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Properties of dehydrated human amnion/chorion composite grafts: Implications for wound repair and soft tissue regeneration

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Received 15 December 2013; revised 6 February 2014; accepted 6 March 2014
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.33141

Abstract: PURION® processed dehydrated human amnion/chorion membrane (dHACM; MiMedx Group, Marietta, GA) tissue products were analyzed for the effectiveness of the PURION® process in retaining the native composition of the amniotic membrane and preserving bioactivity in the resulting products. dHACM was analyzed for extracellular matrix (ECM) composition through histological staining and for growth factor content via multiplex ELISA arrays. Bioactivity was assessed by evaluating endogenous growth factor production by human dermal fibroblasts in response to dHACM and for thermal stability by mechanical tests and *in vitro* cell proliferation assays. Histology of dHACM demonstrated preservation of the native amnion and chorion layers with intact, nonviable cells, collagen, proteoglycan, and elastic fibers distributed in the individual layers. An array of 36 cytokines known to regulate processes involved in inflammation and wound healing were identified in dHACM. When treated with dHACM extracts, bioactivity was demonstrated through an upregulation of basic fibroblast growth factor, granulocyte colony-stimulating factor, and placental growth factor biosynthesis, three growth factors involved in wound healing, by dermal fibroblasts *in vitro*. After conditioning at temperatures ranging from −78.7 to +73.5°C, dHACM retained its tensile strength and ability to promote proliferation of dermal fibroblasts *in vitro*. Elution experiments demonstrated a soluble fraction of growth factors that eluted from the tissue and another fraction sequestered within the matrix. The PURION® process retains the native composition of ECM and signaling molecules and preserves bioactivity. The array of cytokines preserved in dHACM are in part responsible for its therapeutic efficacy in treating chronic wounds by orchestrating a "symphony of signals" to promote healing.
 J Biomed Mater Res Part B: Appl Biomater 00B:000–000, 2014.

Key Words: wound healing, dermal wound dressing, growth factor, amniotic membrane, dHACM

How to cite this article: Koob TJ, Lim JJ, Massee M, Zabek N, Denozière G. 2014. Properties of dehydrated human amnion/chorion composite grafts: Implications for wound repair and soft tissue regeneration. J Biomed Mater Res Part B 2014:00B:000–000.

INTRODUCTION

The amniotic membrane is derived from the inner layer of the placenta and is composed of conjoined amnion and chorion membranes. The amniotic membrane holds the developing fetus and amniotic fluid, so this thin membrane must possess the structural integrity to support the pregnancy through term.[1] It must grow to accommodate the increasing volume of the conceptus, and therefore, it is a metabolically active tissue which continually remodels the extracellular matrix (ECM) through processes governed by paracrine growth factors. Amniotic membranes contain no blood vessels and have no direct blood supply.[2] Required nutrients are supplied to the amniotic membranes directly by diffusion out of the amniotic fluid or from the underlying decidua.[3] Similarly, the membranes also secrete substances both into the amniotic fluid and toward the uterus, influencing both amniotic fluid homeostasis and maternal cellular physiology, respectively.[4]

Amnion is composed of five distinct layers, including the epithelium, basement membrane, compact layer, fibroblast layer, and intermediate or spongy layer. The epithelium, the layer closest to the developing fetus, consists of a single layer of epithelial cells uniformly arranged on the basement membrane.[3] The basement membrane is a thin layer composed of collagens III and IV and noncollagenous glycoproteins laminin, nidogen, and fibronectin.[5] The compact layer is a dense layer almost totally devoid of cells and forms the main fibrous structure of the amnion.[2,5] Interstitial collagens I and III form bundles in the compact layer that maintain the mechanical integrity of the membrane, whereas collagens V and VI form filamentous connections to the basement membrane.[5] The fibroblast layer is the thickest layer of the amnion and consists of fibroblasts embedded in a loose collagen network with islands of noncollagenous glycoproteins.[2,5] The outermost spongy layer forms the interface between the amnion and chorion, composed of a

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Contract grant sponsor: MiMedx Group, Inc. (to T.J.K., J.J.L., M.M., N.Z., and G.D.)

nonfibrillar meshwork of collagen III and an abundant content of proteoglycans and glycoproteins.[3,5]

Chorion is three to four times thicker than amnion.[1,6] Chorion is composed of a reticular layer, basement membrane, and trophoblast layer, which is adhered to the maternal decidua.[5] The reticular layer contacts the spongy layer of the amnion and forms a majority of chorion's thickness.[7] The reticular network is composed of collagens I, III, IV, V, and VI.[5] The basement membrane anchors the trophoblasts to the reticular layer with collagen IV, fibronectin, and laminin.[5,7] The trophoblast layer is the deepest layer, consisting of 2–10 layers of trophoblasts which contact with the decidua.[7]

In addition to physically encasing the amniotic fluid and developing fetus, amniotic membranes play an integral biological role in fetal development and progression of pregnancy; therefore, amniotic membrane grafts harbor significant biological activity, including a number of developmental cytokines. To ensure that safe amniotic tissue allografts can be obtained while also preserving bioactivity for clinical efficacy and stability for long-term storage and off-the-shelf availability, MiMedx Group (Marietta, GA) developed a gentle cleansing and dehydration process (PURION® process) that retains the biological activities inherent in native amniotic tissue.[8–10]

Human amniotic membrane products have become a widely accepted form of treatment in ophthalmic surgery, including corneal ulceration, covering of defects in large conjunctival lesions, and acute chemical burns of the eye.[11] Recently, amniotic membrane products have been effectively applied in healing of chronic cutaneous wounds, including diabetic, venous, arterial, and decubitus ulcers. Treatment of dermal wounds with amniotic tissues promotes healing while reducing pain, preventing scarring, and controlling inflammation.[3,12]

