Topical PDT following excisional wounding of human skin increases production of TGF- β 3, MMP-1 and MMP-9 with associated improvement in dermal matrix organisation

S.J. Mills,^{1,2*} M.D. Farrar,^{2*} G.S. Ashcroft¹, C.E.M. Griffiths,² M.J. Hardman¹ and L.E. Rhodes²

¹Faculty of Life Sciences, University of Manchester, Manchester Academic Health Science Centre, Manchester, UK

²Dermatology Centre, Institute of Inflammation and Repair, University of Manchester, Manchester Academic Health Science Centre, Salford Royal NHS Foundation Trust, Manchester, UK

*These authors contributed equally to this study.

Running head: Modulation of wound healing by MAL-PDT

Correspondence: Lesley E. Rhodes, Photobiology Unit, Dermatology Centre, University of Manchester, Salford Royal NHS Foundation Trust, Manchester, M6 8HD, UK. E-mail: Lesley.E.Rhodes@manchester.ac.uk; Tel: +44 161 2061150; Fax: +44 161 2061156.

Funding source: This study was jointly funded by Galderma Research and Development France and The University of Manchester.

Conflicts of interest: This study was sponsored by Galderma Research and Development France who part-funded the PhD studentship of S.J.M. and contributed to the study design. The authors declare no conflicts of interest.

Word count: 2838

Tables: 1

Figures: 5

What's already known about this topic?

- Photodynamic therapy (PDT) is reported to significantly enhance skin healing after excisional wounding in animal models.
- No equivalent studies have been reported in humans.

What does this study add?

- Topical PDT following excisional wounding of human skin leads to increased production of transforming growth factor β 3 and matrix metalloproteinase-1 and -9. This is accompanied by more orderly dermal matrix deposition.
- This suggests the potential further development of topical PDT to enhance healing of excisional wounds.

Summary

Background Animal studies report photodynamic therapy (PDT) to improve healing of excisional wounds but the mechanism is uncertain and equivalent human studies are lacking.

Objective To explore the impact of methyl aminolaevulinate (MAL)-PDT on clinical and microscopic parameters of human cutaneous excisional wound healing, examining for potential modulation through production of transforming growth factor (TGF)- β isoforms.

Methods In healthy older men (60-77 years; $n=27$), a 4 mm punch biopsy wound was created in skin of the upper inner arm and treated with MAL-PDT three times over five days. An identical control wound to the contralateral arm was untreated and both wounds left to heal by secondary intention. Wounds were re-excised at time points examining the inflammatory phase (7 days, $n=10$), matrix remodelling (3 weeks, $n=8$) and cosmetic outcome/dermal structure (9 months, $n=9$). Production of TGF- β 1, TGF- β 3 and matrix metalloproteinases (MMPs), key mediators of matrix deposition and remodelling, was assessed by immunohistochemistry alongside microscopic measurement of wound size/area and clinical assessment of wound appearance.

Results MAL-PDT delayed re-epithelialisation at 7 days, associated with increased inflammation. However, 3 weeks post-wounding, treated wounds were smaller with higher production of MMP-1 ($P=0.01$), MMP-9 ($P=0.04$) and the anti-scarring cytokine, TGF- β 3 ($P=0.03$). TGF- β 1 was lower than control at 7 days and higher at 3 weeks (both $P=0.03$). At 9 months MAL-PDT treated wounds showed greater, more ordered deposition of collagen I, collagen III and elastin (all $P<0.05$).

Conclusions MAL-PDT increases MMP-1, MMP-9, and TGF- β 3 production during matrix remodelling, ultimately producing scars with improved dermal matrix architecture.

