*Skin Research and Technology 2008; **14**: 418–424*
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doi: 10.1111/j.1600-0846.2008.00225.x

Skin Research and Technology

Improved barrier function observed in cultured skin substitutes developed under anchored conditions

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Background/purpose: The current method of producing cultured skin substitutes (CSS) is focused on providing treatments for severe skin wounds/burns. We have developed a modified growth method to make them more suitable for *in vitro* product-testing/toxicity-testing purposes.
Method: CSS grown in Petri dishes were either transferred to Franz diffusion cells on day 5 (modified method) or left in the Petri dish (standard method) and maintained in these environments for the remainder of the growth phase. Mitochondrial metabolism (MTT assay) was measured on days 5, 10 and 14 and histology was studied on days 5, 10 and 14. Barrier function for all tissues was evaluated by transferring them to Franz cells (standard method) and measuring transepidermal water loss (TEWL), $^{3}H_2O$ penetration and ^{14}C-niacinamide permeability on days 7, 14 and 21.
Results: CSS grown by the standard and modified methods showed comparable cell viability and tissue morphology. Barrier function, however, was markedly improved in CSS grown by the modified method. The average improvement at days 7 and 14 was 1.3-fold for TEWL, 2.1-fold for $^{3}H_2O$ penetration and 6.4-fold for ^{14}C-niacinamide permeability. The barrier function of CSS grown by the modified method was still significantly lower than that of human cadaver skin tested by the same methods.
Conclusions: CSS developed using the anchored multi-cell system showed similar cell viability and morphology and improved barrier function compared with CSS produced by the standard Petri dish method, thereby improving its potential as an *in vitro* skin permeability and toxicity model.

Key words: cultured skin substitutes – barrier function – transepidermal water loss – TEWL – permeability

Accepted for publication 12 July 2006

EPIDERMAL AND full-thickness skin substitutes have the potential to replace or supplement traditional native skin models for *in vitro* studies. Reported uses include percutaneous absorption measurements (1), drug efficacy (2) and metabolism testing (3). Their use as a skin irritation model has been heavily investigated (4, 5). These skin substitutes, sometimes termed 'living human skin equivalents,' are valued because they are alive and of human origin. In addition, they provide research opportunities for studies of basic skin biology, wound repair, skin cancer and melanogenesis (6, 7). For example, reconstructed epidermis, containing melanocytes, has been used as a model to study sunscreen efficacy (8). Underdeveloped epidermis *in vitro* has been considered as a wound-healing model (9).

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Cultured skin substitutes (CSS) developed in our laboratories have been successfully used to treat severe burns and chronic wounds (10, 11). However, their use *in vitro* has been restricted to the investigation of barrier properties (12, 13). Barrier function was determined either with skin biophysical instrumentation (transepidermal water loss (TEWL), surface hydration) (14) or by measuring skin permeability to various test compounds (12). This work and that on related tissue models, e.g. reconstructed epidermis and living skin equivalents (7, 12, 15, 16), has shown the need for modifications to the *in vitro* model, i.e. substantially improved stratum corneum (SC) barrier function. The large size graft format of preparing CSS, while useful in the clinical setting, is not optimal for *in vitro* studies. Many skin substitutes are now being developed in cell culture insert format, which enables

high-throughput *in vitro* studies (17). Additionally, anchored skin equivalents have been developed that have been considered as suitable tools for evaluating percutaneous absorption (18). This report describes a similar anchored, multi cell system of producing our CSS model that leads to a substantial improvement in barrier function.

Material and Methods

Standard method of preparing and maintaining CSS (SM)

The standard process of preparing the CSS has been described (19, 20). Human fibroblasts and keratinocytes are inoculated on subsequent days on a collagen–glycosaminoglycan substrate. On day 3, the CSS are raised to the air–liquid interface to allow for keratinization and formation of the SC. CSS are incubated until day 28 in sterile Petri plates at 37 °C and 5% CO_2.

Modified method of preparing and maintaining CSS (MM)

CSS were grown until day 5 by SM mentioned above. Each CSS measured approximately 16 cm^2 (4 cm × 4 cm) in area. On day 5, each CSS was cut into circular pieces having a 2.5 cm diameter. Approximately four circles were obtained from each graft. These pieces were then mounted on a modified Franz diffusion cell, 0.9 cm internal diameter with a cross-sectional area of 0.79 cm^2 (Fig. 1). Sterile media (19) were added to the receptor chamber in order to provide nutrients to the CSS from below, and to simulate maintaining the tissue at an air–liquid interface similar to SM. The donor and receptor chambers were sealed loosely with glass caps to maintain sterility, and the entire setup was maintained at 37 °C and 5% CO_2. Media were changed daily. Media were checked regularly for contamination, both visually and under the microscope.

