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Title

Hyaluronic acid: evaluation as a potential delivery vehicle for vitronectin:growth factor complexes in wound healing applications.

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Abstract

We have previously reported that novel vitronectin:growth factor (VN:GF) complexes significantly increase re-epithelialization in a porcine deep dermal partial-thickness burn model. However, the potential exists to further enhance the healing response through combination with an appropriate delivery vehicle which facilitates sustained local release and reduced doses of VN:GF complexes. Hyaluronic acid (HA), an abundant constituent of the interstitium, is known to function as a reservoir for growth factors and other bioactive species. The physicochemical properties of HA confer it with an ability to sustain elevated pericellular concentrations of these species. This has been proposed to arise via HA prolonging interactions of the bioactive species with cell surface receptors and/or protecting them from degradation. In view of this, the potential of HA to facilitate the topical delivery of VN:GF complexes was evaluated. Two-dimensional (2D) monolayer cell cultures and 3D de-epidermised dermis (DED) human skin equivalent (HSE) models were used to test skin cell responses to HA and VN:GF complexes. Our 2D studies revealed that VN:GF complexes and HA stimulate the proliferation of human fibroblasts but not keratinocytes. Experiments in our 3D DED-HSE models showed that VN:GF complexes, both alone and in conjunction with HA, led to enhanced development of both the proliferative and differentiating layers in the DED-HSE models. However, there was no significant difference between the thicknesses of the epidermis treated with VN:GF complexes alone and VN:GF complexes together with HA. While the addition of HA did not enhance all the cellular responses to VN:GF complexes examined, it was not inhibitory, and may confer other advantages related to enhanced absorption and transport that could be beneficial in delivery of the VN:GF complexes to wounds.

Key words

Vitronectin:growth factor (VN:GF), hyaluronic acid (HA), skin wound healing, delivery vehicle

1. Introduction

Since their discovery late last century, growth factors have been examined for their potential as wound therapeutics. Experimental evidence has shown that transforming growth factor β (TGF-β) and vascular-endothelial growth factor (VEGF) accelerate the healing of wounds in animal models [1, 2]. Platelet-derived growth factor (PDGF) has been shown to accelerate the healing of human diabetic foot ulcers [3], and basic fibroblast growth factor (bFGF) has been approved as a treatment for wounds in Japan [4]. Several other growth factors have exhibited benefits in clinical trials, for example, granulocyte-macrophage colony-stimulating factor (GM-CSF) and epidermal growth factor (EGF) [5, 6]. However, in every report to date, high doses of these growth factors have been applied to obtain successful wound healing results. Significantly, these therapeutic strategies ignore the central

coordinating role of the extracellular matrix (ECM) in orchestrating and enhancing the cascade of cellular events in the wound healing response [7].

Recent studies in our laboratory have found that the binding of the ECM protein vitronectin (VN) to insulin-like growth factor (IGF), IGF-binding proteins (IGFBPs) and EGF significantly enhanced the proliferation and migration of primary skin keratinocytes [8]. In addition, these novel complexes of VN, IGF, IGFBPs and EGF (VN:GF complexes) have been found to significantly enhance re-epithelialization in a porcine deep dermal partial-thickness burn model [9]. In this proof-of-principle study VN:GF complexes were directly delivered to burn injury sites in water. Water is potentially a less than ideal excipient and topical application using alternative delivery vehicles may further improve the healing outcomes, for example, by prolonging the exposure of the complex to the wound bed and/or reducing the dose of VN:GF complexes required.

Hyaluronic acid (HA) was discovered in bovine vitreous humour by Meyer and Palmer in 1934 and is an important constituent of all connective tissues in the body [10]. Its physicochemical properties complement the activity of small molecules, including diclofenac and ibuprofen [11], pilocarpine and tropicamide [12, 13], xylometazoline and vasopressin [14, 15], taxol and manganese [16, 17], cisplatin [18], doxorubicin [19] and insulin [20]. The successful clinical outcomes established with these pharmaceuticals has facilitated development of novel delivery techniques, including liposomes [21], anti-bacterial and anti-inflammatory drugs [22, 23], implantable drugs and DNA [24].

While growth factors have well recognised roles in the wound healing cascade [25], the viscoelasticity, hygroscopicity, biocompatibility and immunocompatability of HA confer it with a facilitating role that may potentiate or maximize tissue responses to growth factors. Hopkinson stated in 1992 that 'effective repair is dependent on the coordinated activities of both regulatory molecules and structural molecules' [26]. In view of this we examined the application of VN:GF complexes in combination with HA to skin cells.

2. Methods

2.1 Pre-binding VN:GF to culture wells

To reflect more accurately the cellular environment *in vivo* we 'pre-bound' the VN:GF complexes to culture dishes [8, 27, 28]. We routinely adopt this approach because cells *in vivo* are likely to encounter growth factors bound to the ECM rather than in 'solution-phase' [29]. Thus, VN:GF complexes were precoated onto 48-well tissue culture plates (Nagle-Nunc, Denmark) and the lower membrane surface of 12-µm pore 12-well Transwells® (Costar, USA). Firstly, 150 µL of DMEM containing 162 ng VN (Promega, Australia), or 600 µL of DMEM containing 648 ng VN, was added to each well of a 48-well plate and lower chamber of a 12-well Transwell® plate, respectively. These were incubated for two hours at 37 ºC, after which the media containing unbound VN was removed by aspiration. Subsequently 125 µL, or 500 µL of HEPES binding buffer (HBB) (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, pH 7.6) containing IGF-I (54 ng) (Novozymes, Australia), IGFBP3 (162 ng) (Upstate Biotech, USA) and EGF (54 ng) (Novozymes), or IGF-I (216 ng), IGFBP3 (648 ng) and EGF (216 ng) was added to the 48-well plates, or 12-well Transwell®, respectively, and incubated for a further two hours at 37 ºC. The media containing the growth factor complexes was removed and the wells were washed with 500 µL, or 1 mL HBB, respectively.

