TISSUE ENGINEERING: Part A
Volume 14, Number 00, 2008

DOI: 10.1089/tea.2007.0237

TEN-2007-0237-Kalyanaraman

Medium Flow Rate Regulates Viability and Barrier Function of Engineered Skin Substitutes in Perfusion Culture

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ABSTRACT

Perfusion culture of engineered tissues improves mass transfer of nutrients and provides flow-mediated mechanical stimulation to the developing construct, thereby improving its anatomy and physiology *in vitro*. In this study, the responses to medium flow rate of engineered skin substitutes (ESSs) incubated in perfusion at the air–liquid interface were investigated. ESSs fabricated with autologous keratinocytes, fibroblasts, and collagen–glycosaminoglycan (GAG) sponges were incubated for 21 days at the air–liquid interface in a custom-built recirculating bioreactor system at flow rates of 5, 15, and 50 mL/min ($n = 8$ per condition). ESSs were evaluated *in vitro* using histology, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay, bromodeoxyuridine (BrdU) incorporation, and surface hydration. ESSs incubated at 5 and 15 mL/min had histological organization comparable with that of control ESSs incubated in static conditions. ESSs incubated at 50 mL/min displayed a disorganized epidermal substitute and, at later time points in culture, showed greater degradation of the dermal scaffold. Cell viability measured using MTT assay was significantly higher in ESSs incubated at 5 mL/min than in static controls at day 14 (mean ± standard error of the mean 1.63 ± 0.11 vs 1.30 ± 0.14, $p < 0.05$) and day 21 (1.66 ± 0.12 vs 1.11 ± 0.15, $p < 0.05$) of culture. Viability of ESSs incubated at 15 mL/min was comparable with that of controls. ESSs incubated at 50 mL/min had significantly lower viabilities than controls at all time points. Results of BrdU incorporation data showed that, although ESSs incubated at 5 and 15 mL/min were comparable with controls, those incubated at 50 mL/min had fewer proliferating keratinocytes per high-power field than controls (2.77 ± 0.48 vs 28.1 ± 0.78, $p < 0.05$). ESSs incubated at 5 mL/min had surface hydration comparable with that of controls, whereas those incubated at 15 mL/min and 50 mL/min had significantly higher surface hydration than static controls at all time points. ESSs incubated at a 5-mL/min flow rate and transplanted onto full-thickness wounds on athymic mice demonstrated wound healing comparable with that of controls. From these results, it can be concluded that perfusion culture of ESSs at lower flow rates increases cell viability and maintains an epidermal barrier suitable for grafting, whereas higher flow rates lead to deterioration of ESS anatomy and physiology *in vitro*.

INTRODUCTION

A CENTRAL AIM of tissue engineering is to generate tissue analogs *in vitro* that can be used to repair, replace, or regenerate diseased tissue *in vivo*.[1] Engineered skin substitutes (ESSs), composed of autologous keratinocytes and fibroblasts inoculated in collagen–glycosaminoglycan sponges, are effective adjuncts to spilt-skin autografts in the treatment of burns involving more than 50% of total body surface area.[2] Engineered tissues, including ESSs,

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typically undergo a period of maturation *in vitro* before transplantation to the host. During this time, the cells proliferate, differentiate, and secrete their own extracellular matrix. Provision of adequate nutrient supply to the developing construct during *in vitro* maturation is critical for maintaining the viability of the cells and therefore for the generation of functional tissue substitutes suitable for grafting.[3] In the most common technique of incubating the developing construct, called the batch-feed technique, depleted medium is removed and fresh medium is added at set intervals under static culture conditions. This is also the traditional method of incubating ESSs and has the advantage of tailoring the timing of medium changes to the availability of personnel. However, the metabolically active cells consume nutrients from, and release waste products into, the surrounding medium continuously, thereby altering the composition and pH of the medium over a period of time.[4] In the case of ESSs, it is known that the culture medium gradually loses glutamine and accumulates ammonia between medium changes.[5] Thus, there is the potential for physiological injury to the developing tissue construct. Also, the batch-feed technique relies on diffusional transport of nutrients to cells in the interior of the construct, which is limited by the distance of cells from the medium surface. Constructs thicker than 500 μm can develop necrosis in the center because of this limitation of diffusional transport.[6,7] Another disadvantage of the batch-feed technique is that it is labor intensive and therefore not advantageous for large-scale production of tissue-engineered products.

Several strategies have been devised to culture 3-dimensional engineered tissues in dynamic fluid flow conditions, including spinner flasks,[8–13] rotating wall vessels,[8,14–16] and perfusion culture.[17] In perfusion culture, there is continuous flow of medium through or around the constructs that closely approximates the physiological conditions in the body.[4] Apart from being more physiological, this method overcomes the limitations of mass transfer that arise when the constructs are more than 500 μm thick, because the medium reaches the interior of the construct more reliably.[18] Additionally, fluid flow in perfusion culture generates shear stresses, thereby providing mechanical stimulation to the developing construct and aiding in tissue maturation.[19]

The benefits of perfusion culture have been investigated for several tissue types, including bone,[19–24] cartilage,[25–27] oral mucosa,[28] cardiac muscle,[29,30] and blood vessel.[31–34] In general, perfusion culture results in more-homogenous cell distribution, greater cell viability and cell proliferation, better extracellular matrix synthesis, and more-differentiated phenotype of the engineered tissue than with static culture. However, these responses are dependent on medium flow rate, and the optimal flow rate varies with tissue type, scaffold composition, medium viscosity, and bioreactor configuration.[35] Studies have shown that lower flow rates have greater benefits than higher flow rates on engineered tissue in perfusion culture.[36] This phenomenon has been ascribed to factors such as the magnitude of shear stress produced by fluid flow[36] and the concentration of dissolved oxygen in the medium.[37]

