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Reconstructive

Functional Properties of a Purified Reconstituted Bilayer Matrix Design Support Natural Wound Healing Activities

David G. Armstrong, DPM, MD, PhD*
Dennis P. Orgill, MD, PhD†
Robert D. Galiano, MD‡
Paul Glat, MD§
Jarrod Kaufman, MD¶
Marco Mehr, MSc‖
Charles M. Zelen, DPM**

Background: Biomaterial engineering has produced numerous matrices for use in tissue repair, utilizing various materials and processing methods, which can impact the ability of the products to encourage wound healing. Recently, we observed favorable clinical outcomes, using a novel purified reconstituted bilayer matrix (PRBM; Geistlich Derma-Gide) to treat chronic diabetic foot ulcers.
Methods: Evaluations of the structural and functional characteristics of PRBM in vitro were performed to assess how this biomaterial may affect the favorable clinical results observed by influencing the wound environment and key physiologic mechanisms necessary for the healing process. Investigations included scanning electron microscopy, cell culture analyses, gene expression assays, matrix metalloproteinase activity assessment, and pH measurement.
Results: Cross-sectional scanning electron microscopy demonstrated a distinct bilayer structure with porous and compact layers. The PRBM structure allowed cell types involved in wound healing to bind and proliferate. Expression analysis of growth factor-responsive genes demonstrated binding and preservation of bioactive growth factors TGF-β1, bFGF, and VEGF by PRBM. Boyden chamber migration assays revealed increased cellular migration compared with controls. In the presence of PRBM, the activity of MMP-1, MMP-2, and MMP-9 was significantly lower compared with control samples. pH of the PRBM in solution was slightly acidic.
Conclusions: Based on in vitro evaluations, it appears that the PRBM processing without deleterious chemical crosslinking results in a suitable ECM possessing characteristics to aid natural wound healing, including cell attachment, migration, proliferation, differentiation, and angiogenesis. These in vitro data support the promising healing rate observed clinically when chronic DFUs are treated with PRBM. *(Plast Reconstr Surg Glob Open 2021;9:e3596; doi: 10.1097/GOX.0000000000003596; Published online 21 May 2021.)*

INTRODUCTION

The burden of treating chronic wounds is significant, with the healthcare cost of US Medicare patients alone estimated from $28 billion to $96 billion in 2014. A dramatic increase in the cost of foot ulcer treatment specifically has been attributed, in part, to the rising prevalence of type 2 diabetes.[1] These demographic and economic indicators have spurred continued development of biomaterials for chronic wound healing.

Successful healing requires the appropriate balance of bioactive factors responsible for repair processes,

*From the *Southwestern Academic Limb Salvage Alliance (SALSA), Keck School of Medicine of University of Southern California, Los Angeles, Calif.; †Division of Plastic Surgery, Brigham and Women's Hospital, Boston, Mass.; ‡Division of Plastic Surgery, Feinberg School of Medicine, Northwestern University, Chicago, Ill.; §Drexel University College of Medicine, St. Christopher's Hospital for Children, Philadelphia, Penn.; ¶Department of Surgery, Temple University School of Medicine and McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Penn.; ‖Geistlich Pharma AG, Wolhusen, Switzerland; and **Professional Education and Research Institute, Roanoke, Va.*

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Table 1. Cell Lines

Cell line	Abbreviation	Manufacturer	Medium
Adult human dermal fibroblasts	aHDF	ScienCell, Carlsbad, Calif.	Dulbecco's modified Eagle's with 10% fetal bovine serum and 1% Penicillin/Streptomycin solution (all Thermo Fisher Scientific)
Neonatal human epidermal keratinocytes	HEKn	CellnTec, Bern, Switzerland	EpiLife medium with 1× Human Keratinocyte Growth Supplement (HKGS, all Life Technologies)
Human umbilical vein endothelial cells	HUVEC	Caltag Medsystems, Buckingham, UK	Human large vessel endothelial cell growth medium (Caltag)

involving inflammation, granulation tissue formation, re-epithelialization, matrix formation, and remodeling.[2] In chronic wounds, the extracellular matrix (ECM), a crucial component in these events, experiences fundamental biochemical abnormalities, leading to interruption of the natural healing progression.[3,4]

Advances in materials engineering research have produced novel ECM materials, with the ultimate goal of creating a biocompatible matrix that can support healing in an unhospitable microenvironment. Adaptation of the ECM structure through processing techniques can produce a matrix within specified architectural design, with properties that closely mimic human dermal ECM and allow for chemotaxis, cell attachment, proliferation, and remodeling.[5] In some cases, however, processing may negatively impact the product's ability to adequately accomplish these crucial functions.[6,7]

Advanced matrices typically take the form of membranes described as having suitable structure and the capacity to promote biologic activities necessary for wound healing.[8,9] Practically, materials should be readily available, shelf-stable, and have trouble-free handling characteristics, allowing for sizing and direct application to the wound bed. Recently, we evaluated a unique purified reconstituted bilayer matrix (PRBM; Geistlich Derma-Gide) and found it easy to apply, safe, and efficacious in treatment of 10 patients chronic diabetic foot ulcers.[10] We now analyzed the intrinsic properties of PRBM to understand its mechanisms of action. To that end, we performed a series of in vitro studies evaluating key material characteristics and their connection to the natural wound healing sequence.

