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Cytokines in single layer amnion allografts compared to multilayer amnion/chorion allografts for wound healing

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Abstract: Human amniotic membrane allografts have proven effective at improving healing of cutaneous wounds. The mechanism of action for these therapeutic effects is poorly understood but is thought to involve the resident growth factors present in near term amniotic tissue. To determine the relative cytokine contribution of the amnion and chorion in amniotic allografts, the content of 18 cytokines involved in wound healing were measured in samples of PURION® Processed dehydrated amnion, chorion, and amnion/chorion membrane (dHACM) grafts by multiplex enzyme-linked immunosorbent assay array. Both amnion and chorion contained similar amounts of each factor when normalized per dry weight; however, when calculated per surface area of tissue applied to a wound, amnion contained on average only 25% as much of each factor as the chorion. Therefore, an allograft containing both amnion and chorion would contain four to five times more cytokine than a single layer amnion allograft alone. Both single layer amnion and multilayer allografts containing amnion and chorion are currently marketed for wound repair. To examine the role of tissue processing technique in cytokine retention, cytokine contents in representative dehydrated single layer wound care products were measured. The results demonstrated that cytokine content varied significantly among the allografts tested, and that PURION® Processed single layer amnion grafts contained more cytokines than other single layer products. These results suggest that PURION® Processed dHACM contains substantially more cytokines than single layer amnion products, and therefore dHACM may be more effective at delivering growth factors to a healing wound than amnion alone.
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Key Words: wound healing, growth factor, amniotic membrane, dHACM

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INTRODUCTION

Biological tissues from a variety of sources have been used to treat non-healing wounds. Skin autografts and human skin allografts have been employed extensively for burns and chronic wounds. A number of animal-derived xenograft tissues including porcine skin, urinary bladder, and small intestinal submucosa (SIS) have been developed and marketed for treatment of non-healing, topical wounds. Both human allografts and xenografts require decellularization to remove immunoreactive cellular components, leaving an acellular extracellular matrix scaffold for repair with varying amounts of biologically active factors. Recently human amniotic membrane allograft tissues have increased in popularity for treatment of non-healing wounds, partly due to their non-immunogenic properties; however, these tissues remain relatively poorly characterized.

Human amniotic membrane is comprised of two distinct but conjoined tissues, amnion and chorion, both derived from the inner layer of the placenta. The amnion faces the fetus and the chorion faces the uterus. The amnion consists of a layer of epithelial cells anchored to a basement membrane that is underlain by a compact, collagen-rich tissue. The chorion is comprised primarily of dense collagen fibers in an interfibrillar matrix containing proteoglycans and elastic fibers. Cells are distributed throughout the amniotic membrane. Neither the amnion nor chorion is vascularized. The amniotic membrane is a metabolically active tissue that continually remodels and grows to accommodate the growing conceptus. Remodeling of the tissue is governed by growth factors, cytokines, chemokines, and related regulatory factors produced by the endogenous cells in the amniotic membrane.

Human amniotic membranes in the form of fresh, frozen, or minimally processed tissue allografts have proven effective at improving healing of ophthalmic injuries, burns, and chronic wounds. Aside from the well-established barrier function, the mechanism of action for these therapeutic effects is poorly understood but is thought to involve the resident growth factors and cytokines normally present in near term amniotic tissue. Little is known about what growth factors and cytokines are present in amniotic membrane; however, growth factors including but not limited to

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epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), transforming growth factor (TGF) alpha and beta, hepatocyte growth factor (HGF), and nerve growth factor (NGF) have been identified within fresh and preserved amniotic tissues.[1–3]

In order to preserve the bioactivity of fresh tissues, amniotic membranes have been frozen or dehydrated to prevent denaturation of the growth factors within the tissue. Once dehydrated, membranes can be stored at room temperature without risk of hydrolysis and degradation. Dehydrated human amnion/chorion membranes (dHACM) contain a number of growth factors that are known to play a role in normal wound healing, including cell recruitment and proliferation, modulation of inflammation, and regulation of ECM degradation/synthesis.[4] Previous analyses have determined that PURION® Processed dHACM contains over 50 growth factors, cytokines, chemokines, and regulatory factors.[5]

Human amniotic membranes have been used extensively to promote healing in ophthalmic injuries,[6–9] and recent study has established that amniotic membranes are an effective therapy for healing of chronic cutaneous wounds.[10,11] Human amniotic membrane allografts have been shown to reduce inflammation, pain, and scarring while promoting accelerated wound healing.[10–18] Amniotic membrane allografts also provide a biological barrier for the wound, as well as a matrix for cell proliferation and tissue growth.

