Ex vivo evaluation of acellular and cellular collagen-glycosaminoglycan flowable matrices

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NOTE

Ex vivo evaluation of acellular and cellular collagen-glycosaminoglycan flowable matrices

Tom Hodgkinson and Ardeshir Bayat

Abstract

Collagen-glycosaminoglycan flowable matrices (CGFM) are increasingly finding utility in a diversifying number of cutaneous surgical procedures. Cellular in-growth and vascularisation of CGFM remain rate-limiting steps, increasing cost and decreasing efficacy. Through in vitro and ex vivo culture methods, this study investigated the improvement of injectable CGFM by the incorporation of hyaluronan (HA) and viable human cells (primary human dermal fibroblasts (PHDFs) and bone marrow-derived mesenchymal stem cells (BM-MSCs)). Ex vivo investigations included the development and evaluation of a human cutaneous wound healing model for the comparison of dermal substitutes. Cells mixed into the Integra Flowable Wound Matrix (IFWM), a commercially available CGFM, were confirmed to be viable and proliferative through MTT assays ($p < 0.05$). PHDFs proliferated with greater rapidity than BM-MSCs up to 1 week in culture ($p < 0.05$), with PHDF proliferation further enhanced by HA supplementation ($p < 0.05$). After scaffold mixing, gene expression was not significantly altered (qRT-PCR). PHDF and BM-MSC incorporation into ex vivo wound models significantly increased re-epithelialisation rate, with maximal effects observed for BM-MSC supplemented IFWM. HA supplementation to PHDF populated IFWM increased re-epithelialisation but had no significant effect on BM-MSC populated IFWM. In conclusion, when combined with PHDF, HA increased re-epithelialisation in IFWM. BM-MSC incorporation significantly improved re-epithelialisation in ex vivo models over acellular and PHDF populated scaffolds. Viable cell incorporation into IFWM has potential to significantly benefit wound healing in chronic and acute cutaneous injuries by allowing a point-of-care matrix to be formed from autologous or allogenic cells and bioactive molecules.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>IFWM</td>
<td>Integra Flowable Wound Matrix</td>
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<tr>
<td>CGFM</td>
<td>collagen-glycosaminoglycan flowable matrices</td>
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<td>PHDF</td>
<td>primary human dermal fibroblasts</td>
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<td>BM-MSCs</td>
<td>bone marrow derived mesenchymal stem cells</td>
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</table>

Introduction

Dermal substitute assisted healing is now an established practice, particularly in the treatment of burns and chronic ulcers [1–5]. The substitutes available to clinicians vary in their material origin (i.e. biological or synthetic), chemical composition, material format and whether or not they contain cellular material.
The aim of these products is however similar; to blast proliferation and protein synthesis [17]. High signal-regulated kinases) pathways resulting in fibro-

[15, 16], which stimulate Akt and ERK1/2 (extracellular signal-regulated kinases) pathways resulting in fibroblast proliferation and protein synthesis [17]. High

molecular weight HA forms a hydrated, pliable provisional healing wound matrix [18] during embryonic development [19] and is central to fetal scarless wound healing [20]. In adult wound healing, a transient accumulation of HA occurs in the early provisional ECM and decreases as the process continues [21]. Previously, HA supplementation in collagen hydrogel systems has been found to have contradictory effects on fibroblast proliferation [17, 22–24].

In human skin, large quantities of HA are found in the dermis and in the basal and spinous strata of the epidermis (concentrations of approximately 2.5 mg ml$^{-1}$) [25–29]. Local concentrations increase rapidly, up to sevenfold, in response to injury [30]. This occurs in a wide margin around the site of injury—indicating a release of HA synthesis-promoting factors [30–33]. Similarly, HA synthase 1 (HAS-1) expression is increased by 5.7-fold when fibroblasts are exposed to a synthetic matrikine mimicking injury [33]. Exogenous delivery of HA reduces scar formation in adult cutaneous and connective tissue wound healing [34]. HA-induced biophysical alterations in peri-cellular environments can also modulate cell shape, cell-substrate adhesion, cell spreading, cytoskeletal organization and tension [35].

Several investigators have demonstrated the healing potential of direct stem and somatic cell transplantation in the treatment of chronic and acute wounds [10–13]. Powerful paracrine signaling of the transplanted cells is likely to be a primary mechanism for this effect, though cell-tracing studies have found direct cellular contribution of transplanted cells into the healed tissue [10–13]. It is clear from these studies that for cells to be effective they should be applied within a protective and instructive matrix, with reported transplanted cell survival rates below 5% if directly inserted into the wound bed [36].

The aim of this study was to determine if the incorporation of viable cells is possible during the mixing process of IFWM. The effect of incorporating viable cells and HA on re-epithelialisation was then examined through an ex vivo wound healing model.