MATERIALS AND METHODS

Material

An FDA 510(k) cleared (K182838) PRBM (Geistlich Derma-Gide, Geistlich Pharma AG, Wolhusen, Switzerland) was evaluated. The PRBM is produced from porcine connective tissue using proprietary processes including extraction and purification steps to remove cells, lipids, undesired proteins, antigens, and to inactivate potential viruses. The material is reconstituted using lyophilization to form a noncrosslinked, 3-dimensional bilayer extracellular matrix that is sized, packaged, and terminally sterilized. (**See pdf, Supplemental Digital Content 1,** which displays PRBM Processing Steps (ref: Geistlich Pharma AG): A: PRBM Processing Steps: Production of the bilayer wound matrix from porcine tissue follows a 3-step process; extraction, purification, and refinement of the material into a bilayer design featuring a compact upper layer and porous lower layer. The material is packaged dry, but its design allows for rapid fluid uptake. B: PRBM in hydrated condition. **http://links.lww.com/PRSGO/B655**.)

PRBM Morphology (SEM, Mercury Intrusion Porosimetry)

PRBM surfaces were coated with Au/Pd alloy (SC7620, Quorum Technologies). Images were recorded at 810× magnification by scanning electron microscopy (Phenom X Pro, FEI). Pore size distribution was determined by mercury intrusion porosimetry (Poremaster 60 GT, Quantachrome) according to ISO 15901 using a contact angle of 145 degrees and surface tension of mercury of 0.485 Nm^{-1}.

Cell Cultures

Table 1 details cell lines, abbreviations, and cell culture media utilized. Expanded aHDF and HEKn were seeded onto the PRBM smooth surface at 50,000 cells/50 µl medium per 8 × 8 mm pieces. Medium was supplemented after 1 hour at room temperature and subsequent conditions maintained (37°C, 5% CO_2, humid atmosphere), with medium changed thrice weekly. For co-culture experiments, human umbilical vein endothelial cells (HUVECs) and aHDF were seeded onto PRBM at 40,000 cells/50 µl medium at a ratio 1:9. Medium was supplemented after 1 hour at room temperature and cells were cultured for 14 days, as described by the manufacturer (Caltag). Skin equivalents were prepared following the manufacturer's instructions (Cellntec). aHDF were seeded onto PRBM smooth surface and allowed to establish a layer (10d). Keratinocytes were seeded atop and grown in submerged culture (3d). Models were air-lifted and grown at the air–liquid interface (12d).

Histology and Microscopy

Scaffolds were washed in phosphate buffered saline (PBS), fixed with 10% neutral-buffered formalin (PFA), permeabilized with incubation in 0.1% or 0.5% Triton X-100/PBS and embedded in 15% gelatin/PBS, fixed with PFA, and sectioned. Immunofluorescence was performed according to the manufacturer's instructions (Table 2). Samples were imaged with a confocal microscope (Yokogawa

Table 2. Reagents and Antibodies Used for Immunofluorescence

Reagent/ Antibody	Species	Manufacturer	Dilution
Alexa Fluor 488 Phalloidin	—	Thermo Fisher Scientific	1:80
DAPI	—	Thermo Fisher Scientific	1:2000
CD-31	Mouse	Dako, Baar, Switzerland	1:50
Anti-mouse Alexa Fluor 546	Goat	Thermo Fisher Scientific	1:1000

CV1000 Cell Voyager, Visitron Systems). Skin equivalents were fixed, and sections from paraffin-embedded samples were deparaffinized, dehydrated, H&E stained, and analyzed by light microscopy.

Tissue Extract Preparation

PRBM 1 × 1 mm squares were extracted in basal media appropriate for cell type (overnight, 37°C). Tissue residue was removed by centrifugation and the extract sterile filtered. The extract was transparent, soluble at physiological conditions, but solid at 4°C.

Trans-well Migration Studies

Migration assays used xCELLigence RTCA DP Real Time Cell Analyzer (Acea Biosciences). HEKn at 70%–80% confluency were starved (3 hours), trypsinized, neutralized, centrifuged and resuspended. PRBM extracts of 0.5, 2, and 5 mg/ml were loaded into the lower chamber of each well. Unsupplemented medium served as the negative control, and addition of 10% HKGS as the positive control. For confirmation, a 2-mm dry PRBM disk was placed in the lower chamber with unsupplemented medium. An estimated 30,000 cells were loaded into the upper wells with 150-µl unsupplemented medium and cultured (37°C, 16 hours). Migrating cells were detected via cell indexing every 5 minutes; 12 hours are reported. After 16 hours, cells were fixed, stained (0.5% crystal violet solution), and imaged.

