Modulation of Cell Attachment, Proliferation, and Angiogenesis by Decellularized, Dehydrated Human Amniotic Membrane in In Vitro Models

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Abstract: *Background*. Decellularized, dehydrated human amniotic membrane (DDHAM) is an extracellular matrix devoid of cells, cell debris, and growth factors. This study examines the effect of cell attachment to the DDHAM and the induced cellular responses. *Materials and Methods*. The cell types employed in this study were human dermal fibroblasts (HDF), human epithelial keratinocytes (HEK), and human dermal microvascular endothelial cells (HDMEC), all of which play critical roles in the wound healing process. Further, the DDHAM was compared to a dehydrated human amnion/chorion membrane (dHACM), which contains and releases biological entities including growth factors and cytokines. The HDF and HEK were cultured on the DDHAM and the dHACM, and cell imaging and proliferation assays were performed to evaluate cell attachment to and the ability to proliferate on the DDHAM relative to the dHACM. In addition, the effect of soluble factors released by the DDHAM and the dHACM on cell survival, attachment, and proliferation were examined. The authors also evaluated the effect of soluble factors produced by culturing cells on the DDHAM in in vitro functional assays, including cell survival and endothelial cell migration in a wound closure angiogenesis assay. *Results*. The HDF and HEK cells readily attached to and proliferated on the DDHAM, while the dHACM did not support cell attachment and proliferation when cultured under the same conditions. Soluble factors secreted when HDF were cultured on the DDHAM enhanced both endothelial cell and keratinocyte survival and endothelial cell migration in a wound closure assay. *Conclusions*. Although DDHAM is only an extracellular matrix and serves primarily as a scaffold, it has sufficient cues to allow for cell attachment and proliferation. Further, the biological entities released as a consequence of cell attachment promote cell survival and migration.

Key words: amniotic membrane, mechanism of action, wound healing, angiogenesis, biology of wound healing, tissue repair

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The amniotic membrane is one of the oldest biomaterials used as a scaffold in the treatment of burns and wounds. It was first used by Davis[1] as a skin transplant and later for multiple applications in ophthalmology,[2] reconstructive surgeries, and burn and wound management.[3] A long history of amniotic membrane use in wound management has resulted in a considerable volume of clinical evidence in its support, including an association with reduced pain, inflammation, and scarring, as well as the ability to provide a nonimmunogenic biological barrier for the wound.[4]

Various methods of preparation of the amniotic membrane allograft are employed today, resulting in a wide variety of allograft configurations. Options range from single-layered to multiple-layered sheets, some containing nonviable cells and growth factors, to completely decellularized membranes with minimal or no growth factors (eg, BIOVANCE, Alliqua BioMedical, Yardley, PA; EpiFix, MiMedx, Marietta, GA; AMNIOEXCEL, Derma Sciences, Plainsboro, NJ, etc.). Each of these allografts differs in surface characteristics, composition, and quantities of biologically active molecules and growth factors. As a result, they may have different wound-healing attributes and mechanisms of action.

Wound healing is a complex, temporally regulated process involving interaction of multiple circulatory and tissue resident cells with extracellular matrix (ECM) components.[5-7] In the present investigation, dehydrated, decellularized human amniotic membrane (DDHAM; BIOVANCE, Alliqua BioMedical, Yardley, PA) was studied in comparison to a dehydrated human amnion/chorion membrane (dHACM; EpiFix, MiMedx, Marietta, GA) in in vitro models. These benchtop studies included an examination of cell attachment, proliferation, and the consequent response of cell attachment in functional cell survival and migration assays. The cell types employed were human dermal fibroblasts (HDF), human epithelial keratinocytes (HEK), and human dermal microvascular endothelial cells (HDMEC), all of which play critical roles in the wound healing process. An intact single-layer amni-

