Aseptically Processed Dehydrated Human Amnion/Chorion Allografts* Promote Proliferation, Migration and New Matrix Deposition of Type II Diabetic Keratinocytes

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INTRODUCTION

Normal wound healing involves a series of events in the wound bed microenvironment between the extracellular matrix, growth factors and cells that orchestrate the repair and regeneration process. Diabetic environments are compromised, thus altering the ability of cells to respond and progress towards healing in a timely manner [1]. Amniotic membranes are rich in matrix proteins, various growth factors and cytokines that support the healing process. We have previously shown that aseptically processed dehydrated human amniotic membrane allografts (dHAMA) support endothelial and fibroblast cell proliferation, angiogenic capacity and new matrix production, which are key activities during granulation.

The focus of this work is to investigate the responsiveness of diabetic cells to dHAMA, *in vitro*; and to evaluate whether their cellular activity and functionality can be enhanced to the extent comparable to those of normal cells. Cellular behavior of normal keratinocytes and type II diabetic keratinocytes that would represent the epithelialization process during wound healing were examined.

MATERIALS AND METHODS

Dehydrated amnion/chorion allografts were procured according to Good Tissue Practices and processed aseptically without terminal sterilization at Musculoskeletal Transplant Foundation (MTF, Edison, NJ, USA).

Normal human epidermal keratinocytes (normal) and diabetic Type II human epidermal keratinocytes (diabetic) (Lonza) were cultured (100,000 cell/7mm disc) on both sides of dHAMA. Cell viability was assessed through the CCK-8 assay (Sigma-Aldrich) at various time-points along with matrix synthesis (laminin), which was evaluated histologically (Premier Laboratory, LLC).

The chemotactic ability of dHAMA was explored qualitatively (scratch test) and quantitatively (transwell). The scratch test compared cell migration of both cell types (250,000 cells/well) upon exposure to dHAMA extracts (20mg/1mL). A confluent layer of cells was scratched (~1mm gap) and exposed to dHAMA extracts over time. Cell migration was visualized through Calcien AM staining (Life Technologies). Transwell studies were conducted on 80% confluent normal and diabetic keratinocytes that were starved for 16-18 hours in basal media prior to exposure to dHAMA. Transwells (Corning) in 24 well plates were coated with fibronectin at 4°C overnight, aspirated the next day and air-dried for 45 minutes. 5mm dHAMA discs were placed under the transwells in basal media. The starved cells were seeded (40,000 cells/300µL of media) in basal media on top of the transwells and incubated for 24 hours. Basal media alone served as the negative control and for the blanks (no cells). Cell migration was evaluated via the CCK-8 assay on the bottom of the each transwell after rinsing with PBS and using a cotton tip applicator (Fisher Scientific) in a circular motion to remove any adhered cells on the top of each transwell.

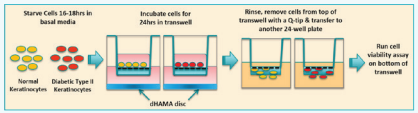


Figure 1: Transwell migration studies on normal and diabetic keratinocytes.

RESULTS

The results indicate that the proliferation trends of diabetic keratinocytes was similar to normal (peaking around day 7) when cultured on dHAMA. In addition, the proliferation patterns were similar on the amnion and chorion facing sides (no sidedness). Furthermore, diabetic cells secreted key extracellular matrix component (laminin) that constitute the basement membrane at a similar level to normal keratinocytes. The scratch studies revealed that normal and diabetic keratinocytes behaved similarly upon exposure to dHAMA extracts. At day 3, both cell types started migrating inward. However, a high seeding density and extensive matrix deposition may have prevented more effective migration in both cell types. A lower seeding density may be requisite to better examine the chemotactic effect of dHAMA, along with using mitomycin-C to inhibit cell growth. However, the quantitative assessment of cell migration through the transwell study demonstrated that both normal and diabetic keratinocytes were responsive to the presence of dHAMA and migrated towards it. Although diabetic keratinocytes exhibited slower overall migration as would be expected, both groups migrated significantly more in the presence of dHAMA when compared to the control (basal media).

