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Research Paper

Distribution of fibroblast growth factor-2 (FGF-2) within model excisional wounds following topical application

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Abstract

Objective To characterise the magnitude and distribution of fibroblast growth factor-2 (FGF-2) following topical application in hypromellose gel and film formulations or a solution in an animal wound model, in order to assess the potential of this route for treatment of chronic wounds.

Method Topical formulations of FGF-2 were applied to punch biopsy wounds, and FGF-2 levels within the wound measured. Each 12 mm diameter wound received 0.3 μg FGF-2 in solution, a 7% (w/w) hypromellose gel, a dried hypromellose film on Melolin-backing or a saline control. After 2, 5 or 8 h the wounds were horizontally dissected into four sections (surface granulation, subcutaneous fat, superficial muscle and deep muscle) which were then analysed for FGF-2 concentration using ELISA. Confocal microscopy was used to evaluate the distribution of FGF-2 within the wound.

Key findings There were significant differences in the mean FGF-2 levels with respect to formulation and time following application ($P < 0.05$). FGF-2 penetrated faster into tissue when formulated as a solution than as a gel or a film. There did not appear to be a significant difference between the gel and the film with respect to total concentrations achieved in the tissue, although confocal microscopy showed differences in FGF-2 distribution within the wound.

Conclusions Delivery of FGF-2 to wounds in a solution gave the greatest increase in tissue FGF-2 concentration when measured by ELISA and visualised using confocal microscopy. Gel and film formulations prolonged the release of FGF-2 into the wound, although FGF-2 levels were not significantly different from controls when measured by ELISA. Confocal microscopy highlighted the differences in the penetration and distribution of the FGF-2 within the wound when released from different formulations.

Keywords fibroblast growth factor-2 (FGF-2); growth factors; hypromellose; wound healing

Introduction

It has been suggested that chronic wounds are slow to heal partly because of a relative deficiency of crucial growth factors.[1–5] In an attempt to convert a chronic wound environment into one that more closely resembles an acute wound, researchers have investigated the potential of topically applied growth factors.[1–6] The factors studied include platelet-derived growth factor, epidermal growth factor and the fibroblast growth factor (FGF) family. These experiments have provided conflicting results as to the benefit of topical growth factors.[7] Reasons for this may include the use of different wound models (i.e. animal, human or diabetic), study end points (breaking strength, re-epithelialisation or time to complete wound closure) and delivery vehicles (solution, spray or gel).[8]

For a topical growth factor therapy to be successful, it is likely that the active factor needs to penetrate into the wound to the base of the granulation tissue.[9] Very little work has been conducted on the distribution of topical growth factors within a wound environment following topical application. Cross & Roberts showed that small (molecular weight < 234 Da) hydrophilic solutes penetrated as far as abdominal fat, but penetration of growth factors (epidermal growth factor 6000 Da and FGF-2 17 000 Da) was poor.[9] The role that the delivery system plays in growth factor therapy has often been ignored, but effects may modulate therapeutic outcomes by optimising the input kinetics to match growth factor clearance,[10,11] especially within chronic wounds where high protease levels

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and accelerated clearances are reported.[12,13] Some have hypothesised that a formulation that targets the responsive cells and maintains adequate pharmacological levels within a wound could offer therapeutic advantages.[14]

The FGF family has been widely studied, and members of this family have been reported to play a significant role in wound healing.[15–17] In particular, FGF-1 and FGF-2 have numerous biological activities important in wound healing.[18–21] These include being mitogenic and chemotactic for vascular endothelial cells and being mitogenic for fibroblasts.[22] FGF-2 was chosen for this current study because it has been claimed to be the most potent cytokine stimulant for angiogenesis.[23] FGF-2 normally appears within hours after wounding and stimulates the migration and proliferation of fibroblasts, production of collagenase by fibroblasts, and plasminogen activator and collagenase activity in endothelial cells.[22,24] Although FGF-2 is normally bound to the extracellular matrix, how it is released from cells remains unclear, as it lacks a signal peptide sequence for secretion.[25] It has been suggested that it may simply be released by damaged cells to exert its local activity.[26]

Some case reports have shown success with an FGF-2 spray,[27] and experimental evidence suggests that controlled delivery of FGF-2 gives greater wound healing than a solution of FGF-2.[28] Thus, techniques to control the release of FGF-2 and hence increase the potential benefit of topical FGF-2 have been investigated. Hydrogel and gel formulations are attractive, as they potentially add further benefit by maintaining a moist wound environment, allowing oxygen transport, and often providing analgesic effect by preventing desiccation of the wound. Additionally, these materials do not adhere strongly to the wound as it heals,[29] so removal should not adversely affect the healing wound tissue.

