Dehydrated human amnion/chorion graft bioassay; Indications for use in regenerative therapeutics

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ABSTRACT

AmnioVera® Amnion/Chorion allograft, minimally manipulated via the Verasure® proprietary process, maintains the functions of placental tissue containing extracellular matrix and bioactive cytokines (growth factors, chemokines). It is the combination of the extra-cellular matrix (ECM), cytokines, their immunomodulatory effect and their anti-inflammatory, anti-adhesion and anti-scarring attributes that make their use in regenerative medicine highly effective and safe, providing significant value in wound care and surgical specialties. The bioassay results presented in this paper demonstrate that the Verasure® process is a significant advancement in placental tissue processing providing high levels of cytokines, growth factors and ECM after processing and sterilization.

1. Introduction

The purpose of this whitepaper is to highlight the background, science and potential uses of human amnion/chorion membrane. The Anu Life Science (ALS) team has developed novel proprietary techniques that are well within minimal manipulation guidelines set forth by the FDA in November 2017, yet yield significantly higher concentrations of growth factors, exosomes and miRNA than reported (via literature and marketing materials) by other tissue banks in the placental tissue arena.

This white paper will be focused on the growth factors (GF) found in AmnioVera, a Vera Bioscience Amnion Chorion product. Vera Bioscience continues to assay its placental tissue product line and will follow up with analysis of exosomes and micro RNA concentrations as well.

2. Background: Placental tissue and its components

Placental tissue has proven to be a very powerful, versatile and safe allograft therapy in a wide range of pathologies treated in the regenerative medicine arena (2). The human amnion is a single layer of epithelial cells separating the amniotic cavity from the vascularized chorion. The Amniotic Membrane (AM) is the innermost layer of the placenta and consists of a thin epithelial layer, a thick basement membrane, and an avascular stroma. It contains collagen types III, IV, V, and VII and fibronectin and laminin (3–5). It also contains fibroblasts and growth factors, cytokines, exosomes, alpha2macroglobulin, miRNA and a wide spectrum of growth factors at high concentrations. AM has been shown to have unique properties including the ability to suppress pain, fibrosis, and bacteria and to promote wound healing (6–10).

The AM contains 2 cell types of different embryologic origin; specifically, amnion epithelial cells, derived from the embryonic ectoderm, and amnion mesenchymal cells, derived from embryonic mesoderm (2). The recommendation of the International Society for Cellular Therapy has been that mesenchymal cells derived from amnion be referred to as *amniotic membrane-human mesenchymal stromal cells* (AM-hMSCs) (3).

Early in gestation amniocytes start off flattened and produce the amniotic fluid; however, as pregnancy progresses, they become cuboidal and have increasing numbers of microvilli on their apical surface. Tortuous intercellular channels exist between the tight junctions of amniocytes. Vascular endothelial growth factor (VEGF) in the fetal membranes appears to be a mediator of this process. VEGF promotes blood vessel development within the amnion and influences the permeability of the micro-vessels, which perfuse the fetal and placental surfaces (11). In a study, Moshiri and Oryan (12) demonstrated the effectiveness of FGF (Fibroblast growth factor) in restoring the morphologic and biomechanical properties of injured tendon in rabbits (13,14). The innate immune system is the first line of defense against pathogens and includes anatomic and physiologic barriers, enzymes and antimicrobial peptides, phagocytosis, and release of proinflammatory mediators by neutrophils and macro¬phages. Many of the substances that constitute the innate immune system have been identified in AM and have been shown to have significant antimicrobial properties, including defensins (human neutrophil defensins 1-3), lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin, secretory leukocyte protease inhibitor, RIS-1/psoriasin (expression in epithelial skin cells indicates their selective role in innate immunity and in inflammatory skin diseases including acne), and a Cathelicidin (Cathelicidin-related antimicrobial peptides are a family of polypeptides, found in lysosomes of macrophages and polymorphonuclear leukocytes (PMNs) and keratinocytes)(15). These potent antimicrobials have shown broad-spectrum activity against bacteria, fungi, protozoa, and viruses. Perhaps the most important of these are the defensins (human neutrophil defensins 1-3), which are found in in AM. Furthermore, lactoferrin is a glycoprotein with 2 binding sites for ferric ions. Lactoferrin is likely secreted by neutrophils and amniotic cells. Lactoferrin has both bacteriostatic activity, owing to the sequestration of iron which is then unavailable for microbial growth, and bactericidal activity, by binding to bacterial outer membranes and triggering release of the lipopolysaccharide lactoferricin. Lactoferricin shows antimi¬crobial effects against viruses, protozoa, and fungi (16).

