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A Prospective Study of 20 Foot and Ankle Wounds Treated with Cryopreserved Amniotic Membrane and Fluid Allograft

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ABSTRACT

We reviewed the background information and previous clinical studies that considered the use of allogeneic amniotic tissue and fluid (granulized amniotic membrane and amniotic fluid) in the treatment of chronic diabetic foot wounds. This innovation represents a relatively new approach to wound management by delivering a unique allograft of live human cells in a nonimmunogenic structural tissue matrix. Developed to fill soft tissue defects and bone voids and to convey antimicrobial and anti-inflammatory capabilities, granulized amniotic membrane and amniotic fluid does not require fetal death, because its procurement is performed with maternal consent during birth. In the present investigation, 20 chronic wounds (20 patients) that had been treated with standard wound therapy for a mean of 36.6 ± 31.58 weeks and with a mean baseline area of 10.15 ± 19.54 cm^2 were followed up during a 12-week observation period or until they healed. A total of 18 of the wounds (90%) healed during the 12-week observation period, and none of the wounds progressed to amputation. From our experience with the patients in the present case series, we believe that granulized amniotic membrane and amniotic fluid represents a useful option for the treatment of chronic diabetic foot wounds.

Regenerative medicine, a commonly used phrase in the field of chronic wound management, is the "process of replacing or regenerating human cells, tissues, or organs to restore or establish normal function" (1). The term also refers to a group of biomedical approaches to clinical therapies that involve the use of stem cells. Tissue-engineered skin and platelet-derived growth factors represent substantial advancements in wound healing, because they stimulate the delivery of certain factors considered important to regenerative medicine, including growth factors, fibroblasts, collagen, and a physical scaffold on which wound repair and regeneration can occur. The amniotic membrane (AM) or amnion is a tissue of particular interest because it can provide cells with multipotency. Amnion is easily obtained after cesarian delivery, because the placenta and associated fluid and membrane are typically discarded after childbirth. The cells have been shown to have extremely low immunogenicity, and their procurement avoids the controversies associated with obtaining human embryonic stem cells. Thus, the use of AM and amniotic fluid (AF) has been proposed as a potentially useful allograft cell therapy in regenerative medicine (2).

The 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, modulates cytokine and growth factor levels, and 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).

AF contains nutrients and growth factors that facilitate fetal growth and provides mechanical cushioning and antimicrobial properties that protect the fetus. The human amnion is a single layer of epithelial cells separating the amniotic cavity from the vascularized chorion. Early in gestation these amniocytes are flattened; 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

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blood vessel development within the amnion and influences the permeability of the microvessels, which perfuse the fetal and placental surfaces (11).

AF also contains carbohydrates, proteins and peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones. The concentration of epidermal growth factor in amniotic fluid is fourfold greater than in maternal serum. AF also contains transforming growth factor (TGF)-α, TGF-β1, and fibroblast growth factor (FGF). In a recent study, Moshiri and Oryan (12) demonstrated the effectiveness of FGF 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, and phagocytosis and release of proinflammatory mediators by neutrophils and macrophages. Many of the substances that constitute the innate immune system have been identified in AF and have been shown to have significant antimicrobial properties, including α-defensins (human neutrophil defensins 1-3), lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin, secretory leukocyte protease inhibitor, psoriasin (S100A7), and a cathelicidin (15). These potent antimicrobials have shown broad-spectrum activity against bacteria, fungi, protozoa, and viruses. Perhaps the most important of these are the α-defensins (human neutrophil defensins 1-3), which are found in significant concentrations in AF. Furthermore, lactoferrin is a glycoprotein with 2 binding sites for ferric ions. Lactoferrin is likely secreted into the AF by neutrophils and amniotic cells. Lactoferrin has both bacteriostatic activity, owing to the sequestration of iron, which is then unavailable for microbial growth, and bacteriocidal activity, by binding to bacterial outer membranes, triggering release of the lipopolysaccharide lactoferricin. Lactoferricin shows antimicrobial effects against viruses, protozoa, and fungi (16).

Human AF also contains factors that appear to minimize scarring, such as hyaluronic acid, which is found in high levels in AF and inhibits collagen synthesis. This hyaluronic acid-rich environment results from a relative lack of hyaluronidase in AF and the presence of hyaluronic acid-stimulating factor. In 1 study of the effect of AF on proteases important to wound healing, human AF was shown to enhance collagenase activity but to inhibit activation of hyaluronidase, elastase, and cathepsin (17,18).

It is also important to clarify the different sources of stem cells. Embryonic stem cells are obtained from the embryo and possess the potential for differentiation into a wide range of cell lineages. Although they hold immense promise to differentiate into the cell type of choice, ethical issues are associated with the use of these cells, and these issues need to be resolved before the promise of therapeutic treatment using embryonic stem cells can be brought into general patient therapeutic treatment plans. Currently, bone marrow (BM) is the most common source of adult stem cells for hematopoietic stem cell transplants and cellular therapies. The stem cells obtained from BM are mesenchymal stem cells (MSCs), which are pluripotent adult stem cells that can differentiate into many different cell types, including osteoblasts, chondrocytes, adipocytes, neurons, cardiac myocytes, and vascular endothelial cells. BM harvest is a surgical procedure that usually requires general anesthesia or sedation, and the proliferative potential and differentiation capacity of the BM MSCs from older donors seems to be reduced. Thus, interest has been increasing in using other sources of stem cells from adult and fetal tissue (1).