In order to ensure that safe amniotic tissue allografts can be obtained while also preserving bioactivity for clinical effectiveness and stability for long-term storage and off the shelf availability, MiMedx Group, Inc. (Marietta, GA) developed a gentle cleansing and dehydration process (PURION® Process) to preserve and maintain the biological activities inherent in native amniotic tissue.[19–21] Both single layer amnion allografts (AmbioDry2®) and amnion/chorion composite grafts (EpiFix® and AmnioFix®) are produced using the PURION® Process.

Previous reports have established that PURION® Processed dHACM grafts contain a large cohort of regulatory factors involved in inflammation and tissue regeneration, and that dHACM retains biological activity that causes dermal fibroblasts and microvascular endothelial cells to proliferate. It has also been shown to induce recruitment and migration of mesenchymal stem cells both *in vivo* and *in vitro*.[4,22] dHACM is a laminate of amnion and chorion; however, the relative contribution of the amnion and chorion in promoting bioactivity *in vitro* and *in vivo* has not yet been established. Therefore, the objective of this study was to determine the relative contribution of each layer to the overall growth factor and cytokine content as well as biological activity in the allograft. In addition, since there is little information available on other marketed amniotic membranes, analyses are presented which compare the structure and composition of several amnion products.

MATERIALS AND METHODS

Dehydrated human amnion/chorion membrane (dHACM)

dHACM is a dehydrated human allograft comprised of amnion and chorion layers derived from the placenta.[19–21] Human placentas were donated under informed consent, following Caesarean 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 HIV, HTLV, Hepatitis B and C, syphilis, and 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 either processed separately, or laminated to form a two layer graft, and the tissues were dehydrated under controlled drying conditions.[21] EpiFix® (MiMedx Group, Marietta, GA) was used as the bilayer dHACM in this study.

Human amnion allografts sold in a dry configuration were obtained commercially and directly prepared for enzyme-linked immunosorbent assays (ELISAs) and histology. The products tested were BioD DryFlex ($n = 5$), BioD AmnioExCel ($n = 5$), Bone Bank SteriShield Single Layer ($n = 1$), and Bone Bank SteriShield II Dual Layer ($n = 2$).

ELISAs

Samples of amnion and chorion were PURION® Processed separately, sterilized, weighed, and prepared for growth factor analyses. Weighed, minced samples were placed in lysis buffer containing protease inhibitors for 24 hours at 4°C. Tissues were then homogenized, centrifuged to remove tissue residue, and standard ELISAs were used to measure the content of each growth factor/cytokine (RayBiotech, Inc., Norcross, GA). Fifteen growth factors/cytokines were measured in each sample. Three metalloproteinase inhibitors were also measured. Growth factor content was normalized to the dry mass of starting tissue or to the surface area of the tissue.

Multiplex ELISA arrays

Content of growth factors in EpiFix, DryFlex, AmnioExCel, SteriShield, and Sterishield II was measured with multiplex ELISA arrays (RayBiotech, Inc.), which are capable of simultaneous quantitative measurement of up to 40 growth factors, cytokines, and other regulatory proteins*. Weighed, minced samples were placed in lysis buffer containing protease inhibitors for 24 hours 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 multiplex ELISA arrays (RayBiotech, Inc.). Growth factor content was normalized to the dry mass of starting tissue or to the surface area of the tissue.

Histology

Samples of EpiFix, DryFlex, AmnioExCel, SteriShield, and Sterishield II 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

*Growth factor analyses were performed by an independent CRO, RayBiotech, Norcross, GA.
†Histology was performed by an independent CRO, Premier Laboratory, Longmont, CO, and analyzed by an independent histopathologist.

