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The regenerative potential of fibroblasts in a new diabetes-induced delayed humanised wound healing model

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Abstract: Cutaneous diabetic wounds greatly affect the quality of life of patients, causing a substantial economic impact on the healthcare system. The limited clinical success of conventional treatments is mainly attributed to the lack of knowledge of the pathogenic mechanisms related to chronic ulceration. Therefore, management of diabetic ulcers remains a challenging clinical issue. Within this context, reliable animal models that recapitulate situations of impaired wound healing have become essential. In this study, we established a new *in vivo* humanised model of delayed wound healing in a diabetic context that reproduces the main features of the human disease. Diabetes was induced by multiple low doses of streptozotocin in bioengineered human skin engrafted immunodeficient mice. The significant delay in wound closure exhibited in diabetic wounds was mainly attributed to alterations in the granulation tissue formation and resolution, involving defects in wound bed maturation, vascularisation, inflammatory response and collagen deposition. In the new model, a cell based wound therapy consisting of the application of plasma derived fibrin dermal scaffolds containing fibroblasts consistently improved the healing response by triggering granulation tissue maturation and further providing a suitable matrix for migrating keratinocytes during wound re epithelialisation. The present preclinical wound healing model was able to shed light on the biological processes responsible for the improvement achieved, and these findings can be extended for designing new therapeutic approaches with clinical relevance.

Key words: animal models – delayed wound healing – diabetic wounds – fibroblasts – tissue engineering

Introduction

Tissue repair is accomplished by the close coordination of the processes of inflammation, re epithelialisation, granulation tissue formation and dermal remodelling that overlap in space and time (1 3). Chronic non healing wounds, such as diabetic ulcers, fail to proceed through these sequential events (4), leading to diminished quality of life, frequent hospitalisation and increased morbidity and mortality (5,6). Moreover, this pathologic healing condition causes significant socio economic consequences for patients, their families and the healthcare system (7).

A peripheral nerve dysfunction induced by sustained hyper glycaemia, with or without coexisting ischaemia, combined with repeated minor trauma seem to be the prime causes of diabetic skin ulcers (8 10). In conjunction with the diabetic neuropathy, several altered cellular and molecular processes, mainly related to granulation tissue formation and resolution, have been linked to impaired diabetic cutaneous wound healing (11).

Due to the limited understanding of the mechanisms responsi ble for poor healing, conventional therapies are unable to guaran tee an adequate and sustained regeneration of the damaged tissue (12). More recently, fibroblasts administered as a part of bioengi neered dermal or dermo epidermal substitutes offer new therapeu tic possibilities in both animal models and clinical studies (13 15). In particular, fibroblasts are able to grow and provide multiple growth factors and extracellular matrix proteins (16 18) that might be altered in diabetic wound beds. Consequently, tissue engineering approaches are emerging as smart strategies for the treatment of chronic cutaneous wounds such as diabetic ulcers (19).

The study of diabetic wound healing in patients is limited by obvious ethical considerations and the heterogeneity of the disease. Thus, apart from a few studies performed on volunteers (20 22), current knowledge of wound healing mainly stems from the use of a broad variety of transgenic and mutant murine models (23 26). Despite the unquestionable value of murine models, innovative approaches must been explored in a human ised context to obtain results of clinical relevance. To this end, chimeric systems composed of skin of human origin, vascularised by murine vessels, have been exploited by several groups, includ ing ours (27 29). The skin humanised mouse model developed by our laboratory is based on the permanent engraftment of a fibrin based bioengineered human skin onto the back of

immunodeficient mice (29,30). The orthotopic bioengineered skin transplantation methodology enables the generation of numerous mice engrafted with a significant homogeneous area of single donor derived stable human skin (31,32) and offers multiple possibilities to faithfully recreate diverse cutaneous pathological (33 35) and physiological processes (36,37).