Another source of regenerative cells has been the relatively recent practice of preserving umbilical cord tissue and cells. With AF cells, it takes 20 to 24 hours to double the number of cells collected, faster than for umbilical cord stem cells (28 to 30 hours) and BM stem cells (more than 30 hours) (19). The progenitor cells that are derived have shown a high self-renewal capacity with more than 300 population doublings. In addition, although scientists have been able to isolate and differentiate, on average, only 30% of MSCs extracted from a child's umbilical cord shortly after birth, the success rate for AF-derived MSCs has been close to 100% (20). Furthermore, extracting cells from the AF bypasses the problems associated with a technique termed *donor–recipient human leukocyte antigen (HLA) matching*, which involves transplanting cells (19).

Thus, the amnion has been used as a physiologic wound dressing and as a graft for skin wound coverage (7–10). Heil et al (21) demonstrated that patients with occlusive vascular disease developed a prominent collateral vascular network below the occlusive site through *spontaneous arteriogenesis* (the remodeling of existing arterio-arteriolar anastomoses to developed functional arteries) and *angiogenesis* (new capillary growth induced by hypoxic conditions) after being injected. 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 John Hopkins Hospital for burns and ocular wounds in 550 cases. Sabella (23), in 1914, 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 Kim and Tseng (25), in 1995, presented their findings. Subsequently, published research has been increasing. In 2007, a technique was developed that allowed for aseptic recovery and preservation of viable cells and proteins (26). 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 acute rejection, 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 of the volunteers. These results suggest that acute immune rejection does not occur after allotransplantation of human amniotic epithelial cells. These investigators suggested that an immune response to the graft occurred, it was low grade and chronic rather than active and ineffective, because the amniotic epithelial cells appeared to survive and proliferate (28–30). AF progenitor cells do not form teratomas in vivo (31). 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 β_2-microglobulin (34,35). Ucakhan et al (33) evaluated safety and efficacy of nonpreserved 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-β and basic FGF (36). Zhang et al (37) described the presence of MSCs in human placenta that were able to differentiate into osteogenic, adipogenic, and chondrogenic lineages and able to suppress T-cell proliferation. In't Anker et al (20) first showed that the AM contains a high number of MSCs with bipotential osteogenic and adipogenic differentiation. Bailo et al (38), in 2004, researched how to induce AM-hMSCs to differentiate into a variety of cell types. Chondrogenic differentiation, inferred after a 3-week immunohistochemical analysis, demonstrated the presence of human type II collagen. Osteogenic differentiation, the formation of mineralized matrix when cells become flattened and show calcium deposits, was revealed as early as the first week of induction. Adipogenic differentiation resulted in single adipocytic multivacuolar cells, together with small and large colonies, with the size increasing with the length of induction. Large-size aggregates displayed an intensive secretion of large, neutral, lipid drops. This was never observed with BM-hMSC adipogenic cells (39). Nonstimulated cells were able to give rise to capillary-like structures following the same kinetics but with less organizational efficiency than the induced cells. Cell treatment with VEGF increased the expression levels of both VEGF receptors and was associated with a clear cytoplasmatic granular positivity for von Willebrand factor compared with untreated cells. Loh et al (40) demonstrated that AM-hMSCs expressed mRNA at greater levels than BM-hMSCs, increasing the potential for cell differentiation and the capacity of cells to be induced to form tissue structures far beyond that of BM-hMSCs. Brichard et al (41) demonstrated that AM-hMSCs yield more organized myogenic differentiation than those derived from adipocytes. In't Anker et al (20) demonstrated that AM-hMSCs maintained the potential to differentiate in a multitude of cell types at a greater and more organized rate than MSCs from any other source.

Patients and Methods

Allograft Procurement

AF and AM were harvested from females undergoing cesarian section. The donors and AM and AF were tested to ensure the absence of virus and other abnormalities. After testing, the tissues were processed and cryopreserved, thereby preserving cell viability. The tissue was collected and procured through BioDlogics (BioD LLC, Cardova TN). All the patients who participated in our investigation were informed and voluntarily signed a consent to research form (Supplemental Fig. S1), after receiving full disclosure. The allograft used was gAM-AF (AmnioMatrix™), which have been approved by the Food and Drug Administration as void filler and are obtained from human amnion tissue that has been tested and aseptically processed. Both allografts are processed and packaged at a Food and Drug Administration (FDA)–registered tissue bank accredited by the American Association of Tissue Banks (AATB) and maintains International Organization for Standardization 13485 certification for contract manufacturing and International Organization for Standardization 9001 certification for testing. The name and location of the tissue bank were not disclosed by the FDA to the authors. AmnioMatrix™ is regulated by the Food and Drug Administration under the Code of Federal Regulations Title 21, Part 1271 and Section 361, of the Public Health Service Act.

