Protein Characterization of Dehydrated Placenta Membrane Products: Artacent Wound and Artacent AC

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Introduction

Human Amniotic Membrane (hAM) is a readily available tissue that is widely used as a wound covering in both chronic and acute wounds. Dehydration is commonly used as a processing step to create a product that is stable at room temperature and has a long shelf life. Two dehydrated membrane products that are widely being used in the clinic are: (i) Artacent Wound ®, that consists of bi-layer of amnion dried membrane, and (ii) Artacent AC ®, which is tri-layer composed of chorion held between two layers of amnion. In general, dehydrated hAMs have been reported [1] to contain a variety of proteins, growth factors and cytokines which help to not only promote and stimulate cell proliferation and migration, but also alter gene and protein expression profiles of cells. Here, we characterize the protein content in Artacent Wound ® and Artacent AC ® using Microarray. The statistical analysis will be conducted to determine the combination of biomarkers that are present in various products, and to compare the biomarkers in Artacent products to that of clinical controls. In addition, immunohistochemistry (IHC) was used to demonstrate various protein composition of extracellular matrix (ECM), growth factors, and immune modulating cytokines. Finally, cell attachment and proliferation of human fibroblasts and keratinocytes was assessed in vitro on Artacent AC ® and Artacent Wound ® products.
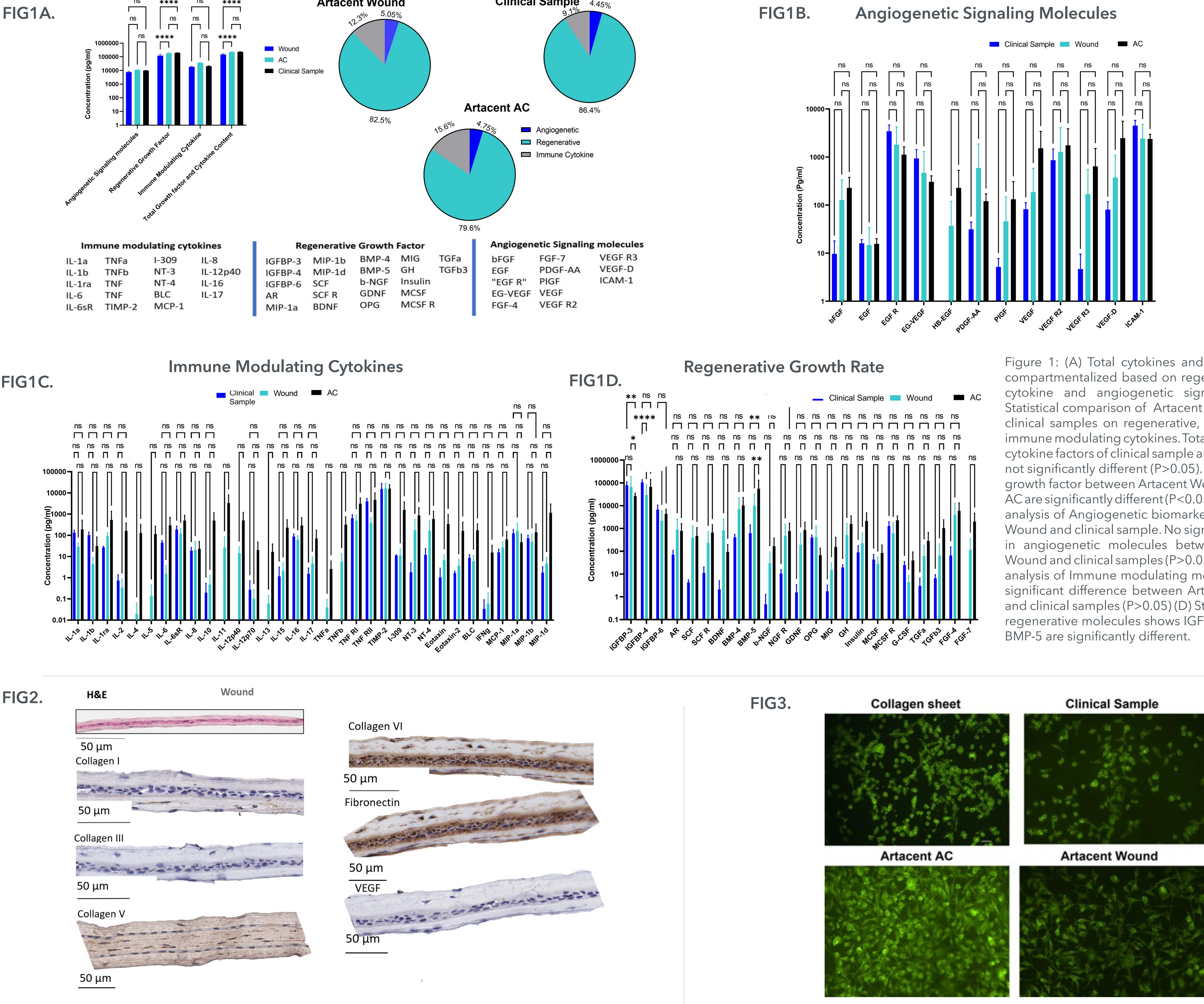
Materials and Methods

1 HISTOLOGY
A 20 x 20 mm section of each tissue was prepared and sent to Histowiz Inc. for histological analysis. Samples were paraffin embedded and stained with Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) labels of Collagen I, Collagen III, Collagen V, Collagen VI, Fibronectin, and VEGF for different extra cellular matrix proteins and chemokine cues.