Native human amnion/chorion membranes contain an array of growth factors, which play critical roles in regulating tissue development and growth *in utero*. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), transforming growth factors alpha and beta (TGF-α,-β), nerve growth factor (NGF), and hepatocyte growth factor (HGF) are some of the growth factors that have been identified in fresh and preserved amniotic membrane tissues.[13,14] A number of these, as well as additional growth factors, have also been previously identified in significant quantities in PURION® processed dehydrated human amnion/chorion membrane (dHACM) tissues, including TGF-α, TGF-β1, bFGF, EGF, HGF, placental growth factor (PlGF), granulocyte colony-stimulating factor (GCSF), angiogenin, angiopoietin-2 (ANG-2), heparin binding epidermal growth factor (HB-EGF), platelet-derived growth factor-AA (PDGF-AA), PDGF-BB, and vascular endothelial growth factor (VEGF), along with other interleukins (ILs) and tissue inhibitors of metalloproteinases (TIMPs).[15,16]

Despite the increasing use of amniotic membrane products in a clinical setting, the characterization of these tissue products remains fairly superficial, including identification of the vast array of growth factors present in these tissues, which likely contribute to the tissues' bioactivity and clinical efficacy. Therefore, the goal of these studies was to characterize PURION® processed dHACM, including the effectiveness of the PURION® process and the stability of the resulting products. Processed dHACM tissues were analyzed histologically and for growth factor content via multiplex enzyme-linked immunosorbent assay (ELISA) array. Then, the processed tissues were characterized, including thermal stability for mechanical integrity, *in vitro* cell proliferative capability, and growth factor content. Finally, the elution of growth factors out of the tissue products was characterized for insight into growth factor delivery profiles within a hydrated wound environment.

MATERIALS AND METHODS

Dehydrated human amnion/chorion membrane (dHACM)

dHACM is a dehydrated human allograft composed of laminated amnion and chorion membranes derived from the placenta.[8–10] Human placentas were donated under informed consent, after Cesarean sections, as regulated by the Food and Drug Administration's (FDA) Good Tissue Practice and American Association of Tissue Banks (AATB). All donors were tested to be free of infectious diseases, including human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV), hepatitis B and C, syphilis, and cytomegalovirus (CMV). Amnion and chorion were isolated from placenta and processed with a proprietary PURION® process that involves gentle cleansing of the layers. The amnion and chorion were then laminated to form the graft, and the graft was dehydrated under controlled drying conditions.[10] Specific versions of dHACM (EpiFix® and AmnioFix®, MiMedx Group) were used as the test material in these studies; therefore, the results of these studies apply only to PURION® processed dehydrated human amnion/chorion composite grafts (dHACM). EpiFix® and AmnioFix® are composed of similar amnion/chorion tissue composites; however, in AmnioFix®, the amnion epithelial layer is removed before dehydration, whereas the epithelial cells are intact in EpiFix®.

Histology of PURION® processed dHACM

EpiFix® and AmnioFix® dHACM products were fixed in 10% formalin, and samples were embedded in paraffin, sectioned into 5-μm thick cross-sections, and stained by Premier Laboratory (Longmont, CO). Tissues were stained with hematoxylin and eosin (H&E) for cell nuclei, Alcian blue for acidic polysaccharides/proteoglycans (nuclear fast red counterstain), Masson's trichrome for collagen, and Verhoeff's stain for elastic fibers (Van Gieson counterstain), according to standard protocols. Stained tissue sections were then analyzed by an independent histopathologist.

Multiplex ELISA array

The content of specific growth factors in samples of processed dHACM (EpiFix®) from five donors was measured with multiplex enzyme-linked immunosorbent assay (ELISA) arrays. Quantibody multiplex ELISA arrays (RayBiotech,

Norcross, GA) are sandwich ELISA-based array systems capable of simultaneous quantitative measurement of up to 40 growth factors, cytokines, proteases, and other proteins. Weighed, minced dHACM samples were placed in lysis buffer containing protease inhibitors for 24 h at 4°C. Tissues were then homogenized, centrifuged to remove tissue residue, and the amount of each factor in the lysis buffer was measured in diluted aliquots with a Quantibody multiplex ELISA array by RayBiotech. Growth factor content was normalized to the dry mass of starting tissue.

In vitro production of growth factors by human dermal fibroblasts

To obtain dHACM extracts for cell culture experiments, sterilized grafts were minced and extracted in Dulbecco's modified Eagle's medium (DMEM) without serum at a concentration of 10 mg of tissue per milliliter of medium. After 24 h of extraction at 4°C, the tissue was removed by centrifugation and the extract was sterile filtered. Previous studies have established that a significant proportion of the growth factors and cytokines in dHACM elute from the tissue under these conditions.[15]

Adult human dermal fibroblasts (HDFa; Gibco, Life Technologies Corp. C-013-5C, Carlsbad, CA) were plated at 2,500 cells per well of a 96-well plate for 24 h in DMEM containing 10% calf serum (Gibco, Life Technologies Corp.). After 24-h incubation to allow for cell adhesion, the cells were treated with dHACM extract in basal DMEM at 10, 5, and 1 mg/mL concentrations. After 72-h treatment, the supernatant from three independent wells per sample group was recovered, pooled, and tested for the presence of growth factors with standard ELISAs (RayBiotech), including bFGF, GCSF, PlGF, EGF, PDGF-AA, PDGF-BB, and TGF-β1.

A CyQuant assay (Molecular Probes, Life Technologies Corp. C7026) was subsequently performed on the adherent cells to quantify DNA content, and DNA content was then translated to cell number, using a standard curve of known cells as determined by counting on a hemocytometer.