Perfusion culture of skin substitutes poses a unique challenge. This is because the skin substitute needs to be incubated at the air–liquid interface for epidermal maturation.[38] Therefore, unlike other engineered tissues, in which medium is perfused through or around the construct, medium can only contact the dermal portion of the skin substitute. Although bilayered skin substitutes cultured in perfusion in a submerged format were shown to recapitulate the stratified anatomy of native epidermis and heal full-thickness wounds on athymic mice after 2 weeks of *in vitro* maturation,[39] those incubated at the air–liquid interface had higher cell viabilities than those that were batch-fee or submerged in perfusion culture.[40] In this study, the responses to medium flow rate of microanatomy, cell viability, keratinocyte proliferation, and epidermal barrier formation of ESSs incubated in perfusion culture at the air–liquid interface have been investigated using a recirculating bioreactor system. Furthermore, quantification of wound area contraction and engraftment of human cells has characterized the ability of ESSs cultured in perfusion to heal full-thickness wounds on athymic mice.

MATERIALS AND METHODS

Fabrication of ESSs

ESSs were fabricated as described in earlier studies,[5,41] with syngeneic normal human fibroblasts (5×10^5/cm^2) and keratinocytes (1×10^6/cm^2) derived from primary cultures of surgical discard skin (abdominoplasty) and collagen–glycosaminoglycan (GAG) sponge scaffolds. One day after keratinocyte inoculation, each ESS was divided in two, and half was incubated at the air–liquid interface in a Petri dish for 21 days with daily medium changes of approximately 240 mL (static control). The other half of the ESS was incubated at the air–liquid interface in perfusion culture for 21 days in the bioreactor system described below ($n=4$ ESSs/cell strain$\times2$ cell strains; total $n=8$ ESSs per condition). This experimental design ensured that the variability in collagen–GAG sponges attributable to the freeze-drying process was controlled.[42]

Bioreactor for perfusion culture of ESSs

The bioreactor system consisted of an incubation vessel, peristaltic pump, medium reservoir, and sterile silicone tubing (Fig. 1). The incubation vessel was fabricated from polycarbonate and measured $12"\times6"\times2.5"$. Slots were machined in the inside wall at a height of $1.5"$ from the base to hold a perforated stainless steel platform on which a cotton pad was placed, and the ESS was placed over it during culture (Fig. 2). The cotton pad served to convey medium to the ESS by capillary action. Ports were drilled into the base

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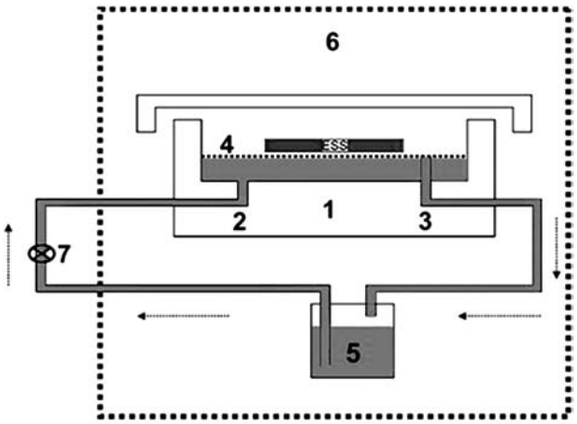


FIG. 1. Schematic diagram of the bioreactor system used for perfusion culture of engineered skin substitutes (ESSs). The polycarbonate culture vessel (1) has inlets and outlets drilled in the base. The inlets (2) are flush with the base, whereas the outlet (3) reaches the level of the perforated steel platform (4) on which the ESSs are placed for culture at the air–liquid interface. The culture vessel and medium reservoir (5) are kept in the incubator (6). The peristaltic pump (7) circulates medium to the culture vessel by means of sterile silicone tubing. Dashed arrows indicate direction of medium flow.

of the vessel to serve as inlets and outlets to medium. The inlets were flush with the base, and the outlets were connected to hose barbs that were flush with the steel platform under the ESS and maintained medium at that level. This design assured incubation of ESSs at the air–liquid interface. The incubation vessel and medium reservoir were placed in an incubator at 37°C and 5% carbon dioxide (CO_2). The inlets and outlets were connected to a medium reservoir using sterile silicone tubing. The peristaltic pump (Cole Parmer) pumped medium from the reservoir to the vessel, and medium returned to the reservoir by passive drainage. The total volume of medium in the bioreactor system was 1200 mL, and this was replaced every 7 days with fresh medium. Medium flow rates of 5, 15, and 50 mL/min were investigated for the responses of ESS quality *in vitro*.

◀AU1

Quality analysis of ESS in vitro

Microanatomy. On days 7, 14, and 21 after keratinocyte inoculation, samples were collected from test and control ESSs for histological analysis. The samples were embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined using a bright-field microscope for cellularity, organization, and epidermal maturation ($n = 8$ per condition).

Cell viability. On days 7, 14, and 21 after keratinocyte inoculation, samples were collected from test and control ESSs for assay with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT),[43] which measures mitochondrial metabolism as a surrogate end point for cell viability. At each time point, four 4-mm punch biopsies each were collected from test and control ESSs ($n = 8$ per condition) and incubated in 0.5 mg/mL MTT solution for 3 hours at 37°C in an atmosphere of 5% CO_2. After 3 hours, 2-methoxy-ethanol was added to the samples, which were then incubated for an additional 3 hours on a rocking platform at room temperature. After this step, the biopsies were removed, and 300 μL of the reaction product was transferred to a 96-well plate. MTT conversion for each ESS was measured for absorbance at 590 nm using a plate reader and expressed as mean ± standard error of the mean (SEM) of the measurements at each time point.