Amnion donors were prescreened. Testing was performed by an AATB-licensed recovery company, which harvested the tissues. The tests were performed by the Medical Director, which confirmed eligibility through behavioral risk assessment, donor medical history, review of blood test results, and communicable disease testing. Procurement of the amnion tissues was done with an aseptic recovery technique during cesarean section, which was undertaken using a standard sterile technique. It is important to understand that the procurement of gAM-AF does not require fetal death, and its recovery was performed with maternal consent during a live birth. Culturing and toxicity testing were performed before "lot release" to eliminate, as much as technologically possible, the risk of disease transmission. Thus, cryopreservation of the gAM-AF yielded a multipotential tissue matrix that contained live amnion cells, cytokines, proteins, growth factors, and multipotent cells essential for fetal growth and development. Multipotent cells are known to serve as cellular "factories" that secrete mediators that stimulate tissue repair and regeneration and elicit other effects beneficial to wound healing. The main purpose of developing the allograft was to fill, cover, and protect recipient wounds in vivo. Furthermore, in our investigation, it was used as an anti-inflammatory, antibacterial, and antiviral wound covering.

Study Population

To become a participant in the present prospective investigation, potentially eligible patients had to meet the following inclusion criteria: a single, non-infected full thickness foot and or leg wound (diabetic, pressure, arterial, or venous) of at least 12 months duration that was unresponsive to a wide range of therapies, including periodic debridement, moist dressings, eradication of infection and antibiotic therapy, off-loading, restoration of vascularity, glycemic control, edema control, and the use of hyperbaric oxygen. The patients who met these criteria during the study period, beginning March 2010 and ending May 2011, were consecutively enrolled into our prospective investigation. The planned study observation period was 12 weeks.

Surgical Technique

The first step was to aggressively debride the wound such that healthy bleeding tissue remained. This step was necessary to create a relatively clean wound bed and generate an inflammatory signal to induce the migration and proliferation of stem cells and growth factors, those delivered to the wound site by application of the amniotic tissues and those recruited from the patient's own immune system. Chemical wound debridement was not used; rather, surgical debridement was used as the sole method of wound surface preparation. After completion of sharp surgical debridement, a specific dose of amnion allograft was calculated. The wound area was measured using the maximal length and width, and the volume was calculated from measurements of the maximal length, width, and depth of the wound. Wound volume was determined by measurement of the maximum length multiplied by the maximum width by the maximum depth. For a wound with a surface area less than 12.5 cm^2, 0.5 mL of the cryopreserved amniotic tissue and fluid-derived allograft was used. For a wound area of 12.5 cm^2 or larger but smaller than 25 cm^2, 1.25 mL of amnion allograft was used. For every 25-cm^2 of wound area that was larger than 25 cm^2, an additional 1.25 mL of amnion allograft was included. This dosing scheme was determined from previous preliminary dose–response laboratory work performed by the allograft company, which showed that larger lesions responded best to larger amounts of allograft, larger lesions took longer to heal unless more allograft was used, and patients with greater degrees of vascular inflow and/or outflow disease also required greater doses than patients without vascular disease. The allograft treatment was initiated on the day of the first debridement of the wound, during the study period, and this day was designated as 0. The allograft was injected into the peri-wound skin that was normal in texture and turgor, free of infection approximately 0.5 cm from the wound edges, after debridement, at the 12-, 3-, 6-, and 9-o'clock positions and directly into the superficial fascia and the subcutaneous tissue of the wound. To minimize discomfort and to ensure an adequate spread of the amniotic tissue, the allograft was mixed with 1% plain lidocaine in a 1:1 ratio. An 18-gauge needle was used to prepare the mixture in a 3-mL syringe, and a 23-gauge needle was used for injection into the recipient tissue. After the first injection of the amnion allograft, additional doses were administered using the same technique at 14- to 21-day intervals until the wound was declared healed or the planned observation period (12 weeks) had ended. The goal was to inject the allograft into the superficial fascia to the level of the subcutaneous tissue, approximately 10 to 15 mm deep to the wound surface, aiming the needle parallel to the wound margin at each location of injection, and depositing the allograft in equal portions.

After each wound treatment session, the surgical site was dressed with a nonporous dressing (Tegaderm™, 3M, St. Paul, MN) followed by application of a nonstick dressing (Telfa™, Covidien, Dublin, Ireland) and a dry sterile dressing of gauze. Three days after injection of the amnion allograft, the wound was redressed, and standard wound care consisting of saline wet to dry sterile gauze dressing was resumed. Concomitant therapies, determined by the primary care physician,

Table
Statistical description of case series (n = 20 wounds in 20 patients, with no loss to follow-up)

Parameter	Patient Age (y)	Wound Duration before Treatment (wk)	Interval to Healing (wk)	Total Allografts Applied (n)	Wound Area* (cm^2)		Wound Volume† (cm^3)		Decrease from Baseline to 12 wk	
					Baseline	At 12 wk	Baseline	At 12 wk	In Wound Area (cm^2)	In Wound Volume (cm^3)
Mean ± SD	63.05 ± 12.64	36.60 ± 31.58	10.5 ± 12.1	1.90 ± 1.37	10.15 ± 19.54	2.47 ± 10.59	7.89 ± 13.17	0.214 ± 0.804	0.935 ± 0.159	0.977 ± 0.057
Confidence interval	5.54	13.84	5.3	0.60	8.56	4.64	5.53	2.72	0.07	0.03
p value					<.05		<.05			
									93.50%	97.71%

