Carbodiimide Conjugation of Fibronectin on Collagen Basal Lamina Analogs Enhances Cellular Binding Domains and Epithelialization

Katie A. Bush, Ph.D.,^{1,2} and George D. Pins, Ph.D.²

To improve the regenerative potential of biomaterials used as bioengineered scaffolds, it is necessary to strategically incorporate biologically active molecules that promote *in vivo* cellular processes that lead to a fully functional tissue. This work evaluates the effects of strategically binding fibronectin (FN) to collagen basal lamina analogs to enhance keratinocyte functions necessary for complete skin regeneration. We found that FN that was passively adsorbed to collagen–glycosaminoglycan basal lamina analogs enhanced epithelial thickness and keratinocyte proliferation compared with nontreated basal lamina analogs at 3 days of air/liquid (A/L) interface culture. Additionally, we evaluated the availability of FN cellular binding site domains when FN was either passively adsorbed or [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] conjugated to basal lamina analogs fabricated from collagen–glycosaminoglycan coprecipitate or self-assembled type I collagen. It was found that 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride conjugation of FN significantly enhanced FN binding site presentation as well as epithelial thickness. Overall, the results gained from this study will be used to improve the regenerative capacity of basal lamina analogs for bioengineered skin substitutes as well as the development of bioengineered scaffolds for other tissue engineering applications.

Introduction

IN THE DEVELOPMENT OF bioengineered skin substitutes for replacement of skin lost to trauma or disease, the addition of biologically active molecules that promote key events in nonscarring self-healing wounds has the potential to guide epithelialization. In the native wound environment, fibronectin (FN) is part of the provisional matrix that interacts with dermal collagens and promotes the migration of keratinocytes through granulation tissue of the wound.^{1,2} FN is also involved in basement membrane synthesis and organization of the wound site, which are critical for the reestablishment of a healthy functional tissue.³ *In vitro* studies have examined the effect of FN on keratinocyte functions necessary for reepithelialization. It was found that passive adsorption of FN on bacteriological plastic increased percentage of adherent cells,⁴ and adsorption on polystyrene enhanced migration⁵ and inhibition of terminal differentiation.⁶ FN that has passively adsorbed to biomaterials that have the potential for implantation has shown that incorporating FN on the surface of poly(lactic-co-glycolic acid) (PLGA) limited keratinocyte migration and when adsorbed to collagen enhanced migration.⁷ Research investigating

passive adsorption of FN to collagen–glycosaminoglycan (GAG) membranes found an increase in attachment over nonmodified collagen surfaces.⁸

In addition to investigating keratinocyte responses to full FN molecules, the modification of biomaterial surfaces with synthetic peptides located in the central cellular binding domain of FN, specifically the arginine–glycine–aspartic acid (RGD) sequence have been examined. Arginine–glycine–aspartic acid peptides have been covalently coupled to collagen–GAG matrices⁹ and to a hyaluronate synthetic matrix.¹⁰ Both studies found increased keratinocyte attachment and spreading in comparison to those on unmodified matrices. Although this approach allows for more RGD sites to be expressed on the surface of the biomaterials, these short sequences lack full biological activity when compared with the native protein.^{11,12}

During wound healing, as well as in cell culture expansion from healthy skin, keratinocytes express an increase in the integrin receptor $\alpha_5\beta_1$, which is specific for the central cellular binding domain of FN.^{3,5,13} The availability of this FN domain and its full biological activity is highly dependent on the structural orientation of the protein and has been found to be critical in modulating cellular functions.^{14–18} When FN

¹Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, Massachusetts.

²University of Massachusetts Medical School, Graduate School of Biomedical Sciences, University of Massachusetts, Worcester, Massachusetts.

adsorbs to a surface, it undergoes a conformation change, which is highly dependent upon the properties of the surface.^{19–21} Recently, our laboratory investigated the availability of the central cellular binding domain of FN and its role on keratinocyte morphology, attachment, and differentiation using self-assembled monolayers as model biomaterial surfaces.²² A direct relationship was found between keratinocyte spreading area and attachment, and an indirect relationship was found between cellular binding domain availability and cell differentiation. When evaluating focal adhesion formation, it was found that the area density of focal adhesions in individual keratinocytes directly corresponded with the availability of the central cellular binding domain of FN, suggesting that the functions evaluated were integrin mediated.

Although much is known regarding the advantages of using FN to enhance reepithelialization in the wound environment, little work has been performed investigating its presentation on dermal scaffolds. Further, understanding how to strategically modify a biomaterial surface to increase the availability of the central cellular binding domain, which has been shown to promote attachment and subsequent intracellular signaling events, is of great importance for enhancing epithelialization of bioengineered skin substitutes as well for engineering other functional tissues.

The purpose of this study was to evaluate the presence of the central cellular binding domain of FN on collagen membranes and to analyze how the presentation of this binding site effects epithelialization. We hypothesize that by increasing the presentation of the central cellular binding domain of FN on the surface of a dermal scaffold, we will enhance epithelialization. Using an *in vitro* skin model, keratinocyte and graft morphology, epidermal thickness, and proliferation were evaluated on the surface of collagen–GAG membranes containing the GAG chondroitin 6-sulfate. Incorporating this molecule into the collagen membranes increases the degradation resistance of the membranes, stabilizes the open pore structure of the material, and enhances

tissue regeneration.²³ FN was found to promote epithelial layers on dermal scaffolds and was found to be morphologically similar to that of native skin. When evaluating proliferation in this model system, we found that FN-treated surfaces enhanced the number of proliferative cells at 3 days of A/L interface culture. To correlate these findings with the presentation of FN on the surfaces, we evaluated the availability of the central cellular binding domain on collagen–GAG membranes. In effort to further enhance the presentation of FN on the surfaces of basal lamina analogs, we developed self-assembled collagen membranes, fabricated from soluble type I collagen (CI) molecules, and compared their performance to collagen–GAG membranes. We also describe a method to covalently modify the surfaces of self-assembled CI membranes with FN using a carbodiimide conjugation strategy, specifically, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and have evaluated the effect of EDC conjugation on the presentation and bioactivity of FN. Overall, we demonstrate that the EDC conjugation strategy greatly enhances the availability of the central cellular binding domain of FN and that this modification strategy enhances epithelialization on the surfaces of basal lamina analogs.