Thermal conditioning and mechanical testing of dHACM

PURION® processed dHACM was incubated for 72 h at mean temperatures of −78.7°C on dry ice, −18.4°C in a freezer, 24°C at ambient conditions, or 48.1, 56.5, 65.4, and 73.5°C in temperature-controlled ovens, as determined using temperature data loggers (Dickson SP175, Addison, IL). After conditioning, all samples were kept at ambient conditions until testing.

Two 12 × 20 mm dry sterile dumbbell test articles were cut from each of three dHACM lots per group. A dumbbell shape with a width of approximately 8.0 mm at its smallest point was used to ensure failure within the gauge length and not at the grips. dHACM sample thickness was measured using digital calipers with an accuracy to 0.001 mm. Samples were strained in tension at 80 mm/min (Instron 5566 Load Frame, Norwood, MA), and force was measured using a 500-N load cell. The test articles were tested in the dry state, and force–displacement curves were examined to determine the ultimate tensile strength of the conditioned tissues.

In vitro proliferation of human dermal fibroblasts in response to thermally conditioned dHACM

dHACM was thermally conditioned as described above, and bioactivity within thermally conditioned dHACM was assessed using an *in vitro* human dermal fibroblast proliferation assay. The relative number of cells was measured and compared between a negative control group (basal medium with no serum), a positive control group (complete medium with 10% serum), and test groups (dHACM extracts) after 3 days in culture.

dHACM extracts were obtained as described above, and adult human dermal fibroblasts were plated at 3,500 cells per well of a 96-well plate for 24 h in DMEM containing 10% calf serum. After 24 h to allow for cell adhesion, the medium was aspirated from the wells and replaced with one of the following: DMEM lacking serum (negative control), DMEM plus 10% calf serum (positive control), or DMEM containing 10 mg/mL dHACM extract. After 72 h, the plate was washed to remove unattached cells and a CyQuant assay was performed to quantify DNA content, as a relative measure of cell number ($n = 5$).

Elution of growth factors from dHACM

The release rate of cytokines from dHACM was determined by incubating tissue samples for 96 h in normal saline at 37°C. After 1, 8, 24, 48, or 96 h, the incubation fluid was collected and the cytokine content was determined by standard ELISA ($n = 5$) for bFGF, PDGF-AA, and TGF-β1, as described above. After 96 h, the tissue was digested with bacterial collagenase (Worthington Biochemical LS005273, Lakewood, NJ), and the growth factor content in the digestion was measured by ELISA.

Statistical analyses

Statistical comparisons were performed by using two-tailed, unpaired Student's *t*-tests with significance set at $p \leq 0.05$ to compare treatment groups to their respective controls. For thermal conditioning studies, a one-way analysis of variance was conducted to compare the mean tensile strengths for the mechanical testing studies or the mean fluorescence for proliferation assays between conditioning groups with statistical significance set at $p \leq 0.05$ (Minitab, State College, PA). All values were expressed as the mean ± standard deviation.

RESULTS

Histology of PURION® processed dHACM

The microscopic structure of PURION® processed dHACM showed clearly defined amnion and chorion layers essentially identical to that in fresh amniotic membrane. Tissues present in fresh amniotic membrane were present in dHACM, including the epithelial/basement membrane layer, compact layer, fibroblast layer, and the chorion (Figure 1). H&E staining showed the presence of structurally preserved epithelial cells in EpiFix® and intact fibroblasts throughout