Abbreviation: SD, standard deviation.

included standard glycemic control, wound offloading, and edema control. Offloading was performed using stiff soled shoes with appropriate plastizote accomodative insoles. Lymphedema was managed with the use of an intermittent, sequential, positive-pressure foot-ankle-leg pump applied for 20 minutes 2 to 3 times daily and ranging in pressure from 0 to 100 mm Hg.

The data were analyzed with attention to type and distribution, and the demographic and outcome variables were described in terms of the mean ± standard deviation. Categorical outcomes were defined in terms of frequency counts and percentages. Furthermore, statistical comparisons between the baseline and 12-week follow-up wound measurements were made using the Wilcoxon signed rank test of the null hypothesis. Statistical significance was defined at the 5% ($p \leq .05$) level.

Results

A total of 20 consecutive patients (20 wounds) were enrolled in the present investigation from March 2010 to May 2011. All the patients were followed up until either their wound had healed or the end of the observation period (no loss to follow-up). Regarding the etiology, 14 (70%) of the wounds were attributed to diabetic neuropathy and 6 (30%) to arterial insufficiency. Of the 20 patients (20 wounds), 18 (90%) demonstrated 100% closure within the 12-week observation period, and none of the patients required amputation. In the 2 patients (10%) who did not fully heal during the 12-week observation period, the cause of the wound was diabetes and excessive pressure. In 19 (95%) of the cases, the use of gAM-AF appeared to eradicate the presence of sinus tracts and tunneling. None of the patients appeared to experience any adverse events related to the use of the gAM-AF allograft, and no evidence was found to suggest amplification of any existing systemic disease. Regarding concomitant edema control, 6 of the patients (30%) used a positive pressure lower extremity pump on the involved extremity during the course of treatment with the amnion allograft.

A statistical description of the measured outcomes is depicted in the Table. The mean patient age was 63.05 ± 12.64 years at the first administration of the amnion allograft. The mean wound duration was 36.60 ± 31.58 weeks (approximately 9 months), and the mean interval to healing after the first administration of the amnion allograft was 10.5 ± 12.1 weeks (approximately 2.6 months). The mean number of allografts applied during the observation period was 1.9 ± 1.37. The mean wound area at baseline was 10.15 ± 19.54 cm^2. At the 12-week follow-up examination, the wound area was 2.47 ± 10.59 cm^2. The decrease in wound area from baseline to the 12-week follow-up visit was 0.935 ± 0.159 (93.5% ± 15.9%), and the decrease in wound volume from baseline to the 12-week follow-up visit was 0.977 ± 0.057 (97.7% ± 5.7%). All 20 wounds showed a clinically significant (>15%) reduction in wound area.

Two brief explanations of representative cases will aid our description of the use of gAM-AF in the treatment of diabetic foot wounds. Patient 1 was a 64-year-old diabetic patient, who required dialysis because of chronic renal failure. This patient had a 24-month history of a nonhealing lateral midfoot ulcer and was under consideration for a transtibial amputation (Fig. 1*A*). By 57 days (approximately 8 weeks) of observation, the wound had healed (Fig. 1*B*). Patient 2 was an 80-year-old male with peripheral arterial insufficiency, who had undergone interventional vascular reconstruction of his lower extremities, hyperbaric oxygen therapy, and a multitude of topical therapies with no resolution of his painful left leg lesion after 18 months of treatment. The lesion was situated on the anterolateral lower leg and measured approximately 11 mm in length × 7 mm in width × 2 mm in depth at baseline (Fig. 2*A*). It had healed by 60 days (approximately 8.6 weeks) after the initiation of gAM-AF therapy.

Discussion

The wounds described in our report were all intractable and chronic and included those associated with diabetes (i.e., pressure ulcers and venous stasis). Many of them displayed undermining and/or sinus tract or tunneling (ST/T) formation. In general, we have found that lower extremity wounds display shapes characteristic of their etiology. For instance, diabetic foot ulcers typically have a small area and a large volume owing to their depth. In contrast, venous ulcers have a large area but a small volume because of their shallow depth. Pressure ulcers and some diabetic ulcers will also have undermining and/or ST/T formation, which add to the wound volume. The data we have collected and analyzed included multiple measurements of the following parameters: area, volume,

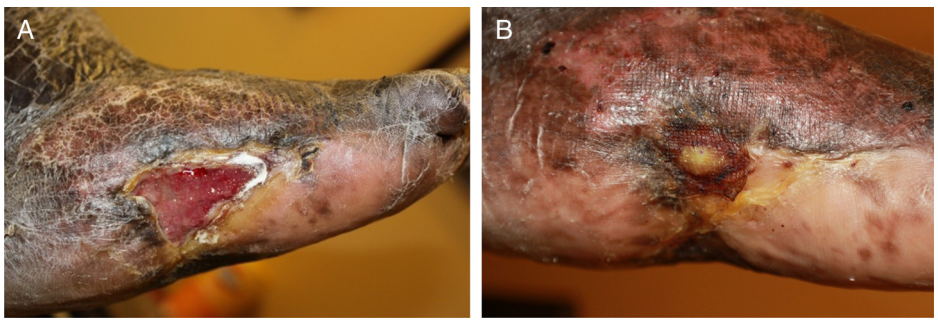

Fig. 1. Patient 1. (*A*) Wound at day 0, measuring 4.9 cm × 3.0 cm. (*B*) Wound healed at day 57.