There are contributing factors that appear to minimize scarring, such as hyaluronic acid and the presence of hyaluronic acid-stimulating factor. In a study of the effect of AM on proteases important to wound healing, human AM was shown to enhance collagenase activity but to inhibit activation of hyaluronidase, elastase, and cathepsin (17,18).

The amnion has been used as a physiologic wound dressing and as a graft for skin wound coverage (7–10). Human AM has proved to be a versatile temporary biologic dressing in studies involving hundreds of patients during the past century. The first reported use of fetal membranes was in skin transplantation in the early 1900s (22,23). AM was also used on burned and ulcerated skin surfaces,

and clinicians reported a lack of infection, a marked decrease in pain, and an increased rate of re-epithelialization of the traumatized skin surfaces. Others have demonstrated the use of AM as a biologic dressing for open wounds, including burns and chronic ulceration of the legs (24). In traditional medicine, the first reported use was by Davis (22) in 1910 at Johns Hopkins Hospital for burns and ocular wounds in 550 cases. In 1914, Sabella (23) reported similar positive findings. Numerous reports were published during the 1940s and 1950s, until the 1970s when the human immunodeficiency virus/acquired immunodeficiency syndrome became epidemic, and its source was unclear (24). AM was no longer favorable as a treatment choice until 1995 when Kim and Tseng (25) presented their findings. Subsequently, published research has been increasing.

The wounds treated with AM responded to a protocol that allowed coverage of tissues as diverse as exposed bowel, pleura, pericardium, blood vessels, tendon, nerve, and bone. Wounds unresponsive to standard therapeutic measures have also responded to application of AM, and human AM dressings have become a useful adjunct in the care of complicated wounds (27).

Reports on the immunogenicity of human amniotic epithelial cells after transplantation into human volunteers have also been published (28–30). Amnion, consisting of a monolayer of epithelium on a basement membrane with an underlying collagen matrix containing a few fibroblasts (which, in theory, would express HLAs, although the epithelium itself lacks them), has been transplanted into subcutaneous pouches in normal human volunteers. None of the volunteers showed clinical signs of rejection (graft-host reaction), and amniotic epithelial cells were demonstrated by biopsy up to 7 weeks after implantation.

HLA antibodies were not detected in serum samples, and no in vitro lymphocyte reaction to the amniotic cells was found in 2 out of 100 volunteers. These results suggest that acute immune rejection does not occur after allotransplantation of human amniotic epithelial cells.

In 1979, Trelford and Trelford-Sauder (32) found that AM transplantation promoted epithelial healing, reduced inflammation, increased comfort, and decreased the severity of insufficient vascularization. In 2002, Ucakhan et al (33) did not find any infectious, inflammatory, or toxic reactions related to AM transplantation. Amnion surface epithelial cells do not express HLA-A, -B, -C, or -DR or b2-microglobulin (34, 35). Ucakhan et al (33) evaluated safety and efficacy of non-preserved AM transplantation with or without limbal autograft transplant in acute and chronic eye injuries. In the transplantation of human organs, whether skin, kidney, liver, or other tissue, the major problem has been rejection of the grafted tissue owing to the host immune response. Despite this risk, amnion has been used successfully as a skin graft without concern for tissue typing and matching of the donor to the host (32). This unique attribute (the lack of immunogenicity) has been described in numerous clinical studies and scientific journals and has led to the characterization of the placental organ as *immune privileged*. Thus, granulized AM and AF (gAM-AF) has been considered by many to be ideal for use in all patients, including the most immunocompromised, such as post-transplant and human immunodeficiency virus-positive patients, and others with compromised immune systems, who could be adversely affected by human tissue transplantation or infection. The unique biologic structure of amniotic tissue, coupled with the low risk of an adverse host immune response, makes gAM-AF ideal for an in vivo wound covering.

Experimental and clinical studies have demonstrated that AM transplantation promotes re-epithelialization, decreases inflammation and fibrosis, and modulates angiogenesis (35). Several growth factors produced by AM are involved in these processes, including TGF-b and basic FGF (36). Additionally, recent published reports have reported that specific soluble factors secreted by human amniotic epithelial cells into AF might be effective in ameliorating liver fibrosis, COPD, and chronic kidney conditions. Extracellular vesicles (EVs) are secreted nanosized (40-100 nm) membrane vesicles that may act as a novel cell-cell communicator. Vera Bioscience will address this more specifically in additional reports to be published.

One needs to also consider the Hyaluronic Acid component of AF. A study by Lockington (1) demonstrates that AM is able to remove reactive oxygen species (ROS) from its environment. Demonstrating total antioxidant capacity in AM provides evidence for its use as a free radical scavenger. An increased awareness of the role of free radicals in corneal disease may lead to treatment strategies utilizing antioxidant agents derived from HA or AM.