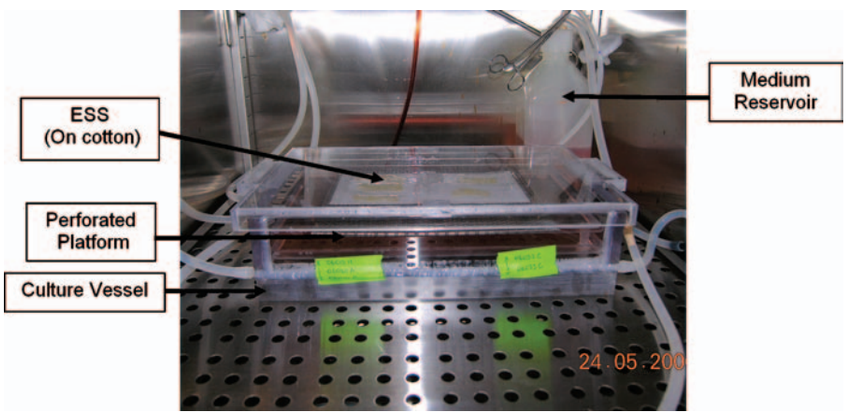


FIG. 2. Perfusion culture of engineered skin substitutes (ESSs) at the air–liquid interface in the custom-built culture vessel. ESSs were placed on cotton pads kept on a perforated steel platform. The cotton serves to wick the medium to the ESSs. Arrows indicate the ESSs, support platform, culture vessel, and medium reservoir. The reservoir in this figure is on the same level as the culture vessel to accommodate it in the field of view, whereas during actual culture it is placed at a lower level to aid gravity-assisted drainage of medium. Color images available online at www.liebertpub.com/ten.

Keratinocyte proliferation. To validate cell viability using the MTT assay, deoxyribonucleic acid synthesis was measured according to incorporation of bromodeoxyuridine (BrdU). The number of actively proliferating keratinocytes in the basal layers of ESSs was quantified according to BrdU immunostaining of ESSs *in vitro*. After 2 weeks of maturation, test and control ESSs ($n = 3$ per condition) were incubated in 65 μM BrdU for 22 h at 37°C and 5% CO_2. After incubation, ESSs were fixed in formalin, paraffin-embedded, and sectioned. The sections were processed for BrdU immunostaining by baking at 60°C for 2 h followed by deparaffinization with xylene and rehydration with graded alcohols. The sections were then co-labeled with 1:10 anti-BrdU fluorescein isocyanate and 1:50 primary anti-pancytokeratin in a procedure described previously.[43] After labeling, the slides were washed with phosphate buffered saline and Milli-Q water and coverslipped with Vectashield hard-mount media containing 4′, 6′-diamidino-2-phenylindole. The slides were examined using epifluorescence microscopy, and 10 unique fields per ESS were analyzed for the number of BrdU-positive cells (total 30 fields per condition). Data were expressed as mean ± SEM of BrdU-positive cells per field.

Surface hydration. On days 7, 11, 14, and 21 after keratinocyte inoculation, surface electrical capacitance (SEC) of the ESSs was measured using the impedance-based Nova Dermal Phase Meter (DPM 9003; NOVA Technology Corporation, Gloucester, MA).[44] At each time point mentioned above, continuous SEC readings for 10 s were collected from 5 different locations in each ESS from the test and control conditions ($n = 8$ per condition) and were expressed as DPM units. The SEC for each condition was expressed as the mean ± SEM of the measurements taken at each time point.

ESS transplantation to athymic mice. All animal care and handling in this study was performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. ESSs were allowed to mature for 2 weeks in static or 5-mL/min perfusion culture *in vitro* before transplantation onto athymic mice (nu/nu, Harlan and Charles River in equal numbers). Briefly, a 2-cm×2-cm full-thickness skin wound was prepared on the dorsolateral aspect of each mouse, sparing the panniculus carnosus. ESSs, along with an overlying piece of non-adherent dressing, were placed orthotopically on the wound and secured to the wound margin with sutures. The grafted ESSs were dressed with a piece of sterile gauze coated with antibiotic ointment (containing equal parts of Neosporin, Bactroban, and Nystatin), and the gauze was held in place with stent sutures. The grafted site was covered with an occlusive dressing (OpSite), and the mice were wrapped in Coban. Two mice from the static group and 1 mouse from the perfusion group died immediately after surgery because of procedure related complications, resulting in $n = 8$ for the control and 9 for the test condition. Dressings and sutures were removed 2 weeks after the surgery. Thereafter, the mice were maintained without dressings until 6 weeks post-surgery, at which time they were euthanized.

Wound areas on athymic mice. Mice were photographed at biweekly intervals from 2 to 6 weeks after surgery. The wound perimeters were traced at the time of surgery and at weekly intervals from 2 to 6 weeks post-operatively. Wound area at each time point was determined from the tracings using image analysis with IMAQ Vision Builder (National Instruments, TX). Percentage of original wound area was defined as the wound area at serial time points divided by the wound area at the time of surgery×100%. Data for each time point were expressed as percentage original wound area (mean ± SEM). ◀AU2

ESS engraftment on athymic mice. Six weeks after surgery, the mice were euthanized, and 2 biopsies of the graft along with adjoining mouse skin were collected. One biopsy was processed using paraffin embedding and the other for cryomicrotomy. The paraffin-embedded specimen was stained using H&E and viewed under the microscope to analyze the histological organization of healed tissue. The frozen sections were stained immunohistochemically for human leukocyte antigen (HLA)-ABC antigens using a procedure previously described[43] to confirm the engraftment of human keratinocytes on the mice. ESS engraftment was expressed as the percentage of animals staining positive for HLA-ABC.