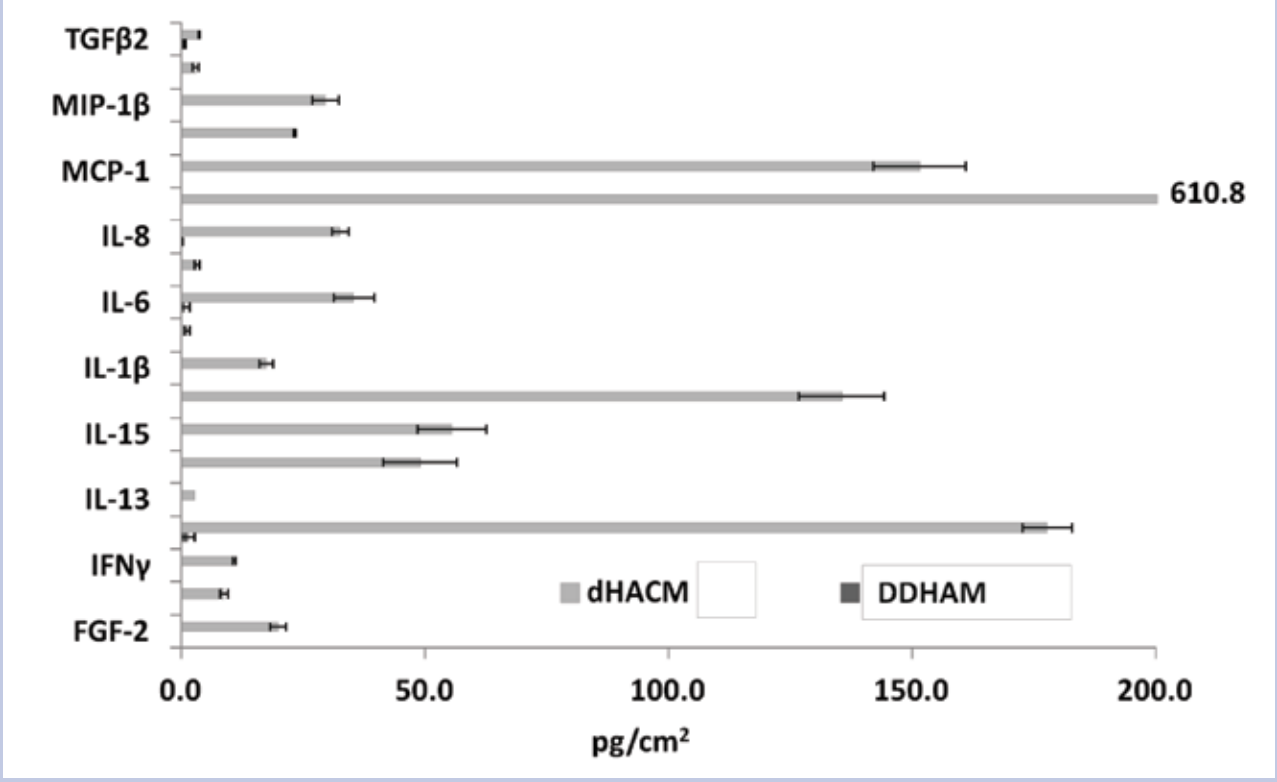

Figure 1. Levels of soluble factors released after a 24-hour incubation of dehydrated human amnion/chorion membrane (dHACM) and decellularized, dehydrated human amniotic membrane (DDHAM) in phosphate buffered saline at 37°C. TGFβ2: transforming growth factor-beta 2; MIP-1β: macrophage inflammatory protein-1beta; MCP-1: monocyte chemoattractant protein-1; IL: interleukin; IFNγ: interferon-gamma; FGF-2: basic fibroblast growth factor

otic membrane depleted of all cells and growth factors, a DDHAM retains a native ECM structure. The dHACM is a laminate consisting of amnion and chorion that retains nonviable cells and active growth factors.[8]

Materials and Methods

Biochemical characterization of DDHAM and dHACM. For the study, DDHAM and dHACM disks were cut into 12-mm diameter pieces, incubated in phosphate-buffered saline (PBS) for 24 hours at 37ºC and centrifuged at 16 000 g for 10 minutes. The supernatant was collected and used for the quantification of soluble factors. Soluble fibronectin and laminin were measured using ELISA kits (Takara Bio Inc, Shiga, Japan) on a SpectraMax i3 Multi-mode Microplate Detection Platform (Molecular Devices, Sunnyvale, CA). Growth factors were measured using the MILLIPLEX MAP human cytokine magnetic kit on the FlexMap 3D system (MILLIPLEX, EMD Millipore, Billerica, MA). All experiments were conducted with 3 samples isolated from different placentas of the DDHAM with 3 analytical replicates.

Cell attachment and proliferation. The HDF, adult HEK, and HDMEC, as well as respective growth media, were purchased from ScienCell Research Laboratories (Carlsbad, CA). The cells were thawed according to manufacturer protocol. The HDF and the HEK were expanded in Corning T-75 CellBind flasks (Corning, NY), and the HDMEC were cultured on fibronectin-coated Corning T-75 flasks up to passage 4.

For initial cell attachment and proliferation experiments, the DDHAM and the dHACM membranes were cut into 12-mm disks, and each disk was inserted into individual wells of a 24-well ultra-low binding plate (Corning, Corning, NY). Teflon inserts (11 mm ID) were inserted into the wells to secure the disks. The teflon inserts were inserted into wells of a 24-well tissue culture-treated plate (TCP) without the DDHAM as a control (TCP). Before cell seeding, 200 µL of growth medium was added to each well. Plates were incubated at 37°C for 30 minutes. The HDF and the HEK were trypsinized with 0.25% trypsin/EDTA (Life Technologies, Carlsbad, CA) from T-75 flasks and counted. The HDF or the HEK were seeded on the DDHAM, the dHACM, or the TCP at a respective density of 3000 cells/cm^2 or 15 000 cells/cm^2 in 200 µL of growth medium and cultured under standard cell culture conditions. Additional plates with scaffolds only were incubated under the same culture conditions and used as the assay control.

After 24 hours of cell culture, Calcein AM (Life Technologies) was added to selected wells from each of the 3 groups and incubated at 37°C for 15 minutes. Cells were then visualized and imaged using EVOS Cell Imaging Systems (Life Technologies).

At the same time, 3 samples from each group were

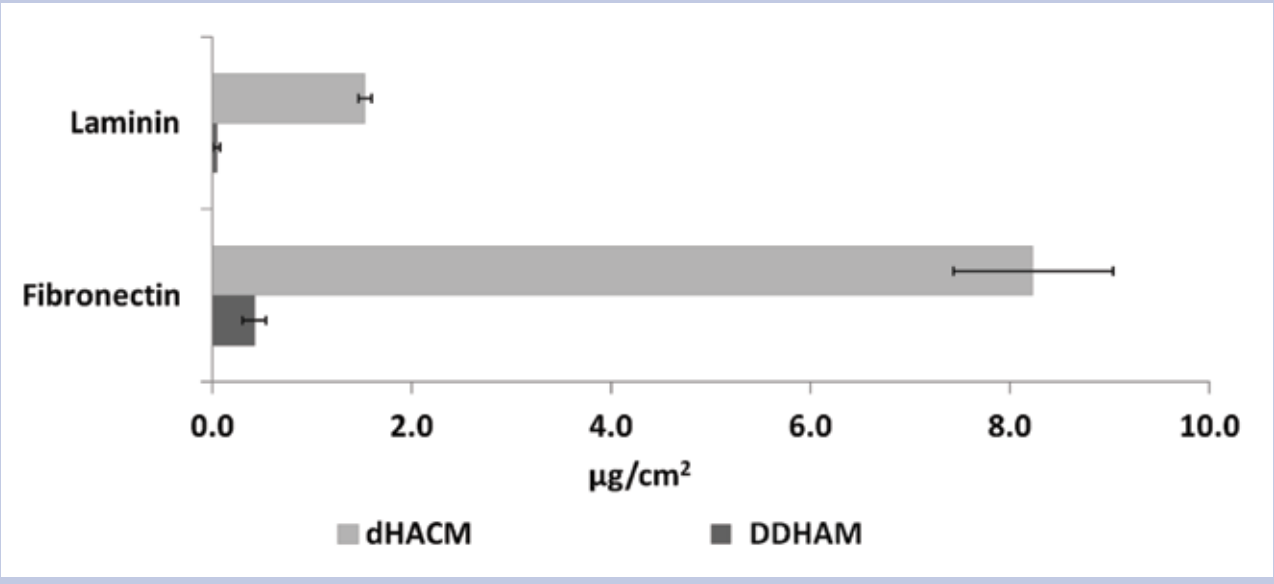

Figure 2. Levels of soluble extracellular matrix proteins released from dehydrated human amnion/chorion membrane (dHACM) and decellularized, dehydrated human amniotic membrane (DDHAM) after a 24-hour incubation in phosphate-buffered saline at 37°C.