MMP Activity Assay

Human recombinant MMPs (R&D Systems) were activated with p-aminophenylmercuric acetate. In total, 100 ng activated rhMMP (at 2 ng/µl in buffer)/mg PRBM was added and incubated (37°C, agitation). Residual enzymatic activity in the supernatant was measured by adding fluorogenic substrate Mca-K-P-L-G-L-Dpa-A-R-NH2 (R&D Systems). Relative fluorescence units were read in kinetic mode (Synergy H1 reader, BioTek). Residual MMP activity was determined by comparing Vmax of the PRBM supernatant relative to Vmax of control (untreated MMP solution incubated in parallel), expressed as percentage activity.

Gene Expression Assays

PRBM 8 × 8 mm squares were incubated with recombinant human transforming growth factor-beta 1 (TGF-β1; 50 ng/ml), basic fibroblast growth factor (bFGF; 50 ng/ml), or vascular endothelial growth factor (VEGF; 100 ng/ml) (R&D Systems) diluted in PBS or complete culture medium. After incubation, membranes were vigorously washed (4×, 15 minutes). An estimated 50,000 cells/sample were seeded on the PRBM smooth surface: aHDFs on TGF-β1- and bFGF-treated membranes and HUVECs on VEGF-treated membranes, respectively. Nonincubated PRBM seeded with cells served as controls. Additionally, cells were seeded directly onto a plate, and membranes were placed in inserts, avoiding contact with cells to evaluate washing. After incubation of 1 hour (for VEGF), 24 hours (for bFGF), and 48 hours (for TGF-β1), cells were lysed and RNA was extracted.

Table 3. Growth Factor Response Genes Selected for Analysis (Applied Biosystems)

Short Name	Gene	Identifier
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1
KANK4	KN motif and ankyrin repeat domains 4	Hs01057354_m1[11,12]
EGR3	Early growth response 3	Hs04935588_m1[13]
MMP-1	Matrix metallopeptidase 1	Hs00899658_m1[14]

qRT-PCR Analysis

Growth factor target genes selected were: KANK4 for TGF-β1, EGR3 for VEGF, and MMP-1 for bFGF with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) utilized as reference. Total RNA was prepared with TRIzol, and 500 µg used for reverse transcription (Aurum Total RNA Mini Kit, BioRad, Cressier, Switzerland). qRT-PCR was performed (Light Cycler 480 Probes Master, Roche; CFX PCR System, Bio-Rad). TaqMan probes were selected (Table 3; Thermo Fisher Scientific).

pH Assessment

pH was measured per Pharmacopoea Europea (2.2.3)/USP 791. Sterile dry PRBM samples were placed into either CO_2-free water for 10 minutes (0.01 g/ml) or PBS for 48 hours, 37°C (chosen to mimic physiological conditions, as suggested in ASTM F2150-13). pH was measured after incubation.

Statistical Analysis

Data from multiple test groups were analyzed using GraphPad Prism (version 7.04; GraphPad Software) to perform analysis of variance (ANOVA) with pairwise comparisons performed using Tukey's test. Two test groups were analyzed using Microsoft Excel to perform Student's *t*-test.

RESULTS

Material Morphology

Scanning electron microscopy demonstrated that PRBM had a macroscopic structure similar to the organization of human skin matrix, with an uppermost compact layer and a thick lower porous layer. (**See pdf, Supplemental Digital Content 2**, which displays Scanning Electron Microscopy. A–C: Dry human skin and PRBM imaged by scanning electron microscopy. Lateral view on cross section of human skin (A) and PRBM (B) and top view of upper compact layer of PRBM (C) are shown. The dashed line demarcates the boundary of epidermis and basement membrane in the human skin and the upper boundary of the compact layer in PRBM. The compact layer of PRBM is characterized by a compact network of arranged ECM fibers. Small (pore size < 10 µm) and large (pore size > 200 µm) pores are visible with most having a diameter in the range of 50 to 90 µm. Copyright SEM human skin (A): Keystone/Science Photograph Library/Steve Gschmeissner. The scale bar is 200 µm. CL, compact layer; DE, dermis; EP, epidermis; PL, porous layer. **http://links.lww.com/PRSGO/B656**.) The upper layer

was characterized by a compact network of arranged ECM fibers forming a sheet-like structure, whereas the lower layer appeared as a loose orientation of fibers forming a sponge-like structure (**See pdf, Supplemental Digital Content 2**. **http://links.lww.com/PRSGO/B656**.). Pore size was determined by mercury intrusion porosimetry. The overwhelming majority of pores observed were between 10 and 400 μm. The modal pores size averaged 73 μm ± 9 μm (n = 10 per batch, 3 batches). PRBM pores were larger than the reported dimensions of fibroblasts and other cells involved in the wound healing cascade, which range from 10 to 15 μm.[15]