Introduction

Wound healing is a complex, organised process by which the body responds to injury and is characterised by a series of overlapping events including clotting, inflammation, re-epithelialisation and tissue remodelling.¹ Wound repair is incompletely understood at the mechanistic level, and much current research is focussed on identifying ways to improve the rate and/or quality of healing (i.e. reducing scarring). Scarring can have a significant effect on skin function including reduced tensile strength and restriction of movement.² In addition, scarring can have psychological impact with cutaneous scars causing high levels of anxiety and negatively impacting on personal relationships, work and leisure activities.³

Topical photodynamic therapy (PDT) is a widely used in non-melanoma skin cancer.^{4,5} A prodrug i.e. aminolaevulinic acid (ALA) or a lipophilic derivative, methyl aminolaevulinate (MAL), is administered followed by its relatively selective uptake by the target tissue and metabolism to protoporphyrin IX. In the presence of oxygen, photochemical activation of protoporphyrin IX generates reactive oxygen species, leading to altered cell signalling, cell damage and/or death. Clinical observations suggest PDT may be beneficial in wound healing, and it produces good-excellent cosmetic outcome.⁶⁻⁸ The potential of PDT in wound healing has been investigated in a limited number of *in vitro* and animal studies. Jayasree *et al.* reported an increase in wound closure rate, fibroblast proliferation and re-epithelialisation following ALA-PDT in a rat excisional wound model.⁹ Other animal studies using topical photosensitisers demonstrated more rapid re-epithelialisation and increased collagen deposition when PDT was applied following skin biopsy or burn.^{10,11} Human studies are scarce, but a positive influence on wound healing is suggested in leg ulcers^{12,13} and in a patient with a basal cell carcinoma treated with MAL-PDT following Mohs micrographic surgery.¹⁴

Transforming growth factor (TGF) β isoforms are important in all stages of wound healing, with TGF- β 1 and TGF- β 2 promoting inflammatory cell recruitment and collagen production, and TGF- β 3 associated with reduced scarring through promotion of collagen organisation.^{15,16} *In vitro* studies have shown PDT to increase endothelial cell proliferation via reduced production of TGF- β presumed to be TGF- β 1.¹⁷ Moreover, matrix metalloproteinases (MMP)-1, 2 and 9 play an important role at the matrix remodelling stage and their activity can be modulated by TGF- β .¹⁸ Thus, PDT could potentially influence wound healing through modulation of TGF- β isoforms. The aim of our novel study was to examine the effects of topical MAL-PDT on wound healing and cosmetic outcomes following excisional wounding in humans, and to explore underlying mechanisms with particular focus on production of TGF- β isoforms. Impact of PDT on the early inflammatory response to wounding was also explored. While in general inflammation is associated with delayed healing,¹⁹ the influence of the acute inflammatory events associated with PDT is unknown.

We examined the effect of topical PDT on the wound healing process in acute excisional wounds in human skin *in vivo*. Wounds were created bilaterally in the upper inner arm skin, and 3 MAL-PDT treatments were given to the actively treated wound while the untreated wound on the contralateral arm provided intra-subject control. Wounds were re-excised at 3 weeks in a subgroup of volunteers to permit examination of the matrix remodelling phase, and at 7 days and 9 months in further subgroups, to assess effects on inflammation (early) and scarring (late), respectively. Healing was quantified by image analysis of histological sections, and wound expression of TGF- β isoforms, MMPs, and deposition of dermal matrix proteins was explored following PDT.

Materials and methods

Participants

This study was approved by the Central Manchester Research Ethics Committee (Ref. 05/Q1407/214) and was conducted according to the Declaration of Helsinki principles.

Participants gave their written informed consent. Twenty-eight healthy white Caucasian men of sun-reactive skin type I-IV were recruited (median age 68 years, range 60-77 years) with one subject later requesting to withdraw from the study. Older men were selected for study as their skin shows slower healing²⁰ and thus PDT impact on wound healing may be more evident. The volunteers were not taking immunosuppressive or anti-inflammatory drugs, steroids or any drugs affecting blood coagulation; none had used any topical therapy on the skin area to be studied in the previous 3 months, and none had a history of wound healing/bleeding disorders.