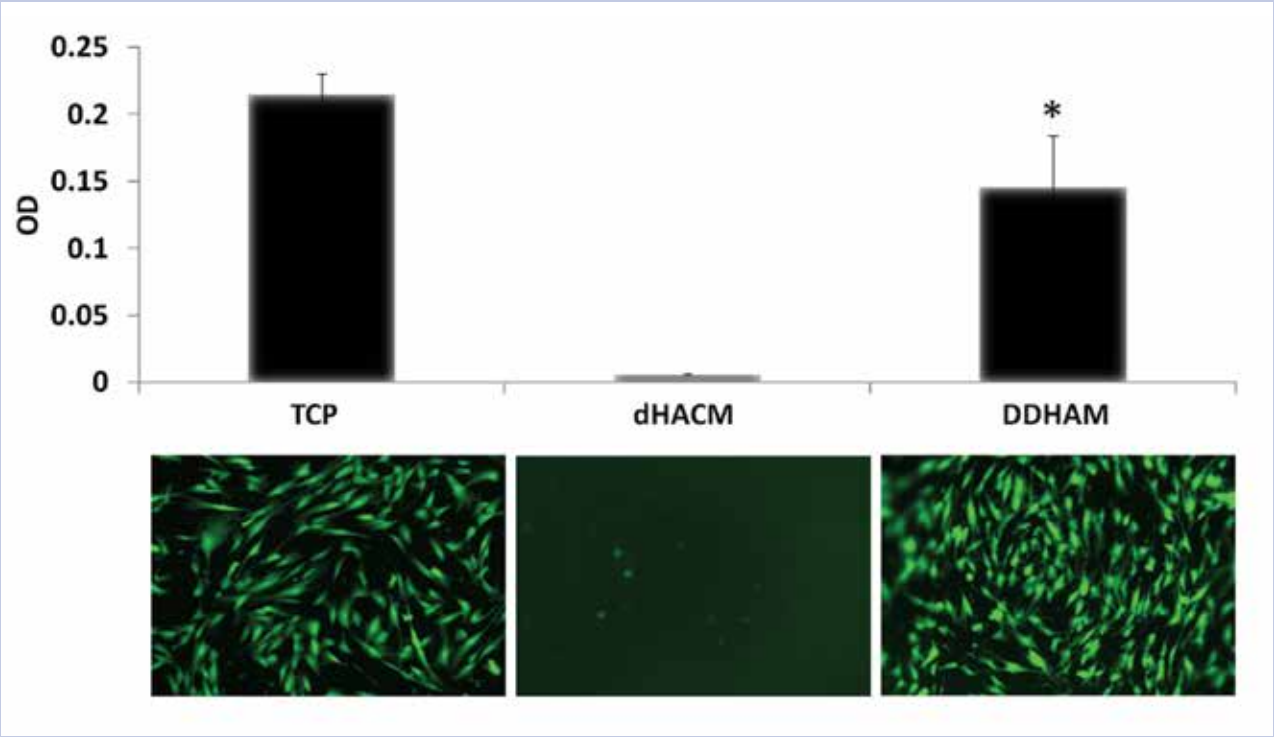

Figure 3. Levels of metabolic activity (MTS assay) of fibroblasts attached to a tissue culture-treated plate (TCP), dehydrated human amnion/chorion membrane (dHACM), and decellularized, dehydrated human amniotic membrane (DDHAM) at 24 hours (n = 3) and corresponding images of Calcein AM-stained cells at 10x magnification. OD: optical density
* indicates a significant difference compared to dHACM, $P < 0.05$.

subjected to CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), and cell metabolic activity/proliferation was assessed using protocols as described. In brief, culture media from the wells were first aspirated, and 100 µL of complete media and 20 µL of MTS reagent were added to each well. Samples were then incubated at 37°C for 1 hour, 100 µL from each well was transferred into a 96-well plate, and absorbance was read at 490 nm using a plate reader (BioTek, Winooski, VT). For each time point, samples from scaffolds-only wells were used as the control, and optical density was respectively subtracted from the total absorbance values.

To evaluate the effect of potential biological factors released from the scaffold extract on cell attachment and proliferation, DDHAM and dHACM were incubated in fibroblasts complete or basal medium at 37°C and 5% CO2 for 24 hours, and supernatant was collected. Human dermal fibroblasts were resuspended either in the scaffold extracts or media controls and seeded in culture plates at 10 000/cm^2. Cells were incubated for 24 hours under standard culture conditions. At 4 hours of culture, the Caspase-Glo 3/7 assay (Promega) was performed to evaluate levels of apoptosis. At 24 hours, the MTS assay was performed to evaluate cell metabolic activities/proliferation. Both assays were performed according to manufacturer protocol. Cells were also fixed at 4 hours and stained for nuclei (4',6-diamidino-2-phenylindole [DAPI]), actin (phalloidin 488), and fibronectin (AF594 IgG I, Life Technologies, Carlsbad, CA). Images were taken with a 40x objective.

To further assess cell attachment and proliferation on DDHAM for longer culture periods, the HDF or the HEK were seeded on the DDHAM and the TCP as previously described. After 4 hours, 1 day, and 3 days of cell culture, Calcein AM and MTS assays were performed as previously described.

In vitro functional assays. Conditioned media for the functional assays were generated as follows: The HDF were seeded on the DDHAM or the TCP at a density of 6000 cells/cm^2 in complete medium and cultured as previously described (fibroblasts on DDHAM and fibroblasts on TCP groups, respectively). Under the same conditions, the DDHAM disks and the TCP without cells were also