In the present work, we report a new *in vivo* model of delayed wound healing in a diabetic context using the skin humanised mouse model that resembles a clinically meaningful skin repair deficient condition. Furthermore, the model has been used as a preclinical platform to evaluate the effectiveness of fibroblast containing fibrin based dermal matrices which considerably enhanced tissue repair.

Methods

Primary culture of human keratinocytes and fibroblasts

Cells from skin biopsies of three healthy donors were isolated by mechanical and enzymatic digestion as previously described (30,38,39). Donors were subjected to standard serological tests according to national regulations (RD 1301/2006). Donors gave their written informed consent for biopsies, and all experimental procedures were conducted in accordance with the World Medical Association Declaration of Helsinki and subsequent revisions.

Grafting of bioengineered human skin equivalents: the skin humanised mice

Six week old female immunodeficient nude mice (Rj: NMRI Foxn1nu; Elevage Janvier, Le Genest Saint Isle, France) were orthotopically grafted with bioengineered cutaneous equivalents. This setting contains human keratinocytes (epidermal component) seeded on a plasma derived fibrin matrix populated with live human fibroblasts (dermal component). Grafting was performed as previously described (29,30) under sterile conditions at the CIEMAT Laboratory Animals Facility (European registration number ES280790000183; Spanish registration number 28079 21 A). All handling was carried out according to European and Spanish laws and regulations on the protection and use of animals in scientific research. Experimental procedures were approved by the Animal Experimentation Ethical Committee of CIEMAT.

Wound healing experimental design in the diabetic skin humanised mouse model

Diabetes was induced in skin humanised mice at 10 weeks post grafting by intraperitoneal injections of streptozotocin (STZ; Sigma Aldrich, St. Luis, MO, USA), dissolved in sodium citrate buffer (0.01 M; pH 4.5). Blood glucose levels were routinely measured under non fasted conditions by tail vein sampling using an Accu Check Blood Glucose Monitor (Roche Diagnostic, Indianapolis, IN, USA). Mice whose blood glucose levels exceeded 250 mg/dl on two consecutive measurements separated by an interval of 48 h were considered diabetic. Normoglycaemic mice were used as controls.

Sustained release bovine insulin pellets (Linbit, LinShin, North Scarborough, ON, Canada) were provided to severely diabetic mice (blood glucose levels higher than 500 mg/dl) to make hyperglycaemia compatible with life. Further information is provided in Fig. S1.

Full thickness 2 mm excisional wounds were created in the stable human skin graft using a biopsy punch (Pfm Medical Ag., Cologne, Germany). Circular excisioned tissue was harvested and used as a reference for anatomopathological analysis. Clinical follow up of the healing process was performed by using a camera coupled to a stereomicroscope (Olympus, Barcelona, Spain).

Anatomopathological analysis was performed at three ($n = 4$ mice per experimental group), seven ($n = 10$) and 14 days ($n = 3$) postwounding. Sample harvesting and processing were carried out as previously described (36,40). Serial 4 μm cross sections were obtained. The whole sample was sectioned to determine the centre of the wound and adequately monitor the healing process. Haematoxylin and eosin staining (Thermo Shandon GmbH, Darmstadt, Germany) was routinely performed in tissue sections.

Wound healing analysis

The re epithelialisation percentage was calculated by the formula: 100× [(wound diameter epidermal gap)/wound diameter] (41). Immunohistochemical and immunofluorescence procedures were performed in tissue sections adjacent to the centre following standard protocols.

To determine the human origin of epidermal and dermal cells, species specific antibodies against involucrin (dilution 1:100; clone SY5 mAb, Sigma, St Louis, MO, USA) and vimentin (dilution 1:50; clone V9 mAb, BioGenex, San Ramon, CA, USA) were used, respectively.

Mature blood vessel density was determined using an antibody specific for α smooth muscle actin (SMA; dilution 1:400; clone 1A4 mAb; Sigma).