Study design

Subjects were randomised to receive active treatment on the left or right arm. The randomisation schedule was generated using the RANUNI routine in SAS (SAS Institute Inc., Cary, USA). A 4 mm punch biopsy was taken from each inner (sun-protected) upper arm of each participant, under local anaesthetic. One wound was dressed and allowed to heal without intervention. The other wound was treated with MAL-PDT immediately after wounding (day 0) and again on days 2 and 4. After 7 days ($n = 10$ subjects), 3 weeks ($n = 8$ subjects) or 9 months ($n = 9$ subjects), wounds were evaluated macroscopically then removed with a 6 mm punch biopsy. Biopsies were formalin fixed and wax-embedded for histology and immunohistochemistry. MAL-PDT was performed by the study nurse while other investigators, including those performing the clinical wound assessments, were blinded to the nature of the treatment.

MAL-PDT procedure

For MAL-PDT, 1.5 g MAL (Metvix[®], 160 mg g⁻¹, Penn Pharmaceuticals, Gwent, UK) was applied to a 3 x 3 cm area over and around the wound, then dressed and left for 3 h. Following cleaning with normal saline the treatment area was exposed to 80 mW cm⁻² red light (Aktilite CL128 LED lamp, Galderma, Paris, France) for 9 min (total dose 37 J cm⁻²).

Macroscopic and microscopic assessments

At the specified time points, wounds were scored clinically for erythema, oedema, colour, contour and texture based on the scheme described by Beausang *et al.*²¹ (Table 1). Scoring was performed blind by 2 examiners directly assessing the participants' skin. For microscopic examination, formalin-fixed, paraffin-embedded biopsy samples were sectioned (5 µm) and dried onto Vectabond[™] (Vector Laboratories, Peterborough, UK)-coated slides at 60°C overnight. Sections were stained with haematoxylin and eosin and the wound width, area and percentage re-epithelialisation determined by image analysis (Image Pro-Plus, Media Cybernetics, Finchampstead, UK). Wound width was defined as the straight-line distance between normal tissue either side of the wound. Wound area was the area under the epidermis that was different from the surrounding normal tissue.

Immunohistochemistry, dermal matrix staining and antibodies

Antigen retrieval of slide-fixed biopsy sections was carried out in 10 mM citrate buffer (pH 6.0) using a microwave. Endogenous peroxidase activity was blocked by incubation in 0.3% (v/v) hydrogen peroxide for 30 min. Immunostaining by the peroxidase method was performed using

the Vectastain ABC kit according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). The antibodies for detection of CD15, CD68, MMP-1, MMP-2 and MMP-9 were from Millipore (Watford, UK). Collagen I, collagen III and fibronectin antibodies were from Sigma-Aldrich (Poole, UK). Antibodies for TGF- β 1, TGF- β 2 and TGF- β 3 were from Santa Cruz (Wiltshire, UK). Antibody for macrophage migration inhibitory factor (MIF) was from R&D Systems (Oxon, UK). Elastin fibres were stained using Weigert's solution (Sigma-Aldrich, Poole, UK). Isotype controls were used in all immunostaining experiments. Quantification was performed by counting positively-stained cells in 5 random areas throughout the wound bed, in each of 2 biopsy sections and the mean number of cells/mm² calculated. Matrix deposition was scored on a scale from 0 to 4 with 0 representing deposition throughout the wound that resembles that of normal skin, and 4 representing little or no matrix deposition.

Measurement of trans-epidermal water loss

Skin barrier function of wounds and adjacent unwounded skin was assessed by measuring trans-epidermal water loss using a Tewameter TM 210 (CK Electronic, Cologne, Germany) according to the manufacturer's instructions.

Statistical methods

Differences between treated and untreated wounds were tested using the paired t-test. Non-parametric data were compared using the Wilcoxon matched pairs test. Significance was accepted at $P \leq 0.05$. Data were analysed using Microsoft Excel (Microsoft Corporation, Washington, USA) and StatsDirect (v2.7.7. StatsDirect Limited, Altrincham, UK). Data are mean $\pm$ SEM.