The aims of this study were to quantify intra-wound FGF-2 concentrations in an animal wound model following topical application in hypromellose gel and film formulations, to determine the magnitude and duration of FGF-2 delivery with these materials. Confocal microscopy was used as a complimentary technique to visualise the distribution of FGF-2 within wounds following topical application.

Materials and methods

Recombinant human basic FGF derived from *Escherichia coli* and Quantikine human FGF basic immunoassay kits were obtained from RnD Systems (Minneapolis, MN, US). Alexa Fluor 555 dye was purchased from Molecular Probes Invitrogen (Eugene, OR, US). Triton X-100 and 4,2-aminoethylbenzenesulfonylfluoride were purchased from Sigma-Aldrich Pty Ltd (Sydney, Australia). Hypromellose, Methocel, E4M CR Premium EP was obtained from Colorcon (Dartford, UK). Melolin was a gift from Smith and Nephew Ltd (Auckland, New Zealand). All other chemicals were analytical grade and were purchased from BDH Chemicals New Zealand Ltd (Palmerston North, New Zealand).

Fluorescence labelling of FGF-2

FGF-2 (25 μg) was dissolved in 25 μl phosphate-buffered saline (PBS), then 2.5 μl 1M $NaHCO_3$ and 1.9 μl dye (Alexa Fluor carboxylic acid/succinimidyl ester at 8.11 nmol/μl) were added. The resulting solution was incubated at room temperature for 15 min. The contents of the reaction tube were pipetted onto a resin bed in microdialysis vials before being centrifuged at 16 000*g* for 1 min.

Preparation of control and active topical formulations

Formulations were prepared as described previously.[30] Briefly, the control formulation consisted of a solution of PBS (20 mM, pH 6.5, ionic strength 150 mM). The solution formulation consisted of PBS with FGF-2 tagged with Alexa Fluor dye at a final concentration of 6 μg/ml. The gel formulation consisted of 7% (w/v) E4M CR hypromellose gel with FGF-2 tagged with Alexa Fluor dye at a concentration of 7 μg/ml. The film formulation was prepared from a 1% (w/v) E4M CR hypromellose gel with FGF-2 tagged with Alexa Fluor dye at a concentration of 2 μg/ml; 150 μl was dried onto a Melolin backing circle (diameter 1.4 cm) with the cotton removed. This gave a dried film with a loading of 0.02% FGF-2 (w/w) in E4M CR hypromellose.

Experimental wounds in Lewis rats

Ethical approval was obtained from the University of Otago, Wellington Animal Ethics Committee (Wellington, New Zealand).

Lewis rats, approximately 20 weeks of age, were used in this study. Rats received buprenorphine 0.075 mg/kg body weight (Reckitt Benckiser, Auckland, New Zealand) by subcutaneous injection 30 min before wounding and were then anaesthetised with 3% halothane in oxygen. The hair was removed from the dorsal surface, from the base of the skull to the hind limb area, using electric clippers. The skin was disinfected with povidone–iodine 10% (Orion Laboratories, Balcutta, Australia) and 70% ethanol. A full-thickness excisional wound was made using a 12 mm diameter biopsy punch. This wound was created 6 cm below the base of the skull and 1 cm to the right of the spinal axis. The wounds were photographed before formulations were applied.

Animals were housed singly and received food and water *ad libitum* during the study.

Wound FGF-2 disposition studies

Following wounding, one of the three FGF-2 formulations or the control formulation was applied: 50 μl PBS, 50 μl solution, 43 μl gel or a single film. This gave a dose of FGF-2 per wound of 0.3 μg, or control. At 2, 5 or 8 h, rats were euthanised by inhalation of carbon dioxide and cervical dislocation. Any excess formulation was removed from the wound surface, and underlying wound tissue was excised to a depth of approximately 5 mm, snap frozen in liquid nitrogen, and then stored at −80°C before analysis.