Comparison of standard and modified method

Eight CSS were prepared and maintained until day 5 by SM. On day 5, each graft was divided and part was grown by SM and the rest by MM until day 14. Tissue biopsies were taken on days 10 and 14 to study tissue viability mitochondrial metabolism (MTT assay) and to compare structure (histology). Baseline MTT and histology were performed initially on day 5. Care was taken at each time point to make measurements on the same CSS grown by the different techniques so as to minimize any inter-tissue variability.

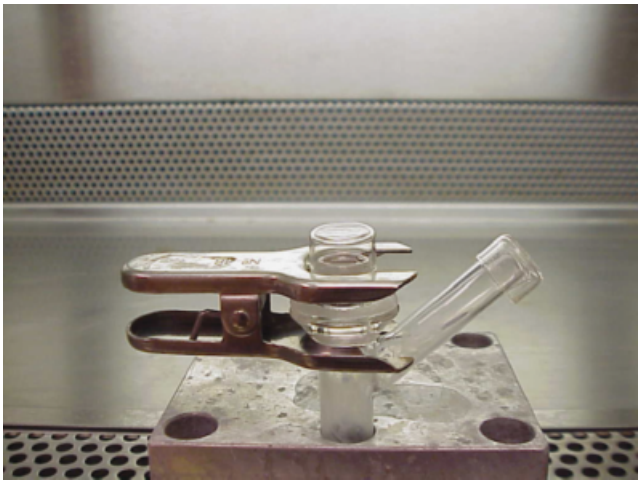

Fig. 1. Illustration of the modified method setup for development of cultured skin substitutes.

MTT assay

Six millimeter punch biopsies ($n = 4$ per method) were collected. Biopsies were incubated for 3 h at 37 °C with 0.5 mg/mL MTT (Sigma Chemical, St Louis, MO, USA). The mitochondria of viable cells cleave the tetrazolium salt MTT to formazan (21, 22). The MTT–formazan reaction product was released by incubating the biopsies in 2-methoxy-ethanol for 3 h on a rotating platform. The optical density of the MTT–formazan product was read at 590 nm on a microplate reader (Cambridge Technologies, Watertown, MA, USA).

Histological evaluation

CSS tissue collected for light microscopy was fixed with 2% glutaraldehyde/2% paraformaldehyde for a minimum of 1 h. Tissue biopsies were then processed, embedded in glycol-methacrylate resin, sectioned and stained with toluidine blue using standard techniques. Sections were examined using a Nikon Microphot-FXA microscope (Nikon Inc., Melville, NY, USA) and photographed using a Spot-Jr. Digital Camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Barrier function measurement of tissue grown by MM

The barrier function of CSS grown by MM was evaluated by measuring three parameters –

TEWL, ^3H_2O penetration and ^{14}C-niacinamide flux. These measurements were made on days 7, 14 and 21 of incubation *in vitro*.

TEWL measurements

A DERMALAB® evaporimeter (Dermalab Inc., Philadelphia, PA, USA) was used to measure water loss *in vitro*. On the days of barrier measurements, the receptor chamber of the diffusion cell was filled with Dulbecco's phosphate-buffered saline (PBS), pH 7.4. After a 1-h equilibration, the DERMALAB probe was placed on the short (0.5 cm) donor chamber opening. A continuous measurement was taken for 1 min, and the 20-s mean was reported. Although this method does not permit direct contact with the skin, a good seal was obtained between the Franz cell and the probe.

Water permeation

The method has been described previously (23, 24). The protocol provides a permeability screen without exposing the tissue to prolonged hydration. Following the TEWL measurements, 150 µL of ^3H_2O, specific activity 0.4 µCi/mL, was applied to the epidermal surface. After 5 min, the tissue was blotted dry with a cotton swab. At 60 min post-dose, the receptor chamber contents were removed for scintillation counting and replaced with fresh buffer. Residual ^3H_2O was thoroughly rinsed from the tissue by several receptor fluid exchanges.