Statistical analysis

Data for cell viability, keratinocyte proliferation, epidermal barrier formation, wound area, and ESS engraftment were analyzed using one-way repeated-measures analysis of variance followed by Student-Neuman-Keul's test for pair-wise comparisons. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

RESULTS

Microanatomy

Representative H&E-stained sections of ESSs after 14 days of *in vitro* maturation in static and perfusion culture are shown in Figure 3. ESSs incubated in perfusion at 5 mL/min (Fig. 3B) showed a well-stratified epidermal substitute overlying a fibroblast-populated dermal substitute. This anatomy was comparable with that of ESSs incubated in static conditions (Fig. 3A). At 15 mL/min perfusion culture, ESSs displayed well-stratified but thinner epidermis than controls and a dermal substitute similar to controls (Fig. 3C). ◀F3

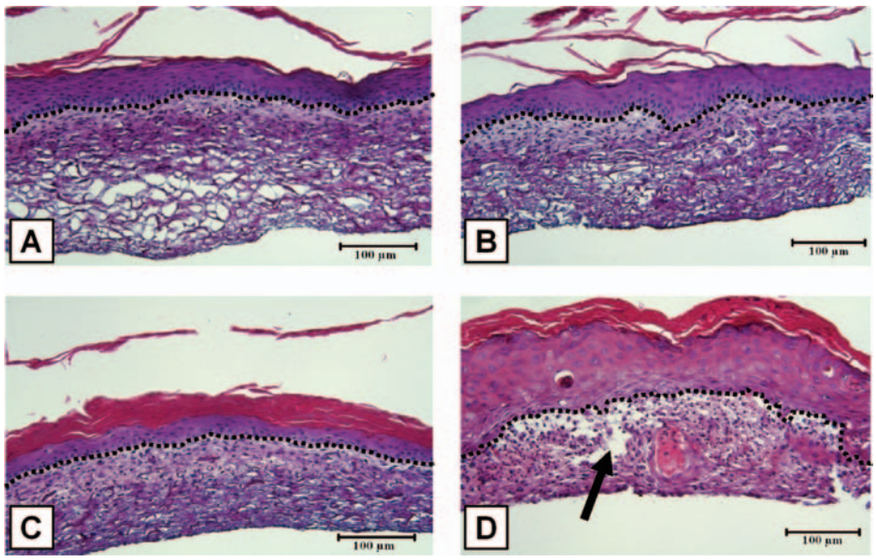


FIG. 3. Representative hematoxylin and eosin–stained paraffin sections of engineered skin substitutes (ESSs) incubated for 2 weeks in static culture (**A**) and in perfusion culture at 5 mL/min (**B**), 15 mL/min (**C**), and 50 mL/min (**D**). ESSs incubated at 5 mL/min (**B**) had anatomy comparable with that of controls (**A**), with a well-stratified epidermal substitute and a fibroblast-populated dermal substitute. ESSs incubated at 15 mL/min (**C**) had thinner epidermal substitute than controls. ESSs incubated at 50 mL/min (**D**) had disorganized epidermal substitute, and the dermal substitute shows premature degradation (arrow). Color images available online at www.liebertpub.com/ten.

However, at 50 mL/min, the epidermal substitute was irregular and disorganized, with poor stratification. The dermal substitute had low density of fibroblasts, and the dermal scaffold showed signs of degradation (Fig. 3D).

Cell viability

Results of cell viability according to MTT assay of ESSs incubated in static and perfusion culture are illustrated in Figure 4. At 5 mL/min, ESSs had cell viabilities comparable with those of controls after 7 days of *in vitro* maturation (1.29 ± 0.12 vs 1.32 ± 0.19). The cell viabilities in ESSs incubated at 5 mL/min were significantly greater than those of controls at day 14 (1.63 ± 0.11 vs 1.30 ± 0.14, $p < 0.05$) and day 21 (1.66 ± 0.12 vs 1.11 ± 0.15, $p < 0.05$) of *in vitro* maturation (Fig. 4A). ESSs incubated at 15 mL/min had cell viabilities comparable with those of controls at all time points during *in vitro* maturation (Fig. 4B). ESSs incubated at 50 mL/min showed significantly lower cell viabilities than controls at all times during *in vitro* maturation (0.74 ± 0.20 vs 1.75 ± 0.06, 0.48 ± 0.13 vs 1.58 ± 0.15, and 0.76 ± 0.25 vs 1.48 ± 0.26 at days 7, 14, and 21, respectively) (Fig. 4C).

Keratinocyte proliferation

Results of quantification of keratinocyte proliferation according to BrdU incorporation are shown in Figure 5. ESSs incubated in perfusion at 5 and 15 mL/min had comparable numbers of actively proliferating keratinocytes per high-power field (HPF) in the basal layers of the epidermis as controls. However, ESSs incubated in perfusion at 50 mL/min had significantly fewer proliferating keratinocytes per HPF than controls (2.77 ± 0.48 vs 28.1 ± 0.78).

Epidermal barrier formation

Results of epidermal barrier formation of ESSs incubated in static and perfusion culture are shown in Figure 6. ESSs incubated in perfusion at 5 mL/min had surface hydration values comparable with those of controls at all times during *in vitro* maturation (Fig. 6A). However, ESSs incubated in perfusion at 15 and 50 mL/min had significantly higher surface hydration than controls at all time points *in vitro* (Fig. 6B, C).

