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Research Article

Structural Dermal Proteins Collagen I and Elastin Influence Fibroblast Migration in an *In Vitro* Wound Assay

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Abstract

In an effort to determine the proper proportions of proteins required to emulate a dermal extracellular matrix (ECM), digital morphometric analysis (DMA) was conducted on human skin biopsy samples. These results were then compared to literature reported values for various skin locations.

The scratch assay, an *In vitro* model for wound healing was used to evaluate the effect different protein concentrations may have on dermal fibroblast migration. The scratch assay was used to evaluate whether the ratio of proteins has an effect on cell mobility. The digital analysis quantified the amount of collagen I and elastin present in the dermis of the skin. The literature reports variable values of collagen and elastin in the dermis. The digital morphometric analysis revealed that collagen I had a composition of 85.1 ± 9.0% (n = 4), while elastin had a composition of 6.0 ± 1.6% (n = 4). These data represent a discrepancy in the amount collagen present in the dermis; the DMA calculated results are much higher than literature reported values. However, the elastin content results are similar to what is reported in the literature for skin (peri-auricular). The scratch assay was used to evaluate if using various collagen/elastin ratios would have an effect on the rate of wound closure over time. The addition of proteins to the cell culture media in the scratch assay led to an effect on the motility of the dermal fibroblasts. In the scratch assay the addition of 100% collagen I had the greatest influence on increasing the percent of scratch closure over time. The next largest influencer on cell motility was a collagen I: elastin blended at a ratio of 9:1; followed by the 1:1 collagen: elastin blend, and controls. The addition of 100% TE to the cell culture media had a positive effect at four hours but slowed in hours 12 and 20. At twenty hours, there were no significant differences between 100% Collagen, 9:1 collagen: TE, 1:1 collagen: TE and the controls. These data suggests the addition of proteins to the cell culture media has an influence on the motility of the dermal fibroblasts. Translationally, in the wound site this may have a positive influence in the early phases of the wound healing cascade. By accelerating the recruitment of fibroblasts to the wound site, progression through the phases of wound healing can be facilitated at a more rapid rate.

Keywords: Elastin; Collagen; Digital Morphometric Analysis; Dermis; Protein Concentration

Abbreviations

DAB+ - 3: 3-Diaminobenzidine Positive; DEEB - Dual Endogenous Enzyme Block; DMA: Digital Morphometric Analysis; DMEM: Dulbecco's Modified Eagle's Medium; ECM: Extracellular Matrix; FBS: Fetal Bovine Serum; hDFn: Human Neonatal Dermal Fibroblasts; IRB: Institutional Review Board; MMPs: Matrix Metalloproteinase; PBS: Phosphate Buffered Saline; ROI: Region of Interest; TE: Tropoelastin.

Introduction

Tissue engineering is an interdisciplinary field in bioengineering that focuses on the generation of new bio-inspired materials for replacing diseased or damaged tissues or organs, such as the integument. In order to achieve this for the skin, fabricating an ECM with attributes of the native dermal ECM would encourage the native cells of the body to inhabit. Currently there are no models of artificial skin that completely replicate the normal uninjured skin (Metcalfe and Ferguson, 2006). Producing protein-based products to close full- and partial- thickness wounds requires a knowledge of the native proteins located in the dermal ECM and their relative amounts. When creating a scaffold that replicates the uninjured dermal ECM it is important to consider the protein composition of the native organ. The primary structural proteins in humans are collagen and elastin [1-6]. Collagen is the primary structural protein providing the strength to the dermis [6]. Elastin is an integral protein providing elasticity and structural integrity to the dermal ECM [7]. Collagen is reported to be found in varying concentrations depending on the anatomical location. Within the normal dermis, collagen composition varies between approximately 32-84% of the integument depending on anatomical location [1,2,5,6,8]. Elastin, on the other hand, comprises 0.6 -7.9% of the dermal ECM depending on anatomical location, gender and age [1-3,5,8].