Results

Treatment tolerability

MAL-PDT was well tolerated by all subjects. One volunteer had an adverse event that was unrelated to the study treatment and he continued to participate in the study. One subject in the 9 month group withdrew before the end of the study. This was not related to an adverse event. Not all analyses were based on the full number of subjects due to availability of tissue.

MAL-PDT treatment delays early wound re-epithelialisation but healing is improved at 3 weeks

Wound size (histological area and width) was unaffected by MAL-PDT, while re-epithelialisation was delayed at 7 days post-wounding ($34.8 \pm 9.0\%$) compared with control wounds ($94.3 \pm 5.8\%$, $P = 0.02$; Fig. 1a,b). By 3 weeks, all wounds were fully re-epithelialised with no significant difference between treated and control groups for wound width (3.1 ± 0.2 mm and 3.1 ± 0.1 mm respectively) or area (3.1 ± 0.2 mm² and 3.8 ± 0.3 mm² respectively). Trans-epidermal water loss (TEWL), an indicator of skin barrier function, was significantly higher from wound sites than from normal skin for both MAL-PDT treated (43.9 ± 5.6 g m⁻² h⁻¹ and 8.6 ± 5.9 g m⁻² h⁻¹ respectively, $P < 0.001$, $n = 10$) and control wounds (21.9 ± 2.8 g m⁻² h⁻¹ and 10.7 ± 2.5 g m⁻² h⁻¹ respectively, $P = 0.03$, $n = 10$) after 7 days, with TEWL from MAL-PDT treated wounds being higher than that from untreated wounds ($P = 0.002$). After 3 weeks these differences were no longer significant indicating barrier function had been restored. Visual examination and scoring of wound appearance at 3 weeks indicated MAL-PDT-treated wounds to be significantly closer in appearance to normal skin than untreated wounds with respect to

erythema ($P = 0.01$) and colour ($P = 0.008$; Fig. 1c,d). An overall assessment of treated v untreated wounds showed the treated wound to be superior in appearance in 7/8 subjects ($P = 0.04$).

MAL-PDT induces increased production of TGF- β 3

Of crucial relevance to the previously reported anti-scarring potential of PDT, differences in the levels of TGF- β isoforms were observed between MAL-PDT and control wounds. Following MAL-PDT, wounds showed a profile of significantly reduced TGF- β 1 ($P = 0.03$; Fig. 2a,b) at 7 days post-wounding. Moreover, at 3 weeks post-wounding, MAL-PDT treated wounds had a profile of significantly higher TGF- β 1 and TGF- β 3 production with treated wounds containing twice the number of TGF- β 3 producing cells ($P = 0.029$; Fig. 2c,d) compared to control wounds. Levels of TGF- β 2 were unchanged at both 7 days and 3 weeks.

MAL-PDT treatment alters the local inflammatory response

To investigate the effect of MAL-PDT on the post-wounding infiltration of inflammatory cells, neutrophils and macrophages were quantified by immunohistochemistry in wound biopsy sections at 7 days and 3 weeks. An apparent augmentation of neutrophil numbers occurred at 7 days in MAL-PDT treated wounds (604 ± 147 cells mm^{-2} compared with 342 ± 120 cells mm^{-2} in control wounds; Fig. 3a,b). Macrophage numbers were significantly higher in MAL-PDT treated wounds (294 ± 68 cells mm^{-2}) compared to control wounds (69 ± 25 cells mm^{-2} , $P = 0.03$; Fig. 3c,d) at 3 weeks post-wounding. However, the number of cells producing the pro-inflammatory cytokine MIF was lower in MAL-PDT treated wounds at 3 weeks (154 ± 116 cells mm^{-2}) compared to control wounds (330 ± 65 cells mm^{-2} , $P = 0.06$, $n = 6$).