Similar Proliferation Trends of Normal & Diabetic Keratinocytes Cultured on dHAMA

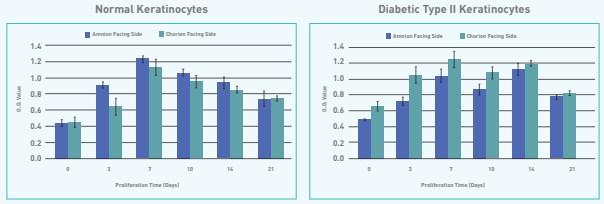


Figure 2: Diabetic keratinocytes cultured on dHAMA exhibited similar proliferation trends to normal keratinocytes plateauing around day 7. Cell viability was monitored via the CCK-8 assay. The number of living cells is proportional to the amount of dye converted from tetrazolium salts generated by the activity of dehydrogenases in cells. In addition, the patterns were similar on both the amnion facing and chorion facing sides (no orientation effects).

Diabetic Type II Keratinocytes Secreted Matrix Proteins Similar to Normal Keratinocytes

Figure 3. Immunohistochemical imaging revealed that diabetic keratinocytes (seeded on the chorion side) are functional on dHAMA, and have started secreting their own extracellular matrix (laminin). This is similar to normal keratinocytes by day 7 (magnification 40x); comparable results were found on the amnion side. These secreted proteins help establish a new matrix network to support cell migration, re-epithelialization and wound closure [2, 3].

Similar Migration Patterns in Normal and Diabetic Keratinocytes Upon Exposure to dHAMA Extracts

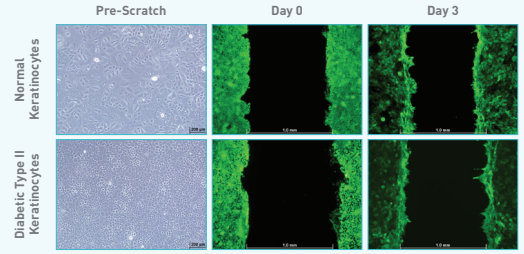
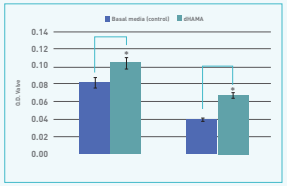


Figure 4: Chemokines present in the extracts of dHAMA are capable of directing cell migration. This is critical for successful epithelialization. Migration patterns were similar in normal and diabetic type II keratinocytes at day 3 (magnification 10x). However due to inconsistent cell growth and excessive matrix deposition, by day 7, there were sheets of cells that lifted off. Further work is needed to optimize seeding densities and media configuration.

dHAMA has Chemotactic Properties Directing Migration in Normal and Diabetic Keratinocytes

Figure 5: Chemokines present in the dHAMA directed cell migration. This is critical for successful epithelialization. Migration was increased upon exposure to dHAMA compared to the control (basal media) for both normal and diabetic keratinocytes. The response to the presence of dHAMA was significantly enhanced in both normal (p=0.0117) and diabetic (p=2.424E-06) keratinocytes, where p<0.05 is considered significant (two tailed T-test, α=0.05).



CONCLUSION

Aseptically-processed dHAMA are ideal substrates for supporting diabetic cellular activities. Similar cell proliferation to normal keratinocytes was observed, along with significantly better quantitative assessment of normal and diabetic keratinocyte migration compared to the control. This demonstrated that dHAMA can help promote proliferation and re-epithelialization activities in diabetic keratinocytes in a similar manner to normal keratinocytes. This is critical in facilitating the wound healing processes in diabetic wound sites.

REFERENCES

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2. Vulnar T, *et al. J Int Med Res.* 2009
3. Reinke JM & Sorg H, *Eur Surg Res.* 2012

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