TABLE I. Cytokine Content in PURION® Processed Amnion and Chorion ($n = 5$)

	Content (pg/mg)			
	Amnion		Chorion	
Cytokine	Average	Standard Deviation	Average	Standard Deviation
TIMP-4	5992.97	2414.42	5958.01	665.55
bFGF	4455.74	1382.91	4276.23	1354.65
TGF-α	3207.53	728.49	4215.82	343.1
PDGF-AA	2151.35	1382.41	4564.91	1465.36
TIMP-2	227.86	104.84	377.95	91.3
HGF	132.05	13.63	147.79	17.47
PlGF	118.72	27.77	114.63	28.28
PDGF-BB	82.93	60.67	151.47	55.82
EGF	77.08	7.26	5.13	4.05
VEGF	29.74	64.24	186.49	354.15
IL-8	28.36	21.01	84.88	22.13
SDF-1α	26.64	0	26.64	0
TGF-β1	16.64	0	25.77	27.08
TIMP-1	4.49	1.68	18.77	20.54
IL-6	2.96	0.92	9.93	2.13
IL-10	1.27	0.91	1.69	0.61
IL-4	0.86	0.1	0.99	0.51
GCSF	0.69	1.08	0.91	0.46

The amount of each cytokine in each sample was normalized to the dry mass of tissue.

(H&E) for cell nuclei and tissue structure and Verhoeff's stain for elastic fibers, both according to standard protocols.

Cell culture—proliferation assays

Adult human dermal fibroblasts (HDFa; Life Technologies Corp., Carlsbad, CA) were plated at 2500 cells per well on a 96 well plate for 24 hours in Dulbecco's Modified Eagle's medium (DMEM) containing 10% calf serum (Gibco, Life Technologies). After 24 hours, the medium was aspirated from the wells and replaced with one of the following: DMEM lacking serum (control), DMEM plus 10% calf serum (positive control), or DMEM containing extracts of PURION® Processed amnion, chorion, or EpiFix dHACM at concentrations equivalent to 3.0, 1.5, or 0.7 cm^2 of tissue. After 72 hours, the plate was washed to remove unattached cells and a CyQuant assay (Molecular Probes CyQuant, Life Technologies C7026) was performed to quantify the number of cells.

To obtain dHACM extracts, PURION® Processed, sterilized single layer amnion, single layer chorion grafts, and EpiFix containing both amnion and chorion with an intact epithelial cell layer were minced and extracted at 4°C in DMEM without 10% calf serum, at a concentration of 20 mg of tissue per milliliter of medium. After 24 hours of extraction at 4°C, the tissue was removed by centrifugation and the extract was sterile filtered. The extract was serially diluted with DMEM without serum to the desired concentrations.

RESULTS

Growth factors in amnion and chorion

We previously reported on the content of over 50 growth factors, chemokines, cytokines, and regulatory factors in PURION® Processed dHACM grafts, specifically EpiFix®, a bilayer laminated graft containing both amnion and chorion.[4,5,22] A representative cohort of eighteen of these regulatory factors were identified in PURION® Processed amnion and chorion. The factors identified in both layers were bFGF, EGF, granulocyte colony stimulating factor (GCSF), HGF, interleukins 4, 6, 8 and 10 (IL-4, IL-6, IL-8, IL-10), platelet derived growth factor AA and BB (PDGF-AA, PDGF-BB), placental growth factor (PlGF), stromal derived factor 1 alpha (SDF-1α), tissue inhibitors of metalloproteinases 1, 2, and 4 (TIMP-1, TIMP-2, TIMP-4), TGF alpha and beta 1 (TGF-α, TGF-β1), and vascular endothelial growth factor (VEGF).

Growth factor content in amnion and chorion

Eighteen representative growth factors and cytokines were quantified in samples of PURION® Processed amnion and chorion (Table I). The measured picograms of protein was normalized to the dry weight of the starting tissue. The relative amount of each growth factor per dry mass of tissue varied within each tissue type, ranging from less than 1 pg/mg dry weight (GCSF, IL-4) to over 4000 pg/mg dry weight (bFGF, PDGF-AA). However, amnion and chorion contained comparable amounts of each growth factor, except for EGF, which was significantly higher in amnion, and PDGF-AA and VEGF, which were significantly higher in chorion.