MAL-PDT influences MMP production early in healing with a subsequent improvement of dermal matrix structure after 9 months

Wound-specific induction of matrix metalloproteinases (MMPs) is important for the processes of re-epithelialisation and matrix remodelling. Quantification of MMP-1, -2 and -9 expression in MAL-PDT versus control wounds revealed little difference at 7 days post-wounding (Fig. 4a,b), when an overt delay in re-epithelialisation was observed. By contrast, production of MMP-1 and MMP-9 was significantly higher in the dermal granulation tissue of MAL-PDT treated wounds at 3 weeks with 2082 ± 489 MMP-1 positive cells mm^{-2} and 1843 ± 397 MMP-9 positive cells mm^{-2} in MAL-PDT treated wounds compared to 631 ± 160 MMP-1 positive cells mm^{-2} ($P = 0.01$) and 745 ± 173 MMP-9 positive cells mm^{-2} ($P = 0.04$; Fig. 4c,d) in control wounds. Histological analysis of 9 month wound tissue revealed a significant improvement in the architecture of deposited matrix following MAL-PDT. Scoring of wound matrix deposition revealed the amount and distribution of collagen I ($P = 0.02$), collagen III ($P = 0.02$) and elastin ($P = 0.008$; Fig. 5a,b) in MAL-PDT wounds to be more similar to that seen in normal, unwounded skin than in control wounds. Visual assessment and scoring of wounds at 9 months showed MAL-PDT-treated tended to be closer in appearance to normal skin than controls and on overall assessment of treated v untreated, PDT-treated wounds were superior in appearance to controls in 7/9 subjects although this did not reach statistical significance ($P = 0.08$; Fig. 5c,d).

Discussion

To our knowledge this is the first reported study to directly investigate the effect of PDT on healing of acute excisional wounds in humans *in vivo*. We have shown that treatment with MAL-

PDT modulated clinical and microscopic parameters of healing and ultimately produced scars with improved dermal matrix architecture. Pivotaly, the number of TGF- β 3 producing cells was significantly higher in MAL-PDT treated wounds than controls after 3 weeks, with an elevated TGF- β 3: β 1 ratio. At 9 months MAL-PDT treated wounds showed improved deposition and organisation of dermal matrix protein at the histological level. Thus PDT treatment of wounds appears to mediate an anti-scarring phenotype with TGF- β 3 as a potential key modulator.

The TGF- β family was assessed in our study in view of its central role in wound healing and scar formation. The isoform TGF- β 1 can have pro- or anti-inflammatory activity depending on the differentiation state of target cells and presence of other cytokines.²² It promotes matrix formation and remodelling by upregulating production of extracellular matrix proteins (particularly collagens I and III) and inhibiting the activity or expression of MMPs.¹⁵ As a potent inducer of collagen production, higher TGF- β 1 in treated wounds could be expected to promote matrix deposition early in healing. However, in the current study, concomitantly increased MMP-1 and MMP-9 expression may account for unaltered matrix staining scores between treated and untreated wounds at 3 weeks. The role of TGF- β 2 in wound healing is similar to that of TGF- β 1, stimulating recruitment of inflammatory cells and fibroblasts to the wound site, and promoting collagen deposition. However, we found no effect of MAL-PDT on TGF- β 2 production at either the inflammatory stage or matrix remodelling stage of the wound healing process. TGF- β 3 facilitates keratinocyte migration and crucially, and in contrast to TGF- β 1, inhibits scar formation and improves collagen organisation *in vivo*.^{16,23} Thus, the increased TGF- β 3 production seen at 3 weeks may have influenced the latter stages of healing in MAL-PDT treated wounds, mediating more ordered matrix deposition. An interesting parallel to this is healing of the oral mucosa which exhibits accelerated healing and reduced scarring following

excisional wounding compared to normal skin.²⁴ Wounds in the gingiva of both pigs and humans have been shown to express higher levels of TGF- β 1 and TGF- β 3 than in skin wounds, associated with increased integrin α v β 6 expression, a possible activator of TGF- β .²⁵ Studies of mucosal healing and earlier studies of TGF- β in human skin indicate an increased ratio of TGF- β 3 to TGF- β 1 to be pivotal in mediating reduced scarring.^{16,25,26} Our data support this association and further investigations to explore the effect of PDT on regulators of TGF- β expression and activation in the context of wound healing in human skin are warranted.