Excised wounds were cut longitudinally into halves. One half was then dissected horizontally into the respective tissue sections (surface granulation, subcutaneous fat, superficial muscle and deep muscle) along the lines of fascia, as illustrated in Figure 1. These sections were weighed and their thickness measured with digital vernier calipers (Kincrome AF/Metric Victoria, Australia) before analysis of FGF-2 by ELISA as described below. The other half was placed in a TissueTek Cryomold (Sakura, Victoria, Australia) with TissueTek Optimal

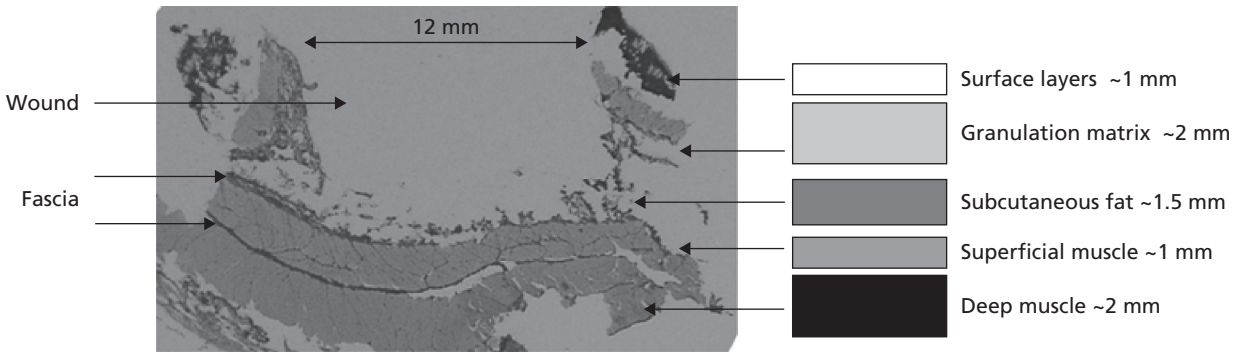

Figure 1 Photograph of a representative excised wound, indicating the tissue sections used for analysis and their approximate thicknesses.

Cutting Temperature compound (Sakura, Victoria, Australia) and then snap frozen in liquid nitrogen. During processing for histology, the wound sample was sliced longitudinally from the centre outwards in a cryostat, generating 7 μm thick sections. These sections were then mounted onto microscope slides and stained as described below for confocal microscopy.

FGF-2 concentrations

Following a similar process to that described by Gardner-Thorpe and colleagues,[31] the tissue sections were homogenised in 0.5 ml extraction buffer consisting of PBS, 0.05% Triton X-100 and 1 mM 4,2-aminoethylbenzenesulfonylfluoride (protease inhibitor). Following homogenisation, the samples were centrifuged at 1500*g* for 10 min at 4°C.

Human FGF basic immunoassay kits were used according to the manufacturer's instructions with standards of known concentrations. The FGF-2 concentration extracted from each tissue sample was determined using ELISA. The ELISA assay was linear over the FGF-2 concentration range 40–640 pg/ml ($R^2 > 0.99$). Experiments showed that addition of the fluorescent tag to FGF-2 had no significant effect on the assay. FGF-2 concentrations were expressed per mass of wet tissue.

Confocal microscopy

The prepared slides were stained with pontamine sky blue (0.1% aqueous) to identify collagen, as described by Vardaxis *et al.*[32] All imaging was conducted on a Zeiss LSM 510 confocal laser scanning microscope (Otago Centre for Confocal Microscopy). Channel one was set with an excitation of 488 nm and capture at 505–630 nm, which showed collagen as green. Channel two was set with an excitation of 543 nm and capture at greater than 650 nm to show the labelled FGF-2 as red.