Growth factor content in grafts containing amnion and chorion

EpiFix® contains both amnion and chorion. The chorion layer is four to five times thicker than the amnion layer, and therefore will contribute disproportionately to growth factor content in amnion/chorion grafts. The relative amount of each growth factor/cytokine in EpiFix that is contributed by the amnion versus the chorion is shown in Figure 1. Chorion contributes on average 82% of the growth factors in

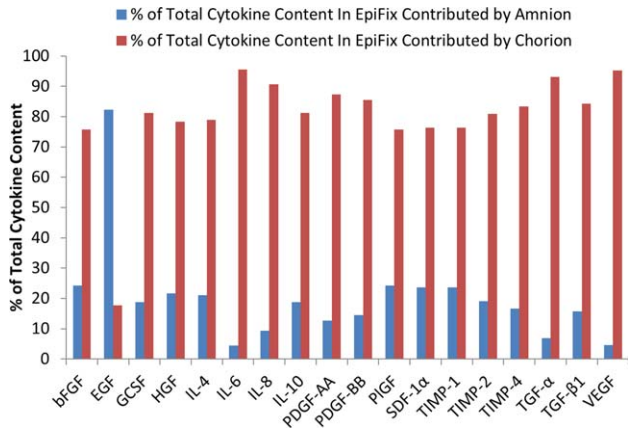


FIGURE 1. Percentage of each cytokine in EpiFix® contributed by amnion versus chorion. Chorion contributed on average 82% of the total growth factors in the tissue, with the exception of EGF, where it only contributed 20% while the amnion contributed 80%. ($n = 20$ for each bar).

the product, except for EGF, where it contributes 20% and the amnion contributes 80%. In some cases, for example, VEGF and TGF-α, the chorion contributes over 90% of the growth factor in the bilayer product.

The results of these analyses establish that multilayer amnion/chorion grafts (EpiFix® and AmnioFix®) contain five times the amount of growth factors and cytokines than contained in single layer amnion grafts. The only exception is EGF, which in multilayer grafts is derived primarily from the amnion layer. These results indicate that a single layer graft derived from amnion only will deliver less growth factors and cytokines than an amnion/chorion multilayer graft of the same surface area. To verify this conclusion, the growth factor content per square centimeter in PURION® Processed AmbioDry2® (single layer amnion graft) was compared with that in the PURION® Processed bilayer EpiFix® and AmnioFix® (Figure 2). For all growth factors except IL-4, the

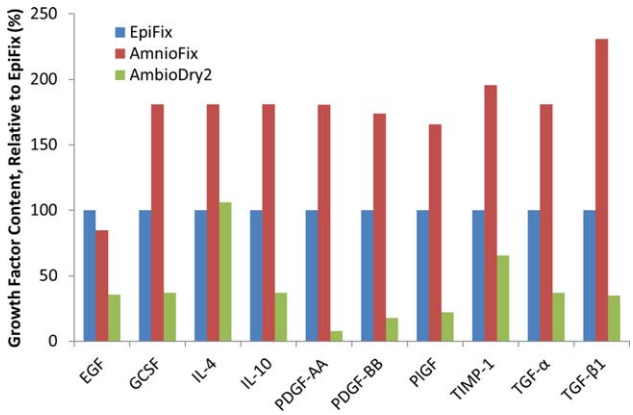


FIGURE 2. Relative amount of representative cytokines in PURION® Processed Ambiodry2® (single layer amnion) and AmnioFix® (multilayer dHACM), compared with EpiFix® (multi-layer dHACM). The relative content of each of the indicated cytokines was determined by dividing the product's cytokine content in picograms per square centimeter by EpiFix's cytokine content in picograms per square centimeter, multiplied by 100. For all cytokines measured, except for IL-4, the growth factor content in the single layer amnion graft (AmbioDry2) was substantially lower than in the multi-layer grafts.