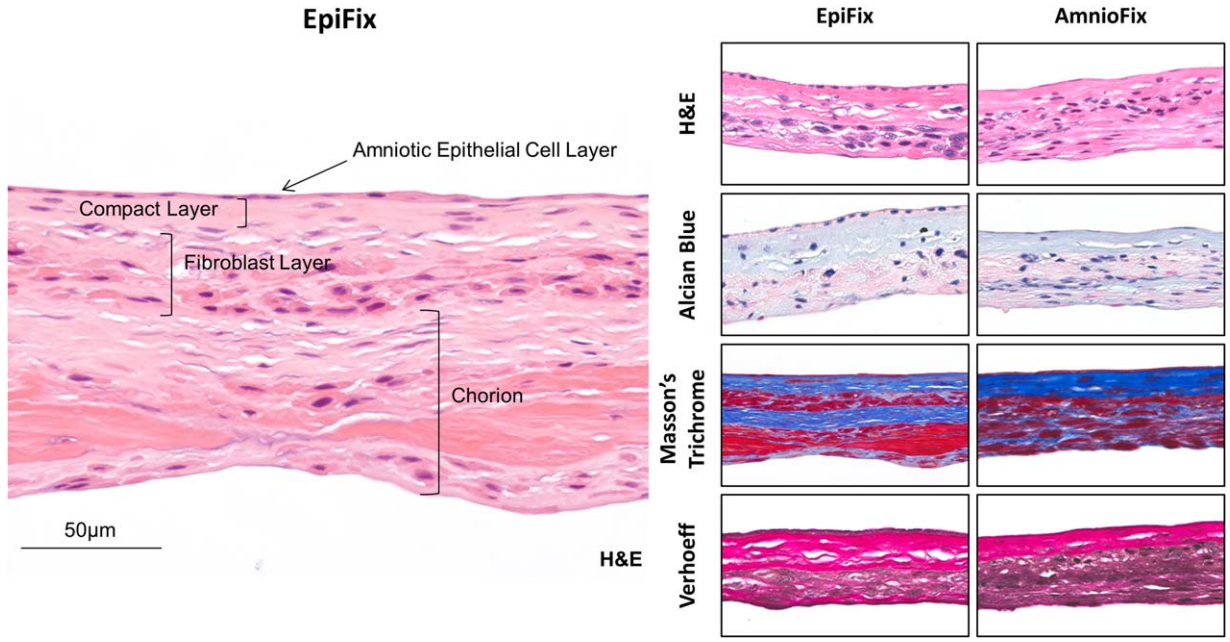

FIGURE 1. Histology of EpiFix® and AmnioFix® dHACM allografts. The left micrograph illustrates the principal layers of amniotic membrane that are present in EpiFix®. The right panel shows differentially stained micrographs of EpiFix® and AmnioFix® with hematoxylin and eosin, Alcian blue, Masson's trichrome, and Verhoeff's stains. Amnion and chorion layers were visible in EpiFix® and AmnioFix®, as well as epithelial cells in EpiFix®. H&E stains cell nuclei dark blue. Alcian blue stains glycosaminoglycans and proteoglycans blue. Masson's trichrome stains collagen blue. Verhoeff's stains elastic fibers black. Tissue sections were counterstained with varying shades of pink or red.

the amnion and chorion layers in both EpiFix® and AmnioFix®. Alcian blue staining showed that the glycosaminoglycans/proteoglycans were present predominantly in the amnion compact and fibroblast layers in both EpiFix® and AmnioFix®. Masson's trichrome stained the dense collagen fiber networks deep blue in the amnion compact and fibroblast layers in both EpiFix® and AmnioFix®. Elastic fibers stained by Verhoeff's stain were found almost exclusively in the chorion, although slight Verhoeff's staining was observed in the epithelial layer. On the basis of this microscopic structure and histochemical analysis, PURION® processing of amniotic membrane seems to preserve the native structure of amniotic membrane.

Growth factors present in dHACM

We previously reported that the following regulatory factors, growth factors, cytokines, and chemokines are present in dHACM: PDGF-AA, PDGF-BB, TGF-α, TGF-β1, bFGF, EGF, HGF, PlGF, GCSF, angiogenin, angiopoietin-2 (ANG-2), HB-EGF, and VEGF, along with IL-4, −6, −8, and −10, and TIMP-1, −2, −4.[15,16] In this study, we report the presence and amount of an additional 36 signaling molecules, including 14 cytokines and 10 chemokines known to regulate inflammation, and 12 cytokines known to regulate wound healing processes (Table I).

Growth factor production by human dermal fibroblasts

The effect of dHACM on selected growth factor production by human dermal fibroblasts was examined by treating cells with extracts of dHACM for 3 days and measuring the amount of each growth factor in the culture medium at the end of the culture period. Because the extract has been demonstrated to cause dermal fibroblasts to proliferate,[15] cell number was measured in each well after the culture medium was collected, and growth factor values were normalized on a per cell number. The starting amounts of each growth factor in the extracts were also measured and subtracted from that in the final culture medium to calculate the amount of additional growth factor produced by the fibroblasts.

The induction of specific growth factor production by dermal fibroblasts in culture caused by extracts of dHACM is shown in Figure 2. Biosynthesis of three of seven selected growth factors measured was upregulated by the tissue extract in a dose-dependent manner. Dermal fibroblasts increased production of bFGF, GCSF, and PlGF when cultured in the presence of dHACM extract, compared with untreated cells cultured without extract in complete medium with 10% serum (Figure 2). Although EGF, PDGF-AA, PDGF-BB, and TGF-β1 were also tested for endogenous production by dermal fibroblasts, increased production of these factors by HDFs was not observed in this experiment.

Thermal stability of physical and biological properties of dHACM

dHACM, thermally conditioned at temperatures ranging from −78.7 to +73.5°C for 72 h, demonstrated no difference in strength in uniaxial tensile tests to failure,

TABLE I. Cytokine and Growth Factor Content in dHACM in pg per mg of Dry Tissue ($n = 5$ Donors)

	Regulators of Inflammation					
Cytokines	Abbreviation	EpiFix	SD	AmnioFix	SD	Function
Growth differentiation factor 15	GDF-15	62.9	6.8	63.25	5.9	Regulates inflammatory and apoptotic pathways in injured tissues.
Granulocyte macrophage colony-stimulating factor	GM-CSF	0.1	0.03	0.12	0.03	Stimulates production of granulocytes and monocytes.
Interferon gamma	IFNγ	0.34	0.33	0.59	0.76	Activator of macrophages
Interleukin 1 alpha	IL-1α	3.23	2.7	3.71	3.36	Activates lymphocyte proliferation and induces fibroblast proliferation.
Interleukin 1 Beta	IL-1β	3.38	3.7	8.03	6.61	Involved in lymphocyte proliferation, differentiation, and apoptosis
Interleukin 1 receptor antagonist	IL-1ra	17.82	23.18	29	16.64	Antagonist of IL-1 signaling
Interleukin 5	IL-5	0.67	0.71	0.78	0.69	Regulates eosinophil growth and activation, stimulates B cell growth, and increases immunoglobulin secretion.
Interleukin 7	IL-7	0.6	0.3	0.47	0.28	Stimulates proliferation, maturation, and survival of B cells, T cells, and natural killer cells
Interleukin 12 p40	IL-12p40	4.32	3.22	3.55	1.96	Subunit of IL-12p70, can act as IL-12 antagonist
Interleukin 12 p70	IL-12p70	0.14	0.11	0.19	0.24	Stimulates growth and differentiation of T cells and natural killer cells.
Interleukin 15	IL-15	0.9	0.45	0.75	0.22	Stimulates proliferation of T lymphocytes and induces proliferation of natural killer cells.
Interleukin 17	IL-17	0.42	0.1	0.48	0.22	Increases chemokine production.
Macrophage colony-stimulating factor	MCSF	1.15	0.33	1.9	0.8	Involved in proliferation, differentiation, and survival of monocytes and macrophages
Osteoprotegerin	OPG	233.05	121.62	319.36	0.11	Soluble decoy receptor that inhibits osteoclast activation
Chemokines	**Abbreviation**	**EpiFix**	**SD**	**AmnioFix**	**SD**	**Function**
B lymphocyte chemoattractant (CXCL13)	BLC	23.41	31.4	14.77	14.91	Selectively chemotactic for B lymphocytes
Eotaxin 2	Eotaxin-2	0.1	0.11	0.07	0.02	Induces chemotaxis in eosinophils and T lymphocytes.
Chemokine ligand 1 (CCL1)	I-309	0.43	0.38	1.09	1.11	Recruits monocytes, natural killer cells, and immature B cells and dendritic cells.
Interleukin 16	IL-16	10.28	6.23	11.9	7.44	Chemoattractant for CD4+ cells, including T cells, monocytes, eosinophils, and dendritic cells
Monocyte chemotactic protein 1 (CCL2)	MCP-1	18.77	13.29	31.95	14.81	Recruits monocytes, memory T cells, and dendritic cells.