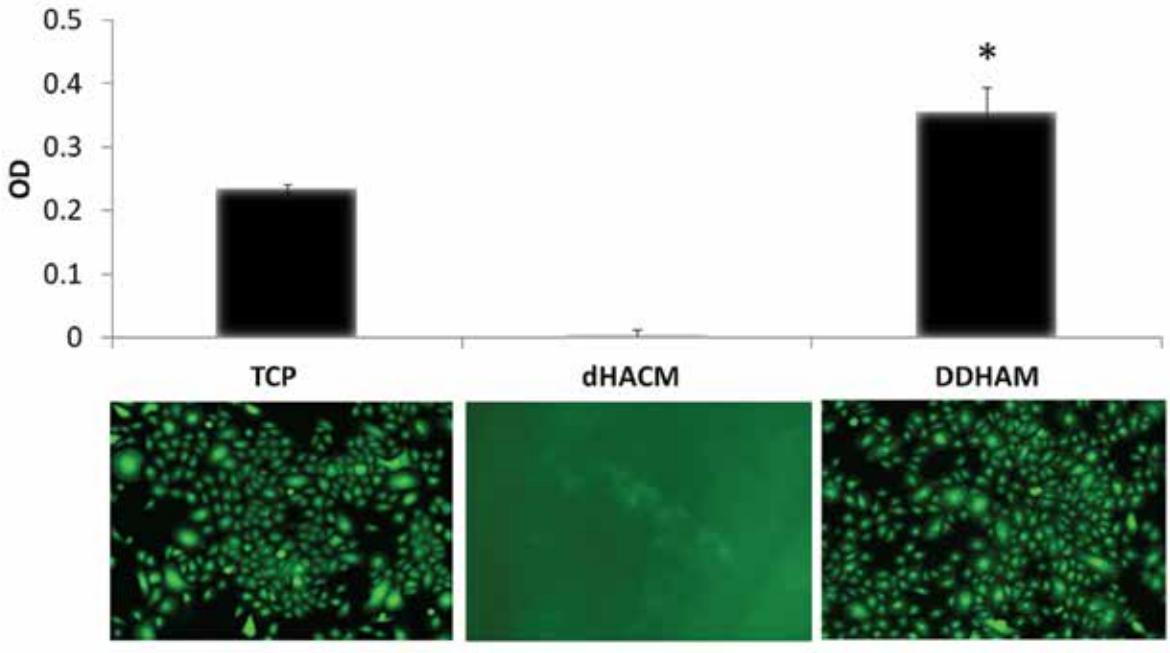

Figure 4. Levels of metabolic activity (MTS assay) of keratinocytes attached to a tissue culture-treated plate (TCP), dehydrated human amnion/chorion membrane (dHACM), and decellularized, dehydrated human amniotic membrane (DDHAM) at 24 hours (n = 3) and corresponding images of Calcein AM-stained cells at 10x magnification. OD: optical density
* indicates a significant difference compared to dHACM, $P < 0.05$.

incubated to serve as controls (DDHAM and TCP groups, respectively). After 24 hours, complete medium was removed from all wells, wells were washed 3 times with Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), and 350 µL of DMEM/0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was added into each well. After 18 hours of incubation at standard culture conditions, conditioned medium from each group was collected, stored at -80°C, and used in the following in vitro functional assays:

1. Cell survival assay: The HDMEC and HEK were expanded to passage 3 and cultured in a Corning 96-well fibronectin-coated plate and a 96-well TCP plate, respectively, until ~70% confluent. Cells were then washed and starved in DMEM without serum or growth factors for 5 hours. A 100-µL aliquot of conditioned media was applied to each well, and cells were incubated for 18 hours under standard culture conditions. A baseline MTS assay was performed before application of the conditioned media and repeated after 18 hours of culture in the conditioned media.
2. Wound healing assay: The HDMEC were expanded to passage 3 and cultured in the 96-well fibronectin-coated plates until ~100% confluent. The cells were washed with DMEM, and a scratch was created in each well using a WoundMaker (Essen Bioscience, Ann Arbor, MI). A 150-µL aliquot of conditioned media was then added to each well, and the cells were incubated for 72 hours under standard culture conditions. Images of wells were taken at 1-hour intervals with IncuCyte ZOOM System (Essen Bioscience), a live-cell imaging system, and analyzed for percent of wound confluence (closure) with IncuCyte ZOOM, Version 2014A software (Essen Bioscience).
3. Tube formation assay: Human umbilical vein endothelial cells (HUVEC) (Lonza, Walkersville, MD) were plated at passage 3 at 20 000 cells/cm^2 in a Corning T-25 flask) and allowed to recover overnight at 37°C, 5% CO2. The cells were serum starved in endothelial basal medium-2 (Lonza) for 3 hours, detached using 0.25% trypsin, and

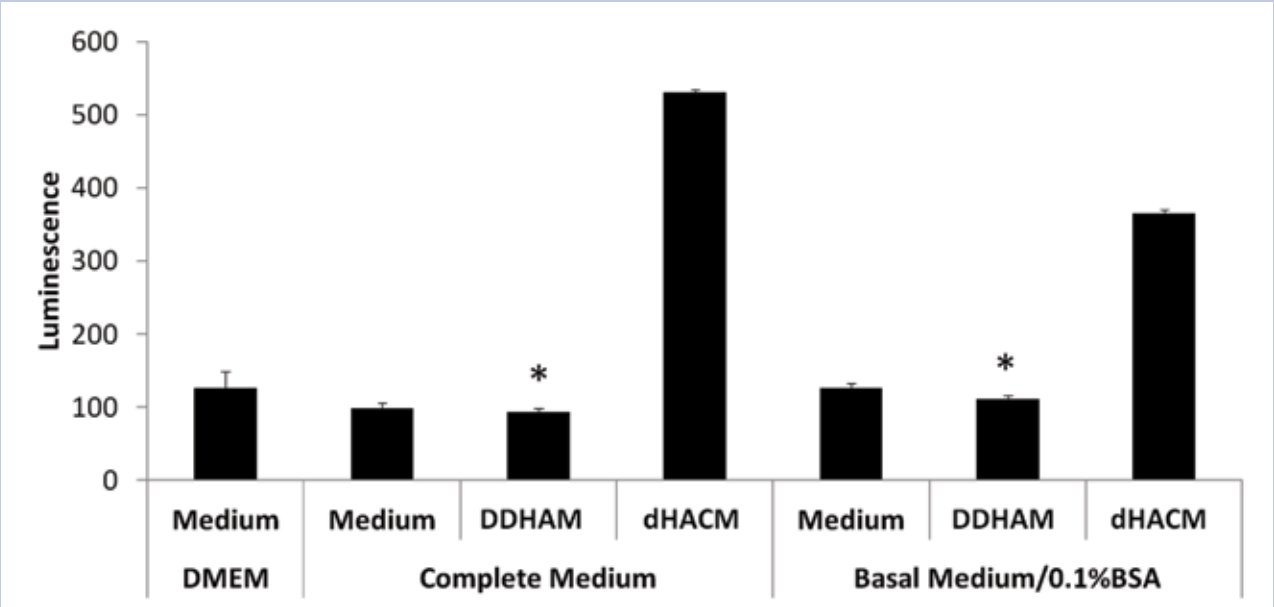

Figure 5. Levels of apoptosis (Caspase 3/7 assay) in fibroblasts seeded on a tissue culture-treated plate and exposed to decellularized, dehydrated human amniotic membrane (DDHAM) and dehydrated human amnion/chorion membrane (dHACM) "extracts" (conditioned medium) at 4 hours of culture (n = 3). DMEM: Dulbecco's Modified Eagle Medium; BSA: bovine serum albumin
* indicates a significant difference compared to dHACM, $P < 0.05$