Materials and Methods

Air/liquid interface culture devices

To evaluate the effect of FN on epithelialization of bioengineered skin substitutes, a custom-designed device was developed to analyze membranes that are precisely conjugated with FN and cultured at the air/liquid (A/L) interface. This system creates an individual well on the surface of a collagen membrane and allows for a tight seal to be made on the surface of the composite assuring that FN placement is in the center (Fig. 1).

Basal lamina analog production

Collagen–GAG membranes. A collagen–GAG dispersion containing acid-insoluble CI (5 mg/mL) and the GAG,

FIG. 1. Custom-built air/liquid (A/L) interface culture devices. To culture keratinocytes on basal lamina analogs, a custom-developed A/L interface culture device was developed by our laboratory. (A) Computer-aided design drawing of individual parts of the device including the base and top pieces with posts on the base piece to allow for alignment of the two pieces and initial stability. A screen sits on the base piece where the membrane is placed on. This screen facilitates diffusion of the cell culture medium from below the membrane and A/L interface culture. A silicone o-ring is fit in the base piece to provide a tight seal that creates a well on the surface of the collagen membrane that allows for protein modification and cell seeding. The complete unit fits in a six-well plate. (B) Computer-aided design drawing of device with base and top piece screwed together and (C) shows a photograph of one of the devices with a collagen membrane placed on top of the screen during assembly. Color images available online at www.liebertonline.com/ten.

chondroitin sulfate (0.18 mg/mL), was prepared as previously described.²⁴ To produce membranes, the fibrillar suspension was cast onto flat polydimethylsiloxane silicone elastomer (PDMS, Sylgard 184; Dow Corning, Midland, MI) molds 9.62 cm² in area, and allowed to air-dry in a laminar flow hood at room temperature. The membrane was then gently peeled from the PDMS surface and dehydrothermally crosslinked according to previously published methods for 24 h.⁸ Membranes were then stored in a desiccator until use.

Self-assembled CI membranes. Acid-soluble CI was extracted from rat tail tendons using protocols previously described.²⁵ Rat tails were received from animals that were euthanized for other protocols, which were approved by Institutional Animal Care and Use Committee, Worcester Polytechnic Institute (Worcester, MA). To produce self-assembled CI membranes, 800 μ L of the acid-soluble CI solution at 10 mg/mL was neutralized using 200 μ L of 5 $\times$ Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 0.22 M NaHCO₃ and 40 μ L of 0.1 M NaOH (Sigma, St. Louis, MO) at 37°C for 18 h on circular PDMS molds.

FN surface modification of collagen membranes

Passive adsorption of FN to collagen membranes. FN (BD Biosciences, Bedford, MA) was resuspended according to manufacturer's recommendations and diluted to desired concentrations (30, 100, and 300 μ g/mL) using Dulbecco's phosphate buffered saline (dPBS). For *in vitro* culture on basal lamina analogs, all collagen membranes were placed in A/L culture devices (Fig. 1) and FN (100 μ g/mL) was placed in the well created on the surface of the collagen membrane and allowed to adsorb overnight at room temperature. For FN cellular binding site evaluation of basal lamina analogs, collagen membranes were placed in a custom high throughput screening device,⁸ and FN was placed into each individual wells at 30, 100, and 300 μ g/mL for self-assembled CI membranes, and at 100 μ g/mL for collagen-GAG membranes overnight at room temperature.

EDC conjugation of FN to collagen membranes. Using protocols previously described to crosslink collagenous materials,^{26,27} the molar ratio of 5:1 (EDC to carboxylic acid groups in collagen) was used to conjugate FN to the surfaces of collagen-GAG and self-assembled CI membranes. The theoretical amount of collagen used for calculations assumed that 1 g of CI contained 1.2 mmol COOH.^{26,27} Collagen-GAG membranes contained 12.5 mg of CI and self-assembled CI membranes contained 8 mg of CI, thus receiving 0.075 mmol EDC and 0.048 mmol EDC, respectively. EDC (Sigma) was dissolved in 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) hydrate (Sigma) dissolved in 40% ethanol (Pharmco Products, Brookfield, CT) at a pH 5.5, and 1.25 mL of solution was placed on collagen-GAG membranes and 0.8 mL was placed on self-assembled CI membranes for 4 h. For *in vitro* culture on basal lamina analogs, the membranes were removed from the EDC solution and immediately placed into the A/L culture devices, and 100 μ g/mL of FN was placed in the well created on the surface of the collagen membrane over night at room temperature. For FN cellular binding site evaluation, the membranes were immediately

placed in a custom high-throughput screening device⁸ and FN was placed into each individual wells at 30, 100, and 300 μ g/mL for self-assembled CI membranes, and at 100 μ g/mL for collagen-GAG membranes overnight at room temperature.

Culture of neonatal human keratinocytes

Neonatal keratinocytes were cultured as previously described.^{8,28} Neonatal foreskins were obtained from non-identifiable discarded tissues from UMass Memorial Medical Center (Worcester, MA) and were approved with exempt status from the New England Institutional Review Board. Keratinocyte isolations were performed using an enzymatic treatment with a dispase (Gibco, Gaithersburg, MD) solution. After 5 days of culture, cells were detached using 0.05% trypsin-ethylenediaminetetraacetic acid (Invitrogen) and then rinsed with serum-free and epithelial growth factor (EGF)-free keratinocyte media. Passage 2 keratinocytes were used in all experiments.