2.2 HA solution preparation

Human recombinant hyaluronic acid (rhHA) (954 kDa) (Novozymes A/S, Denmark) was dissolved in double-distilled water (10 mg/mL) for 2 hr at 37 ºC. This solution was filter-sterilised (0.22 µm) (3 mg/mL) and stored at -20 ºC. The final concentration of HA was determined to be 3 mg/ml after filtration due to difficulties associated with HA's viscosity.

2.3 Migration assay

The human skin keratinocyte (HaCaT) cell line (DKFZ, Germany) and human foreskin fibroblast (HFF) cell line (ATCC, America) were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Australia) supplemented with 5% foetal bovine serum (FBS) (HyClone, USA). All culture media were further supplemented with 0.1 µL/mL gentamicin, 50 µL/mL streptomycin sulfate and 50 units/mL penicillin G (Invitrogen). Migration assays were performed as previously described with the following modifications [8, 28]. HaCaT and HFF cells were starved and seeded into the upper chamber of pre-treated 12-well Transwells® at a density of 1.5 x 10^5 cells in 400 µL serum-free medium (SFM). HA was added in 250 µL to the lower chamber with or without pre-bound VN:GF. Cells were thus exposed to 2, 6, 20, 60, 200, 600 and 2000 µg of HA in the presence or absence of VN:GF complexes in a final volume of 1 mL/well. The vessels were incubated for 10 hr at 37 ºC, 5% CO_2/95% air and cells that had not migrated through the microporous membrane to the lower chamber were gently removed using a flattened cotton bud. Cells located on the lower surface of the porous membrane (migrated) were fixed in 3.7% para-formaldehyde (Univar, Australia) and stained with 0.01% crystal violet (Sigma-Aldrich Pty Ltd, Australia) in phosphate buffered saline (PBS). The crystal violet stain was extracted in 10% acetic acid (BDH Laboratory Supplies, England) and the optical density (corresponding to the number of cells) was determined at 595 nm using a 96-well plate reader (Benchmark Plus, Bio-Rad, UK) [30].

2.4. Proliferation assay

HaCaT and HFF cells were starved and seeded into the 48-well plates at a density of 2.5 x 10^4 cells in 375 µL SFM. Subsequently, various concentrations of HA in 125 µL were added to the wells. Wells were therefore exposed to 1, 3, 10, 30, 100, 300 and 1000 µg of HA in both the absence and presence of VN:GF complexes in a final volume of 500 µL/well. The vessels were then incubated for 48 hr at 37 ^{0}C, 5% CO_2/95% air. Viable cells were quantitated with the CyQUANT® NF Proliferation Assay (Invitrogen) as per the manufacturer's instructions. Fluorescence was determined at λ_{ex} 485nm, λ_{em} 530 nm in black 96-well plates (PerkinElmer, USA) using a POLARstar Optima Microplate Reader (BMG LABTECH GmbH, Germany).

2.5. Statistical analysis of 2D cell culture studies

All experiments were performed in triplicate and each treatment was tested individually three times in each assay. One-way ANOVA with Tukey's post hoc tests (all group comparisons) was applied to determine significance, determined as $p < 0.05$.

2.6. Primary cell isolation and culture

Keratinocytes and fibroblasts were isolated from three unrelated patients and cultured as described previously [31]. *Ex vivo* cultivated keratinocytes and fibroblasts were seeded onto DEDs as described previously [31].

2.7. Assessment of the potential of VN:GF complexes and HA using DED-HSEs

Coincident with elevation to the air:liquid interface, replicate DED-HSEs were treated daily with topical application of 40 µL VN:GF complexes 1x (0.3 µg VN + 0.3 µg IGFBP3 + 0.1 µg IGF + 0.1 µg EGF), VN:GF complexes 3x (0.9 µg VN + 0.9 µg IGFBP3 + 0.3 µg IGF + 0.3 µg EGF), HA (60 µg) (Novozymes, Denmark), HA+VN:GF complexes 1x, HA+VN:GF complexes 3x or serum-free medium (SFM) (negative control). After a further 3 and 7 days of culture at the air-liquid interface, the DED-HSEs were stained with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyletetrazolium bromide (MTT) and fixed in 4% formalin (United Biosciences, Australia) and processed for histological or immunohistochemical analysis [31, 32]. The thicknesses of the proliferative cell layer and differentiated cell layer present in each newly formed epidermis was quantitated using Scion Image software (Scion Corp, USA).