Statistical analysis

Repeated-measures analysis of variance, performed using Minitab software (v 15; www.minitab.com), was used to compare tissue concentrations of FGF-2, because multiple measures (i.e. section) were performed in the same wound. Post-hoc pair-wise *t*-test with pooled SD and using the 'Holm' adjustment[33] were conducted using 'R' statistical software.[34]

Results

Wound tissue wet weight and thickness are given in Table 1. Figure 2 shows the FGF-2 concentrations within tissue sections,

Table 1 Characteristics of wound tissue

Tissue section	Mean thickness (mm)	Mean weight (g)
Surface granulation	1.28 ± 0.40	0.254 ± 0.074
Subcutaneous fat	1.20 ± 0.54	0.282 ± 0.140
Superficial muscle	1.07 ± 0.37	0.204 ± 0.076
Deep muscle	1.37 ± 0.43	0.201 ± 0.080

Values are means ± SD of wound tissue excised at 2, 5 and 8 h ($n = 71$).

as determined by ELISA. The analysis showed that only the main effects of formulation and time were significant ($P < 0.05$). Tissue section within time only marginally failed to reach significance ($P = 0.054$).

It can be seen that the solution formulation typically gave the highest FGF-2 tissue level (Figure 2). A post-hoc analysis showed that mean FGF-2 concentration was significantly higher with the solution formulation than with all other formulations and control ($P < 0.001$).

A post-hoc analysis showed significant differences in the mean FGF-2 concentrations at 2 versus 5 h ($P = 0.033$). No significant differences were seen when comparing the other time points.

Tissue FGF-2 concentrations were not significantly greater than control for either the gel or film formulations. The highest FGF-2 tissue concentrations occurred 2 h after application (Figure 2) and concentrations were highest in the outer two tissue sections (surface granulation and subcutaneous fat layers, Figure 2), although the differences between tissue sections were not statistically significant ($P = 0.054$).

Confocal microscopy allowed visualisation of the fluorescent tag attached to the FGF-2 molecule. Typical appearances of tissue sections are shown in Figures 3–6. Control wound sections (Figure 3) show only the appearance of green-labelled collagen. Following application of FGF-2 in solution (Figure 4), a large amount of red fluorescence was observed in the outer tissue layer at 2 h. At 5 h there appeared to be an accumulation of FGF-2 at the granulation tissue level and a lower concentration on the outer surface; by 8 h there was a more widespread distribution throughout the upper granulation tissue layer. Tissue sections had a similar appearance after application of the gel formulation (Figure 5), although the intensity of the red fluorescence did not appear as strong. For the film formulation (Figure 6) there is no obvious FGF-2 present at