In vitro culture of keratinocytes on basal lamina analogs

After FN adsorption or EDC conjugation of FN to membranes, the membranes were sterilized in composite culture devices using 70% ethanol. Membranes and devices were removed from ethanol and rinsed in sterile dPBS. The A/L culture devices were placed into individual wells of a six-well tissue culture plate and preconditioned for 30 min with the seeding medium consisting of 3:1 mixture of Dulbecco's modified Eagle's medium (high glucose) and Ham's F-12 medium supplemented with 10⁻¹⁰ M cholera toxin, 0.2 μ g/mL hydrocortisone (Calbiochem), 5 μ g/mL insulin, 50 μ g/mL ascorbic acid (Sigma), and 1% penicillin/streptomycin (Invitrogen). Keratinocytes were seeded on the surfaces of the membranes at 500,000 cells/cm² using this medium, and allowed to adhere for 2 h in 10% CO₂ at 37°C. After 2 h, the seeding medium containing 1% fetal bovine serum was placed in each well, completely submerging the grafts. After 24 h, the keratinocyte seeding medium was removed, and the grafts were submerged for an additional 48 h in a keratinocyte priming medium composed of the keratinocyte seeding medium (with fetal bovine serum) supplemented with 24 μ M bovine serum albumin (BSA), 1.0 mM L-serine, 10 μ M L-carnitine, and a mixture of fatty acids including 25 μ M oleic acid, 15 μ M linoleic acid, 7 μ M arachidonic acid, and 25 μ M palmitic acid (Sigma).²⁹ After 48 h in the priming medium, skin equivalents were cultured for 3 or 7 days with an A/L interface medium composed of the serum-free keratinocyte priming medium supplemented with 1.0 ng/mL epithelial growth factor.

Evaluation of epithelialization

To assess epithelialization on the basal lamina analogs, epidermal thickness and proliferation were evaluated after 3 or 7 days of A/L interface culture. Grafts were fixed in a 10% buffered formalin solution (EMD Chemicals, Gibbstown, NJ), dehydrated with increasing concentrations of ethanol, cleared with sec-butyl alcohol (EMD Chemicals), and embedded in the Paraplast tissue embedding medium (McCormick Scientific, St. Louis, MO). Sections of skin equivalents, 6 μ m in thickness, were cut in a plane perpendicular

to the surface of the epithelial layer using a Leica RM 2235 (Leica Microsystems, Bannockburn, IL). Sections were mounted on poly-L-lysine-coated slides (Erie Scientific Company, Portsmouth, NH) for hematoxylin and eosin (H&E) staining and mounted on Superfrost Plus slides (VWR, West Chester, PA) coated with poly-L-lysine (Sigma) to evaluate proliferation.

To evaluate thickness of the epithelial layer, the slides were stained with Harris H&E (Richard-Allan Scientific, Kalamazoo, MI). Thickness measurements were taken in three areas of the image using ImageJ software (downloaded from <http://rsb.info.nih.gov/ezproxy.umassmed.edu/ij/>), and an average value was reported for each graft. For collagen-GAG membranes with and without passively adsorbed FN, at 3- or 7-day culture, seven and four cultured basal lamina analogs were evaluated, respectively. For self-assembled CI membranes with no treatment, passive adsorption of FN, and EDC conjugation of FN, three grafts were evaluated for each condition.

Keratinocyte proliferation was evaluated by detecting the presence of Ki67. Tissue sections were deparaffinized in reverse ethanol-xylene washes, and the antigens were unmasked by placing the slides in boiling Vector Unmasking solution (Vector Laboratories, Burlingame, CA) in a Mantra pressure cooker (Mantra, Virginia Beach, VA) for 1 min after maximum pressure was achieved. Slides were then incubated with blocking solution (10% normal horse serum [Vector Laboratories] in dPBS) for 10 min at room temperature and treated with predilute mouse-antihuman Ki67 (Zymed Laboratories, South San Francisco, CA) overnight in a humidified chamber at room temperature. Slides were incubated with biotinylated anti-mouse IgG (Vector Laboratories) at 1:200 for 30 min at room temperature and then washed with dPBS and stained with Vectastain Elite ABC Kit (Vector Laboratories) for 30 min at room temperature. Stained slides were washed with dPBS and developed using a Vector Nova-Red Substrate Kit (Vector Laboratories) for approximately 1 min. Slides were rinsed in dPBS, followed by a 5-min wash with tap water, and counterstained with Harris hematoxylin for 45 s. The slides were washed with tap water, rinsed with a series of ethanol-xylene washes, and mounted with the VectaMount permanent mounting medium (Vector Laboratories). The number of Ki67-positive cells was counted and divided by the total number of cells in the basal layer to give a percentage of Ki67-positive cells. At 3 or 7 days of A/L interface culture on collagen-GAG membranes passively adsorbed with FN, three different sections of five grafts were evaluated.

FN cellular binding site detection

To measure the availability of the central cellular binding domain of FN, a monoclonal antibody directed toward this domain (HFN 7.1; Developmental Studies Hybridoma Bank, Iowa City, IA) was measured with fluorescence microscopy and image analysis.^{22,30} After passive adsorption or EDC conjugation of FN to CI membranes, the scaffolds were sterilized for cellular culture, and then blocked using 1% heat denatured BSA (in dPBS) for 1 h at room temperature. HFN 7.1 was added to each well for 1 h in 10% CO₂ at 37°C. Each surface was rinsed in blocking buffer (0.05% Tween-20 [Sigma] and 0.25% BSA in dPBS) and incubated with 546 Alexa Fluor-conjugated goat anti-mouse IgG (1:200 in

blocking buffer; Molecular Probes, Eugene, OR) for 1 h in 10% CO₂ at 37°C. ImageJ Analysis software was used to determine the relative amount of cellular binding sites in each well. The relative fluorescence intensity (RFI) was calculated over a region of interest and normalized against fluorescence intensity of non-FN-modified membranes. Eight samples were evaluated for collagen-GAG and self-assembled CI membranes that were treated with 100 µg/mL of FN using EDC conjugation or passive adsorption strategy. For self-assembled CI membranes treated with 30 or 300 µg/mL of FN, four samples were evaluated. Results are reported as averages and standard deviations and each experiment was repeated twice.

Statistical analyses

Sigma Stat Version 3.10 (Systat Software, Richmond, CA) was used to determine statistical differences among the means of experimental groups. To determine if the means of two different samples were significantly different, a Student's *t*-test was performed when the samples were drawn from a normally distributed population with equal variance and a Mann-Whitney Rank Sum Test was used when the data were not drawn from a normally distributed population. For both the Student's *t*-test and the Mann-Whitney Rank Sum Test, a *p*-value <0.05 indicated a significant difference between the means of experimental groups.