Cell Growth and Proliferation

Fibroblasts and keratinocytes appeared to bind to PRBM, proliferate and migrate over time. (**See pdf, Supplemental Digital Content 3**, which displays the supplemental materials and methods. **http://links.lww.com/PRSGO/B657.**) Cultured aHDFs exhibited spindle-shaped, elongated phenotype characteristic of fibroblasts, and processes extending out from the end of the cell body. Consecutive images depicted increased cell density with a confluent cell layer at day 10. F-Actin staining demonstrated a very dense cell layer of viable and aligned cells (Fig. 1A). At day 3, keratinocytes displayed polygonal cobblestone morphology typically observed for this cell type. Consecutive images depicted an increase in cell density and the appearance of a confluent, epithelial-like cell layer at day 10. A dense cell layer and formation of close cell–cell adhesions was observed (Fig. 1B). In co-culture experiments, nonspecific staining of F-Actin bundles (Fig. 1C, green staining) demonstrated that aHDF formed a confluent cell layer on PRBM and supported endothelial cells forming multicellular, vessel-like structures, and staining positive for CD-31 (Fig. 1C, red staining). Sprouting and lateral vessel connections were observed. Fibroblasts appeared to form a dermis-like structure rich in ECM on both the filter membrane and PRBM. A stratified multilayer epithelium was evident for cells cultured on PRBM and less pronounced for cells grown on filter membrane (Fig. 1D). (**See pdf, Supplemental Digital Content 3**, **http://links.lww.com/PRSGO/B657.**)

Incubation of aHDF with soluble PRBM showed a mild increase in proliferation. However, compared with 10% FBS and growth factors supplementation, this effect was rather low, suggesting that soluble PRBM does not contain directly mitogenic factors, and its proliferative effect relies on ECM components only (Supplemental material). These findings align with protein analyses performed by the manufacturer using tandem mass spectrometry (MS/MS), which did not detect peptides in PRBM that could be assigned to growth factors.

Growth Factor Response

Growth factors TGF-β, bFGF, and VEGF are key molecules in wound healing, which are secreted by migrating cells and which bind to extracellular matrix in the wound periphery.[11–15] The ability of these growth factors at physiological concentrations to bind to PRBM and retain their cell signaling activity was assessed. PRBM was incubated with the growth factors and extensively washed to remove any unbound growth factors. Gene expression responses of cells seeded on the PRBM specific to growth factors TGF-β1, bFGF, and VEGF were analyzed. The signaling activity of the growth factors was preserved and retained when washed PRBM was stored (72 hours, 4°C) before seeding (Fig. 2A–C). Response to TGF-β1 was preserved when physiological solution was replaced by cell culture medium containing 10% FBS, but abolished when cells were cultured in indirect contact (data not shown). PRBM bound the growth factors TGF-β1, bFGF, and VEGF, all known to be present in the wound healing environment.[2]

Human Epidermal Keratinocyte (HEK) Migration

Samples containing 0.5, 2, and 5 mg/ml PRBM extracts demonstrated significantly greater HEK migration versus negative control. Groups containing 2 mg/ml and 5 mg/ml PRBM extracts reached or exceeded the number of cells detected in positive controls. Cell indexes were confirmed with crystal violet staining. Keratinocyte migration was further confirmed using PRBM discs. PRBM was capable of directing keratinocyte migration in vitro. **(See pdf, Supplemental Digital Content 4**, which displays effects of extracts of PRBM on HEK migration. HEKs were loaded in wells for transmigration assays and incubated for 12 hours; the lower chamber was loaded with soluble extract (A and B) or intact material (C), respectively. Migrated cells were detected in real-time through determination of cell index. A, Each value represents the average ± SD of extract prepared from 2 batches of PRBM, each analyzed in 3 independent experiments with n = 4 wells per experiment. B, Photographs of cells migrated toward the lower chamber and stained with crystal violet. C, Each value represents the average ± SD of n = 4 wells. $*P \leq 0.05$, $***P \leq 0.001$ compared with basal medium. Error bars represent SD. **http://links.lww.com/PRSGO/B658.)**

MMP Activity

Metalloproteases (MMPs) typically found in high levels in chronic wounds and representative of both collagenase and gelatinase categories were assessed (see supplemental material and methods). In the presence of PRBM, detected activity of MMP-1, MMP-2, and MMP-9 was significantly lower compared with control samples in the absence of PRBM (Fig. 3A-C). PRBM extracts appeared to be inhibitory to these MMPs in vitro.

pH Value

The pH of PRBM/CO_2-free water extraction solution was 4.11 ± 0.14 and pH was 6.32 ± 0.08 in the PRBM/PBS extraction solution. PBS control pH was 7.45 ± 0.01 (Table 4).

DISCUSSION

The mechanism of wound healing is complex, requiring a synchronized interplay of physiologic activities. An imbalance at any step in the healing sequence can interrupt the process and result in a chronic wound. When choosing from numerous options to treat these wounds, clinicians must carefully consider how each product