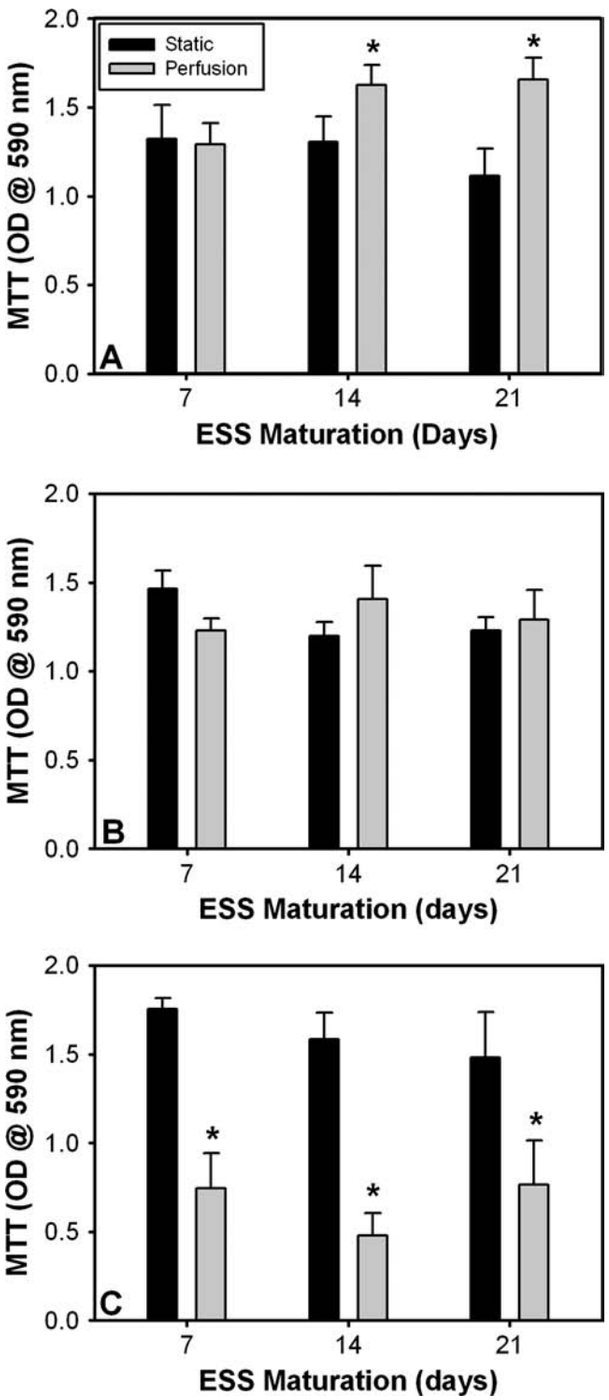
Wound area on athymic mice

Images of athymic mice grafted with ESSs cultured in static or perfusion culture at 5 mL/min, 2 and 6 weeks after surgery, are shown in Figure 7. The wound area decreased over time and there were pigmented areas corresponding to the grafted area, indicating the presence of human-derived cells. In both conditions, the percentages of original wound area were comparable at all times after surgery, with the wound area at 6 weeks being $43.5\% \pm 9.1\%$ and $41.4\% \pm$

5.3% of original wound area in the perfusion and static groups, respectively (Fig. 8). F8 ▶

Engraftment of ESSs on athymic mice

F9 ▶ Figure 9 shows representative H&E-stained sections of ESSs cultured in static or perfusion culture at 5 mL/min and engrafted on athymic mice, 6 weeks after transplantation. Seventy-eight percent (7 of 9) of animals in the perfusion group and 75% (6 of 8) of animals in the static group stained positive for the presence of HLA-ABC antigens, confirming the engraftment of ESSs on those animals (Fig. 10). ◀F10



DISCUSSION

Perfusion culture of engineered tissue is known to result in better construct quality than static culture *in vitro*, possibly because of enhanced mass transfer of nutrients or mechanical stimulation due to fluid flow.[23,36] However, optimization of culture conditions is essential to derive maximum benefit of perfusion culture on construct quality before transplantation. In this study, the responses of ESSs in perfusion culture to medium flow rate were investigated using a recirculation bioreactor system. Although medium did not flow through the interior of the collagen–GAG sponge scaffold because of the culture requirements of ESSs, the term ''perfusion culture'' as used in this study refers to the continuous medium flow that occurred in the recirculation bioreactor. The results obtained indicated that the viability and barrier function of ESSs is greater at low flow rates than at higher flow rates. At a perfusion rate of 5 mL/min, the cell viabilities in ESSs were significantly higher than those of static controls after 2 and 3 weeks of maturation *in vitro*, and keratinocyte proliferation and epidermal barrier formation were comparable with that of controls. At 15 mL/min, the cell viabilities and keratinocyte differentiation in ESSs were comparable with those of controls, but the perfused samples had higher surface hydration. ESSs incubated at 15 mL/min were less uniform than controls, with thin, relatively fewer cellular areas scattered throughout the grafts (data not shown). These thin areas had higher surface hydration and resulted in higher mean surface hydration for the ESSs. At medium flow rate of 50 mL/min, the ESSs had significantly lower viabilities and keratinocyte proliferation and higher surface hydration than controls. The variations between control ESSs in the 3 conditions can be attributed, in part, to variations in pore structure of the collagen–GAG sponge

FIG. 4. Cell viabilities of engineered skin substitutes (ESSs) incubated in perfusion culture at 5 mL/min (**A**), 15 mL/min (**B**), and 50 mL/min (**C**) according to 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Static controls were set up for each flow rate to control for variability in the collagen–GAG sponge ($n = 8$ ESSs per condition). Perfusion culture at 5 mL/min results in greater cell viability than in controls after 2 and 3 weeks of *in vitro* maturation. At 15 mL/min, cell viabilities in perfusion-cultured ESSs are comparable to those of controls. At 50 mL/min, cell viabilities in perfusion-cultured ESSs were significantly less than those of controls at all *in vitro* time points. $^*p < 0.05$ versus control at parallel time points according to 1-way repeated-measures analysis of variance.