Polymorphonuclear neutrophil (PMN) infiltration in the wound bed was determined by labelling myeloperoxidase activity (MPO; dilution 1:50; Hycult Biotechnology, Uden, the Netherlands).

The nuclear antigen Ki67 (Thermo Scientific, Fremont, CA, USA) was selected as a cell proliferation marker. In addition, an antibody against keratin K10 (dilution 1:1000; Covance, Emeryville, CA, USA) was used as an early differentiation marker of keratinocytes. For Ki67 and keratin K10 staining, a heat induced epitope retrieval treatment using 10 mM citrate buffer was required.

Specific biotinylated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). After colour development (Avidin Biotin Complex Vectastain Elite kit; Vector Laboratories, Inc., Burlingame, CA, USA), sections were counterstained with haematoxylin.

Nerve fibres were visualised by immunofluorescence using an antibody against the pan neuronal marker protein gene product 9.5 (PGP 9.5; dilution 1:500; ABD Serotec, Oxford, UK) and a FITC conjugated secondary antibody (Jackson Immuno Research Laboratories). Cell nuclei were stained with DAPI (Merck, Darmstadt, Germany). Microphotographs were taken using a fluorescence microscope (Axioplan 2; Carl Zeiss GmbH, Jena, Germany).

Collagen deposition was assessed by picrosirius red staining following standard procedures (42), and stained sections were analysed by polarised light microscopy (U ANT filter; Olympus).

Treatment of diabetic wounds with bioengineered dermal equivalents in the skin humanised mouse model

Fibrin based bioengineered dermis containing 10^6 live human fibroblasts were applied covering diabetic wounds ($n = 4$) and were kept in place using Tegaderm™ (3M Health Care, ST Paul, MN, USA), as previously described (40). After 7 days, wound samples were collected and analysed. An identical number of diabetic wounds were treated with acellular fibrin gels.

Statistical analysis

Wound healing parameters were analysed in the different experi mental groups, and means were compared using the two tailed unpaired Student's t test. Differences were considered statistically significant when $P < 0.05$. All values were expressed as the mean ± SD.

Results

Establishment of a diabetic skin humanised mouse model

Skin humanised mice were treated with STZ, a drug that selec tively destroys β pancreatic cells (43). Diabetes was induced 10 weeks after grafting, an adequate time period to achieve both epidermal maturation and complete fibrin remodelling to collage nous dermis in the regenerated interfollicular human skin (29,30,32). Within the following 2 weeks, 85% of mice receiving STZ developed hyperglycaemia (250 600 mg/dl) and displayed polyuria, polyphagia and polydipsia, classical clinical symptoms of diabetes. As the survival of mice with hyperglycaemic levels over 500 mg/dl was compromised, exogenous insulin was administered by subcutaneous bioerodible implants and dose was adjusted by applying one or two microimplants (Fig. S1A).

In this way, a chemically induced diabetic skin humanised mouse model was established, and mature human skin perma nently engrafted on immunodeficient mice was exposed to sustained hyperglycaemia for 6 weeks (Fig. 1a). At this time, histological evidence of pancreatic damage was evidenced (Fig. S1B D). Moreover, the main cutaneous features reported in diabetic patients were faithfully recreated in this *in vivo* model, although a normal regenerated human skin in terms of epidermal phenotype and collagenous dermal matrix was observed (Fig. 1b). Specifically, a striking reduction of both blood vessels and nerve fibres depicted by SMA and PGP 9.5 immunostaining was consis tently displayed in all diabetic animals (Fig. 1c,d). In addition, SMA positive vessels appeared disorganised, scattered and located at the reticular dermis in the skin of diabetic animals while vessels were mainly found in the subepidermal vascular plexus in control mice (Fig. 1c).

Delayed wound healing response in the diabetic skin humanised mice

Healing studies were performed by creating full thickness 2 mm excisional wounds on the mature stably regenerated human skin of hyperglycaemic mice. Samples were harvested at 3, 7 and 14 days postwounding. Although the diabetic healing process was delayed at all the studied time points, the maximum differences in the rate of re epithelialisation were detected at seven after injury (Fig. S2). Therefore, all the studies were performed at that time point.