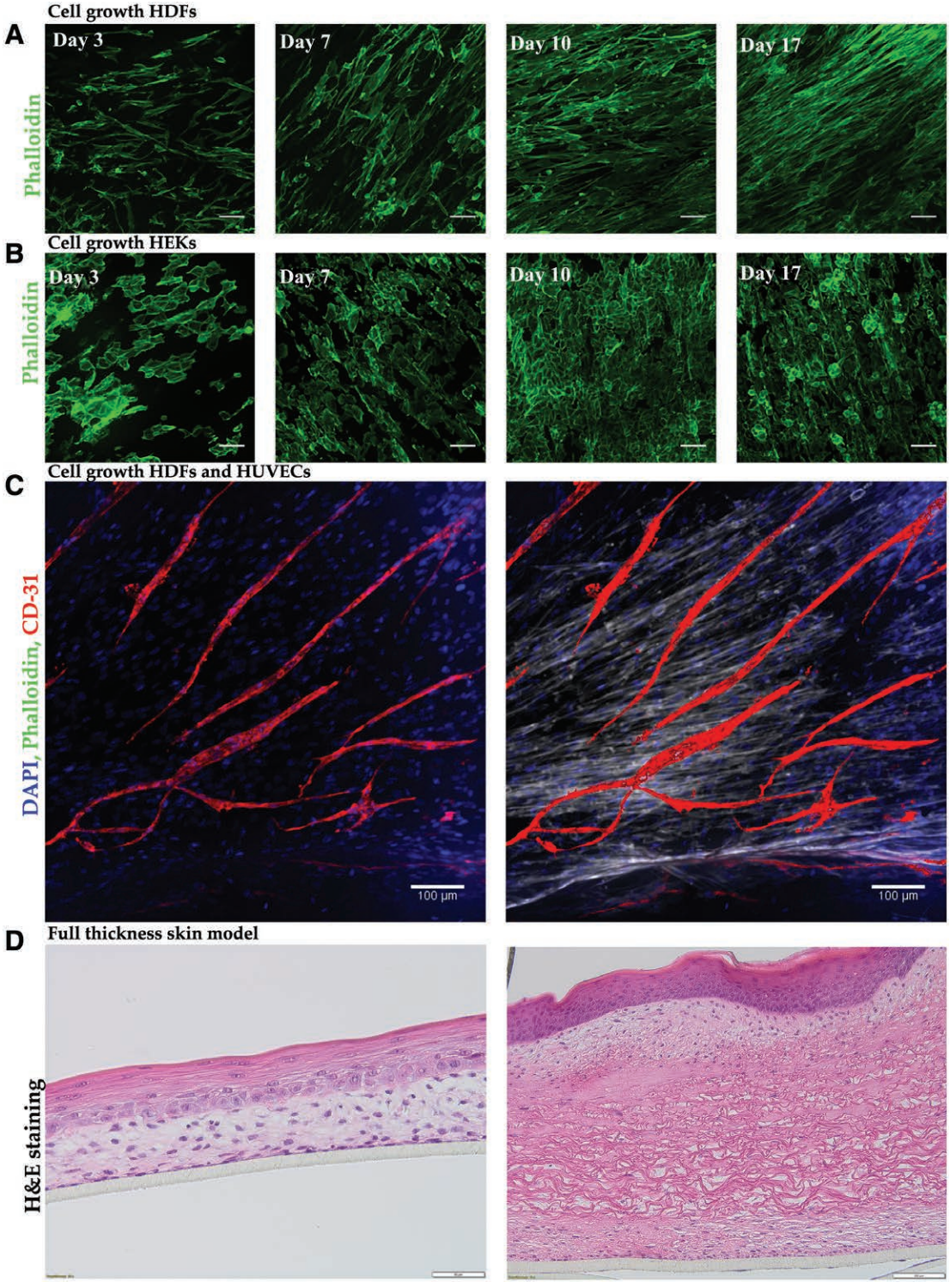

Fig. 1. Cell growth on PRBM. PRBM was seeded with human dermal fibroblasts (HDFs) (A) or HEKs (B) and fluorescently labelled with Phalloidin after indicated time points. Fibroblasts demonstrated a characteristic spindle-shaped, elongated phenotype (A). Keratinocytes showed typical polygonal cobblestone morphology (B). Over time, cell density increased, and both cell types reached confluency at day 10. The scale bar is 100 µm. C, Co-culture of HDFs and HUVECs, fluorescently labelled with DAPI (blue), Phalloidin (green) and anti-human CD31 antibody (red) after 14 days in culture. HDFs formed a confluent cell layer, and endothelial cells formed multicellular, vessel-like structures, positive for CD-31, and were sprouting and formed lateral connection. The scale bar is 100 µm. D, Co-culture of a full thickness skin model consisting of human dermal fibroblasts and HEKs on filter membrane (left) and PRBM (right). Paraffin-embedded sections were stained with H&E and images acquired using a light microscopy. The scale bar is 50 µm (left) and 200 µm (right), respectively.