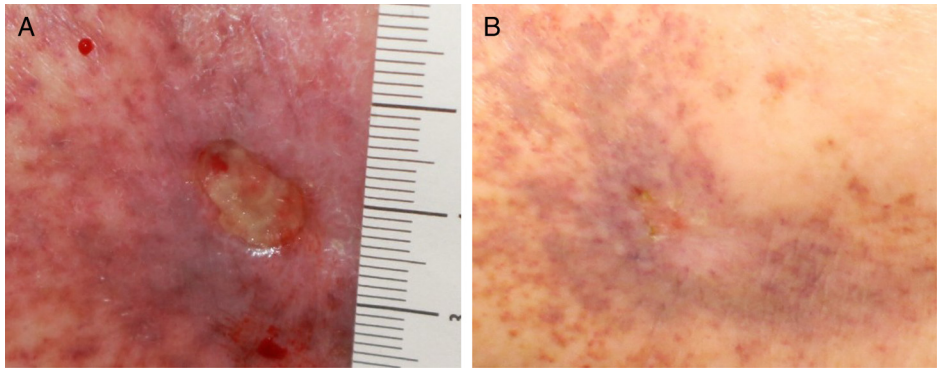

Fig. 2. Patient 2. (*A*) Wound at day 0. (*B*) Wound healed at day 60.

undermining, and ST/T formation. This strategy provided sensitivity to the changes in wound progress for each of the considered parameters.

The decrease in the mean wound size documented in the present case series was more rapid than that reported in previous studies. For example, in a retrospective study of 400 persons with pressure ulcers, diabetic ulcers, and venous ulcers receiving a wide variety of treatment modalities, Jones et al (42) documented that 12.75% of the wounds had healed within 3 months. In the present case series, most of the patients demonstrated significant comorbidities, including diabetes, immunosuppressive drug use after transplantation, and severe lymphedema, all of which have been equated with diminished wound healing. The results from our investigation suggest that wounds in compromised patients can progress more quickly toward wound healing using gAM-AF. Subjective clinician observations were also noted. Many reported seeing the undermining and ST/T formation respond first, with volume second and area last. In addition, during the first week of treatment, a noticeable decrease in exudate and an increase in healthy granulation tissue were observed. The results we observed in the present case series suggest that gAM-AF can be helpful in achieving healing of difficult wounds, despite their etiology, the presence of complicating comorbidities (e.g., elderly age, diabetes, renal failure, venous insufficiency), and very long durations of wound presence before application of the amnion allograft. We believe that these observations are important because, although prospective random controlled trials remain the reference standard for determining treatment efficacy, such studies rarely include medically compromised patients similar to those in our case series. Carter et al (43) reviewed the inclusion and exclusion criteria from 17 wound-care random controlled trials and found that more than 50% of the 3,210 patients from 18 outpatient wound centers would have been excluded from 15 of the 17 studies because of common comorbidities and wound complexity.

During the past 30 years, multiple studies have evaluated various growth factors and how they affect wound healing (44). The published data have suggested that single growth factors, combinations of growth factors in releasates derived from purified platelets, and growth factor-rich products from platelet-rich plasma produced using various methods, can accelerate wound healing. Approaches to therapies using single growth factors have included the clinical evaluation of platelet-derived growth factor (becaplermin), basic FGF, epidermal growth factor, granulocyte-macrophage colony stimulating factor, and keratinocyte growth factor-2. Most displayed promising preclinical data related to the influence of the agent on wound healing in animal models; however, with the exception of becaplermin, the clinical benefits were not fully realized. Braund et al (45) reported that epidermal growth factor, basic FGF, acidic FGF, and platelet-derived growth factor have all shown mixed results. However, TGF-β showed no significant wound improvement, and human growth hormone actually impeded wound healing. Keratinocyte growth factor-2 also showed mixed results (45). A number of growth factors, including VEGF, epidermal growth factor, platelet-derived growth factor, FGF-2, and dozens of others are released by platelets in wounds. In vitro analyses have shown that each growth factor is a signaling molecule responsible for a specific activity in the wound healing cascade of events necessary to natural, organized wound healing (46–48).