TABLE I. *Continued*

Chemokines	Abbreviation	EpiFix	SD	AmnioFix	SD	Function
Monokine induced by gamma interferon (CXCL9)	MIG	211.6	207.6	359.3	139.13	Chemoattractant for T cells
Macrophage inflammatory protein 1 alpha (CCL3)	MIP-1α	1.92	0.84	4.98	2.7	Chemotactic for neutrophils and monocytes
Macrophage inflammatory protein 1 beta (CCL4)	MIP-1β	2.43	1.94	4.61	0.65	Chemoattractant for natural killer cells and monocytes
Macrophage inflammatory protein 1D (MIP-5, CCL15)	MIP-1d	2.32	1.21	1.29	1.14	Chemoattractant for neutrophils, monocytes, and lymphocytes
Regulated on activation, normal T cell expressed and secreted (CCL5)	RANTES	26.76	29.23	68	45.46	Chemotactic for T cells, eosinophils, and basophils

	Regulators of Wound Healing					
Cytokines	Abbreviation	EpiFix	SD	AmnioFix	SD	Function
Brain-derived neurotrophic factor	BDNF	28.27	24.54	18.03	9.14	Supports the growth, differentiation, and survival of neurons.
Bone morphogenetic protein 5	BMP-5	17.79	10.71	30.55	19.17	Plays a role in bone and cartilage development.
Endocrine gland-derived vascular endothelial growth factor	EG-VEGF	210.25	111.83	247.38	83.15	Stimulates endothelial cell migration, proliferation, and survival, potent stimulator of angiogenesis.
Fibroblast growth factor 4	FGF-4	241.54	67.28	233.37	39.84	Broad mitogenic and cell survival activity
Keratinocyte growth factor	FGF-7	11.38	3.46	20.15	8.85	Promotes proliferation and migration of epithelial cells and keratinocytes.
Growth hormone	GH	24.77	24.17	36.23	26.11	Stimulates body growth through IGF-1 production, involved in anabolic activity.
Insulin-like growth factor 1	IGF-I	8.01	4.16	12.65	5.19	Stimulates body growth through broad mitogenic activity.
Insulin-like growth factor binding protein 1	IGFBP-1	353.2	0	353.15	0.12	Binds and stabilizes IGF-1 as a carrier protein, alters interactions with surface receptors.
Insulin-like growth factor binding protein 2	IGFBP-2	1014.75	127.12	1071.44	0.36	Binds and stabilizes IGF-1 as a carrier protein, alters interactions with surface receptors.
Insulin-like growth factor binding protein 3	IGFBP-3	5846.61	989.71	6246.19	486.3	Binds and stabilizes IGF-1 as a carrier protein, alters interactions with surface receptors.
Insulin-like growth factor binding protein 4	IGFBP-4	340.09	190.65	717.24	745.71	Binds and stabilizes IGF-1 as a carrier protein, alters interactions with surface receptors.
Insulin-like growth factor binding protein 6	IGFBP-6	1856.34	1276.28	2312.16	953.06	Binds and stabilizes IGF-1 as a carrier protein, alters interactions with surface receptors.