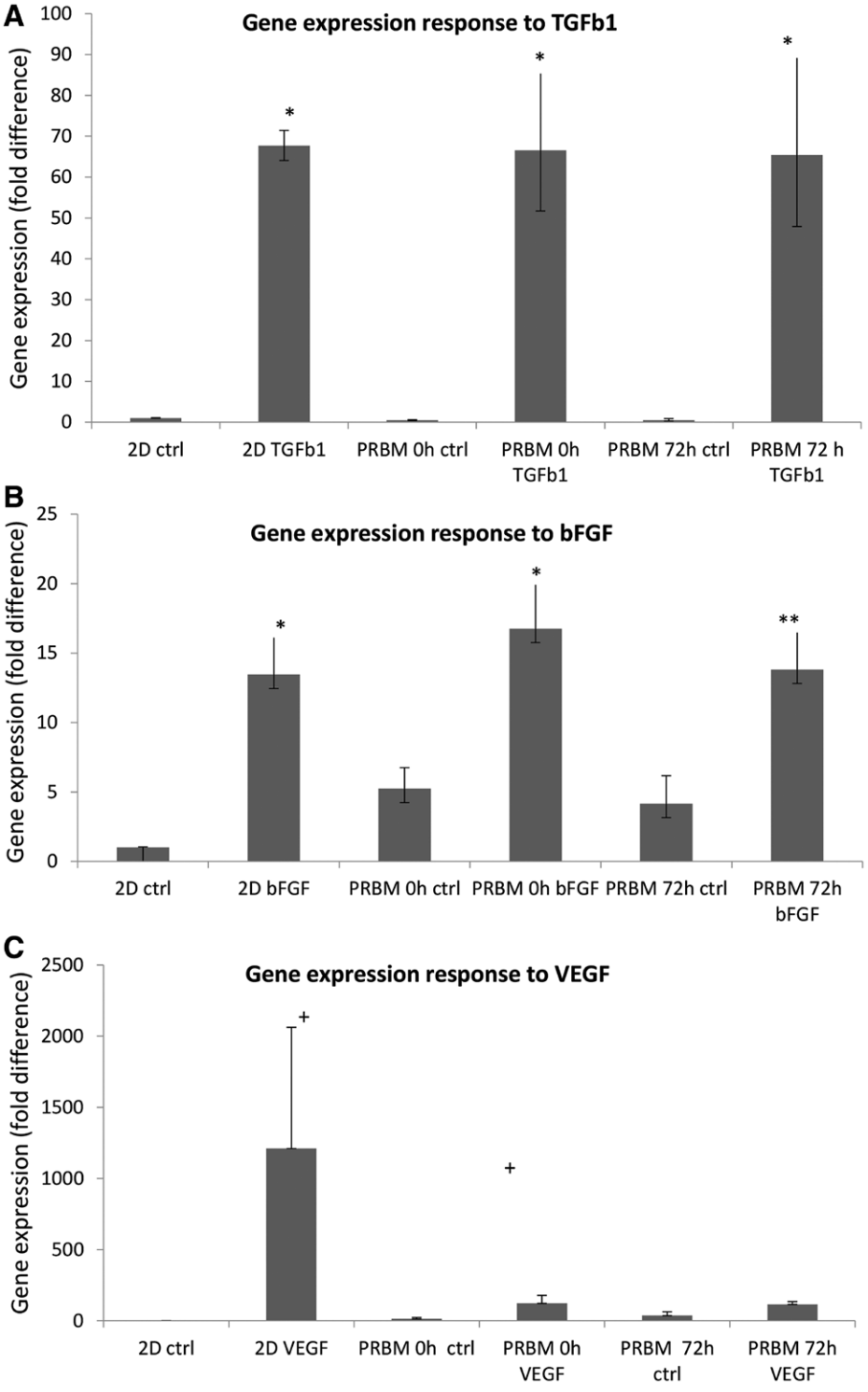

Fig. 2. Gene expression response to growth factors adsorbed to PRBM. Gene expression of growth factor-responsive genes was induced in adult human dermal fibroblasts (aHDF) and HUVECs grown on washed PRBM previously soaked in physiological solutions containing recombinant human growth factors. A, KANK4 response to TGF-b1 in aHDFs. B, MMP-1 response to bFGF in aHDFs. C, EGR3 response to VEGF in HUVECs. "PRBM 0h"—cells seeded onto PRBM immediately after washing; "PRBM 72h"—cells seeded onto PRBM after washing and storage for 72 hours. Before washing, PRBM was soaked with PBS/medium (ctrl) or PBS/medium containing growth factor. 2D, 2-dimensional, meaning cells grown on plastic and treated with medium (ctrl) or growth factor, as positive control of the gene expression effect. $^{**}P \leq 0.01$ compared with control; $^{***}P \leq 0.001$ compared with control. Error bars represent SD (SD).