To determine statistical differences among the means of three or more experimental groups, a one-way analysis of variance (ANOVA) was used when the samples were drawn from a normally distributed population with equal variance, and a Kruskal-Wallis one-way ANOVA on ranks was performed when the data were not drawn from a normally distributed population. When a statistical difference was detected among the group means, a Tukey *post hoc* analysis was performed for both the one-way ANOVA and Kruskal-Wallis one-way ANOVA on ranks. A *p*-value <0.05, for both variance tests, indicated a significant difference between the groups.

Results

FN enhances epithelialization of keratinocytes on basal lamina analogs

Graft morphology and epidermal layer thickness on collagen-GAG basal lamina analogs. The effect of passively adsorbed FN on graft morphology and epithelial layer thickness of keratinocytes was evaluated using custom-built A/L interface culture devices (Fig. 1). FN (100 µg/mL) was passively adsorbed to the surfaces of collagen-GAG membranes, and keratinocytes were cultured on these basal lamina analogs for 3 or 7 days at the A/L interface. Figure 2A shows histological results of control grafts compared with grafts that were passively adsorbed with FN, cultured for 3 or 7 days, and stained with H&E. A thicker epidermal layer formed on membranes modified with FN when compared with control membranes at 3 or 7 days of culture at the A/L interface. At 3 days, 17.7 ± 0.9 and 52.4 ± 6.3 µm were found for control and FN-treated membranes, respectively; at 7 days, 59.6 ± 7.4 and 89.6 ± 4.2 µm were found for control and FN-treated membranes, respectively. These differences in epithelial thickness between control and FN-treated surfaces were statistically different at both time points (Fig. 2B).

FIG. 2. Thicknesses of epidermal layers on collagen-glycosaminoglycan (GAG) membranes. Keratinocytes were cultured for 3 or 7 days at the air/liquid (A/L) interface on collagen-GAG control (nonmodified) membranes or collagen-GAG membranes that were modified by passively adsorbing fibronectin (FN) to the surfaces of the scaffolds. (A) Histological representation of grafts at 3 or 7 days of A/L interface culture. The thickness of the epithelial layer on collagen-GAG membranes treated with FN was greater than that on untreated collagen-GAG membranes. Scale bar represents 30 μ m. (B) Epidermal thickness on collagen-GAG membranes at 3 or 7 days of A/L interface culture was measured on control (nonmodified) collagen-GAG membranes or collagen-GAG membranes that were modified by passively adsorbing FN to the surfaces. At both 3 and 7 days there was a significant difference between untreated and FN-treated surfaces ($*p < 0.05$, Student's *t*-test). Samples for 3-day culture are $n = 7$ and for 7-day culture $n = 4$. Color images available online at www.liebertonline.com/ten.

Keratinocyte proliferation on collagen-GAG basal lamina analogs. To analyze keratinocyte proliferation, the presence of Ki67 in basal keratinocytes was measured on the surfaces of cultured basal lamina analogs. This protein is present during active phases of the cell cycle and absent from resting cells.³¹ Figure 3A shows histological images of control collagen-GAG membranes and membranes that were passively adsorbed with FN, cultured for 3 or 7 days at the A/L interface and immunoassayed for Ki67. Quantitative analyses of these images are depicted in Figure 3B. At 3 days of culture, positive Ki67 basal keratinocytes were counted, and control surfaces and FN-treated surfaces had $24.3 \pm 2.5\%$ and $37 \pm 3.9\%$ Ki67-positive basal cells, respectively; at 7 days, control surfaces and FN-treated surfaces had $23 \pm 2.7\%$

FIG. 3. Proliferation of keratinocytes on collagen-GAG membranes. Keratinocytes were cultured on collagen-GAG membranes for 3 or 7 days at the A/L interface on control (nonmodified) collagen-GAG membranes or collagen-GAG membranes modified by passively adsorbing FN to the surfaces. (A) Histological representation of grafts at 3 or 7 days of A/L interface culture immunostained for Ki67 (brown stained nuclei) to evaluate proliferation of basal keratinocytes. Scale bar represents 30 μ m. (B) The percentage of positive Ki67 basal keratinocytes at 3 or 7 days of A/L interface culture was measured on control (nonmodified) collagen-GAG membranes or collagen-GAG membranes modified by passively adsorbing FN to the surfaces. At 3 days statistical differences were found between keratinocytes cultured on control surface and FN-treated surfaces ($*p < 0.05$, Student's *t*-test). For all experimental conditions, $n = 5$ samples were measured at both 3 and 7 days of culture. Color images available online at www.liebertonline.com/ten.

and $21.9 \pm 2.1\%$ Ki67-positive basal cells, respectively. The percentage of basal keratinocytes expressing Ki67 on FN-modified membranes was statistically different than on control membranes at 3 days of culture; however, at 7 days of culture, no differences were detected.

Availability of cellular binding domain of FN corresponds to keratinocyte attachment on collagen-GAG basal lamina analogs

The availability of the cellular binding domain of FN, specifically the domain that encompasses both the RGD and PHSRN binding sequences, was analyzed on the surfaces of collagen-GAG basal lamina analogs using an antibody directed toward this site. RFI measurements were made on several regions of interest, and an average value was

FIG. 4. Availability of cellular binding domains for FN passively adsorbed on collagen–GAG basal lamina analogs. The availability of the cellular binding domain of FN on collagen–GAG basal lamina analogs was evaluated using a quantitative immunofluorescent assay. FN concentrations of 0, 30, 100, and 300 µg/mL were evaluated, and it was determined that at a concentration of 100 µg/mL the average relative fluorescence intensity (RFI) was statistically different from 0 µg/mL and 30 µg/mL, but did not statistically differ from 300 µg/mL, indicating that a saturation plateau was achieved. Data are reported as averages, and error bars indicate standard error of the mean with $n=3$ (*statistical difference, one-way analysis of variance [ANOVA] with Tukey *post hoc* analysis, $p < 0.05$).