2.8. Assessment of the effects of VN:GF complexes and HA on incorporation of fibroblasts into the DED

Fibroblasts were seeded onto the reticular face of each DED as described above to create the DED-HSE. Coincident with elevation to the air:liquid interface, individual DED-HSEs were treated with; 1) 20 μL of VN:GF complexes (0.015 μg/μL VN + 0.015 μg/μL IGFBP3 + 0.005 μg/μL IGF + 0.005 μg/μL EGF); 2) 20 μL of HA (1.5 μg/μL); 3) HA+VN:GF complexes (0.015 μg/μL VN + 0.015 μg/μL IGFBP3 + 0.005 μg/μL IGF + 0.005 μg/μL EGF + 1.5 μg/μL HA); or 4) serum-free medium (SFM) (negative control). After 3 days culture at the air-liquid interface, the DED-HSEs were fixed in 4% formalin for histological analysis. The fibroblast population was quantitated using Scion Image software.

2.9. Statistical analysis of the 3D DED-HSE studies

Duplicate DED-HSEs from three unrelated patients were tested individually with each treatment within each assay, unless otherwise indicated. One-way ANOVA with Tukey's post hoc tests (all group comparisons) was applied to analyse the data. Statistically significant differences were accepted when $p < 0.05$.

3. Results

3.1. Effect of VN:GF and HA on HaCaT and HFF migration and proliferation

We investigated if the presence of HA would affect VN:GF complexes-induced migration and proliferation of HaCaT cells. As shown in Fig. 1a, HaCaT cell migration is unaffected by the presence of HA alone. Moreover, the effect of VN:GF complexes on HaCaT migration is not altered by the presence of HA. Furthermore, the HA treatment alone has little effect on the proliferation of HaCaT cells. Likewise, the presence of HA does not alter the effect of VN:GF complexes on HaCaT proliferation (Fig. 1b).

We further examined the effect of HA on the migration and proliferation of HFF. As shown in Fig. 1c, HA alone has little effect on the migration of HFF. Moreover, the effect of VN:GF complexes on HFF migration is not affected by HA. Furthermore, the highest concentration of HA (1000 μg/500 μL) significantly increased HFF proliferation, 433% ± 26% above control SFM ($p < 0.01$). Similarly, cell proliferation was stimulated two-fold by HA (> 600 μg/mL) in the presence of VN:GF complexes (Fig. 1d). These data demonstrate HA significantly stimulates HFF proliferation and confers additional enhancement to VN:GF complex-stimulated proliferation, albeit only at the higher concentrations of HA tested.

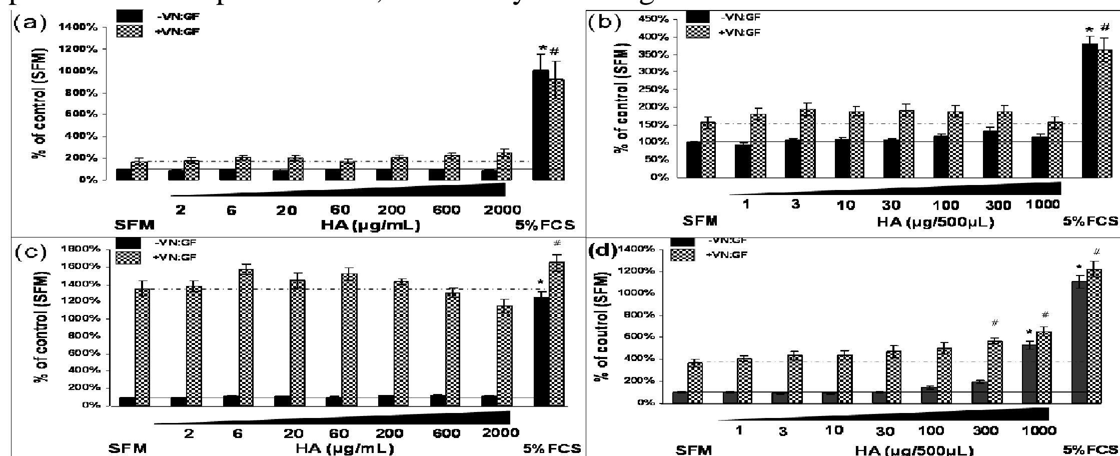

Figure 1. Migration (a) and proliferation (b) of HaCaT keratinocytes and HFF fibroblasts (c and d) exposed to HA in the absence and presence of the VN:GF complex. Responses of HaCaTs and HFFs seeded into 12-well Transwell® (a and c) and 48-well plates (b and d) containing increasing concentrations (μg/mL and μg/500 μL) of HA in the absence and presence of the VN:GF complex are depicted. The data, expressed as the average percentage of control wells containing serum free medium (SFM) alone for 10 hr and 48 hr, respectively, were pooled from three replicate experiments in which each treatment was tested independently in triplicate. The response of cells exposed to wells treated with HA in the absence of VN:GF complex (black bars) are compared with the response obtained in the control SFM wells. The response of cells exposed to wells treated with HA in the presence of VN:GF complex (checked bars) are compared with the effects obtained from wells with the control SFM plus the VN:GF complex. Error bars indicate SEM. *$P < 0.05$.

3.2. Effect of VN:GF complexes and HA on the outgrowth of keratinocytes in DED-HSE model

We employed the 3D DED-HSE model to investigate the influence of VN:GF complexes and HA on keratinocyte outgrowth (Fig. 2a). No statistically meaningful differences were found between DED-HSEs treated with any of the different treatments ($p > 0.05$) (Fig. 2b). These results indicate that HA alone enhances keratinocyte outgrowth to a small extent and that the addition of HA does not alter the effect of VN:GF complexes.