The human origin of both keratinocytes involved in re epitheli alisation and fibroblasts responsible for dermal matrix remodelling after excisional wounding was certified (Fig. S3).

Experimental diabetes distressed the progression of the healing process, leading to a statistically significant reduction of the re epithelialisation rate in 7 day wounds (Fig. 2a c). Indeed, only 12% of the wound was re epithelialised in hyperglycaemic animals compared to 47% in controls.

Biological alterations underlying delayed wound healing in the diabetic skin humanised mice

A representative panel of parameters was evaluated to investigate the alterations underlying diabetes wound repair impairment in the model developed herein.

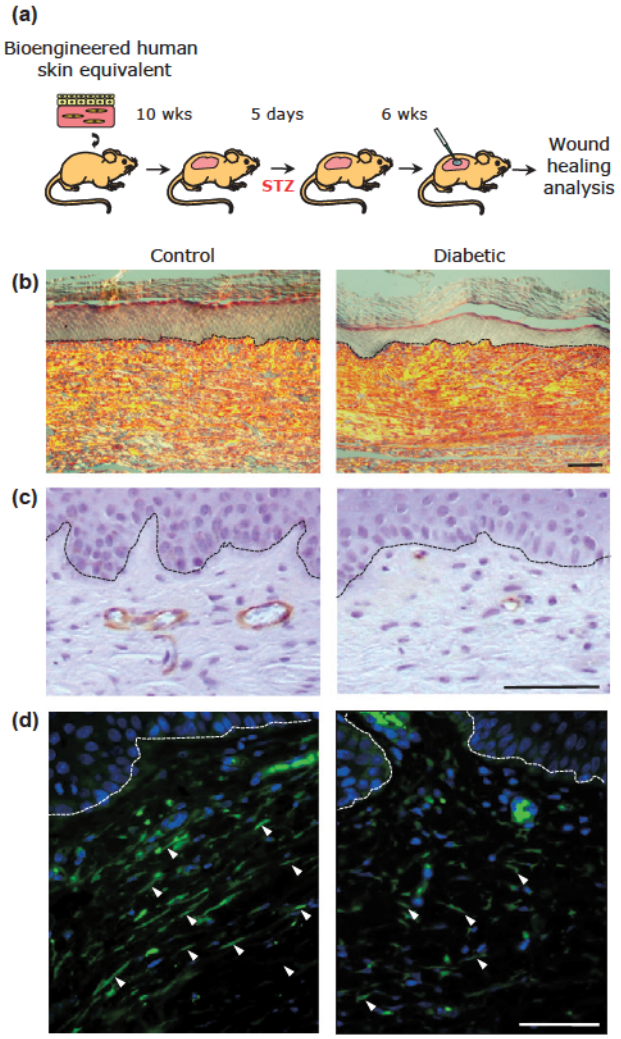

Figure 1. Reduced cutaneous vascularisation and innervation in the regenerated human skin of diabetic mice. (a) Schematic representation of the streptozotocin induced diabetes experimental protocol. Diabetes was induced at 10 weeks postgrafting, after both epidermal maturation and dermal remodelling were completed in the regenerated human skin. STZ was administered on five consecutive days (four doses of 50 mg/kg followed by a single dose of 100 mg/kg). (b) Picrosirius red staining. A normal collagenous dermal matrix was found in diabetic mice after 6 weeks of STZ administration. Scale bar = 100 μm. (c) SMA immunoperoxidase staining. Mature blood vessel density was substantially reduced in the STZ treated mice. Scale bar = 75 μm. (d) PGP 9.5 immunofluorescence. Considerable qualitative changes in cutaneous innervation were detected in STZ treated mice. Scale bar = 50 μm. Experiments were performed with 10 mice in each group. In panels B, C and D, dermo epidermal boundaries are outlined by dashed lines. Abbreviations: wks, weeks; STZ, streptozotocin; SMA, α smooth muscle actin; PGP 9.5, protein gene product 9.5.