Niacinamide permeation

The donor chamber was filled with 0.5 mL of buffer containing ^{14}C-niacinamde (1 µCi/mL) and sufficient unlabeled niacinamide to achieve a chemical concentration of 10 µg/mL and a dose of 6.3 µg/cm^2. Both radioactive and unlabeled niacinamide were obtained from Sigma Chemicals. Radiochemical purity of the ^{14}C-niacinamide was stated by the manufacturer to be $\geq$ 95%. The receptor solutions were exchanged periodically with fresh buffer and analyzed for radioactivity by liquid scintillation counting. Ultima Gold™ liquid scintillation fluid was purchased from Packard Instrument Company (Downers Grove, IL, USA). The permeability coefficient (k_p) was calculated from the steady-state portion of the plot of cumulative amount of radioactivity absorbed vs. time according to the following equation:

$$J_{ss} = k_p C_v \quad (1)$$

where J_{ss} is the steady-state flux in DPM/cm^2/h and C_v is the vehicle concentration in DPM/cm^3.

Statistical comparison

Treatments were compared by Student's *t*-test at a significance level of $P = 0.05$ (two tailed). In cases where the data failed the normality test, a Mann–Whitney rank sum test was performed and $P < 0.05$ was considered significant. For the MTT assays, as the same tissue was analyzed on days 10 and 14, a paired *t*-test was performed.

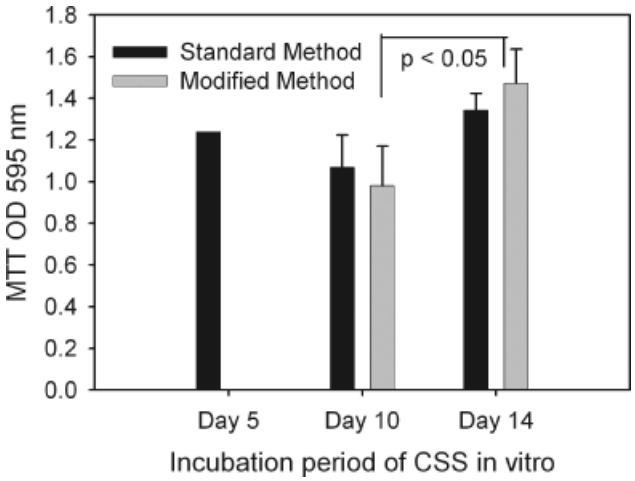

Fig. 2. Comparison of cultured skin substitutes (CSS) viability (mean SE) when grown by the standard method (n = 12) and the modified method (n = 4).

Results

Comparison of SM and MM

No significant difference was observed between MTT in tissue grown by SM and MM (Fig. 2). However, MTT in CSS grown by MM was significantly higher on day 14 than on day 10 ($P = 0.04$). The increase in MTT conversion between CSS grown by SM over this period was not significant ($P = 0.24$). Differences in cellular morphology on day 14 were very subtle (Fig. 3). The tissue showed improvement in structure and organization from days 5 to 14 by both methods. On day 14, an analog of the SC was formed in both tissues, which appeared to be thicker in tissue grown by SM (Fig. 3a). The toluidine blue staining was much more prominent in CSS incubated by MM (Fig. 3b). Nucleated keratinocytes attached to the dermal substitute were seen

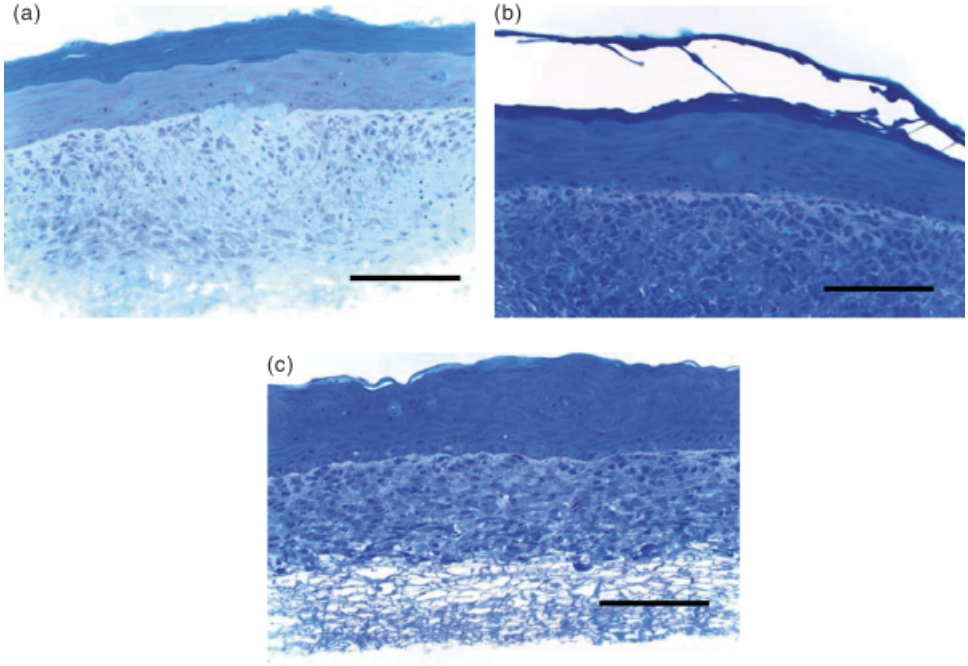

Fig. 3. Comparison of morphology on day 14 for cultured skin substitutes (CSS) grown by (a) the standard method and (b) the modified method. (c) Morphology of CSS on day 5. Scale bar = 100 μm.