Inflammation is a key, tightly regulated stage in wound repair characterised by a complex milieu of cytokines and growth factors²⁷ which influence cell behaviour and differentiation. An inappropriate inflammatory response, either excessive or reduced, can result in impaired healing.^{19,28} Our data show an altered inflammatory response following PDT, with increased neutrophil infiltration associated with early impaired healing (delayed re-epithelialisation). However, ultimately healing was improved at later time points with improved deposition and distribution of dermal matrix. While this initially appears paradoxical, it actually mirrors findings in the skin healing of elderly subjects where an inappropriately excessive, but ineffective inflammatory response results in a more reparative healing phenotype.²⁹ A further observation of note was apparently reduced levels of MIF in MAL-PDT treated wounds at 3 weeks. This proinflammatory cytokine is integral to the inflammatory response and an important regulator of inflammation in wound healing.^{30,31} Thus a key area for further study is to determine the mechanism by which MAL-PDT influences the phenotype of inflammatory cells and the balance of cytokines in the wound healing process. This includes exploration of macrophage phenotype as alternatively activated macrophages play an important role in the resolution of inflammation, promoting cell proliferation and collagen production.³²

In conclusion, this novel study has demonstrated that MAL-PDT treatment of excisional wounds in otherwise healthy human skin has positive effects on histological aspects of wound healing. The observed increased production of TGF- β 1 and TGF- β 3, and increased TGF- β 3:TGF- β 1 ratio may be important in directing a more orderly deposition of extracellular matrix proteins. Further work is indicated to confirm these findings and optimise protocols with a view to examining the effect of PDT in the analogous clinical situation of surgical excision.

Acknowledgements

The PhD studentship of S.J.M. was jointly funded by Galderma Research and Development France and The University of Manchester.

References

1. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008; **453**: 314-21.
2. Larson BJ, Longaker MT, Lorenz HP. Scarless fetal wound healing: a basic science review. *Plast Reconstr Surg* 2010; **126**: 1172-80.
3. Brown BC, McKenna SP, Siddhi K, *et al.* The hidden cost of skin scars: quality of life after skin scarring. *J Plast Reconstr Aesthet Surg* 2008; **61**: 1049-58.
4. Morton CA, Brown SB, Collins S, *et al.* Guidelines for topical photodynamic therapy. *Br J Dermatol* 2002; **146**: 552-67.
5. Morton CA, McKenna KE, Rhodes LE. Guidelines for topical photodynamic therapy: update. *Br J Dermatol* 2008; **159**: 1245-66.
6. Wang I, Bendsoe N, Klinteberg CAF, *et al.* Photodynamic therapy vs. cryosurgery of basal cell carcinomas: results of a phase III clinical trial. *Br J Dermatol* 2001; **144**: 832-40.
7. Rhodes LE, de Rie MA, Enstrom Y, *et al.* Photodynamic therapy using topical methyl aminolevulinate vs surgery for nodular basal cell carcinoma. *Arch Dermatol* 2004; **140**: 17-23.
8. Stebbins WG, Hanke CW. MAL-PDT for difficult to treat non-melanoma skin cancer. *Dermatol Ther* 2011; **24**: 82-93.
9. Jayasree RS, Gupta AK, Rathinam K, *et al.* The influence of photodynamic therapy on the wound healing process in rats. *J Biomater Appl* 2001; **15**: 176-86.
10. Garcia VG, de Lima MA, Okamoto T, *et al.* Effect of photodynamic therapy on the healing of cutaneous third-degree-burn: histological study in rats. *Lasers Med Sci* 2010; **25**: 221-8.
11. Silva JC, Lacava ZG, Kuckelhaus S, *et al.* Evaluation of the use of low level laser and photosensitizer drugs in healing. *Lasers Surg Med* 2004; **34**: 451-7.