reported. When FN was passively adsorbed to collagen–GAG membranes at 30, 100, or 300 µg/mL, cellular binding sites plateaued at a concentration of 100 µg/mL (Fig. 4). The fluorescence intensity values obtained at 100 and 300 µg/mL were statistically greater than those at 30 µg/mL of FN. These data directly correspond with keratinocyte attachment measurements made on FN-treated collagen–GAG membranes in a previously published study.²²

EDC conjugation of FN on self-assembled CI basal lamina analogs promotes increased cellular binding site availability

To determine whether we could increase the presentation of FN cellular binding sites on the surfaces of collagen–GAG basal lamina analogs, we analyzed the effects of covalently binding FN to the surface using an EDC conjugation strategy

(Fig. 5). Since it was found that 100 µg/mL of FN saturated the surfaces of the collagen–GAG membranes, we chose to evaluate this concentration using both adsorption and EDC modification strategies. When analyzing collagen–GAG membranes, the difference between passive adsorption and EDC conjugation of 100 µg/mL of FN was that EDC conjugation statistically increased the average RFI on the surfaces of FN-treated membranes, suggesting that these membranes have a greater capacity for cellular binding (Fig. 6).

The availability of cellular binding domains on the surfaces of self-assembled CI basal lamina analogs was evaluated for both passive adsorption of FN and EDC conjugation of FN. These findings were compared with both passive adsorption and EDC conjugation of FN on collagen–GAG collagen basal lamina analogs (Fig. 7). When we compared the passive adsorption of 100 µg/mL of FN on collagen–GAG and self-assembled CI membranes, a significant increase in average RFI was observed on the self-assembled CI membranes. Similarly, when we compared the binding efficiency of FN using an EDC conjugation strategy at a concentration of 100 µg/mL of FN on collagen–GAG and self-assembled CI membranes, a significant increase was found in average RFI on the self-assembled CI membranes. Additional concentrations were analyzed, for both passive adsorption and EDC conjugation of FN on self-assembled CI membranes, to evaluate whether saturation levels of RFI were obtained. When evaluating lower and higher concentrations of FN (30 and 300 µg/mL, respectively), we found that there were no statistical differences between FN concentrations of 100 and 300 µg/mL and that the 100 µg/mL concentration had statistically increased values over the 30 µg/mL concentration, regardless of the conjugation strategy that was used. These data indicate that the presentation of cellular binding domains on the surfaces of self-assembled CI membranes saturated at an FN concentration of 100 µg/mL. This saturation trend is similar to the results obtained for collagen–GAG membranes, which also saturated at 100 µg/mL.

EDC conjugation of FN on self-assembled CI basal lamina analogs enhances epidermal layer thickness

FN was covalently bound to the surface of self-assembled CI membranes using EDC, and keratinocytes were cultured on the surface of basal lamina analog for 3 days at the A/L interface to determine whether increased cellular binding sites on our new model system promoted an increase in

FIG. 5. Schematic representation of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-mediated conjugation of FN to collagen. The carbodiimide EDC was added to basal lamina analogs at a 5:1 molar ratio (EDC molecules to carboxylic acids in collagen). The EDC reacts with a carboxylic acid from the collagen molecule to form an unstable reactive o-acylisourea ester that can either couple with an amine group in collagen from the basal lamina analog or an amide group in FN that is added to the collagen membrane. If the unstable reactive o-acylisourea ester does not interact with an amine, it hydrolyzes and the carboxyl group is regenerated, thus returning back to its native state.

FIG. 6. Availability of cellular binding domain of FN on collagen-GAG and self-assembled type I collagen (CI) basal lamina analogs. A quantitative immunofluorescent assay was used to measure the availability of the cellular binding domain of FN on the surfaces of modified collagen membranes. At 100 µg/mL of FN, an increase in the cellular binding domain of FN was found when EDC conjugation was used in comparison to passive adsorption on collagen-GAG membranes (* $p < 0.05$, Student's t -test). At this concentration, an increase in the cellular binding domain of FN was also found on self-assembled CI membranes in comparison to collagen-GAG membranes, regardless of membrane modification strategy. When comparing modification strategies on self-assembled CI membranes, the binding domain of FN was statistically greater using EDC conjugation than when passive adsorption was used (* $p < 0.05$, Student's t -test). When evaluating the differences between concentrations of passively adsorbed and EDC-conjugated FN on self-assembled CI membranes, it was found that 100 µg/mL of FN was statistically greater than 30 µg/mL, but not 300 µg/mL for both modification strategies (** $p < 0.05$, one-way ANOVA with Tukey *post hoc* analysis). For collagen-GAG membranes, $n = 8$ for passive adsorption and $n = 8$ for EDC conjugation. For self-assembled CI membranes $n = 4$ for 30 and 300 µg/mL for both passive and EDC, and $n = 8$ for 100 µg/mL for both passive and EDC. Experiments were repeated and similar trends were found.

epithelial layer thickness. Figure 7 shows typical images of a cultured epithelial layer on an untreated self-assembled CI membrane (Fig. 7A), a basal lamina analog with FN passively adsorbed to the surface (Fig. 7B), and a basal lamina analog with FN that was EDC conjugated to the surface (Fig. 7C). These images suggest that the thickness of the epidermal layer on the scaffold corresponds to the presentation and availability of FN central cellular binding domains. Morphometric analyses of these epidermal layers (Fig. 7D) showed that there were statistical differences between all surfaces analyzed.

Discussion

To improve the regenerative capacity of biomaterials scaffolds, biomolecules have been incorporated to present biochemical cues that direct cellular functions. This approach requires that the biomolecules are precisely tailored to the

surface of the biomaterial to ensure that the appropriate cellular binding domains are presented for maximum bioactivity. To improve the compatibility and regenerative potential of biomaterials scaffolds, FN is a protein of interest to adsorb to the surfaces, based on its role in cell adhesions, migration, and differentiation.^{4,6-8,20} However, several studies indicate that when FN is passively adsorbed to the surface of biomaterials, its conformation is effected by surface properties, which modulate cellular binding site presentation as well as biological activity.^{20,21,32,33} In this work we evaluated the effect of passive adsorption of FN on epithelialization of a basal lamina analog. Additionally, we investigated the presentation sites of the central cellular binding domain of FN based on the preparation technique of the basal lamina analog and the conjugation strategy. Overall, we determined that EDC conjugation of FN to the surface of self-assembled CI membranes improved binding site availability and epithelialization.