resuspended in DMEM/0.1% human serum albumin (Octapharma, Hoboken, NJ) and counted. Cells (25 600) were then resuspended in 0.4 mL of conditioned media. Next, 100 µL of cell suspension was plated into a 96-well plate precoated with 50 µL of Cultrex Reduced Growth Factor (Trevigen, Gaithersburg, MD). The plates were then placed into the live-cell imaging system incubator, and the cells were imaged at 36 hours of culture. ImageJ software (public domain) was used to quantify the number of tubes and tube length per image.[9]

4. The same conditioned media from the HDF cultured on the DDHAM were analyzed for the presence and levels of pro-angiogenic factors potentially contributing to tube formation.
5. The same conditioned medium from 4 separate wells were analyzed using a Human Angiogenesis/Growth Factor Magnetic Bead Panel 1 multiplex kit (EMD Millipore, Billerica, MA). Analyses were performed per manufacturer protocol. In brief, 25 µL of standards, controls, and samples were added to the appropriate wells of 96-well plate in duplicates. To all wells, 25 µL of 1X bead solution was added. To the standard and control wells, 25 µL of assay matrix was added, and 25 µL of assay buffer was added to the sample wells. The plate was incubated at 4°C overnight on a shaker plate. Using a magnetic plate, all wells were washed 3 times with wash buffer. For each well, 25 µL of detection antibody solution was added and incubated at room temperature for 1 hour with gentle agitation. After the incubation period, 25 µL of streptavidin-phycoerythrin was added to each well and incubated for 30 minutes at room temperature with gentle agitation. At the end of incubation, the plate was washed 3 times and run on the 3D system with xPONENT software (EMD Millipore). The data was analyzed using MILLIPLEX Analyst 5.1 software (EMD Millipore).

Results

Decellularized, dehydrated human amniotic membrane biochemical composition. Results for the biological entities released from DDHAM and dHACM are shown in Figure 1 and Figure 2. Among the selected 29 cytokines and growth factors tested, dHACM contained

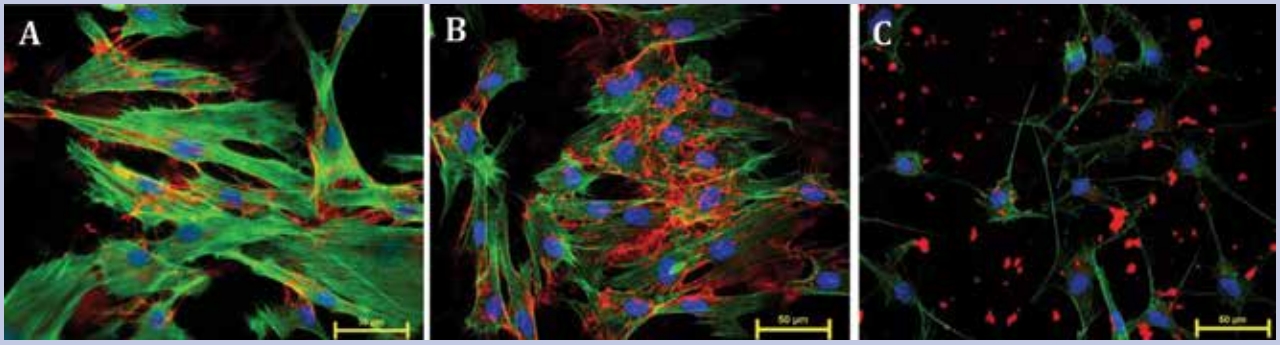

Figure 6. Representative immunostaining images of fibroblasts cultured in complete medium. (A) Decellularized, dehydrated human amniotic membrane "extract"; (B) dehydrated human amnion/chorion membrane "extract"; and (C) blue: nuclei (DAPI), green: actin (phaloidin 488), and red: fibronectin network (AF594 IgG I).