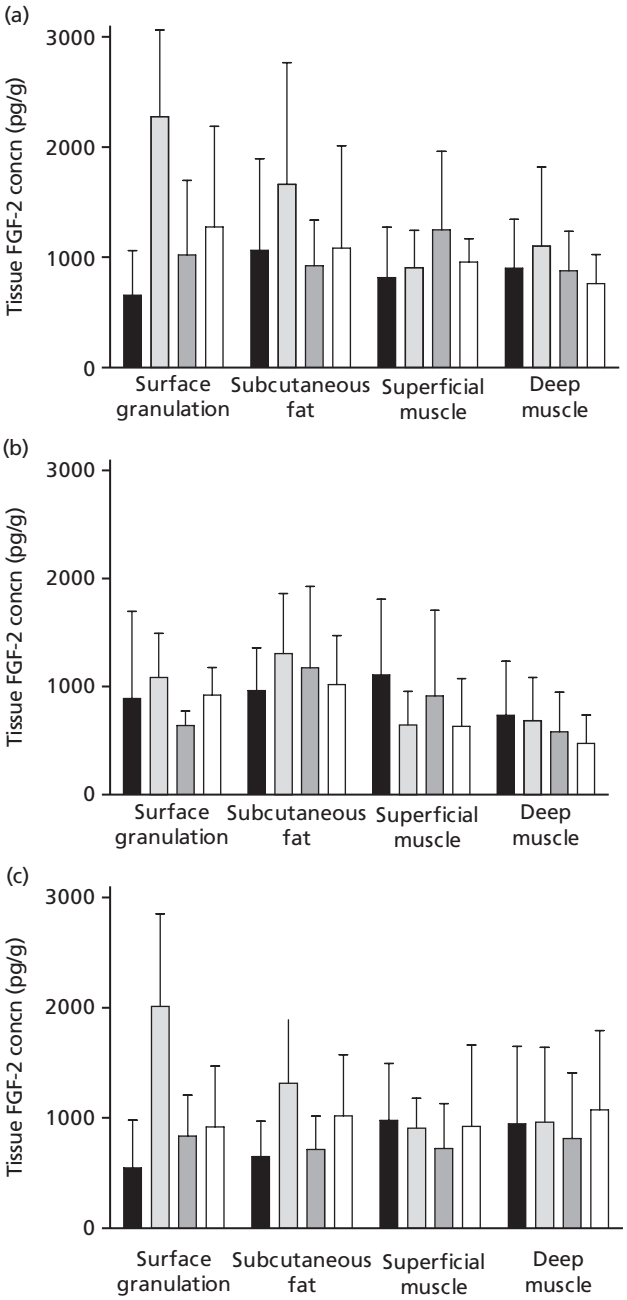

Figure 2 Concentrations of fibroblast growth factor-2 (FGF-2) within tissue sections at (a) 2 h, (b) 5 h and (c) 8 h after application of control (■), solution (▫), gel (▪) and film (□) formulations of FGF-2. Values are mean ± SD (n = 6 except film at 5 h where n = 5).

2 h, but some accumulation appeared at 5 h and a low-intensity diffuse distribution was observed at 8 h.

Discussion

As discussed previously, there is evidence that reduced levels of key growth factors within chronic wounds may be one reason why wounds fail to heal.[1–5] To address this, there have been some attempts to replace such deficits through topical application of growth factors, in both animal models of impaired wound healing and patients with chronic wounds. However, variable results of effectiveness have been reported.[8,10] Some explanations proposed for conflicting efficacy results have included differences in doses applied, the use of different formulation vehicles and comparison of results using different animal models.

The success of topical growth factor therapy may be improved by choosing an appropriate delivery system. With FGF-2, wound healing efficacy is increased when it is applied using a controlled-release system such as gelatin microspheres compared with a solution.[28,35] In addition, the use of hydrogels may prolong growth factor release and prevent wound desiccation. This has led to the development of several delivery systems for FGF-2.[29,36,37] However, there has been little investigation into the distribution of the growth factor following application of these various hydrogel-based systems.

Cross and Roberts[9] have shown that the permeability of wounds to large molecules such as growth factors decreases with wound healing. Therefore, before any clinical assessment of wound healing effects, it is important to characterise whether a topical formulation achieves delivery into the wound tissue. To address this question with hypromellose gel and film formulations, FGF-2 levels within the wounds at tissue depths similar to those used by Cross and Roberts were investigated. We used two techniques in this study: ELISA and confocal microscopy. A commercially available ELISA assay was used, as this assay is rapid, reproducible and measures FGF-2 concentrations as low as 100 pg/ml, which is in line with the FGF-2 concentrations that may be expected in the wound.[38] A limitation of ELISA is that it does not differentiate between endogenous and exogenous FGF-2; however, control experiments determined the normal concentration of endogenous FGF-2 following wounding. For this reason, a secondary technique was used in which FGF-2 was tagged with a fluorescent label and tissue sections were analysed by confocal microscopy. The images that resulted from the confocal analysis showed the relative distribution of the applied FGF-2, allowing qualitative assessment of the tissue distribution of topically applied FGF-2.

This experiment examined FGF-2 distribution in wounds following application of different topical formulations. The solution gave the highest FGF-2 concentrations, which may be explained by the FGF-2 being readily available in the wound environment immediately after application. Interestingly, the exogenously applied FGF-2 was still observed by confocal microscopy in wound tissues 8 h after application of the solution. In comparison, application via the gel and particularly the dried films delayed penetration of FGF-2 into the wound. Analysis by ELISA showed no significant difference in tissue FGF-2 concentrations between the gel, film and control formulations. However, differences were seen by confocal microscopy, with greater FGF-2 concentration in the wound tissue following application of the gel formulation. These differences may be explained by the potential fate of exogenously applied growth factors, including binding to sites within the wound (to both activate targets and to the extracellular matrix), degradation, especially if high protease