Methods

Primary human dermal fibroblast (PHDF) extraction and bone-marrow derived stem cell (BM-MSC) source

Primary human dermal fibroblasts were obtained from 5 Caucasian male and female patients undergoing elective surgery with appropriate written consents and full ethical approval (North West Research Ethics Committee (Ref1/1/NW/068). Full thickness tissue biopsies were processed to extract viable cells according to previously established protocols [37]. Fibroblasts were expanded in culture and cells up to passage 5 utilised. Cells were cultured under standard culture conditions in Dulbecco’s modified eagle medium (DMEM) supplemented
Note with 10% (v/v) fetal bovine serum (FBS) (PAA, Germany). BM-MSCs were purchased from Applied Biological Materials Inc. (Cat no. T4019), expanded in culture under standard conditions in low-glucose DMEM (PAA, Germany), supplemented with 10% (v/v) FBS and cells up to passage 5 utilised. BM-MSCs were shown to be able to differentiate along adipogenic, chondrogenic and osteogenic lineages in vitro (supplementary figure 1; stacks.iop.org/BMM/10/041001/mmedia).

Preparation of integra flowable wound matrix

The lyophilised IFWM was hydrated by mixing thoroughly with a total of 3 ml of complete DMEM (10% fetal bovine serum). To incorporate PHDFs and BM-MSCs into the matrix, the IFWM was partially rehydrated by vigorously mixing with an initial 1.5 ml DMEM. The remaining 1.5 ml of hydrating media was added as a high-density cell suspension (1 $\times$ 10$^6$ cells ml$^{-1}$) and mixed gently but thoroughly. For hyaluronan incorporated scaffolds, Lyophilized sodium hyaluronate (NovaMatrix, Norway) (MW 1 400 000–1 800 000 g mol$^{-1}$) (source: Streptococcus zooepidemicus grown in non-animal material) was dissolved with slow stirring for a minimum of two hours in sterile DMEM (10% fetal bovine serum). This solution was then used to hydrate the IFWM, so that the final concentration was 1.5 mg ml$^{-1}$ HA.

Ex vivo full thickness cutaneous artificial wound model

Full thickness cutaneous tissue was obtained from three male and female Caucasian patients undergoing elective surgery with appropriate ethical approvals. Tissue was trimmed of excess adipose tissue and 8 mm punches taken from main tissue sample. In the centre of these circular skin biopsies, a 4 mm full thickness artificial wound was created by a further punch biopsy. These doughnut shaped tissue samples were washed with sterile PBS containing 1% (v/v) penicillin/streptomycin and inserted into the 24-well plate inserts (Corning, USA). Into the wounded area, prepared IFWM with and without incorporation of viable cells and HA was inserted. The well outside the insert was supplemented with complete DMEM (10% (v/v) FBS) so that the epidermis of the ex vivo tissue was air exposed (figure 1(a)). Tissue was cultured for 14 d with media changed every day.

Post-mixing cell proliferation and ex vivo tissue viability

Cellular IFWM scaffolds were injected immediately after mixing into 24-well plate-inserts (Corning, USA) and cultured submerged in complete DMEM. Cell proliferation after mixing was assessed through the MTT colorimetric assay (Roche, UK), performed to the manufacturer’s instructions. Absorbance was

Figure 1. (A) Schematic diagram of human skin wound model. Full thickness 8 mm skin biopsies were wounded in the centre with 4 mm biopsies. These were placed into well inserts and the artificial wound filled with the relevant flowable scaffold. Media was added to the outside of the insert, so that the epidermis was air exposed. (B) Cell proliferation after mixing. Cells continued to proliferate after the mixing process with PHDFs proliferating to a greater extent than MSCs. HA supplementation increased proliferation of PHDF but not BM-MSC. (* represents $p < 0.05$; ** represents $P < 0.001$.)
measured using a spectrophotometer at OD 550 nm (corrected for OD 690 nm) and background subtracted in acellular scaffolds and wells. Results represent means of three independent assay reactions, for three populations of cells.

To assess the viability of *ex vivo* tissue during the culture period the colorimetric LDH (lactate dehydrogenase) assay was utilised (Roche, UK). According to the manufacturer’s instructions, cell culture media up to 12 d was removed from *ex vivo* cultures. 100 μl of the removed media was aliquoted into new 96-well plates. 100 μl tetrazolium test mix was added and the reaction incubated in the dark with gentle shaking at 20 °C for 30 min. Absorbance was measured using a spectrophotometer at OD 492 nm (corrected for OD 690 nm). Results represent means of three independent assay reactions, for three *ex vivo* cultures.