Streptozotocin treated animals exhibited impaired formation and maturation of the granulation tissue. Thereby, picrosirius red staining in 7 day control wounds showed a collagen rich wound bed, whilst a sparse collagen deposition was noticeable in the dia betic wound beds (Fig. 2d). Consistent with poor dermal matrix remodelling, a significant detrimental change in vascular density within the granulation tissue was detected in diabetic wounds (Fig. 3a,b). This impaired angiogenic response conceivably reflected the defective cutaneous vascularisation observed in diabetic skin humanised mice (Fig. 1c).

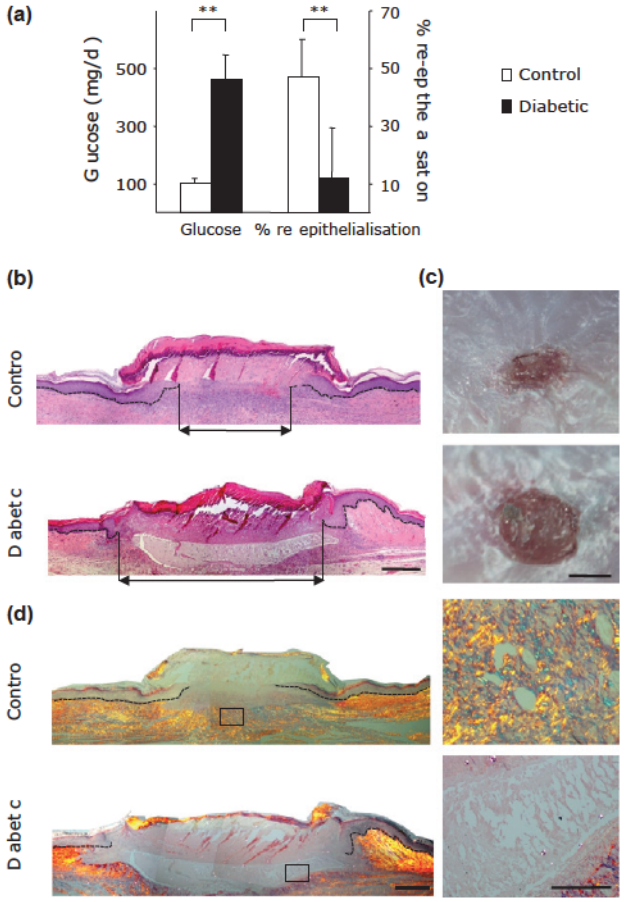

Figure 2. Impaired granulation tissue formation and dermal remodelling contributed to delayed wound healing in the diabetic skin humanised mouse model. (a) Percentage of re epithelialisation calculated by the formula: 100× [(wound diameter epidermal gap)/wound diameter]. Values are expressed as mean ± SD (n = 10 mice in each group). $^{**}P < 0.01$. A statistically significant reduction of re epithelialisation percentage was associated with hyperglycaemia. (b) Composite pictures of H&E stained 7 day wounds. Diminished wound re epithelialisation was evidenced by a larger epidermal gap distance as indicated by double arrowhead. Scale bar = 300 μm. (c) Representative photographs of excisional wounds at 7 days postwounding. Scale bar = 1 mm. (d) Collagen deposition analysed by picrosirius red staining. Panoramic view (scale bar = 250 μm) and close up view (scale bar = 100 μm) of the centre of 7 day wounds. Note the sparse collagen deposition in the 7 day diabetic wound beds while bi refringent collagen type I fibres (orange yellow colour) were predominantly observed in the granulation tissue of control wounds. Collagen type III (green colour) was also distinguishable in 7 day control wounds. Dashed black lines show the migratory tongues at the wound margins in panels B and D. Abbreviations: H&E, haematoxylin and eosin.