in both tissues. Thus, both methods showed similar trends in tissue morphology and cell viability from days 5 to 14, indicating that the MM is a suitable alternative for growing CSS *in vitro*.

Barrier function measurements

Barrier function measurements on tissue grown by MM were compared with previous data for tissue grown by SM (12). The results are shown in Fig. 4. The MM-grown tissues showed significantly lower values of $^{3}H_2O$ and ^{14}C-niacinamide penetration, throughout the incubation period and significantly lower TEWL at day 21. Barrier formation was optimum on day 14 by both methods. At this point, TEWL values were 1.5-fold lower; $^{3}H_2O$ permeation was three-fold lower; and ^{14}C-niacinamide permeability was two-fold lower for tissue grown by MM. Table 1 gives the barrier function on day 14 of tissue grown by both methods compared with human cadaver skin. Post-day 14, the barrier in CSS grown by SM showed some degradation that was not seen in the MM. This indicates that a relatively stronger and less permeable barrier was formed by MM, which remained intact for a longer duration of time.

Discussion

When compared with tissues grown in Petri dishes according to the SM, CSS grown on Franz cells after day 5 developed better barrier function (Fig. 4) while maintaining comparable epidermal architecture (Fig. 3) and MTT rates (Fig. 2).

One important difference between the two methods is that tissue grown by MM is anchored to the diffusion cell rather than floating freely during the incubation period. This anchoring gives rise to tensile stress across the tissue. It has long been recognized that stress either externally applied (25) or internally induced (26) is important for tissue homeostasis, especially in bone, teeth and cartilage. Human skin also reacts to stress *in vivo*. Common examples are the formation of callus under mechanical pressure and scar formation. However, the effect of mechanical stress on skin cells in culture is still not clearly understood. Recent work has shown that collagen I production *in vitro* is strongly down-regulated in relaxed vs. stretched fibroblasts when examined on the mRNA and protein levels (27, 28). Similarly, fibroblasts synthesize tenascin-C and collagen XII to a greater extent under stretched conditions (29, 30). Both keratinocytes (31) and melanocytes (32) have shown increased cell growth under stretched conditions. Applica-