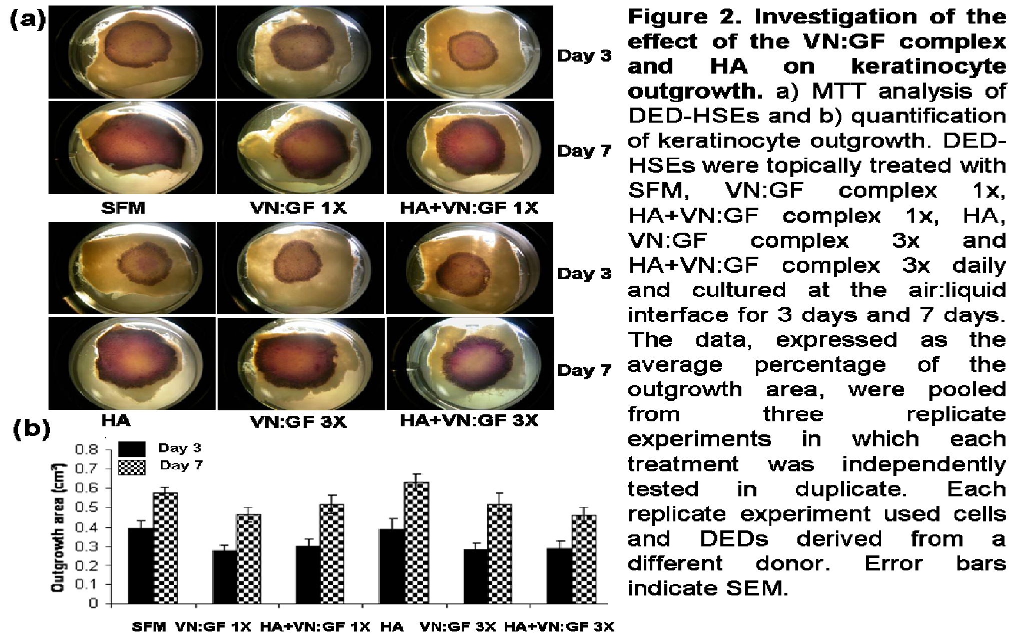

Figure 2. Investigation of the effect of the VN:GF complex and HA on keratinocyte outgrowth. a) MTT analysis of DED-HSEs and b) quantification of keratinocyte outgrowth. DED-HSEs were topically treated with SFM, VN:GF complex 1x, HA+VN:GF complex 1x, HA, VN:GF complex 3x and HA+VN:GF complex 3x daily and cultured at the air:liquid interface for 3 days and 7 days. The data, expressed as the average percentage of the outgrowth area, were pooled from three replicate experiments in which each treatment was independently tested in duplicate. Each replicate experiment used cells and DEDs derived from a different donor. Error bars indicate SEM.

3.3. Effect of VN:GF complexes and HA on growth and differentiation of the epidermis in DED-HSE model

Immunohistochemistry was undertaken to detect the expression of specific markers on the surface of cells in the DED-HSE. Immunohistochemical analysis with a monoclonal antibody raised against the nuclear transcription factor p63 revealed the presence of undifferentiated proliferating cells. At day 3, intense immunoreactivity of p63 was detected in keratinocytes within the suprabasal and basal layers of the DED-HSEs treated with VN:GF complexes, HA or HA+VN:GF complexes. However, decreased immunoreactivity was detected in keratinocytes within the deeper basal layers of the DED-HSEs treated with VN:GF 1x and HA+VN:GF 3x complexes at day 7 (Fig 3, 4). Immunohistochemical analysis with a monoclonal antibody raised against keratins 1, 10, 11 (K1/10/11) revealed the presence of differentiated cells. Positive immunoreactivity of K1/10/11 was present in DED-HSE treated with VN:GF complexes, HA or HA+VN:GF complexes at both day 3 and day 7 (Fig 3, 4). Immunohistochemical analysis with a type IV collagen antibody revealed the presence of the basement membrane. Positive immunoreactivity of collagen IV was present in DED-HSE treated with VN:GF complexes, HA or HA+VN:GF complexes at both day 3 and day 7 (Fig 3, 4). Immunohistochemical analysis with a keratin 14 (K14) antibody revealed the presence of basal cells. Intense immunoreactivity of K14 was detected DED-HSEs treated with HA+VN:GF 1x and VN:GF 3x complexes, but not HA+VN:GF 3x complexes at either day 3 or day 7 (Fig 3, 4).

The epidermis partitions into two layers: a proliferative layer and a differentiated layer. The thickness of these two layers was quantitated (Fig. 3b and 4b) as a surrogate indicator of homeostasis. The thickness of the proliferative layer in DED-HSEs exposed to VN:GF complexes 1x, HA+VN:GF complexes 1x, HA, VN:GF complexes 3x and HA+VN:GF complexes 3x were significantly greater than control SFM. The thickness of the differentiated layers treated with VN:GF complexes 1x, HA+VN:GF complexes 1x, HA, VN:GF

complexes 3x and HA+VN:GF complexes 3x were also significantly thicker than the control SFM, with a depth of 29.23 ± 2.57 µm, 20.51 ± 1.32 µm, 12.56 ± 1.13 µm, 35.52 ± 0.13 µm and 27.78 ± 1.72 µm above, respectively, observed ($p < 0.05$) (Fig. 3b). These results suggest that VN:GF complexes, HA and HA+VN:GF complexes significantly enhance keratinocyte proliferation and differentiation in the *ex vivo* DED-HSE model.