Protein	Average Amount (%)	SD	Anatomic Location	Quantification Method	n	Age (yrs)	Sex	Reference
Collagen								
	48.47	6.96	Skin (prearuicular, SMAS)	DMA	17	26-72	1M, 16F	Har-Shai., *et al*. 1998
	38.70	5.90	Skin (preauricular)	DMA	17	26-72	1M, 16F	Har-Shai., *et al*. 1998
	77.70	5.90	Skin (abdomen)	% Dry weight	47	25-90	Male	Weinstein and Boucek, 1960
	75.90	6.40	Skin (abdomen)	% Dry weight	36	21-78	Female	Weinstein and Boucek, 1960
	71.90	N/R	Skin	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	10.00	N/R	Lung	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	18.00	6.00	Ligamentum Nuchea	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	86.00	N/R	Achilles tendon	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	3.90	N/R	Liver	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	68.00	N/R	Skin (Limbs)	Stereology	45	1-74	26M 19F	Viellaro-Zuccarello., *et al*. 1994
	71.00	N/R	Skin (trunk)	Stereology	45	1-74	26M 19F	Viellaro-Zuccarello., *et al*. 1994
Elastin								
	6.10	1.80	Skin (preauricular, SMAS)	DMA	17	26-72	1M 16F	Har-Shai., *et al*. 1998
	4.71	1.20	Skin (preauricular)	DMA	17	26-72	1M 16F	Har-Shai., *et al*. 1998
	4.50	1.40	Skin (abdomen)	% Dry weight	17	25-96	Male	Weinstein and Boucek, 1960
	4.00	0.90	Skin (abdomen)	% Dry weight	10	21-74	Female	Weinstein and Boucek, 1960
	2.10	1.10	Skin (location unknown)	DMA	10	N/R	N/R	Uitto ., *et al*. 1983
	2.10	0.75	Skin	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	5.00	2.00	Lung	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	30.00	2.00	Ligamentum Nuchea	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	4.40	N/R	Achilles tendon	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	0.24	0.06	Liver	% Dry weight	N/R	N/R	N/R	Uitto, 1986
	2.02	0.28	Skin (arms)	DMA	8	N/R	N/R	Mutsuoka., *et al*. 1985
	2.66	N/R	Skin (Limbs)	Stereology	45	1-74	26M 19F	Viellaro-Zuccarello., *et al*. 1994
	2.50	N/R	Skin (trunk)	Stereology	45	1-74	26M 19F	Viellaro-Zuccarello., *et al*. 1994

Table 1: A table of anatomical locations, gender and age of human protein composition, collagen and elastin content in the ECM. SMAS = superficial muscular aponeurotic system, an area of musculature of the face containing the platysma muscle. This tissue is manipulated during facial cosmetic surgery. N/R = not reported.

Current tissue engineering research focuses on creating wound healing matrices that are composed of collagen, collagen derivatives, cadaveric tissue, and more recently porcine intestinal submucosa proteins [9,10]. Most collagen-based dermal matrices for wound care use bovine and porcine collagen, which are inexpensive and readily available [9]. These experiments utilize bovine type I collagen. The mature elastin protein is highly crosslinked and insoluble [11,12]. Therefore, tropoelastin (TE), the soluble precursor protein to elastin was used throughout these experiments. The TE used in the current experiments is a recombinant derived human dermal isoform, obtained from Protein Genomics, Inc (Sedona, AZ).

In order to determine the optimal protein composition to use in the creation of a bio-inspired wound healing scaffold, a quantification of collagen I and elastin was performed using DMA techniques on human skin samples and these results were compared to the published literature. In the literature, Har-Shai., *et al*. [5] report a collagen variation of 32 – 55% and an elastin variation of 3.5 – 7.9% composition in pre-auricular skin (in front of the ear). Based on these values the dermis of the facial skin must be comprised of a great deal of other proteins and ground substance. The skin in this area has muscular insertions onto the integument in order to allow for the production of facial expressions in individuals. Therefore, this skin must be more resilient and have greater

elasticity than most other anatomical locations. This skin requires a greater amount of elastin than other anatomical regions which are reported to be 0.6 – 7.9% [1-3,5,8]. The range of elastin content throughout the dermis is relatively narrow. Greater variations occur in the amount of collagen present. Therefore, elastin appears to be more tightly regulated than collagen as evidenced by the apparent lower amounts of elastin present in the dermis, compared to collagen, with appropriate functionality maintained. This is also supported by the silencing of the elastin gene at a young age in humans [12] and the relative amount of elastin serving the organism for its lifetime. The half–life of elastin is approximately 70 years, this means that elastic fibers in organs would have survived several billion stretch-relaxation cycles by the start of the seventh decade of life [13]. This explains why the skin begins to sag and loses its elastic properties with aging. To quantify the protein composition of collagen and elastin in the facial region, DMA evaluations were performed on human dermal biopsies, post-auricular. These data were used to validate previous reports of collagen and elastin in the skin of the facial region. When designing a tissue engineered scaffold to close full- and partial-thickness wounds the scaffold should be able to be used in various anatomical skin locations. The goal of this research is to generate a scaffold versatile enough to be used in various skin locations. Therefore, it is advantageous to measure and mimic the elastin content of the facial region where it is reported to have the highest elastin content.