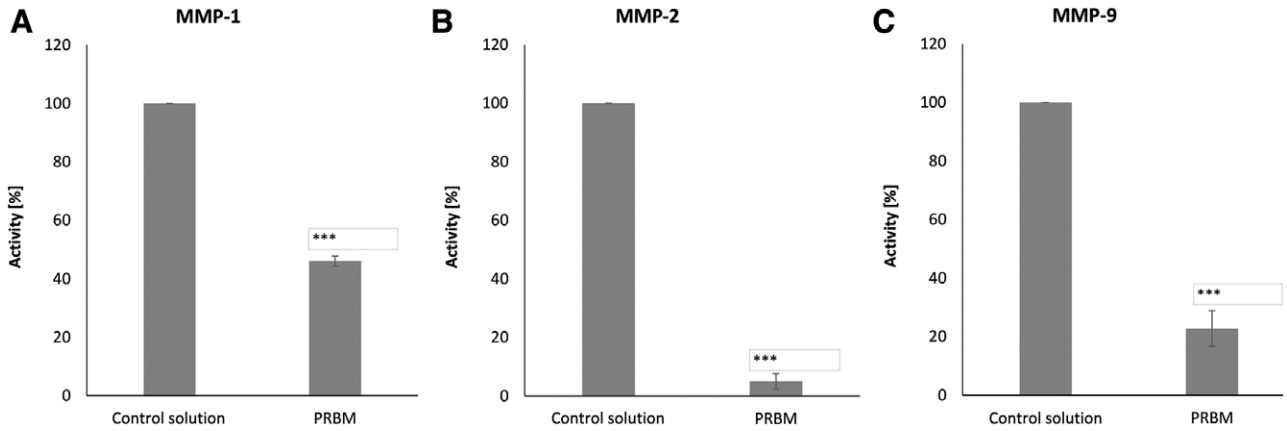

Fig. 3. Effects of extracts of PRBM on protease activity. Samples of PRBM were incubated in solutions containing human recombinant MMP-1 (A), MMP-2 (B), and MMP-9 (C). After 2 hours, supernatant extracts were mixed with fluorogenic substrate and protease activity was measured kinetically. Each value represents the average ± SD of 1 batch of PRBM, each analyzed in 2 independent experiments with n = 2 wells per experiment. ***$P \leq 0.001$ compared with control solution. Error bars represent SD.

functions under a specific combination of conditions. The healing of chronic wounds is an unpredictable process; however, the correlation between advanced wound care device characteristics and their clinical performance provides insights into the mechanism by which a product such as PRBM achieves its clinical goal. The classification, origin, composition, and characteristics of a range of available wound care products as well as PRBM are summarized in Table 5.

A recent 10-patient observational study demonstrated that Geistlich Derma-Gide PRBM, a bilayer matrix derived from porcine ECM, is safe and efficacious in treatment of hard to heal chronic diabetic foot ulcers.[10] In an effort to provide evidence for this material's mechanism of action, in this study we selected in vitro evaluations focusing on the material structure and fundamental bioactivities in the context of the wound healing cascade.

Structurally, the high porosity in the range of 10–400 µm observed via SEM suggests that the PRBM construct is suitable to support cellular ingrowth, attachment, and proliferation as well as mature vascularized tissue formation.[23] A scaffold with pore size much smaller than 10 µm can impede cell penetration. In a scaffold with excessively large pores, the density of ligands available can be insufficient for cell receptors to bind a minimum number of cells residing in the wound bed.[24] Our studies demonstrated the ability of PRBM to support fibroblast, keratinocyte, and endothelial cell attachment, and proliferation within the matrix itself under cell culture conditions. Furthermore, PRBM extracts enhanced fibroblast proliferation and keratinocyte migration. In comparison with full thickness skin, and supported by data presented, the PRBM uppermost compact layer functionally mimics the basement membrane, supporting attachment and growth of keratinocytes and allowing re-epithelialization. The lower porous layer accommodates ingrowth of fibroblasts and endothelial cells that would originate from the surrounding tissue in a patient's wound. These cells may support wound healing through the production of new ECM and through provision of nutrients to the epithelial layer.

Table 4. pH of Solutions after Incubation with PRBM

Solution	pH of PRBM in Solution
Water	4.11 ± 0.14
Phosphate buffered saline (PBS)	6.32 ± 0.08
Control PBS alone	7.45 ± 0.01 (no PRBM)

PRBM was incubated in water and PBS, respectively. Subsequently the pH of the solution was determined.

The association between excessive elevated MMP activity and the lack of healing in chronic diabetic wounds has been well described.[3,25,26] MMPs are sub-grouped as collagenases (MMP-1, 8, and 13) or gelatinases (MMP-2 and -9) or others based on the specificity of their substrates.[27] To simulate conditions that wound dressings typically encounter, we purposely assessed critical MMPs that are found in excessive levels in chronic diabetic wound fluids[25,27] from both collagenase and gelatinase categories (MMP-1, -2, -9). Collagen-based dressings designed to simulate normal ECM have demonstrated the ability to modulate MMPs, thus returning the compromised wound environment to a state considered more conducive to resume the normal healing progression.[28] Reduced protease activities in the presence of PRBM under in vitro conditions were observed in this study, leading us to hypothesize that the specific action in a clinical scenario may be entrapment into the matrix, thereby rendering an inability of proteases to degrade patient tissue as discussed by Tati et al[28] or competitive inhibition whereby competition between PRBM-derived peptides and patient tissue for the cleavage site of MMPs as reasonable mechanisms. Further experiments utilizing dose response methodology evaluating extract only, thereby excluding the possibility of entrapment, pointed to the latter as the more likely scenario, suggestive of a lower "MMP-threat" to patient tissue. Through targeting multiple proteases in the proteolytic cascade, the PRBM may prove to be clinically effective in modulating MMP activity over time particularly in the hyper-proteolytic environment of chronic wounds.