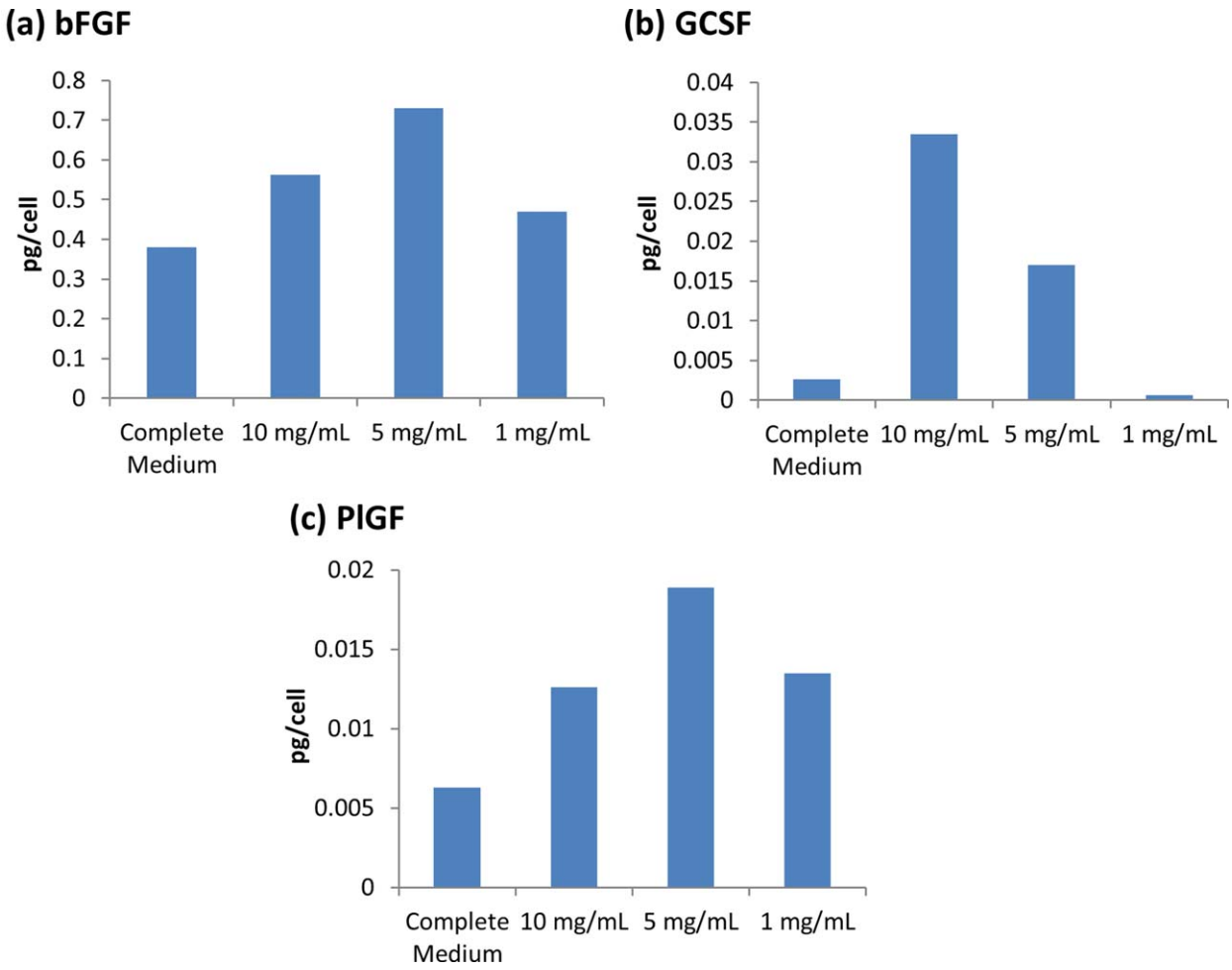

FIGURE 2. Endogenous growth factor production by human dermal fibroblasts when cultured in the presence of varying concentrations of dHACM extract. Dermal fibroblasts increase production of bFGF, GCSF, and PlGF when cultured in the presence of 5 and 10 mg/mL dHACM extract, compared with untreated controls in complete medium containing 10% calf serum. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

suggesting that the physical properties of dHACM did not significantly degrade at these extreme temperatures (Figure 3). No combination of two groups was statistically significantly different ($p \leq 0.05$).

Thermal conditioning did not significantly change the abilities of these tissues to promote HDF proliferation, indicating that the biological activity of dHACM was preserved at extreme temperatures as well. Human dermal fibroblasts were cultured in the presence of extracts of thermally conditioned dHACM at 10 mg/mL, and all conditioned dHACM groups except for the 24°C group significantly increased proliferation compared with the negative control groups (Figure 4). In addition, all dHACM groups were not statistically different than the positive control group.

Elution of growth factors from dHACM

Release profiles of bFGF, PDGF-AA, and TGF-β1 indicated that there are at least two pools of each growth factor in the amnion that can be released into 0.9% saline. One is only weakly bound in the tissue and will gradually diffuse out of the tissue over the course of 96 h when suspended in 0.9% saline. A second pool of growth factors remains bound in the tissue. The relative proportion of the two pools varies with each growth factor. The release kinetics of bFGF, PDGF-AA, and TGF-β1 eluted from dHACM in saline at 37°C are shown in Figure 5. After an initial burst release of these growth factors, the rate of release declined. Significant amounts of these growth factors were not released into saline after 96 h, indicating that the loosely bound fraction had eluted from the material. Collagenase digestion of the tissue released the remaining bound growth factors from the ECM.

DISCUSSION

These studies demonstrate that PURION® processed dehydrated human amnion/chorion membrane (dHACM) allografts retain the native composition of ECM and signaling molecules normally present in amniotic membrane, and that the PURION® process preserved bioactivity that could potentially support wound healing processes. Histological staining of dHACM tissue sections demonstrated preservation of the layers of the amnion and chorion, including the

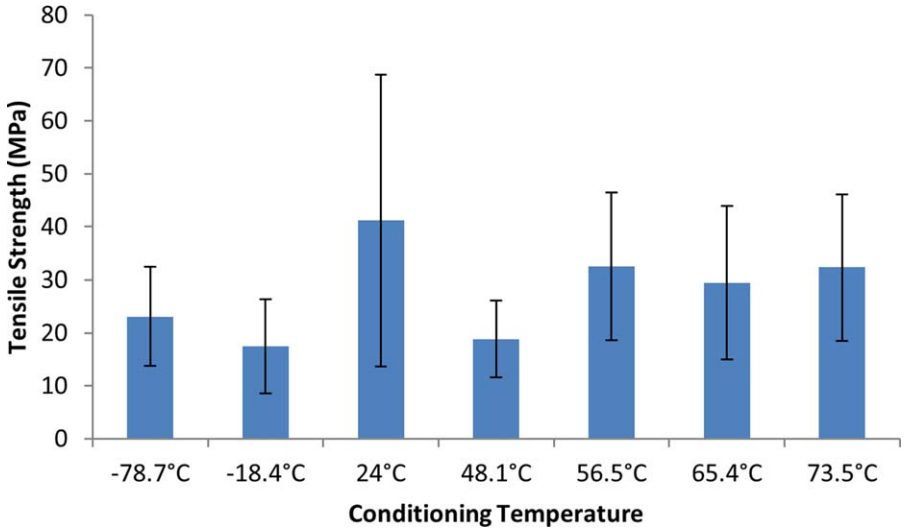

FIGURE 3. Mechanical properties of thermally conditioned dHACM. Tensile strength of dHACM tissue did not change after thermal conditioning at a wide range of temperatures for 72 h, demonstrating thermal stability of physical properties (n = 5). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