FN enhanced epithelial thickness and keratinocyte proliferation on the surfaces of collagen-GAG basal lamina analogs. When FN was passively adsorbed at a saturation density previously determined on the surface,⁸ epithelial thickness was enhanced in comparison to untreated membranes at both 3 and 7 days of A/L interface culture. The morphology of basal keratinocytes on the FN grafts exhibited a more native columnar morphology than those on the scaffolds without FN. When keratinocyte proliferation was examined using Ki67, a nuclear marker for proliferation, it was found that the percentage of Ki67-positive cells at 3 days of A/L interface culture on FN-treated membranes was greater than on untreated membranes, ~35% to ~20% of total basal cells, respectively. At 7 days of A/L interface culture, no differences were found between percentages of Ki67-positive basal keratinocytes, with both membranes having ~20% of total basal cells.

In unwounded epidermis, between 10% and 20% of basal keratinocytes are proliferative, based on the location of the skin.³⁴⁻³⁶ In an acute wound environment, keratinocyte proliferation is increased. Within hours after injury, keratinocytes at the wound edge become activated and undergo a phenotypic change that facilitates migration over the wound bed.^{5,15} A proliferative burst occurs 24 to 72 h postinjury and after wound closure, the proliferative capacity of the basal layer returns to normal status.³⁷ Our results suggest that FN-treated scaffolds closely mimic the wound environment, and provide the appropriate signals for proliferation to occur. Once the cells sense that a monolayer is formed, proliferation returns to normal and the cells begin to undergo differentiation and migrate upward to create a stratum corneum that provides protection from the environment.

After evaluating the effects of FN on graft morphology and proliferation, we investigated the presentation of the cellular binding domain of FN that was passively adsorbed on the collagen-GAG basal lamina analogs. We determined that FN cellular binding site presentation directly corresponded with previously published values for keratinocyte attachment to collagen-GAG membranes.⁸ We concluded that we were saturating our collagen-GAG membrane surfaces using passive adsorption since there were no differences between membranes that were treated with 100 or 300 µg/mL of FN. To increase the number of FN presentation

sites, we evaluated different sources of collagen to fabricate membranes as well as conjugation strategies to covalently link FN to the surfaces.

In this study, we analyzed the presentation of FN cellular binding domains on collagen-GAG basal lamina analogs and compared it with the FN cellular binding domains on self-assembled CI basal lamina analogs. Our initial studies focused on the use of collagen-GAG membranes fabricated from a Food and Drug Administration-approved commercially available product, to facilitate a rapid translation from benchtop to bedside.³⁸ Although this product has many advantageous properties, the starting collagen material is considered insoluble when placed in an acidic environment and does not completely dissolve into individual collagen fibrils. When a suspension of these collagen fibrils is air-dried, the aggregates of fibrils come together and form a membrane with random orientation. In contrast, the self-assembled CI membranes developed for these studies are fabricated from a solution of acid solution CI molecules. When neutralized, these collagen molecules self-assemble into individual fibrils, and aggregate laterally and linearly to form collagen fibers with structural morphology comparable to native tissue constructs.³⁹ Our studies indicate that when 100 μg/mL of FN is passively adsorbed to the surfaces of the different collagen membranes, the self-assembled CI basal lamina analog has significantly more FN cellular binding site availability than the collagen-GAG basal lamina analog. With CI, the FN binding site is found on the $\alpha 1$ chain¹ between amino acid residues 757 and 791.⁴⁰ When the soluble collagen self-assembles, it exposes the FN binding site, similar to that in native tissue, unlike the collagen-GAG fibers that do not have all FN binding sites exposed, because of the random packing of the fibrillar aggregates. Additional analysis was performed evaluating the cellular binding site availability of FN on self-assembled CI basal lamina analogs at varying concentrations of FN to determine the saturation limit. We found that the availability of FN on the surfaces of the self-assembled CI membranes at 100 μg/mL of FN was the optimal concentration for binding site availability, similar to the evaluation of binding site availability on collagen-GAG membranes.

Various investigations have evaluated covalent conjugation strategies to improve the presentation and bioactivity of FN over passive adsorption on various surfaces.^{41–43} The

FIG. 7. EDC conjugation of FN on self-assembled CI basal lamina analogs enhances epidermal thickness. Histological images of surfaces of self-assembled CI basal lamina analogs treated with Dulbecco's phosphate buffered saline (dPBS) (controls) (A), passive adsorption of FN (B), or EDC conjugation of FN (C), and keratinocytes cultured on the membranes at the A/L interface for 3 days. Scale bars represent 50 μm. Conjugation of FN to the surfaces of the self-assembled CI basal lamina analogs using EDC caused an increase in epidermal thickness in comparison to control surfaces and surfaces treated by passively adsorbing FN to the scaffolds (D). All surfaces were statistically different from each other (* $p < 0.05$, one-way ANOVA with Tukey *post hoc* analysis). Bars indicate mean values, and error bars indicate standard error; sample numbers are $n = 3$ for control and passive adsorption, and $n = 5$ for EDC conjugation. Color images available online at www.liebertonline.com/ten.

use of a carbodiimide conjugation strategy was evaluated to crosslink our membranes as well as to covalently bind FN. This crosslinking agent has been highly successful in crosslinking collagen and improving its degradation resistance and mechanical properties,^{44,45} as well as coupling chondroitin sulfate, heparin sulfate, and heparin to the surface of collagen scaffolds.^{46–48} We investigated its use as a potential method to covalently conjugate FN to the surface of both collagen-based scaffolds and found a significant increase in cellular binding site availability of FN when compared to that of using passive adsorption. When keratinocytes were cultured at 3 days at the A/L interface on self-assembled basal lamina analogs with no FN, passively adsorbed FN, and EDC-conjugated FN, an increase in epithelial thickness was found between all surfaces. These data also correspond with the data from the FN cellular binding domain availability analysis. Future studies will evaluate EDC conjugation of FN on epithelialization of a basal lamina analog laminated to a dermal scaffold to determine the effects of this conjugation strategy on proliferation and differentiation in a composite model of bioengineered skin substitute.