Everts et al (49) postulated that the need for these multiple growth factors to drive effective healing might explain the limited effectiveness of single growth factors in improving wound healing. Early efforts to develop a multiple growth factor therapy considered that platelets—as first responders in the wound healing process—release hundreds of growth factors, chemokines, and cytokines that regulate angiogenesis, new tissue deposition, and regeneration (50–56). It has been demonstrated that a subpopulation of cells in amniotic fluid produces Oct4 mRNA, which is used to maintain pluripotency (57). Unlike previous efforts, in which researchers had to purify platelets to access the natural complement of all growth factors, using the AM and AF provides a method for delivering all the growth factors and cells that will respond and differentiate into the different cell lineages necessary for wound closure.

The 1995 edition of *Diabetes in America* documented a mortality rate of 38% to 68% within 5 years after lower extremity amputation (58). The monthly cost of managing an uncomplicated ulcer, as reported in published studies, has been assumed to reflect the standard of care (59). The monthly cost of wound care for an ulcer not complicated by infection has been estimated to be $942 compared with the monthly cost of $2,492 for an ulcer complicated by cellulitis and $4,619 for an ulcer complicated by osteomyelitis (these costs have been updated to 2006 dollars using the medical care component of the Consumer Price Index) (59). An understanding of these costs makes clear the need to heal lower extremity wounds in as rapid and lasting fashion as possible.

AM has many additional advantages, because amniotic tissue is usually discarded, yet it is easily accessible and allows a very high recovery of cells. Under experimental conditions, from day 7 of culture onward, the cellular yield of human AM-MSCs obtained from a very small area (about 4 cm^2) of AM was considerably greater than the yield obtained from the same number of BM-derived cells (60). The number of cells yielded by the primary culture after our procedure (considering 4 cm^2 of AM) ranged from 1.3 to 1.5 $\times$ 10^6. Considering the whole area of the AM (1,300 cm^2), ideally the cell number obtained could be about 4 $\times$ 10^8, a suitable amount for cell therapy in a human clinical setting (60).

Myocardial infarction, peripheral arterial occlusive disease with critical ischemia (with resultant, difficult to heal, lower extremity wounds), and stroke are the most important clinical consequences of end-stage occlusive vascular disease, for which current therapies often prove inadequate, and treatment remains palliative. Thus, the development of regenerative methods using stem cell therapy could hold unprecedented prospects (61). Additional studies are required to fully understand the potential of AM-hMSCs in the realm of cell therapy strategies. Their inducible angiogenic potential could result in a new therapeutic approach, including tissue-engineered vascular grafts, to treating various forms of ischemic vascular disease.

Therapeutic angiogenesis might be a strategy to restore tissue damaged because of myocardium infarction, peripheral occlusive vascular disease, and other angiopathies. To develop future treatments using regenerative medicine, the currently reported yield of AM-hMSCs results from a very small area, about 1/325 of the total area of the AM. This result, and that both collagenase and trypsin, which have been used throughout the initial steps of cell isolation, are currently available as guanosine monophosphate (GMP)-grade enzymes, suggests that a remarkable potential for upscaling the overall procedure for clinical use can be envisioned (62). Cryopreserved gAM-AF was developed using a proprietary technique—now called AmnioGenic therapy—that granulated the amnion in an effort to preserve its structural properties in an injectable form. This "microscaffold" created by the granulated tissue matrix includes the proteins, carbohydrates, lipids, hyaluronic acid, growth factors, and other chemical compounds naturally present in AF and amniotic tissue to provide an in vivo wound covering derived from those components essential for fetal growth and development.

As with most observational investigations, we recognize a number of methodological limitations that could threaten the validity of our conclusions. For instance, we did not compare the use of the amnion allograft with that of any other therapy. Also, comparisons with historical (previously published) controls are always subject to bias and difficult to interpret. Furthermore, the surgeons applying the intervention also performed the wound measurements and were fully aware of the treatment and aims of the investigation. We could not distinguish between the therapeutic influence of other interventions, such as the use of the lymphedema compression pump, and that of the allograft. Finally, we did not measure subjective patient satisfaction or foot-related quality of life using a valid health measurement instrument, although our aim was to measure wound healing in the clinical setting.

In conclusion, gAM-AF is a cryopreserved structural tissue matrix derived from AM and AF developed for clinical use to fill soft tissue defects and bone voids and as an anti-inflammatory and antibacterial wound covering. From our understanding of the existing data, and our experience with the patients described in the present report, we believe that gAM-AF represents a safe and useful treatment alternative for the management of recalcitrant wounds of the lower extremities. We also believe that the results of our observational study could be useful in the development of future randomized controlled trials and prospective cohort studies focusing on the treatment of difficult lower extremity wounds.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jfas.org (http://dx.doi.org/10.1053/j.jfas.2013.03.024).