Interestingly, the differences in the depth of the proliferative layers between DED-HSEs treated with VN:GF complexes, HA and HA+VN:GF complexes was lost with prolonged treatment (i.e. 7 days) (Fig. 4b). However, the thicknesses of differentiated layers in DED-HSEs treated with VN:GF complexes 1x, VN:GF complexes 3x and HA+VN:GF complexes 3x, remained statistically thicker than control-treated constructs ($p < 0.05$) (Fig. 4b). These results indicate that VN:GF complexes 1x, VN:GF complexes 3x and HA+VN:GF complexes 3x enhance keratinocyte differentiation.

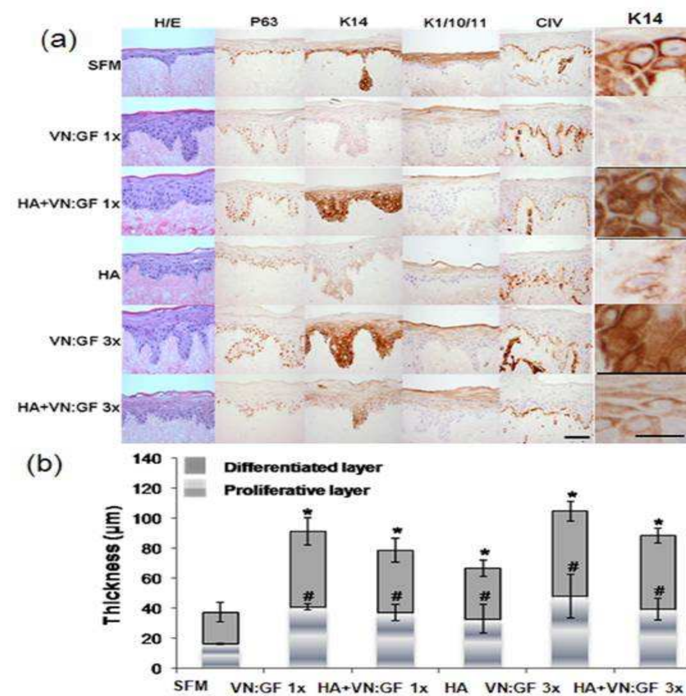

Figure 3. Investigation of the effect of the VN:GF complex and HA on epidermal formation. a) Histological analysis of DED-HSEs and b) quantification of the proliferative and differentiated layers of the epidermis. DED-HSEs were topically treated with SFM, VN:GF complex 1x, HA+VN:GF complex 1x, HA, VN:GF complex 3x and HA+VN:GF complex 3x daily and cultured at the air:liquid interface for 3 days. Scale bars, 100 µm (left) and 20 µm (right). The data, expressed as the average percentage of the thickness of proliferative and differentiated layers of the epidermis, were pooled from three replicate experiments in which each treatment was independently tested in duplicate. Each replicate experiment used cells and DEDs derived from a different donor. The asterisks (#) and (*) indicate treatments which significantly increased the proliferative (checked bars) and differentiating layers (black bars) compared to the responses observed with DED-HSEs treated with the control SFM treatment ($P < 0.05$). Error bars indicate SEM.

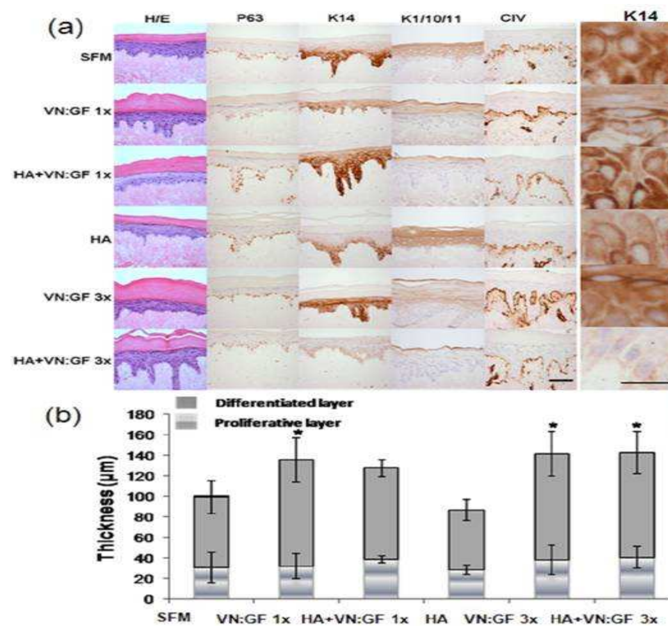

Figure 4. Investigation of the effect of the VN:GF complex and HA on epidermisation. a) Histological analysis of DED-HSEs and b) quantification of the proliferative and differentiated layers of the epidermis. DED-HSEs were topically treated with SFM, VN:GF complex 1x, HA+VN:GF complex 1x, HA, VN:GF complex 3x and HA+VN:GF complex 3x daily and cultured at the air:liquid interface for 7 days. Scale bars, 100 µm (left) and 20 µm (right). The data, expressed as the average percentage of the thickness of proliferative and differentiated layers of the epidermis, were pooled from three replicate experiments in which each treatment was independently tested in duplicate. Each replicate experiment used cells and DEDs derived from a different donor. The asterisks (#) and (*) indicate treatments which significantly increased the proliferative (checked bars) and differentiating layers (black bars) compared to the responses observed with DED-HSEs treated with the control SFM treatment ($P < 0.05$). Error bars indicate SEM.