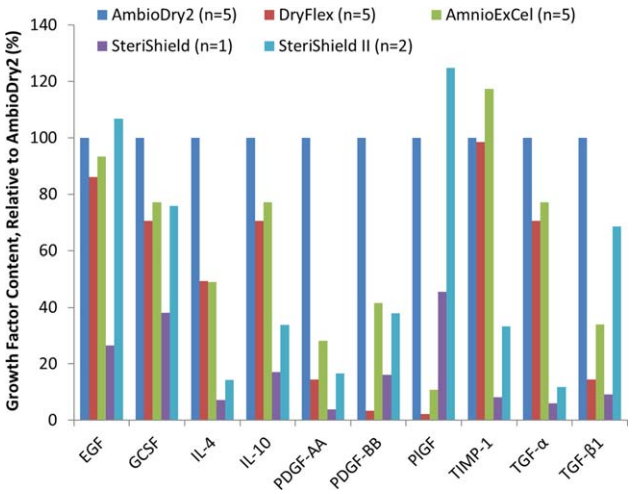


FIGURE 3. Relative amount of representative cytokines in single layer amnion products, compared with AmbioDry2. The relative content of each of the indicated cytokines was determined by dividing the product's cytokine content in picograms per square centimeter by AmbioDry2's cytokine content in picograms per square centimeter, multiplied by 100. PURION® Processed single layer amnion, AmbioDry2, generally contains substantially greater amounts of growth factors than other marketed single layer amnion products.

growth factor content was substantially lower in the single layer amnion graft (AmbioDry2).

PURION® Processed single layer amnion, AmbioDry2, contains relatively small amounts of growth factors compared with EpiFix; however, it still contains substantially greater amounts of growth factors than other marketed single layer amnion products (DryFlex, AmnioExCel, SteriShield, and SteriShield II; Figure 3).

Composition of dehydrated amniotic membrane allografts

Differential staining of commercially marketed amniotic grafts was used to determine the tissue composition of these products. Figure 4 shows the differential Verhoeff's staining in amnion, chorion, and EpiFix. Verhoeff's method stains elastic fibers, which are present in the epithelial layer of the amnion and throughout most of the chorion, but it does not stain the amnion compact layer. Micrographs of Verhoeff stained amniotic membrane allografts are shown in Figure 5. Based on the differential staining, the composition of each product is indicated in Figure 5. EpiFix is composed of amnion and chorion with intact epithelial cells on the amnion surface and intact interstitial cells in both amnion and chorion. AmnioFix contains amnion with disrupted epithelial cells and chorion, both of which contain intact interstitial cells. AmbioDry2, DryFlex, AmnioExCel, and SteriShield Single Layer each consist of single layer amnion with intact cells. SteriShield II Dual Layer is a double layer amnion with both intact epithelial layers facing inward.

Growth factor content in single layer amnion grafts

The contents of representative cytokines in single layer amnion allografts were measured to determine whether all amnion-based grafts are equivalent. The data are shown in

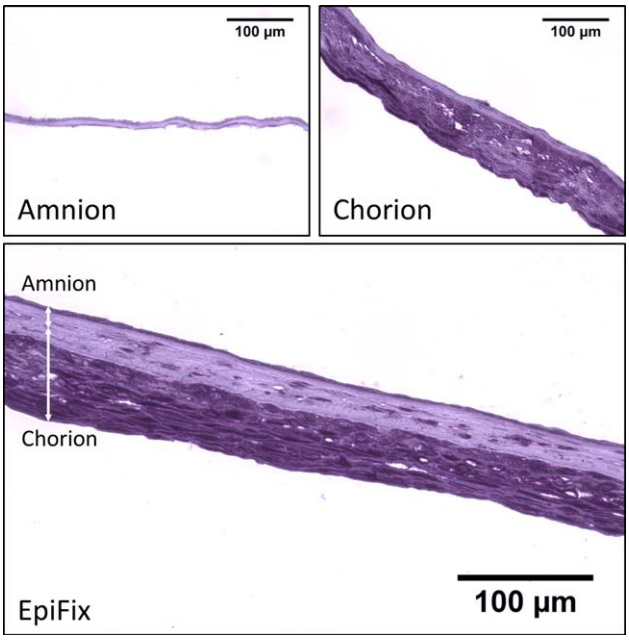


FIGURE 4. Representative micrographs of Verhoeff stained amnion, chorion, and EpiFix. Verhoeff stain can be used to clearly identify the amnion and chorion layers of the amniotic membrane grafts. Verhoeff's method stains elastic fibers, which are present in the epithelial layer of the amnion and throughout most of the chorion, but it does not stain the amnion compact layer.