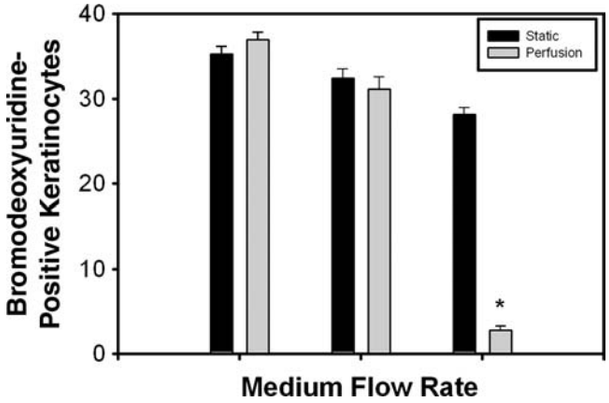


FIG. 5. Keratinocyte proliferation in perfusion culture according to quantification of number of bromodeoxyuridine-positive keratinocytes per high power field (HPF). Static controls were set up for each flow rate to control for variability in the collagen-glycosaminoglycan sponge (n=3 engineered skin substitutes (ESSs) per condition), Keratinocyte proliferation between control ESSs and those incubated at 5 and 15 mL/min were comparable. ESSs incubated at 50 mL/min had significantly less keratinocyte proliferation than controls ($*p < 0.05$ according to 1-way repeated-measures analysis of variance).

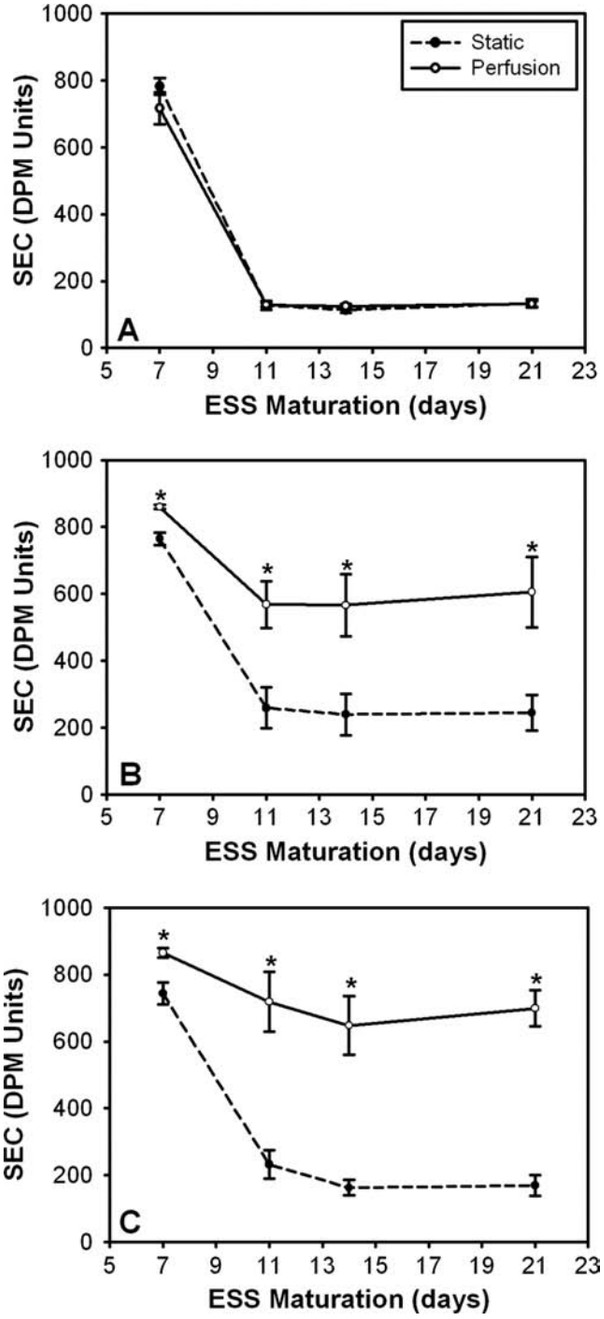


FIG. 6. Epidermal barrier of engineered skin substitutes (ESSs) incubated in perfusion culture at 5 mL/min (**A**), 15 mL/min (**B**), and 50 mL/min (**C**) according to measurement of surface hydration. Static controls were set up for each flow rate to control for variability in the collagen-glycosaminoglycansponge ($n = 8$ ESSs per condition). ESSs incubated at 5 mL/min had surface hydration comparable with that of controls at all time points. At 15 mL/min and 50 mL/min, surface hydration of perfusion-cultured ESSs was significantly higher than that of controls at all times points *in vitro*. $*p < 0.05$ versus control at parallel time points according to 1-way repeated-measures analysis of variance. SEC, surface electrical capacitance; DPM, Dermal Phase Meter.

that resulted from the freeze-drying process used in their fabrication.[42]