Overall, the results from these studies indicate that the cellular binding domain of FN can be enhanced on collagen-based biomaterials and directly influences functions important for epithelialization. The information gained from this study can be applied to other model systems where the enhancement of cellular binding sites of FN on collagenous biomaterials would enhance tissue functionality. Additionally, this information can be used in the design of engineered tissues where the incorporation of a basal lamina analog is necessary to direct epithelial polarity and functions as well as to separate cell types and act as a selectively permeable barrier, such as in the glomerulus of the kidney or the small intestine.

Acknowledgments

This research was funded by the National Institutes of Health (EB-005645) and the U.S. Army Medical Research and Materiel Command (Grant W81XWH-08-01-0422). The HFN 7.1 hybridoma supernatant was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA: R.J. Klebe contributor. The authors would like to thank the Department of Obstetrics and Gynecology at University of Massachusetts Medical School (Worcester, MA) for providing neonatal foreskins for keratinocyte isolations and Russell Kronengold at Kensey Nash Corporation (Exton, PA) for his generous donation of SEMED-S collagen. The authors would also like to thank Jeannine Coburn, Donna Davidson, Christina Mezzone, Kevin Cornwell, Brett Downing, and Stuart Howes for their technical assistance.

Disclaimer

The views, opinions, and/or findings and information contained in this article are those of the authors and should not be construed as an official Department of Defense position or policy, unless so designated by other documentation. No official endorsement should be made.

Disclosure Statement

No competing financial interests exist.

References

1. Woodley, D. Reepithelialization. In: Clark, R.A., ed. *The Molecular and Cellular Biology of Wound Repair*. New York: Plenum Press, 1996, pp. 339–354.
2. Martin, P. Wound healing-aiming for perfect skin regeneration. *Science* **276**, 75, 1997.
3. Grinnell, F. Fibronectin and wound healing. *J Cell Biochem* **26**, 107, 1984.
4. Adams, J.C., and Watt, F.M. Expression of beta 1, beta 3, beta 4, and beta 5 integrins by human epidermal keratinocytes and non-differentiating keratinocytes. *J Cell Biol* **115**, 829, 1991.
5. Guo, M., Toda, K., and Grinnell, F. Activation of human keratinocyte migration on type I collagen and fibronectin. *J Cell Sci* **96** (Pt 2), 197, 1990.
6. Adams, J.C., and Watt, F.M. Fibronectin inhibits the terminal differentiation of human keratinocytes. *Nature* **340**, 307, 1989.
7. Tjia, J.S., Aneskievich, B.J., and Moghe, P.V. Substrate-adsorbed collagen and cell secreted fibronectin concertedly induce cell migration on poly(lactide-glycolide) substrates. *Biomaterials* **20**, 2223, 1999.
8. Bush, K.A., Downing, B.R., Walsh, S.E., and Pins, G.D. Conjugation of extracellular matrix proteins to basal lamina analogs enhances keratinocyte attachment. *J Biomed Mater Res A* **80**, 444, 2007.
9. Grzesiak, J.J., Pierschbacher, M.D., Amodeo, M.F., Malaney, T.I., and Glass, J.R. Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatization. *Biomaterials* **18**, 1625, 1997.
10. Cooper, M.L., Hansbrough, J.F., and Polarek, J.W. The effect of an arginine-glycine-aspartic acid peptide and hyaluronate synthetic matrix on epithelialization of meshed skin graft interstices. *J Burn Care Rehabil* **17**, 108, 1996.
11. Garcia, A.J., Schwarzbauer, J.E., and Boettiger, D. Distinct activation states of alpha5beta1 integrin show differential binding to RGD and synergy domains of fibronectin. *Biochemistry* **41**, 9063, 2002.
12. Pierschbacher, M.D., and Ruoslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30, 1984.
13. Grinnell, F. Wound repair, keratinocyte activation and integrin modulation. *J Cell Sci* **101** (Pt 1), 1, 1992.
14. Pettit, D.K., Hoffman, A.S., and Horbett, T.A. Correlation between corneal epithelial cell outgrowth and monoclonal antibody binding to the cell binding domain of adsorbed fibronectin. *J Biomed Mater Res* **28**, 685, 1994.
15. Grinnell, F., and Feld, M.K. Adsorption characteristics of plasma fibronectin in relationship to biological activity. *J Biomed Mater Res* **15**, 363, 1981.
16. Iuliano, D.J., Saavedra, S.S., and Truskey, G.A. Effect of the conformation and orientation of adsorbed fibronectin on endothelial cell spreading and the strength of adhesion. *J Biomed Mater Res* **27**, 1103, 1993.
17. Garcia, A.J., Ducheyne, P., and Boettiger, D. Effect of surface reaction stage on fibronectin-mediated adhesion of osteoblast-like cells to bioactive glass. *J Biomed Mater Res* **40**, 48, 1998.
18. Underwood, P.A., Steele, J.G., and Dalton, B.A. Effects of polystyrene surface chemistry on the biological activity of solid phase fibronectin and vitronectin, analysed with monoclonal antibodies. *J Cell Sci* **104** (Pt 3), 793, 1993.