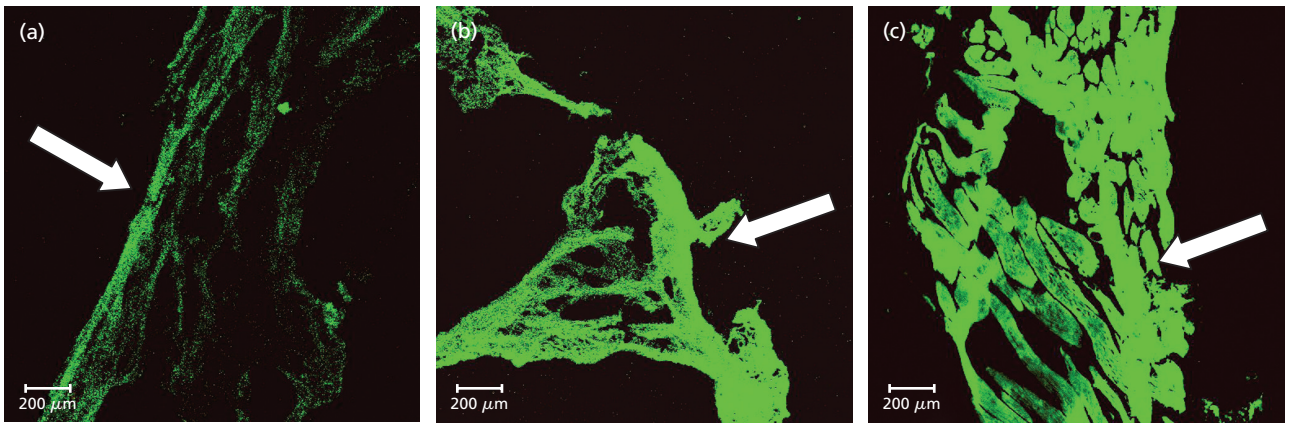

Figure 3 Representative confocal microscopy images of control tissue sections taken after (a) 2 h, (b) 5 h and (c) 8 h. Green represents collagen tissue; red represents exogenous fibroblast growth factor-2 (FGF-2). White arrow indicates wound surface. Scale = 200 μm.

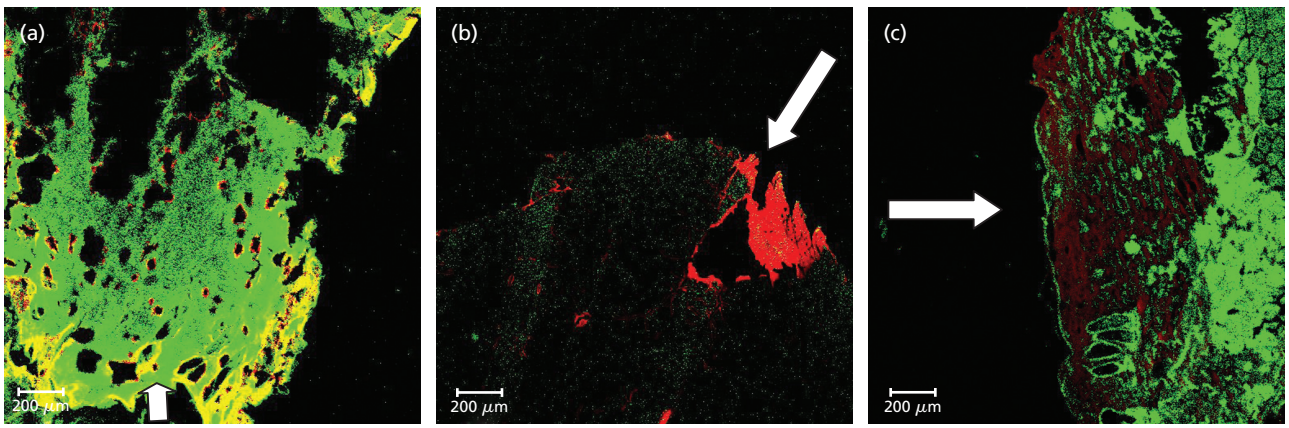

Figure 4 Representative confocal microscopy images of tissue sections treated with the solution formulation taken at: (a) 2 h, (b) 5 h and (c) 8 h. Green represents collagen tissue; red represents exogenous fibroblast growth factor-2 (FGF-2). White arrow indicates wound surface. Scale = 200 μm.