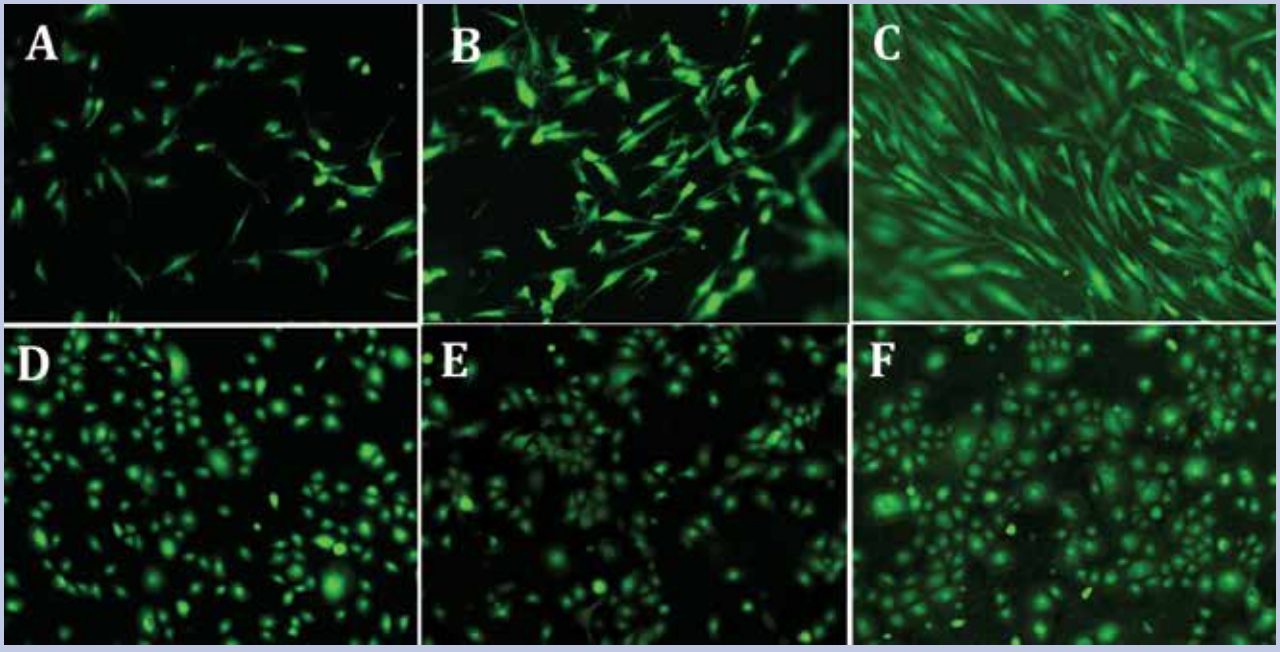

Figure 7. Live stain images of human dermal fibroblasts on decellularized, dehydrated human amniotic membrane (DDHAM) after (A) 4 hours, (B) 1 day, and (C) 3 days of culture. Live stain images of epithelial keratinocytes on DDHAM after (D) 4 hours, (E) 1 day, and (F) 3 days of culture. Cells were stained with Calcein AM and imaged with fluorescence microscopy at 10x magnification.

significant amounts of transforming growth factor-beta 2, macrophage inflammatory protein-1β, monocyte chemoattractant protein-1, IFNy, FGF-2, interleukin 1 beta (IL-1β), IL-6, IL-8, IL-13, and IL-15. In contrast, the DDHAM did not contain any growth factors. Nominal amounts of fibronectin and laminin were detected in the DDHAM, while significantly higher amounts of these proteins were detected in the dHACM (> 10-fold higher quantities relative to the DDHAM).

Cell attachment and proliferation. Dermal fibroblast and keratinocytes seeded on the DDHAM and the tissue culture plastic (TCP) showed attachment and spread within 24 hours. Figure 3 shows fluorescent microscopic images of live staining with Calcein AM of the HDF on the TCP and the DDHAM at 24 hours. The MTS assay confirmed that the HDF were metabolically active on the TCP and the DDHAM. In contrast, there was no visible attachment of the HDF on the dHACM with nominal cel-

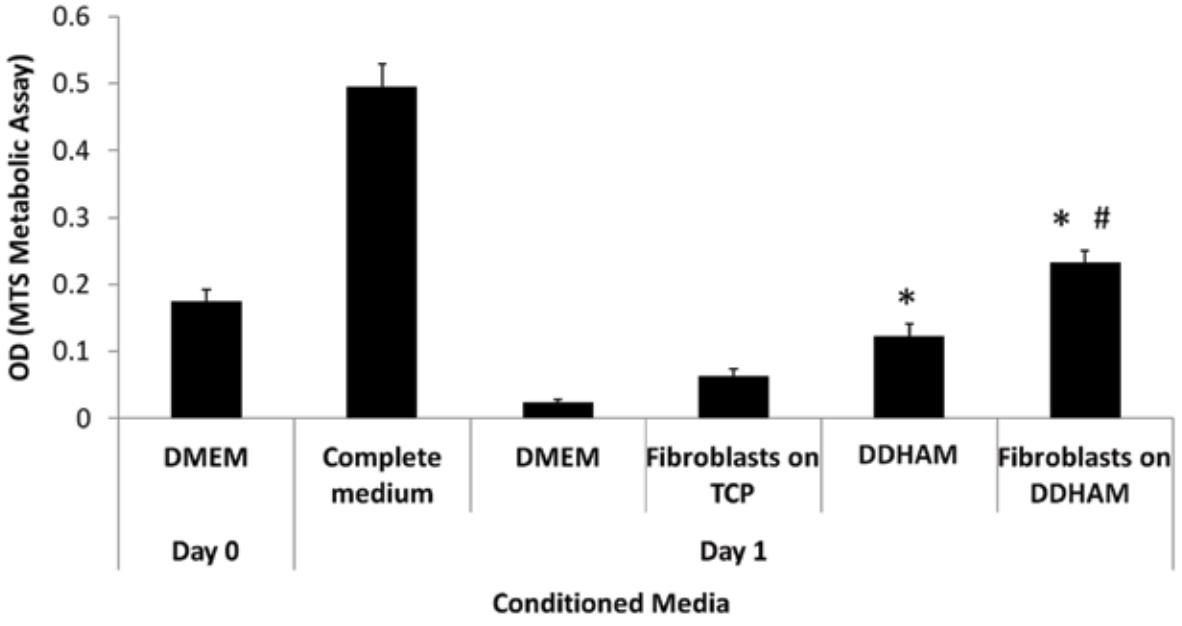

Figure 8. Levels of metabolic activity (MTS assay) of endothelial cells after starvation (day 0) and after "treatment" with conditioned media (day 1) (n = 6). DDHAM: decellularized, dehydrated human amniotic membrane. OD: optical density *: significant difference compared to the tissue culture-treated plate (TCP) groups (Dulbecco's Modified Eagle Medium [DMEM] and fibroblasts on TCP), $P < 0.05$; #: significant difference compared to the DDHAM group, $P < 0.05$.

lular metabolic activity as measured by the MTS assay. Likewise, keratinocytes also attached and spread on the TCP and the DDHAM but not on the dHACM scaffolds (Figure 4). Keratinocytes were metabolically active on the DDHAM, but not on the dHACM.

To further investigate the difference between DDHAM and dHACM in cell attachment, conditioned medium generated from an HDF cell culture on the DDHAM and the dHACM was used to culture HDF on the TCP. As shown in Figure 5, the conditioned medium generated from the HDF culture on the DDHAM did not show any apoptosis of fibroblasts as measured by luminescence in a Caspase 3/7 assay. This was true both for culturing in complete medium as well as basal medium. In contrast, the conditioned medium generated from culturing HDF on the dHACM showed higher luminescence, indicative of apoptosis of the cultured fibroblasts. Immunostaining of the HDF at 4 hours revealed a healthy fibroblast phenotype in the DDHAM extract (Figure 6). The HDF cultured in the DDHAM-conditioned medium were elongated with branched cytoplasm, while cells in the dHACM extract exhibited a contracted and almost nonexistent cytoskeleton. A dense network of insoluble fibronectin was deposited by the HDF grown in the DDHAM-conditioned medium (Figure 6A in red).