12. Clayton TH, Harrison PV. Photodynamic therapy for infected leg ulcers. *Br J Dermatol* 2007; **156**: 384-5.
13. Morley S, Griffiths J, Philips G, *et al.* Phase IIa randomized, placebo-controlled study of antimicrobial photodynamic therapy in bacterially colonized, chronic leg ulcers and diabetic foot ulcers: a new approach to antimicrobial therapy. *Br J Dermatol* 2013; **168**: 617-24.
14. Reddy KK, Hanke CW, Tierney EP. Rapid wound re-epithelialization and basal cell carcinoma clearance after Mohs micrographic surgery with postoperative photodynamic therapy. *J Drugs Dermatol* 2010 ; **9**: 143-8.
15. Barrientos S, Stojadinovic O, Golinko MS, *et al.* Growth factors and cytokines in wound healing. *Wound Rep Reg* 2008; **16**: 585-601.
16. Shah M, Foreman DM, Ferguson MW. Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 1995; **108**: 985-1002.
17. Statius van Eps RG, Adili F, Watkins MT, *et al.* Photodynamic therapy of extracellular matrix stimulates endothelial cell growth by inactivation of matrix-associated transforming growth factor-beta. *Lab Invest* 1997; **76**: 257-66.
18. Schreml S, Szeimies R-M, Prantl L, *et al.* Wound healing in the 21st century. *J Am Acad Dermatol* 2010; **63**: 866-81.
19. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 2007; **127**: 514-25.
20. Ashcroft GS, Mills SJ, Ashworth JJ. Ageing and wound healing. *Biogerontology* 2002; **3**: 337-45.

21. Beausang E, Floyd H, Dunn KW, *et al.* A new quantitative scale for clinical scar assessment. *Plast Reconstr Surg* 1998; **102**: 1954-61.
22. Wahl SM. Transforming growth factor- β : innately bipolar. *Curr Opin Immunol* 2007; **19**: 55-62.
23. Occleston NL, Lavery HG, O’Kane S, Ferguson MW. Prevention and reduction of scarring in the skin by transforming growth factor beta 3 (TGF β 3): from laboratory discovery to clinical pharmaceutical. *J Biomater Sci Polym Ed* 2008; **19**: 1047-63.
24. Szpaderska AM, Zuckerman JD, DiPietro LA. Differential injury responses in oral mucosal and cutaneous wounds. *J Dent Res* 2003; **82**: 621-6.
25. Eslami A, Gallant-Behm CL, Hart DA, *et al.* Expression of integrin α v β 6 and TGF- β in scarless v scar-forming wound healing. *J Histochem Cytochem* 2009; **57**: 543-57.
26. Schrementi ME, Ferreira AM, Zender C, DiPietro LA. Site-specific production of TGF- β in oral mucosal and cutaneous wounds. *Wound Repair Regen* 2008; **16**: 80-6.
27. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003; **83**: 835-70.
28. Ashcroft GS, Horan MA, Ferguson MW. Aging alters the inflammatory and endothelial cell adhesion molecule profiles during human cutaneous wound healing. *Lab Invest* 1998; **78**: 47-58.
29. Gosain A, DiPietro LA. Aging and wound healing. *World J Surg* 2004; **28**: 321-6.
30. Hardman MJ, Waite A, Zeef L, *et al.* Macrophage migration inhibitory factor: a central regulator of wound healing. *Am J Pathol* 2005; **167**: 1561-74.
31. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol* 2003; **3**: 791-800.