The inflammatory response in the stroma of 7 day diabetic wounds was associated with a massive infiltration of myeloperoxi dase positive neutrophils flanking the fibrin plug (Fig. 3c). In con trast, much fewer neutrophils were found in the granulation tissue of control wounds, revealing a practically resolved inflammatory phase.

The Ki67 proliferative index of basal keratinocytes was assessed in unwounded and wounded regenerated human skin. Surprisingly, on day 7, a comparable postwounding proliferative burst was observed in both experimental groups (Fig. 3d,e). However, while well developed migratory tongues, with no stratum corneum and decreasing expres sion of K10, were observed in controls, diabetic wounds exhibited a

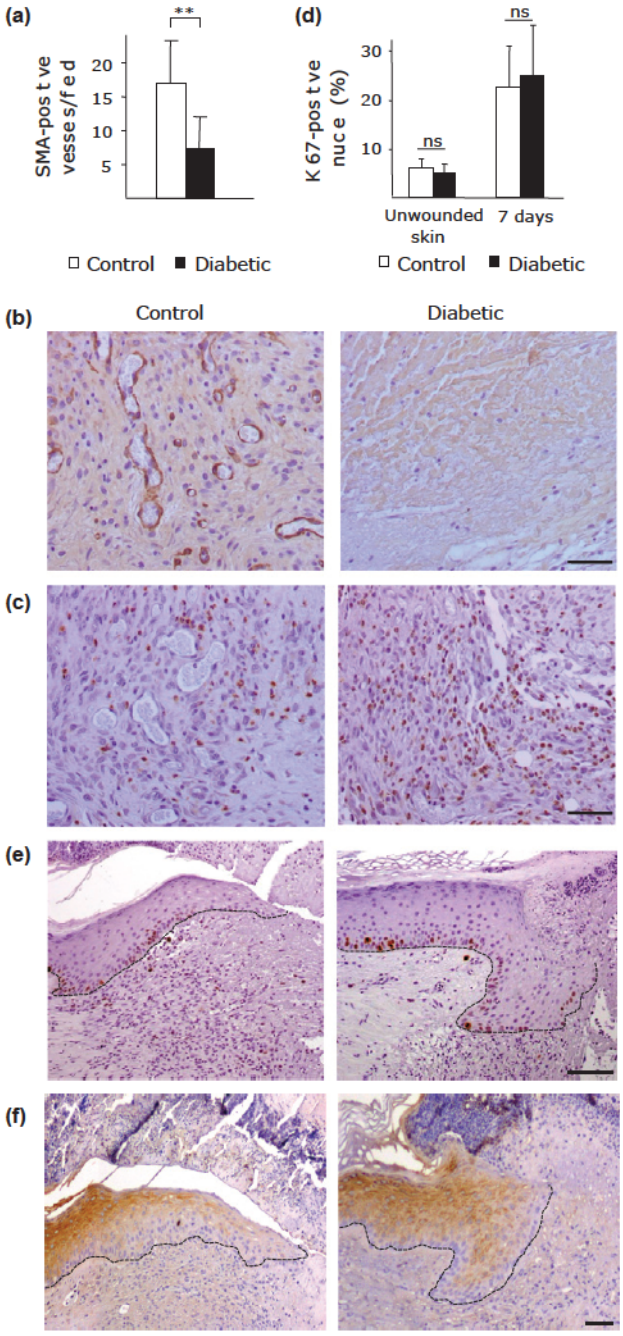

Figure 3. Reduced vascularisation and persistent neutrophil infiltration without epidermal proliferation alteration in diabetic wounds. (a) Quantitative analysis of SMA positive vessels. Four randomly chosen high power fields were counted per wound. Results (mean ± SD) are from 10 mice in each group. $^{**}P < 0.01$. (b) SMA immunoperoxidase staining in the granulation tissue of 7 day control and diabetic wounds. Scale bar = 50 μm. (c) Immunodetection of MPO positive polymorphonuclear neutrophils in areas surrounding the granulation tissue at 7 days after injury. Scale bar = 50 μm. (d) Ki67 quantitative analysis in unwounded skin and in the neo epidermis of 7 day wounds. A total of 300 nuclei were counted, and the proliferation index was calculated as the percentage of Ki67 positive nuclei in relation to the total number of nuclei of the basal keratinocyte layer. Results are expressed as mean ± SD. (e) Ki67 staining of 7 day wounds. Scale bar = 100 μm. (f) Immunoperoxidase staining of keratin K10. Scale bar = 50 μm. In panels E and F, dermo epidermal boundaries are outlined by dashed black lines. Abbreviations: SMA, α smooth muscle actin; MPO, myeloperoxidase; ns, non significant.