3.4. Effect of VN:GF complexes and HA on incorporation of fibroblasts into the DEDs

We also examined the *ex vivo* colonisation of our DED-HSE models by donor-derived fibroblasts (Fig. 5a-d). The average number of fibroblasts successfully incorporated into DED-HSE models treated with VN:GF complexes, HA and HA+VN:GF complexes were 6.42 ± 0.86, 7.67 ± 0.71 and 8.21 ± 0.89 per field of view, respectively. The average number of fibroblasts in control (SFM)-treated DED-HSEs was 5.54 ± 0.60 (Fig. 5e). These results indicate that VN:GF complexes alone, HA alone and HA+VN:GF complexes can enhance the incorporation of fibroblasts into DED-HSE, while HA does not alter the effect of VN:GF complexes on fibroblasts.

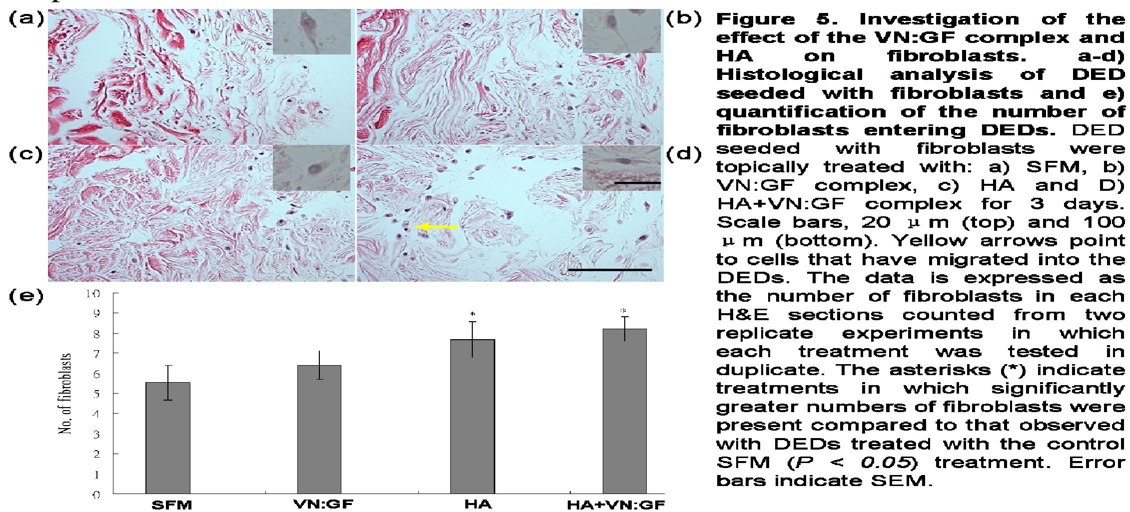

Figure 5. Investigation of the effect of the VN:GF complex and HA on fibroblasts. a-d) Histological analysis of DED seeded with fibroblasts and e) quantification of the number of fibroblasts entering DEDs. DED seeded with fibroblasts were topically treated with: a) SFM, b) VN:GF complex, c) HA and D) HA+VN:GF complex for 3 days. Scale bars, 20 μm (top) and 100 μm (bottom). Yellow arrows point to cells that have migrated into the DEDs. The data is expressed as the number of fibroblasts in each H&E sections counted from two replicate experiments in which each treatment was tested in duplicate. The asterisks (*) indicate treatments in which significantly greater numbers of fibroblasts were present compared to that observed with DEDs treated with the control SFM ($P < 0.05$) treatment. Error bars indicate SEM.

4. Discussion

As growth factors play important roles during the wound healing process, extensive experimental investigations of their activities, both *in vitro* and *in vivo*, have been conducted. One family of growth factors thought to take part in the complex processes involved in skin growth and repair is the insulin-like growth factor (IGF) family, comprised of IGF-I and IGF-II. These growth factors have various biological functions, including promotion of growth and development [33], regulation of cell proliferation and differentiation [34] and deoxyribonucleic acid (DNA) synthesis [35]. Primary keratinocytes normally express both insulin and IGF-I receptors and respond to either ligand during differentiation [36]. The role of epidermal factor (EGF)-stimulated cell motility in promoting wound healing has also been shown both *in vitro* in cell culture and *in vivo* in corneal and skin wounds [37]. Several other growth factors that are also involved in wound repair have been found to interact with components of the extracellular matrix (ECM) [38]. Following on from this, it is now thought that these growth factor:ECM interactions play a key role in most tissue growth and repair processes, including cell attachment, proliferation, migration, differentiation, cell survival and angiogenesis [7].

It has been found that vitronectin (VN), an ECM protein abundant in both the circulation and in tissues, increases epithelial outgrowth from skin organ explant cultures and supports the spreading of keratinocytes freshly isolated from guinea pigs [39]. VN also has been shown to induce enhanced motility of human keratinocytes *in vitro* [40] and provides an essential component of the temporary wound bed matrix [41]. Since the temporary wound bed matrix is rich in growth factors, the interaction between the ECM and growth factors is regarded as very important in wound healing [7]. Several growth factors involved in wound repair have been found to interact with components of the ECM [38]. For example, VN has been found to bind to IGF-family members [9, 27], epidermal growth factor (EGF) [42] and basis fibroblast growth factor (b-FGF) [43].