Because early reconstitution of the epidermal barrier is one of the major functions of ESSs after grafting, those incubated at 15 and 50 mL/min were considered unfit for transplantation. ESSs incubated in perfusion culture at 5 mL/min for 2 weeks and then grafted on full-thickness wounds on athymic mice achieved effective wound closure, thereby providing a basis for clinical translation of the perfusion culture system. The benefits of perfusion culture at this flow rate did not translate into better wound healing outcomes *in vivo*. This can be ascribed to several factors such as the host inflammatory response and the period of *in vitro* maturation before transplantation. Although the former cannot be controlled, further studies are necessary to evaluate the transplantation of ESSs incubated in perfusion culture for longer periods (e.g., $\geq$3 weeks) of *in vitro* maturation. The finding that the viability of ESSs cultured in perfusion remains high at 3 weeks of maturation, whereas that of controls is declining supports the rationale for such a study (Fig. 4). However, results of this study also indicate the need for further optimization of medium flow rate in perfusion culture to achieve better wound than with static culture.

The beneficial effect of perfusion culture at low flow rates on ESS viability *in vitro* has implications for their long-term incubation in culture. According to current protocols, ESSs incubated in static culture are grafted to patients after 10 days of *in vitro* maturation to take advantage of maximum cell viability during this time. This study has shown that

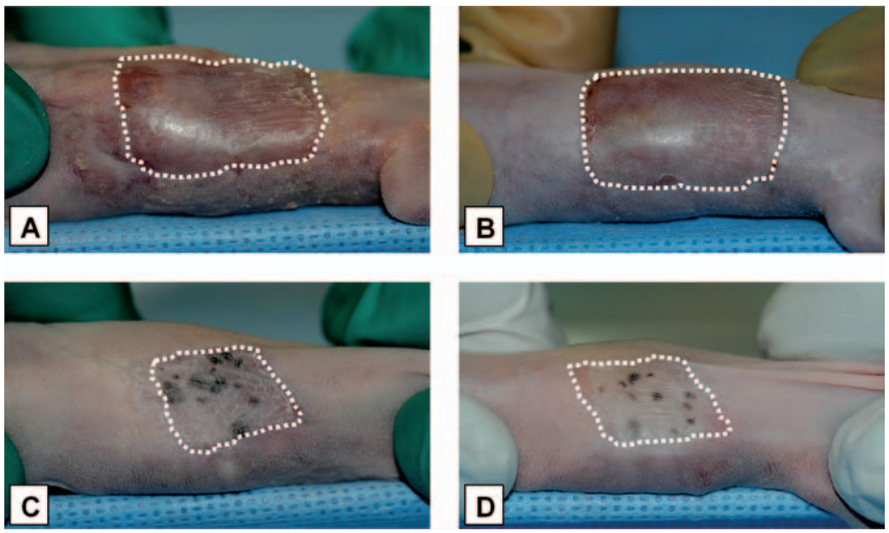


FIG. 7. Representative images at 2 and 6 weeks after surgery, of athymic mice grafted with engineered skin substitutes incubated in static (**A and C**) and perfusion culture at 5 mL/min (**B and D**). The perimeter of the grafted area has been delineated with dashed lines. Note the extent of wound contraction in both conditions. Also remarkable is the presence of pigmented spots within the grafted area at 6 weeks in both conditions, indicating the presence of human-derived melanocytes in those regions. Color images available online at www.liebertpub.com/ten.

perfusion culture of ESSs at 5 mL/min maintains high cell viabilities even after 3 weeks of *in vitro* incubation and therefore that they can be stored longer in culture before transplantation. Perfusion culture also has benefits in reducing labor and materials involved in ESS incubation. Whereas static culture requires daily medium changes, perfusion culture requires medium to be changed only once a week. Also, less medium is needed for perfusion culture than fo static culture, with 1200 mL being consumed in the former and 1680 mL in the latter. The culture vessel and silicone tubing can be resterilized and reused, thereby reducing the use of disposable plastic ware in the process of ESS fabrication.

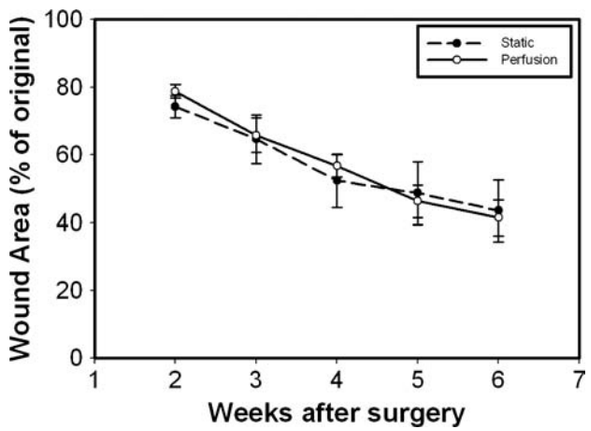


FIG. 8. Wound contraction, expressed as percentage of original wound area, of engineered skin substitutes incubated in static culture and perfusion culture at 5 mL/min and transplanted on athymic mice. There were no significant differences between the 2 conditions at parallel time points.

