	12.7 ± 4.0
Free fatty acids	0.78	0.4 ± 0.3	0.1 ± 0.1	0.1 ± 0.1
Triglycerides, alkanes[b]		36.6 ± 0.3	58.3 ± 3.5	43.8 ± 2.2

[a] Percentage of total lipids. Values are given as mean ± SD.
[b] Identified from Ponec *et al.* (11).
[c] Identified from Ponec *et al.* (11) and considered as ceramide I (2).

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