Figure 6 along with a micrograph of an H&E stained section and a description of the composition of the graft based on the histology. The content of each cytokine was normalized to the surface area of the graft to represent the relative amount of cytokine applied to a wound. These picograms per square centimeter values were then used to calculate the cytokine content relative to the picograms per square centimeter cytokine content in EpiFix, which provides a direct comparison of cytokine content in the single layer grafts versus that in dHACM. The differences between cytokine contents in the grafts can be determined by comparing the percentage values for each cytokine (blue bars, Figure 6) since the same normalizing factor for EpiFix was used for the calculation.

Overall, the cytokine contents per unit surface area in the single layer amnion products were substantially less than that in EpiFix for all of the grafts tested. The content of cytokines in AmbioDry2 was greater than that in the other single layer grafts. DryFlex and AmnioExCel contained the same mixture of cytokines as AmbioDry2, however the contents were in general lower than those in AmbioDry2. SteriShield and SteriShield II contained relatively few cytokines and at low levels, except for bFGF, which was equivalent or higher than what is present in EpiFix.

Cell culture—proliferation assays

Previous analyses have shown that extracts of dHACM (EpiFix®) induce proliferation of human dermal fibroblasts *in vitro*.[4] The effects of extracts of amnion, chorion, and EpiFix on proliferation of human dermal fibroblasts *in vitro* were measured in order to determine whether both amnion and chorion are able to induce fibroblast proliferation, and if this biological activity derives from one or both layers in EpiFix. Following 3 days of exposure to differing concentrations of each of the extracts of amnion, chorion, or EpiFix, the numbers of cells were measured for each treatment. The values were then normalized to the square cm of tissue that the extract represented. The data are shown in Figure 7. Both the amnion and chorion caused dermal fibroblasts to proliferate. However, the chorion per square centimeter was on average five times more effective at inducing proliferation. These results indicate that the biological activity in EpiFix that is responsible for inducing fibroblast proliferation is contained in both the amnion and chorion, but the predominant basis for the bioactivity is derived from the chorion.

DISCUSSION

Amnion and chorion contain a similar array of growth factors, cytokines, and regulatory factors. This diverse combination of factors plays a significant role during fetal development and growth, and it is likely that these soluble factors also play a critical role in facilitating repair and regeneration when applied to chronic wounds. Overall, the amounts of each growth factor in the tissue are practically consistent between amnion and chorion; however, since chorion is four to five times thicker than amnion, the chorion contains substantially more of these factors per square centimeter than the amnion. Therefore, when amnion is combined with chorion to form a graft, as in dHACM or EpiFix, the resulting bilayer graft contains five-fold more growth factors than a single layer graft containing only the amnion, suggesting that dHACM may be more effective at delivering growth factors to a healing wound than amnion alone.

There are no studies or reports comparing the efficacy of single layer amnion allografts to the efficacy of bilayer allografts containing both amnion and chorion for improving wound healing. However, a prospective randomized clinical trial has shown that PURION® Processed dHACM, a bilayer graft comprised of amnion and chorion (EpiFix), for the treatment of diabetic foot ulcers showed that 77% and 92% of the chronic wounds at four and six weeks, respectively, healed with a bi-weekly treatment of dHACM. In contrast, standard of care resulted in healing in only 0% and 8% of patients.[18] Clearly, the amnion/chorion graft (dHACM, EpiFix) significantly improves chronic wound healing, compared with the standard of care. These data suggest that the high abundance of growth factors that results from combining amnion and chorion may contribute to the efficacy of the graft for wound repair and tissue regeneration. While the clinical efficacy of dHACM grafts in treatment of chronic wounds has been thoroughly established in peer-reviewed literature,[18,23–25] further clinical studies are required to demonstrate the relative efficacy of single layer amnion products.