Recent studies undertaken by our laboratory have investigated the functional effects of a substrate-bound complex composed of VN, IGF and EGF. Specifically, we have demonstrated that IGF-I associates with VN through IGFBPs [27]. Moreover, these trimeric complexes (IGF-I + IGFBP + VN) have been shown to enhance keratinocyte protein synthesis and migration [28]. Further, we have observed that the addition of EGF to the substrate-bound VN:IGF:IGFBP significantly increased the proliferation and migration of primary skin keratinocytes [8]. As to the precise mechanisms underlying the enhanced cellular responses to growth factors in the presence of VN, it has been proposed that "cross-talk" occurs between growth factor receptors and αv integrins [29]. Growth factors are known to modulate the expression of integrin family members in tissue repair [44]. Indeed, recent studies from our own laboratory demonstrated that the novel VN:GF complexes enhance breast epithelial cellular migration via increased and sustained activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3-K/AKT) signalling pathway through co-activation of the IGF-1R and αv integrins [29]. In addition, it has been found that antibody inhibition of both the VN-binding αv integrins and the IGF-1R are critical for the enhanced keratinocyte migration observed in response to the VN:GF complexes in cell culture [9]. Moreover, to investigate the potential of the VN:GF complexes as a therapy for chronic wound healing, chronic wound fluid was added to replicate the *in vitro* environment of a chronic wound. We subsequently have demonstrated that the activity of the VN:GF complexes remains substantial, even in the presence of protease-rich chronic wound fluid collected from patients with chronic leg ulcers [9]. Furthermore, we have demonstrated that these VN:GF complexes also enhance the re-epithelialization of deep dermal partial-thickness burn injuries in a porcine model [9].

For our *in vivo* porcine study, VN:GF complexes were applied in water. For human applications water is a less than ideal excipient. Thus we investigated the potential of other approved formulations as a delivery vehicle for the VN:GF complexes. In particular, we examined the response of human keratinocytes and fibroblasts to topically delivered VN:GF complexes in the presence of hyaluronic acid (HA). HA is a high molecular weight polyanionic polysaccharide of the extracellular matrix, composed of repeated N-acetyl-D glucosamine and beta-glucoronic acid units [45]. HA is especially enriched in mammalian skin, in both dermis and epidermis [46]. Although the structure of HA is very simple, it has remarkable rheological, viscoelastic and hygroscopic properties that are critical to dermal tissue function. However, recently the role of HA in the mediation of physiological functions, such as morphogenesis, regeneration and wound healing [47] via its interaction with binding proteins and cell surface receptors has been recognised. It also has been reported that HA dynamically regulates cell signalling and behaviour [48]. Furthermore, interactions between HA and its cognate cell surface receptors, CD44 and hyaladherin (RHAMM), are thought to facilitate cell adhesion, cell motility, and cellular proliferation . It was for these reasons that we examined whether HA held potential as a carrier for the delivery of the VN:GF complexes to wounds.

Of interest to us, Kothapalli and Ramamurthi (2008) have reported that concurrent delivery of hyaluronan and IGF-I increased elastin regeneration by adult rat vascular cells [49]. In addition, a HA pack impregnated with IGF-I was found to enhance re-epithelialisation in a sheep wound model [50], and a wound dressing composed of a HA sponge containing EGF decreased the size of full-thickness skin defects and increased epithelisation in a rat wound model [51]. We therefore hypothesized that HA would have a facilitating role, and may perhaps even potentiate or maximize, the cellular responses to the VN:GF complexes. We tested this hypothesis using our system of pre-binding VN:GF complexes to culture vessels [52], exposing cells to these immobilised complexes and assaying the proliferation and migration of HaCaTs and HFFs in the presence and absence of HA.

We observed that HA alone has little effect on the proliferation of HaCaTs. In addition, the presence of HA does not alter the effect of VN:GF complexes on HaCaT proliferation (Fig. 1b). However, HA stimulated the proliferation of HFF fibroblasts (Fig. 1d), confirming the observations of Greco *et al*. (1998) [53]. Mohapatra et al. (1996) have reported that the progression of fibroblasts through the cell cycle is regulated by the interactions of HA with the cell surface receptor, RHAMM [54]. In particular, HA ligation of RHAMM maintains the level of Cdc2/cyc B1 complex kinase activity, permitting cells to progress through the G2/M phase of the cell cycle [53]. Our observation that VN:GF complexes enhanced the proliferation of HFF, and that this response is further enhanced by HA (Fig. 1d), raises the possibility of cross-talk between RHAMM and the IGF cell surface receptor (IGF-IR). Indeed, RHAMM has previously been associated with growth factor-regulated signalling, including signalling by PDGF [55].

In addition to cell proliferation, cell migration is also critical to the re-epithelialisation of cutaneous wounds. We have previously reported that the migration of keratinocytes and fibroblasts is significantly enhanced in the presence of the VN:GF complexes [56]**.** Thus we assayed the migration of HaCaT and HFF cells to determine whether the presence of HA also affected VN:GF complexes-stimulated cell migration. We observed no significant difference in cell migration when HaCaTs and HFFs were exposed to a range of concentrations of HA alone (Fig. 1a, c). Furthermore, the addition of HA to VN:GF complexes did not further enhance the migration of HaCaTs and HFFs (Fig. 1a, c).