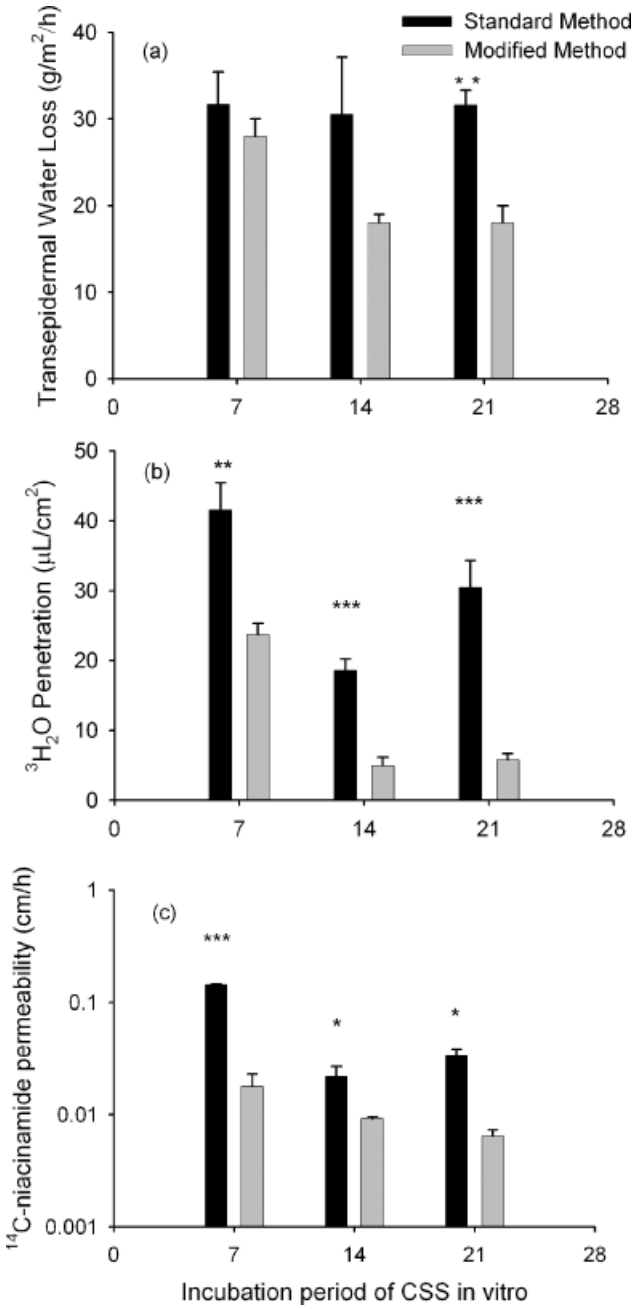

*Fig. 4. Comparison of cultured skin substitutes barrier function when grown by standard and modified methods. (a) Transepidermal water loss; (b) $^{3}H_2O$ penetration and (c) ^{14}C-niacinamide permeability (geometric mean ± SE). Solid bars, standard method (n = 20–27); open bars, modified method (n = 5–6). *P < 0.05; **P < 0.01; ***P < 0.001.*

tion of mechanical pressure has been demonstrated to increase keratinocyte differentiation (33, 34). Akhyari et al. (35) have shown enhanced formation of cardiac muscle grafts under stretched conditions. Michel et al. (18) showed that anchoring improves tensile strength and performance of skin equivalents. Thus, it is generally accepted that tissues under stress develop

TABLE 1. Barrier function of CSS on day 14 grown by the standard and the modified method compared with barrier function of human cadaver skin (mean ± SE)

Barrier measurements	Standard method (n = 21–27)	Modified method (n = 5–6)	Human cadaver skin (n = 8)
Transepidermal water loss (g/m²/h)	30 ± 11	18 ± 1	8 ± 1
$^{3}H_2O$ penetration (μL/cm²)	19 ± 2	4 ± 2	1.0 ± 0.4
^{14}C-niacinamide permeability (cm/h) × 10^3	22 ± 5	9 ± 4	0.2 ± 0.2

CSS, cultured skin substitutes.

an adaptive response/feedback mechanism in order to reduce the stress and attain the original balance between external and internal forces. We postulate that a similar phenomenon may come into play in CSS grown by the MM, leading to improved barrier properties.

Lopez et al. (36) developed anchored dermal equivalents in order to counter the tractional remodeling that occurs when fibroblasts attach to the three-dimensional collagen matrices. These traction forces produce mechanical and biochemical instabilities in the system. In a free-floating dermal equivalent, this contraction could produce dramatic structural disorder. Anchoring was found to be beneficial, especially in maintaining the structure and organization of the dermal equivalent. Michel et al. (18) used these dermal equivalents as a substrate and developed anchored skin equivalents. The anchored dermal equivalent was seeded with keratinocytes and the system was raised to the air–liquid interface to obtain a skin equivalent. They proposed this system as a tool for evaluating percutaneous absorption. Our anchored system follows a similar principle; however, it differs from that of Michel et al. in that the CSS in our model remain free floating during the initial phase of development. It is not yet known whether the fully anchored model would lead to an improvement in barrier properties comparable to MM.

One of the current limitations of tissue grown by SM is the inability of the tissue to desquamate *in vitro* (16, 37). Owing to accelerated cell maturation, a thick cornified epithelium develops *in vitro*. This also happened for CSS grown by MM. A limited comparison showed CSS grown by MM to have fewer cornified layers than those grown by SM. One explanation for this could be that the change in the microenvironment that occurs in the MM leads to slower epidermal

turnover rate. This hypothesis could be tested by studying the effect of desquamating agents (retinol, hydroxyacids) on CSS development. The new experimental model, although effective, is difficult to maintain and use due to its bulkiness. It would seem possible to extend the MM into a multiwell format to enable high-throughput screening. As the CSS maintains viability, a wider variety of physiologically relevant measurements could be made on such a system, in contrast to the limitations of typical systems, e.g. excised human or pig skin (38, 39).

In conclusion, we have developed a method (MM) for growing and maintaining CSS on Franz diffusion cells. CSS grown by this method showed better barrier properties than those grown by standard methods in Petri plates. The tensile stress induced by growing the tissue on a fixed substrate may be a factor in this result.

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