fully keratinised acanthotic epithelium with a concomitant expression of keratin K10 reaching the edge of the wound (Fig. 3e, f), evidencing impaired migratory activity of keratinocytes.

Noticeably, long term analysis revealed that 7 day postwoun ding defects were still evident, albeit attenuated, in completely re epithelialised 14 day diabetic wounds (Fig. S2C).

Bioengineered dermis accelerated wound healing in diabetic wounds

Diabetic wounds in the skin humanised mouse model displayed delayed granulation tissue formation with poor vascular and cellu lar density, mimicking the pathological conditions that predispose diabetic patients to develop cutaneous ulcers (4,44,45). With the aim of correcting these biological alterations, the therapeutic potential of tissue engineered dermal equivalents containing human fibroblasts assembled in a three dimensional fibrin matrix was evaluated in a diabetic context (Fig. S4). In the preclinical model, a significant healing improvement was seen in diabetic wounds at 7 days post treatment (Fig. 4a,b), achieving a re epithelialisation rate comparable to that found in untreated control wounds (Fig. 2a). Moreover, a granulation tissue rich in collagen was observed in diabetic wounds after the treatment with bioengineered dermal equivalents (Fig. 4c). Additionally, fibro blast containing bioengineered dermis seemed to exert an angio genic effect during granulation tissue formation (Fig. 4d,e), and indeed, vascular density in treated diabetic wounds was compara ble to that found in untreated controls (Fig. 3a). Furthermore, no noticeable effects on granulation tissue formation were detected when fibroblasts were not included in the fibrin gels used for wound treatment (Fig. 4c e).

Our results demonstrated, in a preclinical model, the effective ness of bioengineered dermal equivalents in diabetic wounds, particularly through the promotion of wound bed maturation.