The AM anti-inflammatory action may be mediated in part by interleukin-10 **(IL-10)**, of which we detect significant amounts in AM extracts. IL-10 is known to suppress or counteract the actions of pro-inflammatory cytokines such as IL-611 and tumor necrosis factor-alpha (TNF-a) (12). IL-10 also suppresses amniotic cell production of IL-8,13 which is a pro-inflammatory chemokine attracting the migration of neutrophils. TCF-13 superfamily provides the protein substrates for production of inhibin and activin. Activin promotes the production of prostaglandin **PGE2**. (14,15) A low dose of activin stimulates, but a high dose of activin inhibits, the production of IL-6, IL-8, and PGE2 by the AM. (l6) No such effect is noted in the chorion or decidua. TNF-a is significantly inhibited by activin in the chorion and decidua's (l6). The AM contains various protease inhibitors, including anti-trypsin inhibitor (17), which may exert an anti-inflammatory effect (18). Future studies are needed to determine whether IL-10, activin, protease inhibitors, and/or a combination of them are responsible for the anti-inflammatory action of AM when it is transplanted to the ocular surface.

The AM contains IL-1 receptor antagonist **(IL-1)** and helps transport it to the amniotic fluid.19 IL-lRA is a potent inhibitor of lL-1, and thus will suppress the inflammation mediated by IL-1. Data has shown that limbal epithelial cells cultured on the AM stromal matrix downregulate the expression and production of IL-1 but upregulate the expression and production of IL-lRA, resulting in a higher ratio of IL-lRNIL-1 (20). Such an effect withstands the challenge of lipopolysaccharide (20). These findings support the concept that the AM exerts its anti-inflammatory action by suppressing the signaling pathway via IL-1.

As a review of cytokines, chemokines are proteins found in amniotic membrane and fluid allografts. Cytokines are a broad and loose category of small proteins (~5–20 kDa) that are important in cell signaling. They are released by cells and affect the behavior of other cells, and sometimes the releasing cell itself. Some cytokines enhance or inhibit the action of other cytokines in complex ways. Cytokines include chemokines, interferons, interleukins, lymphokines, tumor necrosis factors, but generally not hormones or growth factors.

Chemokines are a family of small cytokines or signaling proteins secreted by cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines.

Growth factor cytokine	Function
Growth Differentiation factor 15	Regulates apoptotic / inflammatory pathways in response to injury
Granulocyte macrophage colony stimulating factor	Immunomodulating – related to developing granulocytes and monocytes
Interferon Gamma	Immunomodulating

Growth factor cytokine	Function
Interleukin 1 alpha	Immunomodulating
Interleukin 1 beta	Immunomodulating
Interleukin 1receptor antagonist	Immunomodulating
Interleukin 5	Stimulates immunoglobulin release
Interleukin 7	Immuno cell development
Interleukin 12 p40	Chemotactic
Interleukin 12 p70	Immuno modulating
Interleukin 15	Immunomodulating defense
Interleukin 17	??
Macrophage stimulating factor	??
Osteoprotegerin	Affects osteoclast activity
Brain neurotrophic factor	Growth
Bone morphogenic 5	Bone- cartilage
Endocrine Vascular Endothelial GF	Angiogenesis
Fibroblast GF 4	Cellular activator
Fibroblast GF 7	Epithelial cell activity
Growth Hormone	Affects IGF-1
Insulin like GF 1	System growth- mitogenic activity
Insulin like GF binding 1	Stabilizes IGF-1
Insulin like GF binding 2	Stabilizes IGF-1
Insulin like GF binding 3	Stabilizes IGF-1
Insulin like GF binding 4	Stabilizes IGF-1
Insulin like GF binding 6	Stabilizes IGF-1

3. Methods

One standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody is arrayed in quadruplicate. Detection Method Fluorescence: with laser scanner: Cy3 equivalent dye. It combines the advantages of the high detection sensitivity & specificity of ELISA and the high throughput of arrays. Like a traditional sandwich-based ELISA, it uses a pair of cytokine-specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different epitope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-labeled Cy3 equivalent dye using a laser scanner. Unlike the traditional ELISA, Quantibody (Ray Biotech) products use array format. By arraying multiple cytokine-specific capture antibodies onto a glass support, quantitative, multiplex detection of cytokines in one experiment is made possible.

For cytokine quantification, the array-specific cytokine standards, whose concentration has been predetermined, are provided to generate a standard curve for each cytokine. By comparing signals from unknown samples to the standard curve, the cytokine concentration in the samples will be determined.