Medium flow rate has been shown to affect cell viabilities and cell differentiation in 3-dimensional bone constructs cultured in perfusion similarly to effects observed in this study.[36] In the case of engineered bone, medium was perfused through the thickness of the construct, exposing cells to flow-mediated shear stresses and leading to cell death at higher flow rates. However, in the present study, ESSs were incubated in perfusion at the air–liquid interface, and the cells were not exposed to direct medium flow. Therefore, it is hypothesized that shear stress may not be a major mediator of the flow-rate-dependent response, especially at the lower medium flow rates. The finding of premature collagen degradation at 50 mL/min indicates that, at this flow rate, shear stress may play a role in regulating the response of ESSs to perfusion culture. At all flow rates, it is believed that the balance between supply of nutrients and the removal of cell-derived products, including growth factors critical for tissue organization and maturation, may play a crucial role in regulating the anatomy and physiology of ESSs. Earlier studies on perfusion culture of human mesenchymal stem cells in 3-dimensional scaffolds have shown that maximal growth rate of the cells is achieved at a specific flow rate because of optimal delivery of oxygen at that flow rate. At medium flow rates lower and higher than the optimal, cell growth is inhibited because of oxygen limitation and tox-

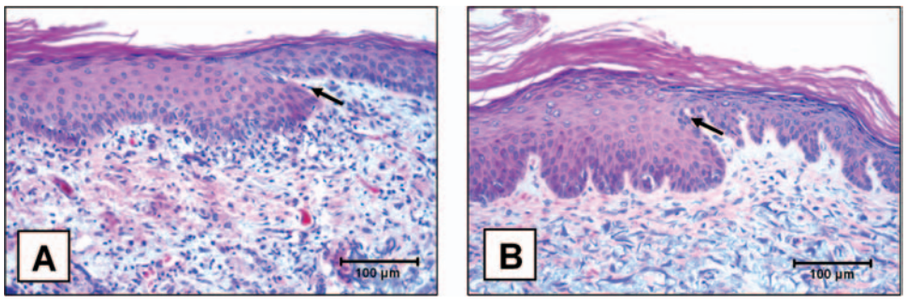


FIG. 9. Representative hematoxylin and eosin–stained paraffin sections of biopsies of grafted engineered skin substitutes (ESSs) incubated in static culture (**A**) and perfusion culture at 5 mL/min (**B**), collected after euthanizing the mice 6 weeks after surgery. In both conditions, the normal mouse epidermis is thin and lacks rete ridges. The thicker epithelium with prominent rete ridges resembles human skin and is derived from the grafted ESSs. Color images available online at www.liebertpub.com/ten.

icity, respectively.[45] A similar mechanism may exist in the case of ESSs whereby medium flow rate regulates the supply of one or more nutrients in perfusion culture. Also, medium flow rate may regulate the residence time of growth factors secreted by cells into the surrounding medium, which can influence ESS viability and organization. At low medium flow rate, the residence time of growth factors in the immediate vicinity of ESSs would be greater than at higher flow rates. Therefore, at a 5 mL/min flow rate, enhanced nutrient supply coupled with favorable balance between nutrients, growth factors, and metabolic waste could have led to the observed beneficial effects on ESS viability and barrier properties. Conversely, the deterioration of ESS quality at 15 and 50 mL/min could be attributed to a combination of suboptimal nutrient delivery and depletion of vital growth factors. Further elucidation of the mass transport and magnitude of shear stress due to fluid flow can be performed using mathematical modeling and computational fluid dynamics studies in this bioreactor system,[35] taking into account active convection in the bulk medium in the culture vessel and passive diffusion across the cotton pad and collagen–GAG scaffold. Such modeling studies will also help to predict optimal flow rates for various configurations of the bioreactor such as shape and size of culture vessel and changes in medium composition. The role of shear stresses in flow-related effects on ESSs can be studied by changing the medium viscosity while perfusing at a constant flow rate.[19] The culture vessel used in this bioreactor also has slots at a lower level than those used in this study, allowing for investigation of the role of medium volume in perfusion culture of ESSs.

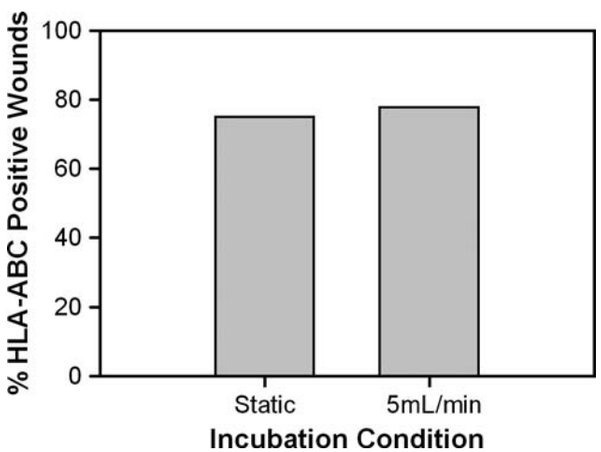


FIG. 10. Percentage of graft biopsies from static and 5-mL/min perfusion mice that stained positive for presence of human leukocyte antigen-ABC antigens. There were no significant differences between the conditions.

As engineered tissue replacements transition from the lab bench to the market, there will be an increasing need for automation and scale-up while maintaining or even improving the anatomy and physiology of the cell-based products. For ESSs, this laboratory has evaluated an automated bioreactor for keratinocyte culture,[46] and the advantages of perfusion culture demonstrated in this study is a further step in this direction. Overall, incorporation of these technologies in the manufacturing process can lead to generation of greater amounts of useful product with lesser expense of labor and materials, leading to lower cost to the patient. At the same time, improvement in the anatomy and physiology of engineered tissue can translate into more-effective wound healing and better *in vivo* performance, thereby leading to better outcomes in patient care.

ACKNOWLEDGMENTS

The authors extend their gratitude to Jenny Klingenberg and Heather Powell, PhD, for assistance with animal surgery and photography; Jodi Miller for providing cell-culture media and assistance with animal surgery; and Chris Lloyd for fabrication of collagen sponges.

This project was supported by a research grant from the National Institutes of Health (GM050509).

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