To further define the capabilities of the PRBM, we evaluated growth factors TGF-β1, bFGF, and VEGF, which function within several phases of healing and are

Table 5. Characteristics of Representative Commercially Available Acellular Skin and Dermal Substitutes for Chronic Wound Treatment

Skin Substitute Category[16]	Product Trade Name	Material Composition Characteristics	Material Composition Characteristics	Ref
Acellular bilayer dermal substitute, natural origin and temporary	Geistlich Derma-Gide (Geistlich Pharma AG)	Purified porcine collagen, native macro-structure (upper layer) and refined collagen (lower layer)	Upper occlusive barrier layer supports fibroblast attachment and proliferation. Refined collagen dermal layer exhibits biological activity: chemotactic effects and MMP inhibition	This article
Acellular bilayer dermal substitute, natural origin and temporary	Miroderm (Miromatrix Medical)	Purified porcine liver using perfusion decellularization	Intact extracellular matrix with an epithelial basement membrane, an open collagen matrix, and vascular ECM	[17]
Acellular bilayer. Epidermal layer is synthetic and permanent until removed. Dermal layer natural and temporary	Omnigraft (Integra LifeSciences)	Bilayer: Silicon; processed bovine tendon collagen and chondroitin-6-sulphate, cross-linked	Silicon moisture barrier. Dermal regeneration matrix with porosity and slower degradation of chemically cross-linked dermis-facing layer	[18]
Acellular single dermal layer (natural from cadaver). Temporary	Allopatch Pliable (MTF Biologics)	Acellular human dermal matrix: aseptically processed human dermis	Dermal substitute. Scaffold properties: Fibroblast attachment, proliferation, and invasion	[19]
Acellular dermal single layer—dehydrated human amnion-chorion membrane (dHACM). Temporary and natural origin	EpiFix (MiMedx)	Human amnion/chorion	Wound covering with endogenous growth factors and cytokines. Chemotactic and proliferative effects of retained GFs, and recruitment of mesenchymal progenitors to wound	[20]
Acellular single layer –and natural origin, temporary, dermal layer substitute	OASIS Wound Matrix (Smith and Nephew)	Porcine, small intestinal-submucosa collagen scaffold (SIS). Retention of growth factors and non-collagenous ECM	Dermal substitute. Scaffold for cellular invasion and growth factors	[21]
Antimicrobial collagen-based wound care product	Puraply (Organogenesis)	Porcine, small intestinal-submucosa collagen scaffold (SIS) with polyhexamethylene biguanide (PHMB)	SIS scaffold with the addition of antimicrobial activity	[22]

characteristically out of balance in chronic wounds. TGF-β1 contributes in the inflammatory phase and is involved in granulation tissue formation, re-epithelialization, matrix formation, and remodeling.[29] bFGF accelerates wound healing and functions in granulation tissue formation, re-epithelialization, matrix formation, and remodeling.[3] VEGF effects multiple components of the wound healing cascade, including tissue granulation, angiogenesis and epithelization, and collagen deposition.[30] While the presence of these important growth factors is decreased in chronic wounds, our experiments indicated that PRBM bound and preserved their bioactivity over 72 hours in vitro. Retention and protection of endogenous growth factors are expected to encourage arrested wound healing to proceed to closure.

Fundamental biochemical conditions in the wound environment should not be overlooked when considering the potential of advanced therapies. pH is an influential factor, with differing pH ranges required for distinct phases of healing.[31] Furthermore, it has been suggested that actively measuring and controlling local pH to bring the wound environment to an acidic condition could be beneficial to healing.[32] By encouraging growth of pathogenic bacteria, an alkaline pH in the wound bed can contribute to an unsuitable environment. In fact, chronic wounds exhibit excessive breakdown of the ECM, particularly in alkaline conditions. It has been reported that shifting pH level from alkaline to acidic may favor the production of healthy granulation tissue by decreasing both growth of bacteria and MMP activity.[33] The pH of PRBM was slightly acidic under physiologic conditions, which we believe may be beneficial in counteracting detrimental effects of an alkaline chronic wound environment. Additionally, an acidic environment can improve tissue oxygenation, which is conducive to successful healing.[34] We believe that combining technologies such as the one studied with companion diagnostics in vivo (ie, measuring pH or other factors) may assist in clinical decision-making and may provide further foundation for development/iteration of these technologies.

CONCLUSIONS

These in vitro analyses suggest the means by which the PRBM (Geistlich Derma-Gide) achieves its clinical goal of healing diabetic foot ulcers. The matrix is compatible with cellular ingrowth, and in media, the scaffold exhibits structural cues of native ECM, allowing attachment, proliferation, and migration of fibroblasts and keratinocytes. Growth factors bind to PRBM and are preserved in

an active state over time, while excessive MMP activity can be modulated in the presence of the material in culture conditions. These findings along with encouraging preliminary clinical results support further investigation and clinical application of this PRBM.

Charles M. Zelen, DPM
Professional Education and Research Institute
222 Walnut Ave.
Roanoke, VA 24016
E-mail: cmzelen@periedu.com

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