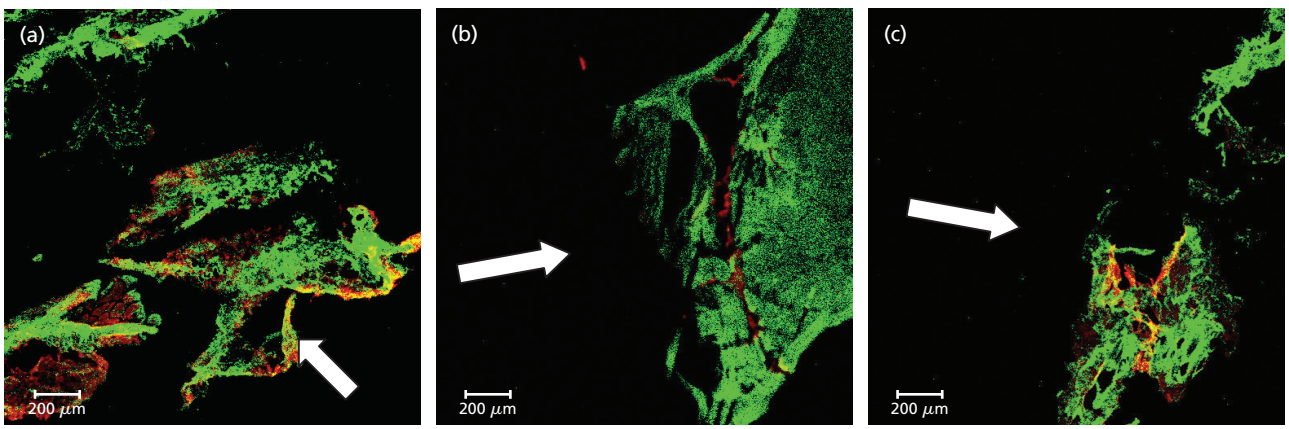

Figure 5 Representative confocal microscopy images of tissue sections treated with gel formulation taken at: (a) 2 h, (b) 5 h and (c) 8 h. Green represents collagen tissue; red represents exogenous fibroblast growth factor-2 (FGF-2). White arrow indicates wound surface. Scale = 200 μm.

concentrations are present in the wound environment, and systemic absorption. In the current study, high initial concentrations were seen with the solution, as there is no rate-limiting step for the FGF-2 to enter the wound, compared with gel and film formulations where adequate hydration is required for release. The fate of the free FGF-2 can be local degradation, systemic absorption and local binding.

Given that one potential fate of the FGF-2 is local degradation, it is possible that the fluorescence seen is the remainder of the tag following this degradation. In addition,

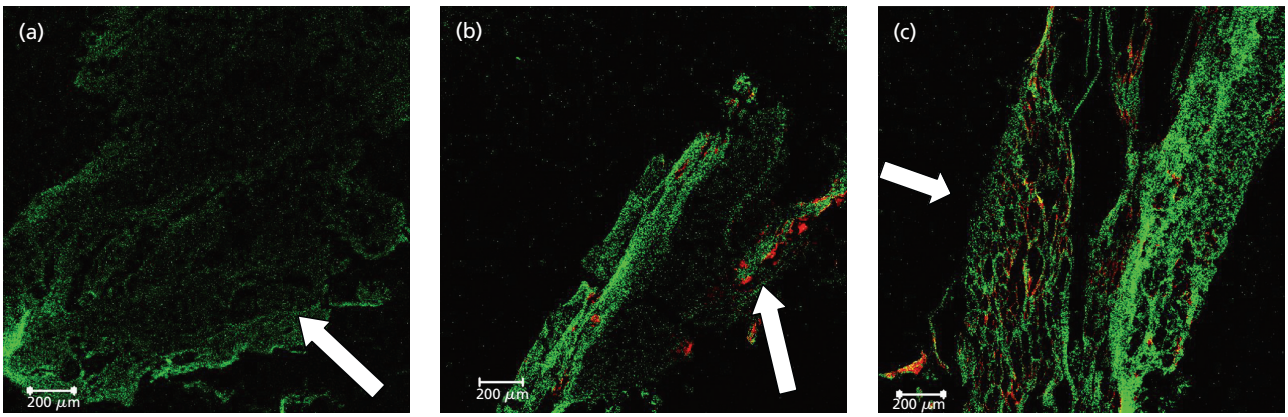

Figure 6 Representative confocal microscopy images of tissue sections treated with the film formulation taken at: (a) 2 h, (b) 5 h and (c) 8 h. Green represents collagen tissue; red represents exogenous fibroblast growth factor-2 (FGF-2). White arrow indicates wound surface. Scale = 200 μm.

this fluorescence may be due to FGF-2 binding to sites within the wound.