**Processing, sectioning, histochemistry and immunohistochemistry of wound models**

*Ex vivo* wound models were removed from culture at 7 and 14 days for analysis of wound healing through histochemistry and immunohistochemistry of wound cross-sections. Tissue was fixed in 4% (v/v) formalin and processed. Wound cross sections were bisected through the center of the wound and placed en face for paraffin embedding. For wound closure analysis, sequential wound cross-sections (5 μm) were taken containing both wound area tissue and adjacent non-injured tissue. Sections were deparaffinised, stained for nuclei with haematoxylin, counterstained with eosin and mounted for microscopic observation.

For immunohistochemical staining, paraffin embedded sections were again routinely deparaffinised in xylene and rehydrated through an ethanol gradient. Antigens were retrieved with citrate buffer (0.01 M, pH 6) at 60 °C for one hour. Primary antibodies were incubated for one hour at room temperature. After washing in tris-buffered saline (TBS) sections were pre-incubated in 10% bovine serum albumin (BSA) for one hour at room temperature. Following washing, the corresponding secondary antibodies were incubated for one hour at room temperature (supplementary table 1 for list of primary and secondary antibodies; stacks.iop.org/BMM/10/041001/mmedia).

**Effect of HA supplementation on gene expression in *ex vivo* wound model—qRT-PCR**

*Ex vivo* models were collected in 1 ml of Trizol (Invitrogen, UK) and incubated at room temperature for five minutes. Tissue was homogenized and RNA extracted using the RNeasy Kit (Qiagen, UK) according to the manufacturer’s instructions. RNA concentration was determined using the NanoDrop ND-1000 UV visible spectrophotometer (Labtech International (UK) and normalized across all cDNA synthesis reactions (qScript™ cDNA SuperMix (Quanta Biosciences, USA)). qRT-PCRs using the Lightcycler 480 II platform (Roche, UK). Gene expression levels were further normalized with an internal reference gene, ribosomal protein L32 (RPL32).

**Statistical analysis**

For the comparison of cell proliferation, cytotoxicity and gene expression data were statistically tested using independent t-tests. All data were analysed using IBM SPSS Statistics Software version 19.0 (SPSS Inc., Chicago, IL, USA). Significant values were considered as those where *p* < 0.05.

**Results**

**Post-mixing proliferative status of incorporated cells**

The main concern with the mixing process was preserving cell viability through the damage caused by shear forces during mixing and any osmotic shock that might be caused by the lyophilised scaffold prior to hydration. The viability and proliferative status of both PHDFs and BM-MSCs was confirmed through the MTT assay (figure 1(b)). The proliferation of cells after the mixing process was confirmed up to 1 week post-mixing. After 3 d culture, PHDF proliferation was enhanced in HA supplemented IFWM in comparison to un-supplemented IFWM (*p* < 0.05). After one week, PHDFs had proliferated to a greater extent (*p* < 0.05) than BM-MSCs. Supplementation of the IFWM with HA increased PHDF proliferation significantly but did not have an effect on BM-MSC proliferation.

**Re-epithelialisation of artificial wound cross-sections**

The re-epithelialisation of IFWM assisted wounds was assessed through stereomicroscopic images and stained wound cross-sections of the centre of the wounds. Haematoxylin and eosin images of artificial wounds show that re-epithelialisation proceeds, albeit at an attenuated rate in comparison to the natural process. Up to 1 week *ex vivo* culture there is a comparable, low-level of migration from the wound margins for all scaffolds. HA-supplementation increased migration in PHDF-IFWM and IFWM scaffolds but had no effect on BM-MSC-IFWM scaffolds at this time point. After 2 weeks culture, a significant difference was observed between IFWM scaffolds (figure 2). HA-supplemented IFWM showed a greater amount of epithelial migration than IFWM, with migration showing a more organized morphology across the surface of the scaffold. Migration into PHDF-IFWM was increased in comparison to IFWM, though without the addition of HA the migration was observed to be less organised. With HA supplementation, the migration became more organised across PHDF-IFWM, with wound closure observed in some scaffolds at this point. The greatest migration was observed in BM-MSC-IFWM scaffolds, the migratory epidermis forming layers several cells thick, and showing stratification. The
addition of HA to BM-MSC-IFWM was observed to decrease epithelial migration at this time point.

The viability of models over the culture period was confirmed through LDH assays (figure 3). Initially cell death was increased, which was attributed to the trauma of surgery and setting up the models. Cell death then fell and steadily rose over the culture period until the termination of culture. The supplementation of scaffolds with BM-MSCs corresponded with a decrease in the level of cell death observed.