Cell proliferation on the DDHAM. Fibroblasts and keratinocytes not only attached but also proliferated on the DDHAM scaffolds. Figure 7 shows fluorescent microscopic images of live staining with Calcein AM after 4 hours, 1 day, and 3 days for HDF (Figure 7A-C). At 4 hours, the HDF had already attached to the DDHAM and became elongated. On day 3, significantly larger numbers of fibroblasts were detected on the scaffolds. Keratinocytes also attached and proliferated on the DDHAM scaffolds (Figure 7D-F). These results were also confirmed with the MTS assay (data not shown).

Effect of conditioned medium from an HDF-DDHAM culture on cell behavior. Nutrient-deprived endothelial cells, when exposed to conditioned medium generated from a culture of HDF on the DDHAM, recovered as demonstrated by an MTS assay as shown in Figure 8. Endothelial cell activity was significantly higher with the conditioned medium from a HDF-DDHAM culture than from a HDF-TCP culture. In the wound closure assay, an in vitro assay to assess endothelial cell migration, conditioned medium from a HDF-DDHAM culture supported endothelial

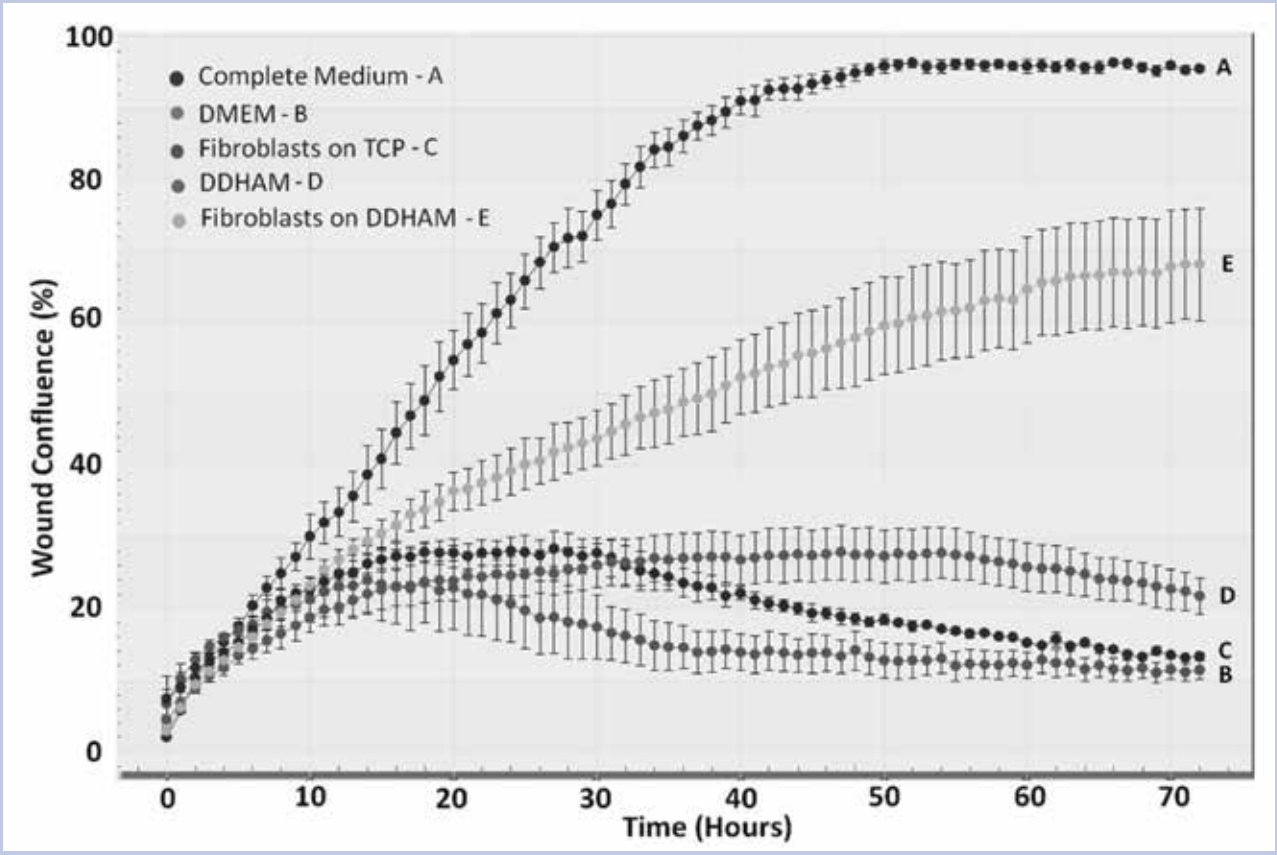

Figure 9. Time course of "wound confluency" (scratch assay) within endothelial cell monolayer treated with conditioned media (n = 4). DMEM: Dulbecco's Modified Eagle Medium; TCP: tissue culture-treated plate; DDHAM: decellularized, dehydrated human amniotic membrane

cell migration at 68% of complete media, which served as the positive control (Figure 9). Culture media from fibroblasts on the TCP (Figure 9, line C) or the DDHAM alone (Figure 9, line D) did not show any endothelial cell confluence over time (lack of migration of endothelial cells).

The tube formation assay is an in vitro assay to model angiogenesis. Figure 10A-E shows images of tube formation after treating human umbilical vein endothelial cells with conditioned or complete media for 36 hours. In the DMEM control group (Figure 10A), only short and sparsely distributed tubes were formed. In the fibroblasts on the TCP and the DDHAM-alone groups (Figure 10B,C), longer and more abundant tubes were observed compared to the DMEM control. However, no networks were visible. In the fibroblasts on the DDHAM group (Figure 10D), longer tubes were formed and a network, similar to the positive control, was produced.

Quantification of the number of tubes per image resulted in a significantly higher density of tube formation using conditioned media from the fibroblasts cultured on the DDHAM-conditioned media compared to the DDHAM alone or fibroblasts cultured on plastic (Figure 10).