The film formulation produced only a low level of tissue fluorescence due to labelled FGF-2. This low tissue concentration of FGF-2 in wounds after application of a film could be caused by a number of factors, including degradation of FGF-2 during the formulation preparation, insufficient wound exudate to fully hydrate the film, and retention of FGF-2 on the wound dressing. Previously reported in-vitro release studies for the film formulation were able to detect 86–100% of FGF-2 released into diffusion cells within 5 h.[30] In that study, FGF-2 release was measured by ELISA. It is therefore unlikely that the low tissue concentrations measured in the wound tissue following application of the gel formulation were due to physical degradation of FGF-2 during preparation of the hypromellose formulations. However, as the in-vitro experiments were conducted in sink conditions, lack of wound exudate that would be found *in vivo* may have been a factor.

It is also possible that the addition of FGF-2 caused a down-regulation of endogenous FGF-2 release,[39] leading to concentrations that were not significantly above those in the control group. This may explain why the absolute concentrations determined by ELISA were comparable to the controls, but differences were seen with confocal microscopy. It is important to note that excess gel and film formulations were removed after the experimental time period and so, as mentioned above, the lack of wound exudate may have delayed the release from the formulations, such that not all of the applied FGF-2 was recovered in the tissue sections.

There are some differences in the results obtained with ELISA and confocal microscopy. It is important to recognise that the ELISA results are averaged and shown as a set of six, whereas the confocal images are analysed individually. Additionally, one-half of the wound was used in the ELISA and the remaining half was used for microscopy, and it is possible that the FGF-2 was not distributed evenly in the two halves. This said, however, the combination of both techniques provides insight into the distribution of the factor within the wound, and not simply the FGF-2 concentrations.

As expected, the concentration of FGF-2 decreased with the depth of tissue, which is related to the freedom of the FGF-2 to diffuse through the tissue.[9] Several factors may affect the rate of this diffusion, including the release rate from the formulation, the degree of binding of the FGF-2 to the extracellular matrix, and exposure to the proteases in the wound environment.[40,41] Although the studied formulations appeared to produce only modest elevation of FGF-2 above control at the 8 h time point, these studies were conducted in healthy animals without impaired healing and able to upregulate production and release of endogenous FGF-2.

While the FGF-2 solution gave the highest tissue levels, it may not be the most appropriate formulation for wound healing. This bolus of FGF-2 within the wound may actually down-regulate receptors or cause only a transient benefit.[39,42] In comparison, a lower concentration over a prolonged period of time may aid the retention of the growth factor within the wound.[43] For these reasons, the use of a commercially available dressing was considered potentially useful and may have additional clinical benefit. The dressing chosen (Melolin (Smith & Nephew, Hull, UK)) is a low-adherent absorbent wound dressing composed of three layers: a thin perforated polyester film, a highly absorbent cotton/polyester pad, and a hydrophobic outer backing layer which can be removed from the wound after FGF-2 release is complete without disrupting the newly forming granulation tissue. Additionally, using a wound dressing as a controlled-release platform may assist uniform coverage of the formulation over the wound surface. To investigate the use of Melolin as a platform for the administration of a prolonged-release system for growth factors to wounds, the polyester film was carefully removed from the cotton layer and hypromellose containing FGF-2 was added onto the polyester film surface before drying. It is proposed that hydration of the polymer on contact with the wound fluid would produce a gel from which the growth factor would be released over an extended period. This would also reduce the potential loss of growth factor due to formulation drainage over adjacent healthy skin, which might be problematic for liquid formulations such as solutions and gels.

Conclusions

This study used two techniques to determine penetration of FGF-2 into wounds from three formulations. It showed that the delivery of FGF-2 to wounds in a solution gave the greatest increase in tissue FGF-2 concentration when measured by ELISA and visualised using confocal microscopy. The gel and film formulations prolonged the release of FGF-2 into the wound, although FGF-2 levels were not significantly different from controls when measured by ELISA. Confocal microscopy images highlighted the differences in the penetration and distribution of the FGF-2 within the wound when released from different formulations.

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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