epithelial, basement membrane, compact, fibroblast, and intermediate layers of the amnion, and the reticular layer of the chorion (Figure 1). Intact, nonviable cells are visible in the membranes with collagen, proteoglycan, and elastic fibers distributed in the individual layers.

PURION® processing of human amniotic membrane retains an array of cytokines, chemokines, and growth factors naturally present in the native tissue. Together with our previous analyses,[15,16] we have identified a diverse array of 57 regulatory proteins present in dHACM allografts. These proteins are known to regulate a variety of processes involved in inflammation, angiogenesis, ECM remodeling, and tissue regeneration (Table I).

Inflammation is critical during the early stages of wound repair,[17] and chronic inflammation is a primary cause of refractive healing of wounds.[18] dHACM contains inflammatory cell chemokines and cytokines that regulate the activity of cells derived from the immune system. Chemokines recruit immune cells to the site of a wound, and cytokines regulate activity of these immune cells. dHACM contains chemokines and cytokines that directly affect T cells, B lymphocytes, monocytes and macrophages, neutrophils, eosinophils, and natural killer cells. Together this balance of inflammatory regulators may modulate the inflammation response within refractory wounds, and together with additional wound healing cytokines also retained in dHACM may provide an ideal balance of cues to promote healing.

When treated with dHACM extracts, adult human dermal fibroblasts upregulated biosynthesis of three growth factors involved in wound healing, bFGF, GCSF, and PlGF *in vitro*.

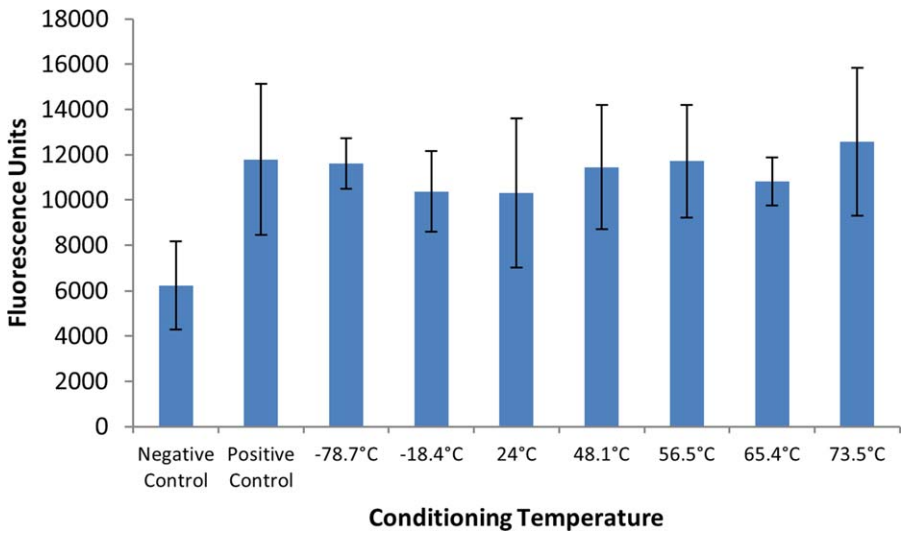

FIGURE 4. Effects of thermally conditioned dHACM on dermal fibroblast proliferation. Human dermal fibroblasts proliferated when cultured for 72 h in the presence of 10 mg/mL extract from thermally conditioned dHACM, as shown by CyQuant DNA assay as a measure of cell number, demonstrating thermal stability of bioactivity. Negative controls were basal medium, and positive controls were complete medium containing 10% FBS (n = 5). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