2 MICROARRAY
Quantibody® Human Cytokine Antibody Array 1000 assay from RayBiotech Inc. The assay is the multiplexed ELISA quantitative platform enabling to detect and quantify up to 1000 human cytokines in a single experiment. Briefly, samples were incubated on captured antibody for 2 hours. The biotinylated antibody cocktail were added and then incubated again for another 2 hours following by streptavidin labeled dye. The result were then analyzed using fluorescent microarray reader.

3 CELL CULTURE
Fibroblast L929 cells were cultured and 10^5 cells/ml were added to each sample of each well with 3 mm diameter. Acti-stain™ 488 phalloidin were added and fixed before microscopy imaging as described previously [2].

4 STATISTICAL ANALYSIS
Statistical analysis on all results was performed using Two-way ANOVA with the test for multiple comparison using Tukey statistical hypothesis testing group of products of all biomarkers. Statistical significance threshold was set at 0.05 (p < 0.05). Error bars represent a standard deviation of the mean (n = 5). An asterisk shows statistically significant magnitude change compared to all other data sets.

Results

FIG1A.

Artacent Wound — 12.9%, 5.05%, 82.5%

Clinical Sample — 4.45%, 86.4%

Artacent AC — 15.6%, 4.75%, 79.6%

- Angiogenetic
- Regenerative
- Immune Cytokine

Immune modulating cytokines

IL-1a	TNFa	I-309	IL-8
IL-1b	TNFb	NT-3	IL-12p40
IL-1ra	TNF	NT-4	IL-16
IL-6	TNF	BLC	IL-17
IL-6sR	TIMP-2	MCP-1	

Regenerative Growth Factor

IGFBP-3	MIP-1b	BMP-4	MIG	TGFa
IGFBP-4	MIP-1d	BMP-5	GH	TGFb3
IGFBP-6	SCF	b-NGF	Insulin	
AR	SCF R	GDNF	MCSF	
MIP-1a	BDNF	OPG	MCSF R	

Angiogenetic Signaling molecules

bFGF	FGF-7	VEGF R3
EGF	PDGF-AA	VEGF-D
"EGF R"	PIGF	ICAM-1
EG-VEGF	VEGF	
FGF-4	VEGF R2	

FIG1B. **Angiogenetic Signaling Molecules**

FIG1C. **Immune Modulating Cytokines**

FIG1D. **Regenerative Growth Rate**

Figure 1: (A) Total cytokines and biomarkers were compartmentalized based on regenerative, immune cytokine and angiogenetic signaling molecules. Statistical comparison of Artacent Wound & AC with clinical samples on regenerative, angiogenetic and immune modulating cytokines. Total regenerative and cytokine factors of clinical sample and Artacent AC are not significantly different (P>0.05). Total regenerative growth factor between Artacent Wound and Artacent AC are significantly different (P<0.05). (B) Comparison analysis of Angiogenetic biomarkers of Artacent AC, Wound and clinical sample. No significant differences in angiogenetic molecules between Artacent Ac, Wound and clinical samples (P>0.05). (C) Comparison analysis of Immune modulating molecules shows no significant difference between Artacent AC, Wound and clinical samples (P>0.05) (D) Statistical analysis in regenerative molecules shows IGFBP-3, IGFBP-4 and BMP-5 are significantly different.

FIG2.

Figure 2. Histological evaluation of Artacent wound. ECM components of Artacent Wound were evaluated using standard histological techniques including Haematoxylin and Eosin (H&E), Immunohistochemistry of Collagen I, III, VI, Fibronectin, VEGF. Images taken with a 40× objective, each scale bar measures 50 μm.

FIG3.

Figure 3. Fibroblast cell biocompatibility of Artacent AC & wound after 24h. Fibroblast on Artacent products were more spreaded and proliferated in comparison with clinical sample. The scale bar represents 50 μm.

Discussion

Protein microarray analysis and quantification shows Artacent AC and Artacent Wound have various protein, biomarker, and chemical cues for angiogenetic and immune modulating cytokines and regenerative biomarkers (Figure 1A). There are no significant differences in angiogenetic and immunomodulating biomarkers in Artacent AC, Artacent Wound, and clinical control (Figure 1B&C). These products posed statistical differences in BMP-5, IGFBP-3 and IGFBP-4. Artacent AC has more BMP-5 compared to Artacent Wound and clinical control (P<0.01). BMP-5 is beneficial to promote wound healing and tissue regeneration [3].

Artacent Wound protein composition posed uniform distribution of immune, angiogenetic, and regenerative molecules. However, the protein distribution in Artacent AC showed more regenerative growth factors compared to angiogenetic and immune modulating cytokines.

Histology H&E and IHC of various ECM biomarkers showed that Artacent Wound consists of various collagen types(I, III, V and VI), in addition to fibronectin and VEGF growth factor. This ECM composition makes the product unique to promote re-epithelization in the wound healing process. Biocompatibility assay of Artacent products improves fibroblast proliferation in comparison with clinical control samples. This may attribute to the fact that Artacent products consist of the enriched biomarker and cytokine molecules.

Conclusions

- Artacent AC shows higher level of BMP-5 biomarker compared to control sample.

- Histological analysis showed Artacent Wound has various ECM proteins and growth factor:
  - Collagen I, III, V, VI
  - Fibronectin
  - VEGF

- Both Artacent AC and Artacent Wound showed better cell proliferation within 24hours when compared to a similar product as well as collagen sheet.

REFERENCES

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