Verhoeff staining of histological tissue sections was able to discriminate amnion from chorion through a combination

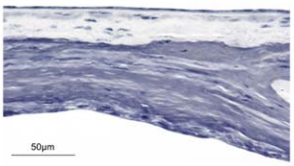
	Verhoeff Stain	Composition
MiMedx EpiFix		Laminate of amnion and chorion with intact cells.
MiMedx AmnioFix		Laminate of amnion and chorion. Epithelial cell layer is disrupted. Intact cells are present in remaining layers.
MiMedx AmbioDry2		Single layer amnion with intact cells.
BioD DryFlex		Single layer amnion with intact cells.
BioD AmnioExCel		Single layer amnion with intact cells.
Bone Bank SteriShield Single Layer		Single layer amnion with intact cells.
Bone Bank SteriShield II Dual Layer		Double layer of amnion with intact cells. Epithelial cell layers are facing inward.

FIGURE 5. Verhoeff stained amniotic membrane allografts. Verhoeff staining clearly distinguishes multi-layer dHACM grafts (EpiFix and AmnioFix) from single layer amnion products. EpiFix and AmnioFix are multi-layer grafts composed of amnion and chorion. AmbioDry2, DryFlex, AmnioExCel, and SteriShield are single layer amnion grafts. SteriShield II is a double layer amnion graft with epithelial layers facing inward.

of the presence of elastic fibers and tissue thickness. The chorion stained positively for elastic fibers, while only the epithelial cell layer of the amnion stained positively with Verhoeff. The chorion was also visibly thicker than the amnion by four- to five-fold.

When comparing growth factor content among several commercially available single layer amnion allografts in this study, the growth factor content in the tissues varied substantially, suggesting that the different parameters used for processing the tissue affects retention of growth factors within the membranes. Preserving the bioactivity of the native amniotic tissues by retaining the diverse array of naturally occurring growth factors and cytokines is critical in developing an effective treatment for healing of chronic wounds. Of the single layer amnion tissues, the highest growth factor contents in this study were measured in the PURION® Processed AmbioDry2, indicating that the PURION® Process is a gentle cleansing method that does not remove the growth factors measured in this study from the tissue membranes. Proliferation of human dermal fibroblasts when treated with soluble tissue extracts verified that these growth factors remained biologically active in PURION® Processed tissues.

When compared with DryFlex and AmnioExCel, the single layer amnion products contained a similar array of growth factors; however, the amounts of these growth