19. Garcia, A.J., and Boettiger, D. Integrin-fibronectin interactions at the cell-material interface: initial integrin binding and signaling. *Biomaterials* **20**, 2427, 1999.
20. Garcia, A.J., Vega, M.D., and Boettiger, D. Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. *Mol Biol Cell* **10**, 785, 1999.
21. Keselowsky, B.G., Collard, D.M., and Garcia, A.J. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *J Biomed Mater Res A* **66**, 247, 2003.
22. Bush, K.A., Driscoll, P.F., Soto, E.R., Lambert, C.R., McGimpsey, W.G., and Pins, G.D. Designing tailored biomaterial surfaces to direct keratinocyte morphology, attachment, and differentiation. *J Biomed Mater Res A* **90A**, 999, 2008.
23. Yannas, I.V., and Burke, J.F. Design of an artificial skin. I. Basic design principles. *J Biomed Mater Res* **14**, 65, 1980.
24. Bush, K.A., Balestrini, J., Walsh, S., Downing, B., and Pins, G.D. Characterization of Keratinocyte Adhesion on Surface Modified Basal Lamina Analogs. Philadelphia, PA: Biomedical Engineering Society, 2003.
25. Cornwell, K.G., Downing, B.R., and Pins, G.D. Characterizing fibroblast migration on discrete collagen threads for applications in tissue regeneration. *J Biomed Mater Res A* **71**, 55, 2004.
26. Olde Damink, L.H., Dijkstra, P.J., van Luyn, M.J., van Wachem, P.B., Nieuwenhuis, P., and Feijen, J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. *Biomaterials* **17**, 765, 1996.
27. Olde Damink, L.H., Dijkstra, P.J., van Luyn, M.J., van Wachem, P.B., Nieuwenhuis, P., and Feijen, J. *In vitro* degradation of dermal sheep collagen cross-linked using a water-soluble carbodiimide. *Biomaterials* **17**, 679, 1996.
28. Pins, G.D., Toner, M., and Morgan, J.R. Microfabrication of an analog of the basal lamina: biocompatible membranes with complex topographies. *FASEB J* **14**, 593, 2000.
29. Boyce, S.T., and Williams, M.L. Lipid supplemented medium induces lamellar bodies and precursors of barrier lipids in cultured analogues of human skin. *J Invest Dermatol* **101**, 180, 1993.
30. Bowditch, R.D., Halloran, C.E., Aota, S., Obara, M., Plow, E.F., Yamada, K.M., and Ginsberg, M.H. Integrin α IIb β 3 (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. *J Biol Chem* **266**, 23323, 1991.
31. Scholzen, T., and Gerdes, J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* **182**, 311, 2000.
32. Allen, L.T., Tosetto, M., Miller, I.S., O'Connor, D.P., Penney, S.C., Lynch, I., Keenan, A.K., Pennington, S.R., Dawson, K.A., and Gallagher, W.M. Surface-induced changes in protein adsorption and implications for cellular phenotypic responses to surface interaction. *Biomaterials* **27**, 3096, 2006.
33. Kowalczyńska, H.M., Nowak-Wyrzykowska, M., Dobkowski, J., Kolos, R., Kaminski, J., Makowska-Cynka, A., and Marciniak, E. Adsorption characteristics of human plasma fibronectin in relationship to cell adhesion. *J Biomed Mater Res* **61**, 260, 2002.
34. Heenen, M., Thiriar, S., Noel, J.C., and Galand, P. Ki-67 immunostaining of normal human epidermis: comparison with 3H-thymidine labelling and PCNA immunostaining. *Dermatology* **197**, 123, 1998.
35. Caldwell, C.J., Hobbs, C., and McKee, P.H. The relationship of Ki67 and involucrin expression in proliferative, preneoplastic and neoplastic skin. *Clin Exp Dermatol* **22**, 11, 1997.
36. Usui, M.L., Mansbridge, J.N., Carter, W.G., Fujita, M., and Olerud, J.E. Keratinocyte migration, proliferation, and differentiation in chronic ulcers from patients with diabetes and normal wounds. *J Histochem Cytochem* **56**, 687, 2008.
37. Usui, M.L., Underwood, R.A., Mansbridge, J.N., Muffley, L.A., Carter, W.G., and Olerud, J.E. Morphological evidence for the role of suprabasal keratinocytes in wound re-epithelialization. *Wound Repair Regen* **13**, 468, 2005.
38. PMA Approval for Integra Dermal Regeneration Template United States Food and Drug Administration, 2002. PMA: P900033 www.fda.gov.
39. Pins, G.D., Christiansen, D.L., Patel, R., and Silver, F.H. Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties. *Biophys J* **73**, 2164, 1997.
40. Kleinman, H.K., McGoodwin, E.B., Martin, G.R., Klebe, R.J., Fietzek, P.P., and Woolley, D.E. Localization of the binding site for cell attachment in the α 1(I) chain of collagen. *J Biol Chem* **253**, 5642, 1978.
41. Vallieres, K., Chevallier, P., Sarra-Bournet, C., Turgeon, S., and Laroche, G. AFM imaging of immobilized fibronectin: does the surface conjugation scheme affect the protein orientation/conformation? *Langmuir* **23**, 9745, 2007.
42. Vallieres, K., Petitclerc, E., and Laroche, G. Covalent grafting of fibronectin onto plasma-treated PTFE: influence of the conjugation strategy on fibronectin biological activity. *Macromol Biosci* **7**, 738, 2007.
43. Zhang, Y., Chai, C., Jiang, S., Teoh, S., and Leong, K. Fibronectin immobilized by covalent conjugation or physical adsorption shows different bioactivity on aminated-PET. *Mater Sci Eng C* **27**, 213, 2007.
44. Powell, H.M., and Boyce, S.T. EDC cross-linking improves skin substitute strength and stability. *Biomaterials* **27**, 5821, 2006.
45. Powell, H.M., and Boyce, S.T. Wound closure with EDC cross-linked cultured skin substitutes grafted to athymic mice. *Biomaterials* **28**, 1084, 2007.
46. Pieper, J.S., Hafmans, T., Veerkamp, J.H., and van Kuppevelt, T.H. Development of tailor-made collagen-glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects. *Biomaterials* **21**, 581, 2000.
47. Pieper, J.S., van Wachem, P.B., van Luyn, M.J.A., Brouwer, L.A., Hafmans, T., Veerkamp, J.H., and van Kuppevelt, T.H. Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats. *Biomaterials* **21**, 1689, 2000.
48. Pieper, J.S., Oosterhof, A., Dijkstra, P.J., Veerkamp, J.H., and van Kuppevelt, T.H. Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. *Biomaterials* **20**, 847, 1999.

Address correspondence to:

George D. Pins, Ph.D.

Department of Biomedical Engineering

Worcester Polytechnic Institute

60 Prescott St.

Worcester, MA 01606

E-mail: gpins@wpi.edu

Received: July 23, 2009

Accepted: September 23, 2009

Online Publication Date: January 7, 2010