Discussion

Chronic and difficult-to-heal wounds are characterized by disruption of ECM growth factor interactions, which are due to biochemical abnormalities of the wound ECM and are often found in patients with diabetes and venous insufficiency.[10] This disruption impairs normal ECM-cell interactions, which in turn could result in abnormal cell proliferation and ECM deposition. A variety of tissue-derived products are widely used for wound management in patients with delayed or nonhealing wounds. These products have been shown to improve wound closure and reduce wound-healing time. However, the mechanisms by which these advanced tissue products work remains to be understood; each product type may work via different mechanisms. In the current study, sev-

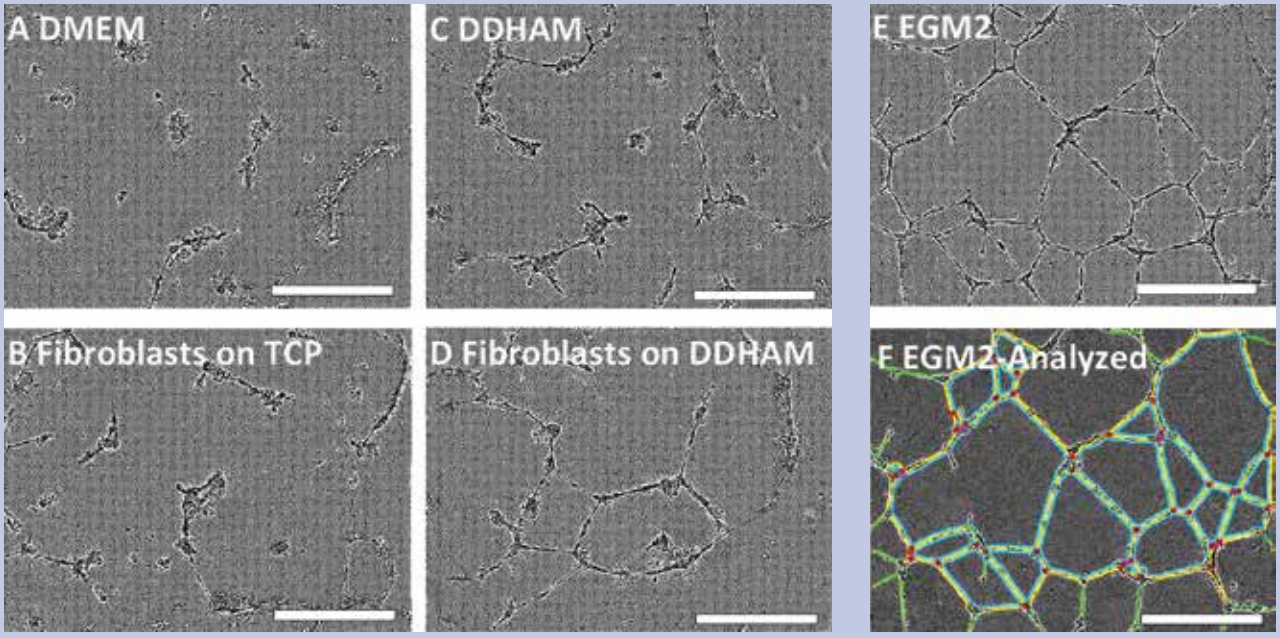

Figure 10. Images of tube formation (A-E) after human umbilical vein endothelial cell treatment with conditioned media. (A) Dulbecco's Modified Eagle Medium (DMEM); (B) fibroblasts on tissue culture-treated plate (TCP); (C) decellularized, dehydrated human amniotic membrane (DDHAM); (D) fibroblasts on DDHAM; (E) endothelial cell growth medium (EGM-2) (positive control) for 36 hours; and (F) tubes selected by software. Scale bar represents 500 µm

eral in vitro model systems were employed to investigate a DDHAM, which does not contain or release growth factors, cytokines, or other bioactive molecules. The results for DDHAM were compared to results for dHACM, which is an amnion-chorion product containing and releasing growth factors, cytokines, and ECM protein residuals. These studies show that DDHAM behaves primarily as an ECM with sufficient biological cues to allow for cell attachment and proliferation.[11,12] Further, conditioned media generated from cultures of fibroblasts on DDHAM are able to enhance cell survival and support endothelial cell migration and tube formation in an in vitro setting.

It is well known that anchorage and attachment of adherent cells to an ECM is a primary requirement for their survival and growth in the wound environment.[13] An ECM contains multiple cell adhesion proteins, such as collagen, fibronectin, and laminin, which are recognized by specific cell surface integrins to mediate attachment to the surface of the matrix.[14,15] Decellularized, dehydrated human amniotic membrane is a decellularized ECM in which the natural composition and structure of the matrix proteins are preserved. These extracellular proteins, comprised of collagen and elastin as well as fibronectin, laminin, and glycosaminoglycans, support cell attachment.[16] The MTS metabolic assay and cell imaging demonstrated that DDHAM supports fibroblast and keratinocyte attachment and proliferation. In contrast to DDHAM, dHACM did not support either fibroblast or keratinocyte attachment or subsequent survival on the scaffold. The release of bioactive molecules from dHACM likely has a negative effect on cell attachment, in that these molecules interfere with cell attachment rather than help it. Further, if cells such as fibroblasts are unable to attach and spread in the first few hours, they become apoptotic and die within 24 hours.

Given that revascularization and wound closure are subsequent events to fibroblast proliferation in the wound-healing cascade, the investigation examined the effect of fibroblasts on DDHAM-conditioned medium on the health and function of endothelial cell and keratinocytes through in vitro cell survival, wound closure, and endothelial cell tube formation assays (Figure 10). Fibroblasts on DDHAM-conditioned medium rescued endothelial cells that were subjected to nutrient deprivation and promoted endothelial cell tube and network formation as well as endothelial cell migration and proliferation. Because cells did not attach to the dHACM (amnion-chorion product) and because conditioned medium from dHACM causes apoptosis, tube formation and endothelial migration studies could not be performed with dHACM. The in vitro functional studies with a HDF-DDHAM culture con-

ditioned medium show sufficient biological signals in the conditioned medium to impact endothelial cell migration and tube formation.

Conclusions

In this work, in vitro models systems that allowed examination of ECM-cell interactions and consequent differentiation between 2 biologic advanced wound-management products were employed. This work leads to the conclusion that DDHAM is only an ECM scaffold, which interacts with key cell types important in the wound healing process. The data showed that ECM-cell interaction is sufficient to allow for the release of key biological entities in the conditioned media and that these biological entities can impact cell function such as endothelial cell migration and vascularization — both important events in wound repair.

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