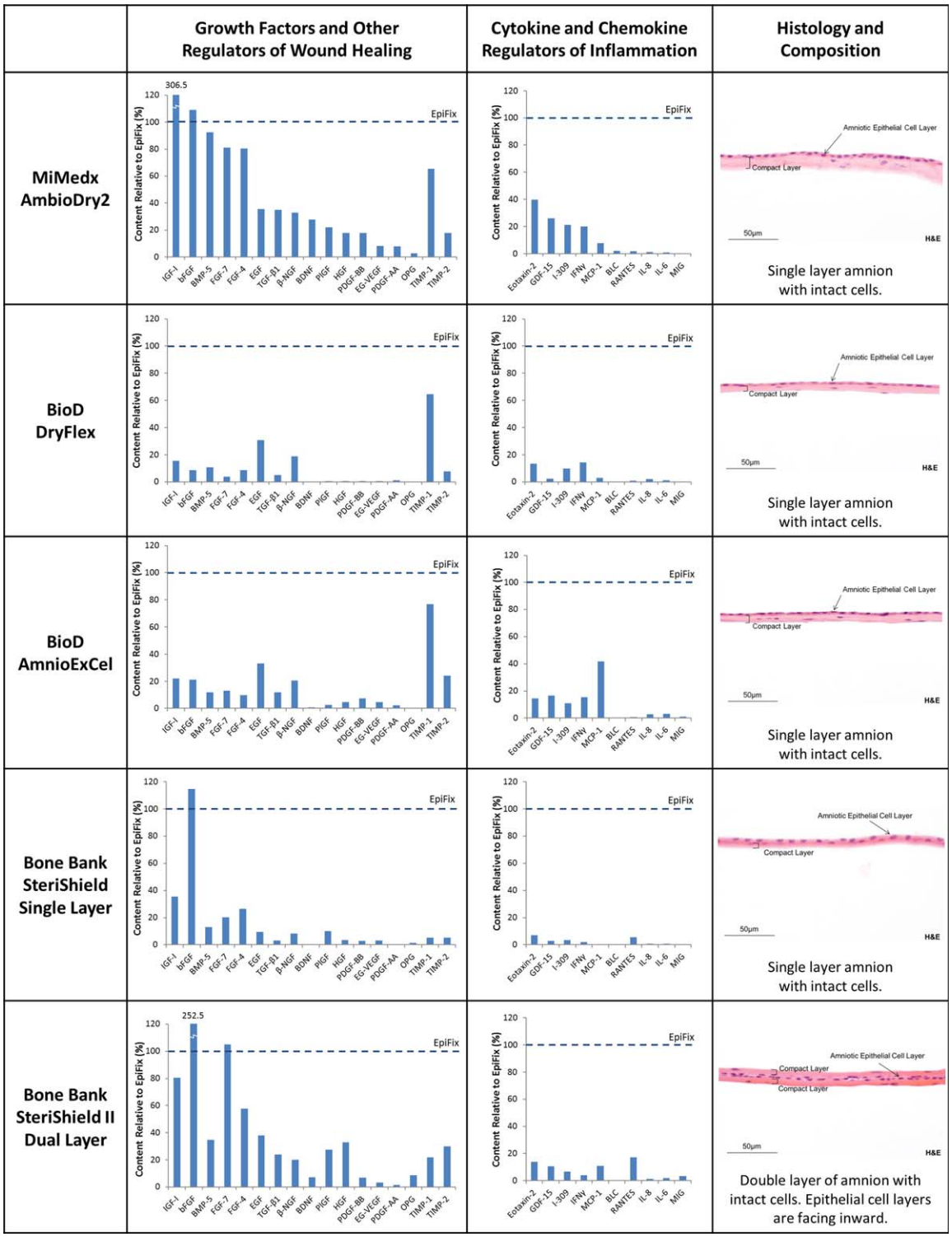


FIGURE 6. Relative cytokine content in single layer amnion allografts. The cytokine contents per unit surface area in the single layer amnion products were substantially less than that in EpiFix (multi-layer dHACM) for all of the grafts tested. The content of cytokines in PURION® Processed AmbioDry2, however, was generally greater than that in the other single layer grafts.

factors in DryFlex and AmnioExCel were consistently less than that measured in AmbioDry2. The SteriShield allografts contained a similar array of growth factors, but once again in lesser quantities than PURION® Processed AmbioDry2, except for a high content of bFGF. The processing methods used for these allografts are not publicly available, so understanding the cause for the differences is not feasible; however, it is likely that respective processing techniques wash away many of these soluble signals, resulting in a low abundance of growth factors in these tissues.

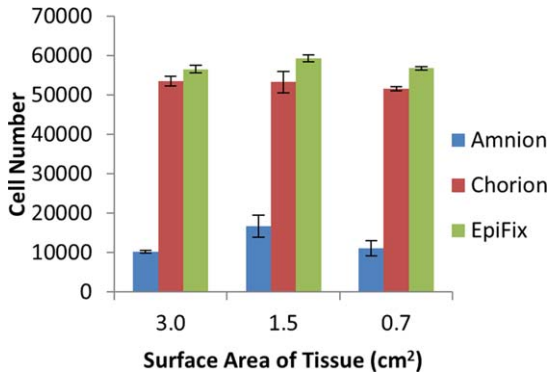


FIGURE 7. Relative effects of PURION® Processed amnion, chorion, and EpiFix on proliferation of adult human dermal fibroblasts *in vitro*. For a given surface area of tissue, chorion and EpiFix (dHACM) grafts were more effective at promoting fibroblast proliferation than amnion alone.

The results of this study suggest that PURION® Processed tissues retain a high content of growth factors within the membrane allografts, and that chorion membranes possess a four- to five-fold higher growth factor content than amnion per equivalent surface area, largely due to chorion's greater thickness. Human dehydrated amnion/chorion membrane (dHACM) laminates are effective treatments to promote healing of refractive wounds, suggesting that the retention of biologically active growth factors during processing of amniotic tissues is critically important in preserving the bioactivity of the native tissues for wound care.

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