Digital analysis of skin biopsy histology facilitated collagen: elastin ratio ranges to test in a cell culture assay. This assay has been used to model the mobility of dermal fibroblasts in a mock wounding event. The scratch assay is an effective tool to evaluate cellular migration using an *In vitro* system [14-16]. This is an *In-vitro* benchtop assay that can be used as a predictor for how human dermal fibroblasts will act in an *In vivo* full thickness dermal wound model [15]. Cell culture assays provide initial data that can be analyzed and repeated in an economical, high-throughput, and timely manner before moving into more complicated physiological systems, models, or entire organisms [15]. The addition of collagen and tropoelastin in various ratios was investigated to determine the effect on fibroblast motility; providing an indication of their ability to influence wound healing.

Materials and Methods

Histology preparation

Access to post-auricular human biopsy skin samples was provided by Dermatology Consulting Services with informed patient consent and IRB Approval (High Point, NC). Three millimeter (3mm) biopsy punches of the post-auricular skin were obtained from human patients (n = 4). The samples were fixed in 2% paraformaldehyde, dehydrated, and paraffin embedded. For histology the tissues were sectioned to 5 µm.

Immunohistochemistry

- **Collagen I:** Slides were deparaffinized, rehydrated, and treated with Citrate Buffer (ThermoScientific) at 95°C for 20 min. then rinsed with DEEB (Dako), for 10 min. The samples were blocked with Protein Block Serum-Free (Dako) for 15 min, then incubated at 37°C with 1:500 dilution of anti-Collagen I antibody (Abcam) for 30 min. This was followed by incubation for 30 min. with species-appropriate biotinylated secondary antibodies (Envision+ rabbit, Dako) for 30 min. Chromogenic detection was performed by development with DAB+ Nuclei were identified via hematoxylin counter stain.
- **Elastin:** Slides were deparaffinized, rehydrated, and treated with proteinase K (Dako) for 2 min. then rinsed with a buffer wash of TBS with Tween20 (Dako). Endogenous peroxidases were blocked with a 3% hydrogen peroxide solution for 10 min. Following buffer rinse, samples were incubated at 37°C with 1:25 dilution of anti-Elastin antibody (Abcam) in antibody diluent for 60 min. This was followed by incubation with species-appropriate biotinylated secondary antibodies (Envision mouse+ kit, Dako) for 30 min. Chromogenic detection was performed by development with DAB+ (Dako). Nuclei were identified via hematoxylin counter stain (Dako).

Slide Scanning

All slides were scanned using a Hamamatsu Nanozoomer with a resolution of 0.46 µm per pixel at 20X magnification (Hamamatsu.com).

Digital Morphometry

The resulting images were annotated to only include the dermis of the sample, which was the ROI (Figure 1). The ROI extended from the basement membrane of the epidermis to the hypodermis. If there was no adipose to represent the hypodermis, then the ROI included all of the dermal tissue present on the section. After the annotations were made, Aperio's Color deconvolution analysis was run on the annotated layer to determine the percent of positively stained tissue that reacted positively for collagen I and elastin antibodies.

Scratch Assay

hDFn, 3rd passage; (Cell Applications, SanDiego, CA) were grown as a monolayer in tissue-culture-treated T25 culture flasks (Corning Inc., Corning, NY). The cells were grown in DMEM supplemented with 10% FBS (Life Technologies, Carlsbad, CA). Cells were in-

Figure 1: Picture of an elastin stained slide highlighting the region of interest (ROI, red outline). All of the area inside the red line was analyzed for elastin-reacted DAB stain. The black area outline was an area excluded from the analysis. The epidermis is the superficial side of the sample and the hypodermis is the deep side of the tissue. Scale bar = 600 µm.

cubated in a humidified incubator at 37°C, 5% CO_2. After 48 hours, the cells were sub-cultured into 6 or 12 well tissue culture plates, seeded at a density of 5,000 cells/cm^2. Cells were grown for another 48 hours until reaching optimal density (70-80%). A 200 ul pipet tip was used to scratch across the monolayer of cells creating a 1-2 mm width between cell margins, and the media was aspirated. Excess cells and debris from the scratch were rinsed with 1xPBS (Life Technologies, Carlsbad, CA). Each treatment was performed in 15 separate wells with controls. The collagen used was bovine type I (Sigma) and the TE used was recombinant derived human dermal isoform of tropoelastin (Protein Genomics, Sedona, AZ). The treatments included 100% collagen (n = 15), 100% TE (n = 15), a 1:1 collagen: TE blend (n = 15), and a 9:1 collagen: TE blend (n = 15). The proteins were diluted (100µg/mL or 0.1%) in serum-free DMEM supplemented with 1% gentamycin and pipetted onto the scratched cell monolayer [17]. Images of cellular migration were captured every 4 hours for 24 hours, or until cells fully closed the scratch site.