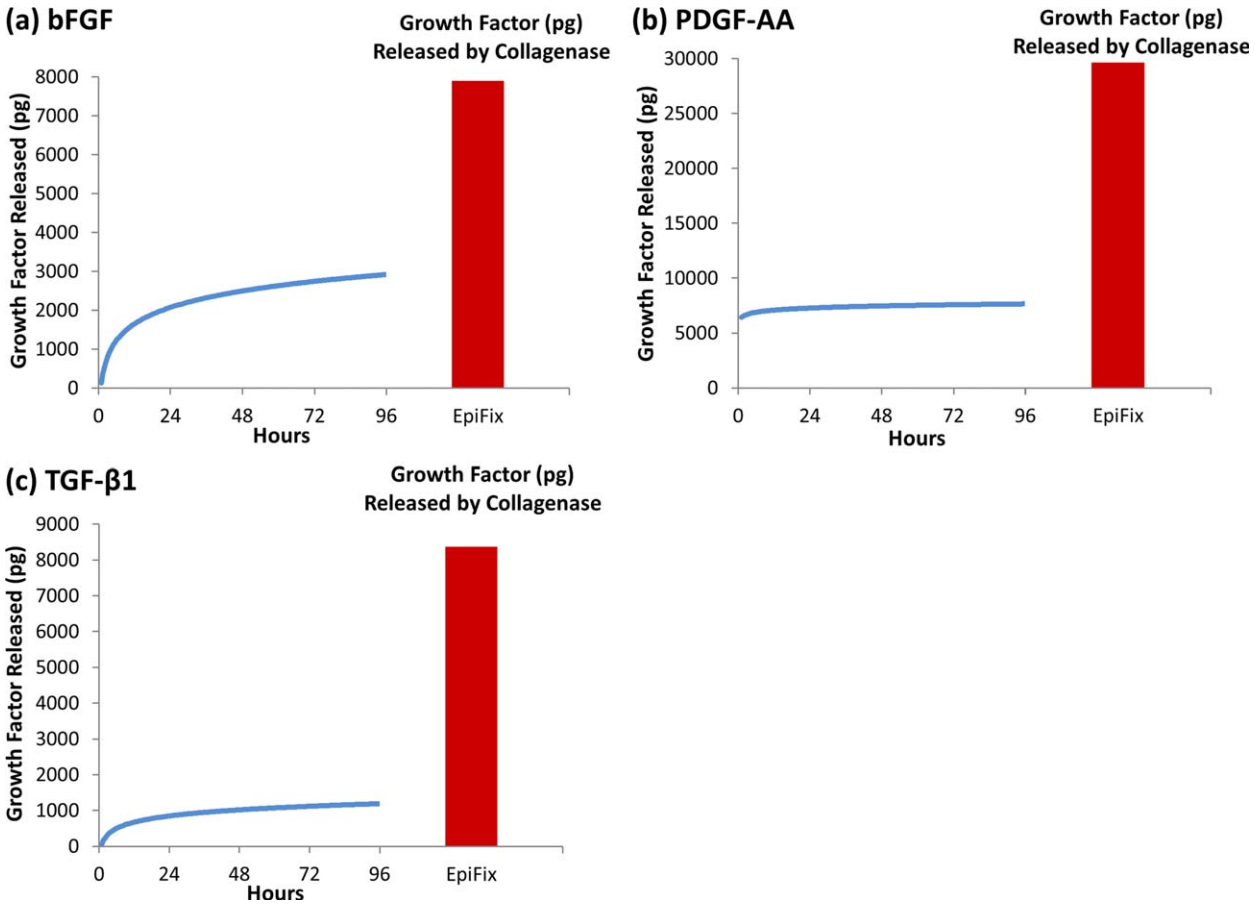

FIGURE 5. Elution of growth factors over 96 h into normal saline. bFGF, PDGF-AA, and TGF-β1 eluted out from dHACM in 0.9% saline at 37°C. After an initial release of these growth factors, the rate of release declined, and significant amounts of these growth factors were not released into saline after 96 h. Collagenase digestion of the tissue released the remaining bound growth factors from the tissue. The trendline illustrates the means for five replicate samples at each time point. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We previously reported a number of biological activities of dHACM, including induction of proliferation of human dermal fibroblasts and microvascular endothelial cells, upregulation of angiogenic growth factors by endothelial cells, chemotactic recruitment of mesenchymal stem cells *in vitro* and *in vivo*, as well as increased neovascularization in a mouse subcutaneous implantation model.[15,16] Thus, dHACM contains an array of regulatory molecules that can positively influence processes critical for the three phases of wound repair: inflammation, angiogenesis, and production and remodeling of the ECM.

Both the biological activity and structural integrity of the matrix in PURION® processed tissues were stable to extremes in temperature. dHACM tissue was conditioned for 72 h at temperatures ranging from −78.7 to +73.5°C, and tensile strength and ability to promote proliferation of dermal fibroblasts was not affected by this thermal conditioning (Figures 3 and 4). These results strongly suggest that the PURION® process not only retains growth factors within the matrix, but also protects these regulatory cytokines from denaturation. These findings have significant implications for the storage and stability of these tissues at ambient conditions for the 5-year shelf life, allowing immediate, off-the-shelf availability.

Finally, dHACM was shown to gradually release a selection of growth factors from the tissue matrix when incubated in saline. The release of these cytokines into the wound environment would be critical for clinical efficacy. In saline, only a fraction of the available growth factor diffused out of the matrix after 96 h, suggesting that an additional population remained sequestered within the tissue matrix. Treatment of the tissue with collagenase released the remaining growth factor, suggesting that through degradation and remodeling of the dHACM tissue within the wound environment, this remaining population of growth factors may be released in the wound for sustained delivery to promote long-term healing.

Reported clinical use of dHACM for nonhealing wounds has demonstrated the efficacy of these dehydrated amnion/chorion membrane allografts. In one small series, patients with a variety of wound types refractory to traditional therapies demonstrated improved healing after dHACM allografts treatment.[19] Sheikh et al.[20] also reported a case series demonstrating that refractory, nonhealing wounds treated

with dHACM healed after dHACM therapy, and the wounds did not recur with long-term follow-up. Finally, a prospective randomized controlled trial for the treatment of diabetic foot ulcers showed that 77 and 92% of chronic wounds at 4 and 6 weeks, respectively, healed with a biweekly treatment of dHACM. In contrast, standard of care resulted in healing in only 0 and 8% of patients at 4 and 6 weeks, respectively.[21] These clinical results clearly demonstrate that dHACM is efficacious for wound healing, and the results from this study help to explain the molecular basis and mode of action of dHACM in promoting healing in chronic wounds.

It is likely that the collection of growth factors present in dHACM tissue is in part responsible for the therapeutic efficacy of these allografts in treating chronic wounds. With the wide of array of cytokines and regulatory molecules preserved in dHACM tissues and released into the wound environment, we believe that delivery of these signals from a natural tissue matrix into a wound environment orchestrates an ideal "symphony of signals" to promote healing of refractory wounds.

ACKNOWLEDGMENTS

Histological staining was performed by Premier Laboratory (Longmont, CO), and growth factor multiplex ELISA arrays were performed by RayBiotech (Norcross, GA).

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