We also evaluated the effects of VN:GF complexes and HA using a 3D cell culture approach and primary cells derived from adult human skin [57, 58]. The 3D approach we employed, the DED – HSE model, exhibits similar morphology, structure and functional biochemistry to native human skin; hence, provides an *ex vivo* tool to investigate the effect of potential therapeutics [32]. Indeed, this 3D culture system allows keratinocytes to grow, differentiate and function as a living organ; with a stratified epidermis composed of a stratum basale, stratum spinosum, stratum granulosum and stratum corneum, as exists *in vivo* [32, 59, 60]. This model therefore enables the migration, proliferation and differentiation of keratinocytes to be studied in a rigorously controlled system.

Experimental results from our DED-HSE studies indicate firstly that VN:GF complexes enhanced keratinocyte proliferation and differentiation and that this response is sustained and dose-dependent. The thickness of the proliferative and differentiating layers of the epidermis, whose formation involves keratinocyte migration, proliferation and differentiation, is significantly increased by application of VN:GF complexes (Fig. 3, 4). Secondly, these results demonstrate that HA alone is a significant stimulus of keratinocyte migration, proliferation and differentiation (Fig. 3). These findings corroborate previous reports, which suggest that HA stimulates keratinocyte migration, proliferation and differentiation through interactions with the cell surface HA receptors CD44 and hyaladherin RHAMM [61-63]. These 3D HSE-DED results are also consistent with previous clinical trials showing that skin lesions could be improved when HA is applied topically [64]. However, the data presented here is different from the 2-D cell culture results that we reported, which indicated that HA alone has little effect on the migration and proliferation of HaCaT cells. This may be explained by the difference between the 2-D and 3-D culture systems, as well as differences between the HaCaT cell line and keratinocytes derived from human skin. Thirdly, HA+VN:GF complexes were found to significantly enhance the development of both the proliferative and the differentiating layers of the DED-HSE model. The immunohistochemical analysis indicated that the addition of HA to the VN:GF complexes has a variable effect on the expression of K14. A stimulatory effect of HA in K14 immuno-reactivity in the presence of the lower concentration of VN:GF complexes was observed, but not at the higher concentration of VN:GF complexes. Therefore, HA may well be enhancing

the delivery of lower concentrations of the VN: GF complexes in skin wound healing, given that the migration and proliferation of basal cells play key roles in re-epithelialisation [65] (Fig 3, 4).

Fibroblasts were also introduced and responses to VN:GF complexes and HA were assayed in our 3D DED-HSE model. Reproducing the experimental outcomes we observed in the 2D (monolayer) model we noted that greater numbers of fibroblasts were present in the DEDs treated with HA alone and HA+VN:GF complexes than we observed in our serum-free control samples (Fig. 5).

In summary, the results we report here indicate for the first time that HA can significantly enhance keratinocyte and fibroblast cellular responses. Although HA does not in the main enhance, nor indeed inhibit, the effect of VN:GF complexes on cellular responses of skin cells, the degradation products of HA, such as the oligosaccharides and very low molecular weight HA, may enhance chronic wound healing in the *in vivo* chronic wound environment. In fact, low molecular weight HA has previously been shown to induce secretion of antimicrobial peptides such as β-defensin 2 [66], human beta defensin (HBD) 1, HBD2, HBD3, human cathelicidin (LL37), secretory leukocyte protease inhibitor (SLPI) and lactoferrin [67]. In addition, oligosaccharides of HA have been found to induce angiogenesis through CD44 and RHAMM-mediated signalling pathways [68]. In contrast, low molecular weight HA may also hinder chronic wound repair due to their reported roles in multiple pro-inflammatory activities [69]. However, we believe that the widely documented positive effects of low molecular weight HA and its associated oligosaccharides are likely to outweigh any potential increase in inflammation. While our data does not indicate an enhancement of the cellular responses to VN:GF, the use of HA as a delivery vehicle for VN:GF in wound healing applications may confer other advantages, including the physicochemical properties of HA noted earlier, which confer it with an ability to sustain elevated pericellular concentrations of these species, thus potentially prolonging interactions with cell surface receptors and protecting them from degradation. Indeed, HA has previously been successfully used as a delivery vehicle for a range of dermal drugs, including diclofenac [11], ibuprofen [70], Clindamycin phosphate [71] and cyclosporine [72]. The beneficial effects of HA in these situations has been reported to include: enhancing both the partitioning of drugs into human skin and its retention and localization in the epidermis; and minimizing percutaneous absorption of drugs and assisting the transport of drugs to the epidermis [73]. HA has also been employed to deliver protein and peptide drugs such as insulin and recombinant human interleukin 11 (rhIL11) to enhance the absorption of the hormone or/and achieve a sustained release [74, 75]. Our future studies will therefore be focussed on determining the underlying mechanisms behind the coordinated regulation between VN:GF and HA, and exploring those parameters mentioned in the studies cited above. In addition we aim to investigate the chemical modification and crosslinking of HA monomers into hydrogel materials [76]. For example, temperature-sensitive HA hydrogels synthesized by photopolymerization of vinyl group modified HA in combination with acrylate group end-capped poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) tri-block copolymer resulted in a sustained release of recombinant human growth hormone [77]. In addition, an injectable HA-tyramine hydrogel system has been shown as a suitable injectable and biodegradable system for the delivery of therapeutic proteins [78]. Such modified or crosslinked HA hydrogels could protect the VN:GF proteins from thermal, pH and proteolytic changes *in vivo*, thus providing more effective and controlled delivery and release *in vivo* [79].

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Disclosure Statement

Some authors (Z.U. and D.I.L.) have a duality of interest and hold shares in Tissue Therapies Ltd.

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