References

1. Scheubel RJ, Zorn H, Silber RE, Kuss O, Morawietz H, Holtz J, Simm A. Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. J Am Coll Cardiol 42:2073–2080, 2003.
2. Sakuragawa N, Kakinuma K, Kikuchi A, Okano H, Uchida S, Kamo I, Kobayashi M, Yokoyama Y. Human amnion mesenchyme cells express phenotypes of neuroglial progenitor cells. J Neurosci Res 78:208–214, 2004.
3. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. Clarification of the nomenclature for MSC: the International Society for Cellular Therapy position statement. Cytotherapy 7:393–395, 2005.
4. Modesti A, Scarpa S, D'Orazi G, Simonelli L, Caramia FG. Localization of type IV and V collagens in the stroma of human amnion. Progr Clin Biol Res 296:459–463, 1989.
5. Fukuda K, Chikama T, Nakamura M, Nishida T. Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. Cornea 18:73–79, 1999.
6. Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 20:173–177, 2000.
7. Trelford JD, Trelford-Sauder M. The amnion in surgery, past and present. Am J Obstet Gynecol 134:833–845, 1979.
8. Colocho G, Graham WP III, Greene AE, Matheson DW, Lynch D. Human amniotic membrane as a physiologic wound dressing. Arch Surg 109:370–373, 1974.
9. Prasad JK, Feller I, Thomson PD. Use of amnion for the treatment of Stevens-Johnson syndrome. J Trauma 26:945–946, 1986.
10. Subrahmanyam M. Amniotic membrane as a cover for microskin grafts. Br J Plast Surg 48:477–478, 1995.
11. Cheung CY. Vascular endothelial growth factor activation of intramembranous absorption: a critical pathway for amniotic fluid volume regulation. J Soc Gynecol Investig 11:63–74, 2004.
12. Moshiri A, Oryan A. Structural and functional modulation of early healing of full-thickness superficial digital flexor tendon rupture in rabbits by repeated subcutaneous administration of exogenous human recombinant basic fibroblast growth factor. J Foot Ankle Surg 50:654–662, 2011.
13. Yoshio H, Tollin M, Gudmundsson GH. Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense. Pediatr Res 53:211–216, 2003.
14. Espinoza J, Chaiworapongsa T, Romero R. Antimicrobial peptides in amniotic fluid: defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. J Matern Fetal Neonatal Med 13:2–21, 2003.
15. Akinbi HT, Narendran V, Pass AK, Markart P, Hoath SB. Host defense proteins in vernix caseosa and amniotic fluid. Am J Obstet Gynecol 191:2090–2096, 2004.
16. Otsuki K, Yoda A, Saito H, Mitsuhashi Y, Shimizu Y, Yanaiha T. Amniotic fluid lactoferrin in intrauterine infection. Placenta 20:175–179, 1999.
17. Ozgenel GY, Filiz G. Effects of human amniotic fluid on peripheral nerve scarring and regeneration in rats. J Neurosurg 98:371–377, 2003.
18. Gao X, Devoe LD, Given KS. Effects of amniotic fluid on proteases: a possible role of amniotic fluid in fetal wound healing. Ann Plast Surg 33:128–134, 1994. discussion 134–135.
19. Tsai MS, Lee JL, Chang YJ, Hwang SM. Isolation of human multipotent mesenchymal stem cells from second trimester amniotic fluid using a novel two-stage culture protocol. Hum Reprod 19:1450–1456, 2004.
20. In't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 22:1338–1345, 2004.
21. Heil M, Eitenmüller I, Schmitz-Rixen T, Schaper W. Arteriogenesis versus angiogenesis: similarities and differences. J Cell Mol Med 10:45–55, 2006.
22. Davis JW. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. Johns Hopkins Med J 15:307–396, 1910.
23. Sabella N. Use of fetal membranes in skin grafting. Med Records NY 83:478–480, 1913.
24. Faulk WP, Matthews R, Stevens PJ, Bennett JP, Burgos H, Hsi BL. Human amnion as an adjunct in wound healing. Lancet 1:1156–1158, 1980.
25. Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 14:473–484, 1995.
26. Koh JW, Shin JW, Oh JY, Kim MK, Ko JH, Hwang JM, Wee WR, Lee JH. The expression of TIMPs in cryo-preserved and freeze dried amniotic membrane. Curr Eye Res 32:611–616, 2007.
27. Gruss JS. Human amniotic membrane: a versatile wound dressing. Can Med Assoc J 118:1237–1246, 1978.
28. Adinolfi M. HLA typing of amniotic fluid cells. Prenat Diagn 2:147, 1982.
29. Adinolfi M, Akle CA, McColl I, Fensom AH, Tansley L, Connolly P, Hsi BL, Faulk WP, Travers P, Bodmer WF. Expression of HLA antigens, beta 2-microglobulin and enzymes by human amniotic epithelial cells. Nature 295:325–327, 1982.
30. Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet 2:1003–1005, 1981.
31. Delo DM, De Coppi P, Bartsch G Jr, Atala A. Amniotic fluid and placental stem cells. Methods Enzymol 419:426–438, 2006.
32. Trelford JD, Trelford-Sauder M. The amnion in surgery, past and present. Am J Obstet Gynecol 134:833–845, 1979.