Scratch assay image analysis

Images were captured using OptixCam image software (OCView V7.3.1.8; The Microscope Store, LLC, Roanoke, VA) on an Olympus CK2 inverted microscope (Olympus Corporation, Waltham, MA). Photographs were analyzed using ImageJ (V1.48) analysis software (National Institute of Health, Bethesda, MD). Wound widths in each well were measured at 10 unique locations along the length of the scratch, and an average value was reported (n = 15 for all treatments).

Statistical Analysis

Statistical analysis of these measurements was performed using JMP pro13. The results of the scratch assay were analyzed using a repeated measures ANOVA. Values evaluated were based on the mean percent values of the scratch closure expressed over four-hour time points. A Tukey post hoc analysis with a p value < 0.05 was used to determine significance.

Results

Digital Morphometric analysis

The results of the color deconvolution analysis measured collagen I composition at 85.1 ± 9.0% (n = 4) of the human dermis while elastin composition was 6.0 ± 1.6% (n = 4) of the human dermis. See figures 2 and 3.

Figure 2: A) Elastin-reacted IHC original image. B) Quantification of the amount of elastin found in human post-auricular dermis. False color mark-up of the image; yellow = weak positive, orange = positive, red = highly positive, blue = negative.
Scale bars = 100 µm.

Figure 3: A) Collagen I-reacted IHC original image. B) Quantification of the amount of collagen I in human, post-auricular dermis. False color mark-up of the image; yellow = weak positive, orange = positive, red = highly positive, blue = negative.
Scale bare = 100 µm.

Scratch assay

In the field of tissue engineering protein composition is an important factor. All values are reported as mean ± standard error of the mean. After the initial four-hour time point, the 100% collagen treatment (27.3 ± 2.75%), was significantly different from the 1:1 Collagen: TE blend (17.73 ± 3.75%) and the control (17.43 ± 1.86%) but not significantly different than 100% TE (18.60 ± 1.88%) and the 9:1 collagen: TE blend (23.03 ± 1.04%). At twelve hours, the 100% collagen treatment (82.44 ± 2.12%) was significantly different from 100% TE (54.57 ± 5.17%) and control (64.86 ± 3.51%). The controls were not significantly different from the 100% TE wells. The 1:1 Collagen: TE blend (71.26 ± 3.04%) and the 9:1 Collagen: TE blend (77.23 ± 4.57%) were not significantly different from the 100% collagen or controls but were significantly different from the 100% TE wells. At twenty hours, the 100% collagen (97.95 ± 0.67%), 1:1 collagen: TE blend (97.89 ± 0.60%), 9:1 collagen: TE blend (98.21 ± 1.20%) and controls (96.0 ± 1.42%) were similar to each other but significantly different from 100% TE (87.35 ± 4.36%). See figure 4 for images of the scratch assay at 4 hours, 12 hours, and 20 hours. See figure 5 for a graphical representation of the scratch assay data at four, 12 and 20 hours post wounding.

Figure 4: Images of human dermal fibroblast scratch assay cultures with treatments and control. These images were used to measure the percent of cell migration at 4 hours, 12 hours and 20 hours. The treatments had 0.1% of the protein solution added to the cell culture media. Measurements were taken from the borders of the wound.

Figure 5: Box and whisker plot of the wound closure assay, demonstrating the motility of human dermal fibroblasts over a twenty-hour assay. Levels not connected by the same letter are significantly different from each other ($p < 0.05$). By 20 hours the 100% TE treatment was significantly different from the other treatments.

Discussion

Performing DMA across an entire slide provides the investigator with a semi-automated, reproducible method to evaluate histology more quickly with higher throughput capabilities [18-22]. DMA reduces analysis time, generates quantitative data, and allows for reproducibility because the exact parameters and assumptions are used across each sample within this particular study with no inter-investigator bias [18-24].