Through stereomicroscope images of wounds it was sometimes possible to observe the migratory keratinocyte sheet over the surface of the scaffold (figure 4(A)). The presence of the incorporated PHDFs and BM-MSCs within the scaffolds was confirmed through immunostaining. Cell nuclei (Dapi) were clearly visible in the center of the scaffold, whilst cells were negative for cytokeratin 14, which would be present if cells were migratory keratinocytes (figures 4(B) and (C)). Similarly, cells did not stain positively for cytokeratin 10 within the scaffold (figure 4(C)). Cells stained positively for the proliferation marker PCNA (proliferating cell nuclear antigen), but did not stain for alpha-SMA, indicating a non-myofibroblastic, contractile phenotype. QRT-PCR analysis of gene expression in cell-incorporated models showed significantly
increased levels of collagen I and III expression in addition to increases in ECM remodeling enzymes such as MMP2 over acellular scaffolds (figure 5).

**Discussion**

One potential benefit of emerging flowable dermal matrices is the ability to customize scaffolds to include viable cells and/or biologically instructive molecules. In this study the feasibility of including viable cells into the IFWM and maintaining the viability through the mixing process was investigated. The effects of cell incorporation on re-epithelialisation of an *ex vivo* human wound healing model was then assessed.

One concern with the direct transplantation of cells remains the maintenance of viability once in the wound environment [36]. It is currently thought that to increase the efficacy of transplanted cells they should be provided within a protective and instructive carrier [36, 38]. Through a modification of the existing manufacturer’s instructions viable cells were incorporated into the IFWM and continued to proliferate in culture. In this case, the primary concerns were osmotic stress, caused by the lyophilized nature of the collagen-GAG scaffold, and shear force during the mixing process itself.

Previous studies in animal models [39–42] have demonstrated the efficacy of MSCs for the treatment of cutaneous defects, with the transplanted cells believed to act through the release of paracrine signals [43] or through direct differentiation and incorporation into the cellular structures of the skin [44]. The results presented here in a human *ex vivo* model are in agreement with previous work in rats by Shumakov *et al* [42], which showed application of both fibroblasts and MSCs improved healing, with a maximal effect observed for MSC application, in comparison to control wounds (no cells). Interestingly, it has been suggested that MSC incorporation may also be able to restore lost skin appendages, with a study in rats demonstrating labeled transplanted-cell incorporation into new appendages within the wound area [45].

Research into the efficacy of skin substitutes is often hindered by the need to use animal models, which are variable and biologically dissimilar to humans in a number of important aspects. Several models have previously been proposed, including organotypic co-cultures, to improve this situation and allow the preclinical evaluation of skin substitutes [46]. The model presented in this study provides a method for preclinical comparison in an environment that closely mimics the natural tissue and in the presence of numerous cell types, including immune cells, arranged in the natural architecture. Though animal models are not replaced through utilization of this model, it could reduce animal work by allowing the examination of specific processes, such as re-epithelialisation in the context of wound healing, in human skin.

In the *ex vivo* model, HA supplementation increased keratinocyte migration in PHDF populated scaffolds but not MSC populated scaffolds. The mechanism for this effect requires further study. HA presented to cells in this allows normal modes of receptor activation and may induce a wound healing phenotype in the PHDFs [23]. The different keratinocyte migratory response in the scaffolds could be due to a difference in receptor expression.

![Figure 4](image-url)
Note on PHDF/BM-MSC surfaces or a different downstream signaling response to receptor activation within cells, resulting in changes to paracrine signaling. Proliferation assays of BM-MSCs showed no significant response to HA supplementation, whilst PHDFs were stimulated to proliferate indicating that this may be the case.

The enhancement of re-epithelialisation of IFWM-HA models supports results from several previous reports indicating that HA promotes proliferation and migration in keratinocytes [4, 32] and is an important contributor in re-epithelialisation [32]. The evidence that HA is important in the re-epithelialisation of cutaneous injuries and in epidermal homeostasis is detailed [25, 47]. However, as with its cellular effects on the dermal fibroblasts [17, 22–24], the mechanisms of action and effect of HA on keratinocyte behaviour remain unresolved within the literature. Several investigators have reported an association between increasing epidermal HA and increasing keratinocyte proliferation and hyperplasia whilst inhibiting differentiation. Through a human organotypic keratinocytes-fibroblast co-culture, Gu et al demonstrated that exogenously delivered HA enhances epidermal proliferation and basement membrane secretion [4, 48]. The findings of this study show that in this system, HA supplementation increases epidermal migration in acellular and PHDF populated scaffolds, but attenuated migration in BM-MSC populated scaffolds.

**Conclusion**

The incorporation of viable cells into the IFWM was possible with minor alterations to the preparation protocol. The inclusion of viable PHDF and BM-MSCs improved the re-epithelialisation of artificial human cutaneous wounds. The incorporation of HA into the IFWM promoted re-epithelialisation in acellular and IFWM scaffolds but had a contrasting effect on BM-MSC containing IFWM. Mixing viable cells with the IFWM may be a promising tool for healing chronic and acute injuries.

**Acknowledgments**

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