33. Ucakhan OO, Koklu G, Firat E. Nonpreserved human amniotic membrane transplantation in acute and chronic chemical eye injuries. Cornea 21:169–172, 2002.
34. Akle CA, Adinolfi M, Welsh KI. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet 2:1003–1005, 1981.
35. Solomon A, Wajngarten M, Alviano F, Anteby I, Elchalal U, Pe'er J, Levi-Schaffer F. Suppression of inflammatory and fibrotic responses in an in-vitro model of allergic inflammation by the amniotic membrane stromal matrix. Clin Exp Allergy 35:941–948, 2005.
36. Perin L, Sedrakyan S, Da Sacco S, De Filippo R. Characterization of human amniotic fluid stem cells and their pluripotential capability. Methods Cell Biol 86:85–99, 2008.
37. Zhang Y, Li C, Jiang X, Zhang S, Wu Y, Liu B, Tang P, Mao N. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. Exp Hematol 32:657–664, 2004.
38. Pasquinelli G, Tazzari P, Ricci F, Orrico C, Vaselli C, Buzzi M, Foroni L, Alviano F, Lucarelli E, Bagnara GP, Stella A, Conte R. Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. Ultrastruct Pathol 31:23–31, 2007.
39. Bailo M, Soncini M, Vertua E, Signoroni PB, Sanzone S, Lombardi G, Arienti D, Calamani F, Zatti D, Paul P, Albertini A, Zorzi F, Cavagnini A, Candotti F, Wengler GS, Parolini O. Engraftment potential of human amnion and chorion cells derived from term placenta. Transplantation 78:1439–1448, 2004.
40. Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38:431–440, 2006.
41. Brichard SM, Delporte ML, Lambert M. Adipocytokines in anorexia nervosa: a review focusing on leptin and adiponectin. Horm Metab Res 35:337–342, 2003.
42. Jones KR, Fennie K, Lenihan A. Evidence-based management of chronic wounds. Adv Skin Wound care 20:591–600, 2007.
43. Carter MJ, Fife CE, Walker D, Thomson B. Estimating the applicability of wound care randomized controlled trials to general wound-care populations by estimating the percentage of individuals excluded from a typical wound-care population in such trials. Adv Skin Wound Care 22:316–324, 2009.
44. Hunt TK, La Van FB. Enhancement of wound healing by growth factors. N Engl J Med 321:111–112, 1989.
45. Braund R, Hook S, Medlicott NJ. The role of topical growth factors in chronic wounds. Curr Drug Deliv 4:195–204, 2007.
46. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 83:835–870, 2003.
47. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen 16:585–601, 2008.
48. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 341:738–746, 1999.
49. Everts PA, Brown Mahoney C, Hoffmann JJ, Schonberger JP, Box HA, van Zundert A, Knape JT. Platelet-rich plasma preparation using three devices: implications for platelet activation and platelet growth factor release. Growth Factors 24:165–171, 2006.
50. Steed DL, Goslen JB, Holloway GA, Malone JM, Bunt TJ, Webster MW. Randomized prospective double-blind trial in healing chronic diabetic foot ulcers: CT-102 activated platelet supernatant, topical versus placebo. Diabetes Care 15:1598–1604, 1992.
51. Knighton DR, Fiegel VD. Regulation of cutaneous wound healing by growth factors and the microenvironment. Investig Radiol 26:604–611, 1991.
52. Knighton DR, Ciresi K, Fiegel VD, Schumerth S, Butler E, Cerra F. Stimulation of repair in chronic, nonhealing, cutaneous ulcers using platelet-derived wound healing formula. Surg Gynecol Obstet 170:56–60, 1990.
53. Keyser JE. Diabetic wound healing and limb salvage in an outpatient wound care program. South Med J 86:311–317, 1993.
54. Glover JL, Weingarten MS, Buchbinder DS, Poucher RL, Deitrick GA III, Fylling CP. A 4-year outcome-based retrospective study of wound healing and limb salvage in patients with chronic wounds. Adv Wound Care 10:33–38, 1997.
55. Atri SC, Misra J, Bisht D, Misra K. Use of homologous platelet factors in achieving total healing of recalcitrant skin ulcers. Surgery 108:508–512, 1990.
56. Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost 91:4–15, 2004.
57. Prusa AR, Marton E, Rosner M, Bernaschek G, Hengstschlager M. Oct4-expressing cells in human amniotic fluid: a new source for stem cell research? Hum Reprod 18:1489–1493, 2003.
58. Shearer A, Scuffham P, Gordois A, Oglesby A. Predicted costs and outcomes from reduced vibration detection in people with diabetes in the U.S. Diabetes Care 26:2305–2310, 2003.
59. Stute N, Holtz K, Bubenheim M, Lange C, Blake F, Zander AR. Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol 32:1212–1225, 2004.
60. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 28:875–884, 2000.
61. Liu L, Sun Z, Chen B, Han Q, Liao L, Jia M, Cao Y, Ma J, Sun Q, Guo M, Liu Z, Ai H, Zhao RC. Ex vivo expansion and in vivo infusion of bone marrow-derived Flk-1+CD31−CD34− mesenchymal stem cells: feasibility and safety from monkey to human. Stem Cells Dev 15:349–357, 2006.
62. Hollander AP, Dickinson SC, Sims TJ, Brun P, Cortivo R, Kon E, Marcacci M, Zanasi S, Borrione A, De Luca C, Pavesio A, Soranzo C, Abatangelo G. Maturation of tissue engineered cartilage implanted in injured and osteoarthritic human knees. Tissue Eng 12:1787–1798, 2006.