32. Loke P, Nair MG, Parkinson J, *et al.* IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol* 2002; **3**: 7.

Table 1. Scoring scheme for visual assessment of wounds

Assessment	Score			
	0	1	2	3
Erythema	None	Slight	Moderate	Intense
Oedema	None	Slight	Moderate	Intense
Colour ¹	Normal	Minor mismatch	Obvious mismatch	Gross mismatch
Contour ¹	Normal	Palpable	Hypertrophic	Keloid
Texture ¹	Normal	Proud/indented	Firm	Hard

¹Relative to normal unwounded skin.

Figure legends

Fig 1. MAL-PDT delays re-epithelialisation but wounds have improved visual appearance

at 3 weeks. (a) Representative H&E-stained sections of wounds at 7 days; arrows indicate wound margins. (b) Quantification showed delayed re-epithelialisation in treated wounds compared to controls. (c) Representative photographs of treated and control wounds (3 weeks). (d) Wound scoring reveals treated wounds to have significantly less erythema and be closer in colour to normal skin than control wounds. Data are mean + SEM, $n = 5$ (B), $n = 8$ (D); $*P < 0.05$; $**P < 0.01$; Scale bar = 0.5 mm.

Fig 2. Modulated production of TGF- β isoforms in MAL-PDT-treated wounds.

(a) Representative immunohistochemistry of TGF- β 1 and TGF- β 3-producing cells in treated and control wounds at 7 days. (b) Quantification showed significantly fewer TGF- β 1-producing cells in treated wounds compared to controls at 7 days post-wounding. (c) Representative immunohistochemistry of TGF- β 1 and TGF- β 3-producing cells in treated and control wounds at 3 weeks. (d) Quantification showed significantly higher numbers of TGF- β 1 and TGF- β 3-producing cells in treated wounds compared to controls 3 weeks post-wounding. Data are mean + SEM, $n = 10$ (b), $n = 6-8$ (d); $*P < 0.05$; Scale bar = 50 μ m.

Fig 3. Increased macrophage infiltration in MAL-PDT-treated wounds at 3 weeks.

(a) Representative immunohistochemistry of neutrophils and macrophages in treated and control wounds at 7 days. (b) Quantification of neutrophils and macrophages in treated and control wounds 7 days post-wounding. (c) Representative immunohistochemistry of neutrophils and

macrophages in treated and control wounds at 3 weeks. (d) Quantification showed significantly higher numbers of macrophages in treated wounds compared to controls at 3 weeks post-wounding. Data are mean + SEM, $n = 10$ (b), $n = 6$ (d); $*P < 0.05$; Scale bar = 50 μm .

Fig 4. Increased production of MMP-1 and MMP-9 in MAL-PDT-treated wounds at 3 weeks. (a) Representative immunohistochemistry of MMP-1 and MMP-9-producing cells in treated and control wounds at 7 days. (b) Quantification of MMP-producing cells in treated and control wounds 7 days post-wounding. (c) Representative immunohistochemistry of MMP-1 and MMP-9-producing cells in treated and control wounds at 3 weeks. (d) Quantification showed significantly higher numbers of MMP-1 and MMP-9-producing cells in treated wounds compared to controls at 3 weeks post-wounding. Data are mean + SEM, $n = 10$ (b) except MMP-2 $n = 5$; $n = 6$ (d) except MMP-2 $n = 4$; $*P < 0.05$; Scale bar = 50 μm .

Fig 5. Improved matrix deposition in MAL-PDT-treated wounds after 9 months. (a) Representative immunohistochemistry (collagen I, collagen III, fibronectin) and Weigert staining (elastin) of treated and control wounds 9 months post-wounding. (b) Visual assessment scoring revealed deposition of collagen I, collagen III and elastin in treated wounds to be significantly closer to that of normal unwounded skin than deposition in control wounds. (c) Representative photographs of treated and control wounds at 9 months post-wounding. (d) Wound scoring of control and treated wounds at 9 months. Data are mean + SEM, $n = 9$ (b,d) except elastin $n = 8$; $*P < 0.05$; $**P < 0.01$; Scale bar = 0.5 mm.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.