In this study, DMA was performed to quantify the amount of collagen I and elastin present in the ECM of the dermis in human biopsies. The results of the DMA on the skin biopsies reacted with antibodies against collagen I demonstrated that collagen I comprises 85.1 ± 9.0% of the dermis and elastin comprises 6.0 ± 1.6% of the dermis. The elastin composition data is similar to what has been published for the facial region, however, the collagen I data differs from what is published for protein composition in the facial region [5]. The literature demonstrates collagen I can vary in composition between 32 – 55% based on location [5]. These are much lower than values calculated using DMA in the current study. The analyzed biopsies were removed from the cephalic region, post-auricular (behind the ear). In the Har-Shai*., et al*. [5] study the samples were removed from the pre-auricular region of the face. This may account for the differences in collagen composition found between these two studies. The facial region is reported to have a higher

composition of collagen and elastin [5]. When attempting to tissue engineer a dermal inspired wound healing device, the composition of the device should emulate the native dermis. The device should also be versatile enough to be used in various anatomic locations without causing any tissue damage or pathologies. By targeting the higher end of reported and measured elastin content, the proposed bio-inspired scaffold could be used in more anatomical locations, including locations that need higher elastin. The limitation for using the lower end of reported or measured elastin in the design is that it would be limited in its ability to treat areas of high native elastin content, such as the face.

The protein composition data generated in the current study was then used in a cell culture study to evaluate how dermal fibroblasts respond to the addition of ECM proteins to cell culture media. While the wells with 100% collagen present had an initial increase in the mobility of the cells in culture, there was no difference from wells with 100% TE and 9:1 collagen: TE blends at four hours. By 12 hours the wells with 100% collagen, 9:1 collagen TE, and 1:1 collagen: TE were similar in their response. This data suggests that the addition of TE to the proposed scaffold would not have deleterious effects but rather have similar effects as 100% collagen, although, a 100% collagen scaffold would not deliver similar ECM properties as what is found in the native dermis. The addition of TE to a scaffold would provide a protein composition that is more similar to the native dermis than a collagen scaffold alone.

This may benefit the overall function of the scaffold when used to treat dermal wounds. The human dermal fibroblast was used in the current study, because this is the predominant cell population in the dermis. Additionally, this cell was utilized due to its importance in the healing progression. Dermal fibroblasts play a significant role in matrix remodeling in the wound healing process. Fibroblasts are collagen- and elastin-secreting cells that simultaneously function with MMPs and other cell types to break down and deposit new extracellular matrix proteins to support adherence and proliferation [16]. It is because of these important features, dermal fibroblasts provide a relevant *In-vitro* simulation of *In-vivo* wound progression when utilizing a single cell type [15,16].

Utilizing the scratch assay in combination with the results from digital morphometry, different blends of collagen I and elastin were used to evaluate if these proteins would have a positive effect on the mobility and proliferation of fibroblasts. In the 4- and 12-hour time points, 100% collagen resulted in increased cell proliferation and motility, evidenced by faster wound closure. Although it was not significantly different that the 9:1 collagen: TE blend, because there is elastin present in the dermis, the addition of TE to the resultant scaffold should provide additional mechanical characteristics that collagen alone cannot provide. A tissue engineered skin substitute should have appropriate physical and mechanical properties of the target organ (Metcalfe and Ferguson, 2006). Using a scaffold of 100% TE would not be advantageous due to the fact that there is no dermal ECM in the body that is 100% elastic tissue. A similar argument can be made for the use of a scaffold that is a 1:1 blend of collagen and TE. While these may not have a negative influence on cell motility in the scratch assay they are not physiologically relevant to a bio-inspired dermal wound healing device. Increasing the amount of tropoelastin beyond physiological composition would make the scaffold too elastic and would not have the stiffness required to mimic the dermal ECM. The addition of TE in a proportion that is physiologically relevant may increase the resiliency of the material. That is, the ability of the material to return to its native state when it is stretched before it reaches plastic deformation [25-33].

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Conclusion

These data suggest, that when creating a tissue engineered skin substitute for use in wound healing a scaffold composed of a blend of collagen I and tropoelastin would mimic the native dermal ECM and promote dermal fibroblast migration. By simulating the protein composition of the skin, incorporating exogenous tropoelastin, the resulting scaffold should have similar mechanics to the ECM of the integument. Translating these results into a full thickness dermal wound, a wound healing device should stimulate dermal fibroblast motility in the wound margin, thus facilitating wound closure. More rapid dermal cell migration (e.g. dermal fibroblasts) will facilitate infiltration into the wound space, allowing the wound to progress more quickly into the proliferative phase of wound healing. In normal human dermal wound healing, fibroblasts typically infiltrate by day three. By adding the proteins to the wound space and increasing cell motility the wound should close in a shorter amount of time.

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Volume 3 Issue 2 February 2019