Vera Bioscience had quantification analysis completed on 41 proteins for Amnion Chorion membrane, utilizing the Ray Biotech Quantibody system.

4. Results

Target Cytokine – Growth Factor	Amnion-Chorion Median Reported Value *picograms per milliliter (pg/mL)*
AR	37.9
BDNF	5.1
bFGF	324.2
BMP-4	*0.8*
BMP-5	*385.7*
BMP-7	281.3
b-NGF	*0.4*
EGF	15.8
EGF R	4,491.9
EG-VEGF	1,194.5
FGF-4	*20.9*
FGF-7	44.2
GDF-15	1,474.4
GDNF	*3.7*
GH	67.1
HB-EGF	*7.2*
HGF	1,229.5
IGFBP-1	2,533.6
IGFBP-2	1,746.2
IGFBP-3	32,545.4
IGFBP-4	15,236.8
IGFBP-6	43,931.5
IGF-1	*8.2*
IL-1ra	-
Insulin	118.5
MCSF R	1,807.3
NGF R	*0.0*
NT-3	*3.2*
NT-4	60.5
OPG	1,031.7
PDGF-AA	216.9
PDGF-BB	21.8
PIGF	7.3
SCF	16.5
SCF R	131.7
TGFa	79.9
TGFb1	4,271.6
TGFb3	84.5
VEGF	*3.8*
VEGF R2	1,931.2
VEGF R3	*52.6*
VEGF-D	*0.0*

5. Discussion

This review and study indicate that our Verasure® proprietary Amnion/Chorion allograft process is successful. With minimal manipulation of the Amnion/Chorion allograft, we dry and retain functional bioactive proteins (cytokines, chemokines, growth factors). The AmnioVera allografts' bioactive proteins, combined with the presence of the extracellular matrix, lamin, and fibronectin, present a solid allograft that is effective in a wide array of applications. The applications range from topical wound healing to enhancing surgical repair by reducing incidence of adhesions, fibrosis and painful scars.

The anti-inflammatory action appears to require contact with its ECM. The mechanism by which AM ECM exerts its anti-inflammatory action plays a role in modulating balance M1 and M2 macrophages. Several authors have reported that human AM can suppress alloreactive responses suggesting that the AM may also suppress acquired immunity.

The anti-fibrosis anti-scarring capacity of the AM is due to the presence of the anti-inflammatory cytokine, IL-10, which can inhibit the production of IL-6 (3,11). Diminished IL-6 production contributes to fetal wound repair without scarring (4,31). The phenomenon of fetal wounds healing without scar was confirmed in a study by Liechty. Three different TGF-beta (1, 2, 3) are the

most potent cytokine, promoting myofibroblast differentiation by upregulating expression of alpha-SMA, integrin alpha5beta1, and EDA containing fibronectin **Fn-46** in a number of cell types, including fibroblasts. These factors contribute to healthy tissue healing to form fully functional, flexible tissue, with strong anatomic layering. TGF-beta also upregulates the expression of such matrix components as collagens and proteoglycans, downregulates proteinase and matrix metalloproteinases (MMPs), and upregulates their inhibitors.

The presence of neurotrophic factors that control the growth and targeting of sensory and autonomic nerves to the peripheral tissues (6) are present: nerve growth factor **(NGF)**, brain-derived neurotrophic factor **(BDNF)**, and neurotrophin-3 **(NT-3)**. The fact that the AM contains these neurotrophic factors suggests that it has a significant role in the development of the fetal nervous system, enabling scarless wound healing. The presence of NGF makes it extremely effective in treating corneal eye lesions (7, 8).

It is the combination of each of these powerful cytokines, chemokines and ECM present in our Amniogen Amnion/Chorion allografts that amplify the healing potential of the recipients.

6. Conclusion

In this review we demonstrate our evidence that regenerative therapeutics require a combination of components to drive the healing regenerative process using healthy processed placental tissues. It is clear that the proprietary Verasure® processing techniques provide enhanced levels of growth factors to our allografts. The growth factors, cytokines, and ECM all need to work together to provide a safe and efficacious pathway to healing traumatized, injured tissue.

Vera Bioscience, in collaboration with its tissue bank, Anu Life Sciences, are on a mission to increase the understanding and awareness of the plethora of proteins and extra-cellular matrix interactions in our placental tissue allografts.

As we develop additional assays of exosomes, miRNA, various secretome vesicles, and Alpha 2 macroglobulin in our minimally manipulated placental allografts, we will bring this information to our clinical studies in multiple pathologies. Vera Bioscience demonstrates that placental tissue allografts processed appropriately bring the whole symphonic orchestra to play in regenerative healing of injured, traumatized and/ or diseased tissue.

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