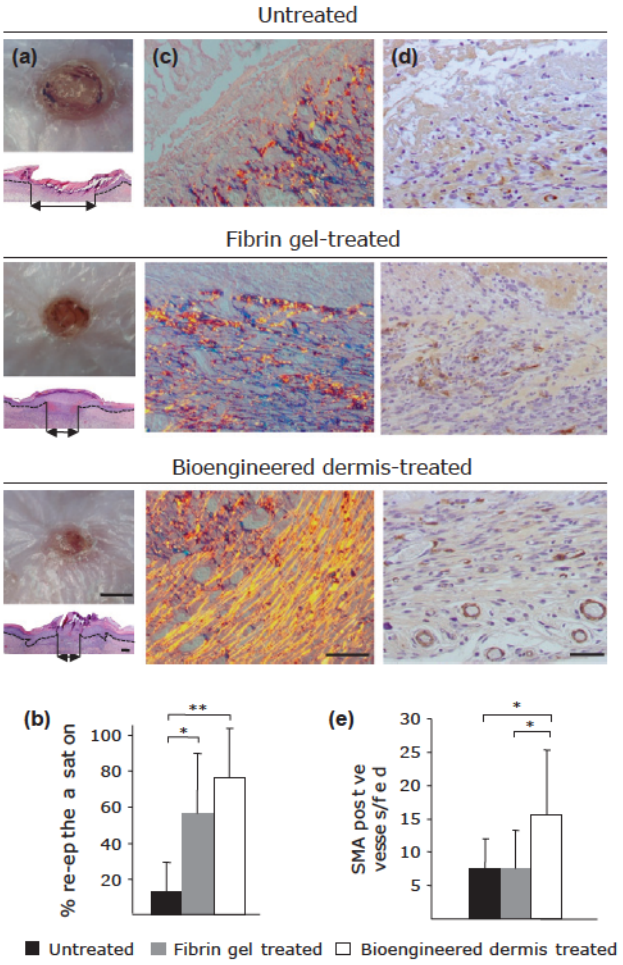

Figure 4. Fibroblast containing bioengineered dermis enhanced wound healing in the diabetic skin humanised mouse model. (a) Representative photographs (scale bar = 1 mm) and composite pictures showing panoramic view of 7 day diabetic wounds stained with H&E (scale bar = 500 μm). Accelerated wound closure was evidenced in diabetic wounds after treatment by a reduction of the epidermal gap distance (double arrowhead). Dashed black lines delimit the wound margins. (b) Percentage of re epithelialisation in diabetic wounds. Results are expressed as mean ± SD (n = 4 mice in each group). **$P < 0.01$; *$P < 0.05$. (c) Picrosirius red staining in centre of 7 day diabetic wounds. A collagen enriched granulation tissue was observed in diabetic wounds after treatment with bioengineered dermis. Scale bar = 50 μm. (d) SMA immunoperoxidase staining in the granulation tissue of 7 day diabetic wounds. A significant increase in the vascular density in diabetic wounds treated with bioengineered dermis was observed. Scale bar = 50 μm. (e) Quantitative analysis of SMA positive blood vessels. Results are expressed as mean ± SD. **$P < 0.01$; *$P < 0.05$. Abbreviations: H&E, haematoxylin and eosin; SMA, α smooth muscle actin.

Discussion

Diabetes affects approximately 2% of the world population in developed countries and its incidence increases in relation to current lifestyle (46). Diabetic patients frequently suffer from impaired wound healing and are consequently prone to develop cutaneous ulcers with a high rate of recurrence that often become chronic (5,6,47). The development of effective treatments for heal ing chronic ulcers largely depends on understanding the patho genic mechanisms involved. This poses the need for reliable animal models that recapitulate situations of impaired wound healing (23,48). The db/db mouse model presents delayed cellular infiltration, granulation tissue formation and collagen deposition, as well as prolonged persistence of neutrophils and reduced angio genesis (24,26). These defects have also been depicted in our model. However, extrapolation of results coming from rodent and other small mammal models should be interpreted with caution, due to major architectural and functional differences with human epidermis (49). In fact, wound healing in mice is a process that occurs mainly at the expense of contraction, a mechanism of minor relevance in human skin (40,50). To overcome these problems, several groups, including ours, have developed a skin humanised mouse model (28 30,51). Specifically, the model devel oped in our laboratory faithfully reproduces the main features of human healing at both structural and functional levels (36,40). Our preclinical humanised wound healing system comprises two processes of skin regeneration. The first entails the grafting of bio engineered human skin onto 12 mm excisional wounds in immu nodeficient mice (29,30). The second regeneration process involves the closure of 2 mm small full thickness wounds per formed in mature, quiescent and stably engrafted human skin (10 12 weeks after grafting). Closure of these small wounds is carried out by human keratinocytes and human fibroblasts with minimal contraction contributing to wound resolution (40).

To establish a model of wound healing impairment in the skin humanised mouse model, a pharmacological induced diabetes protocol based on STZ administration was employed. Both sur vival and hyperglycaemia were modulated by a subcutaneous insu lin implant that guaranteed elevated but life compatible levels of

blood glucose during a period of time adequate to cause an impact on the skin.

Hyperglycaemia induced nerve damage resulting in peripheral neuropathy is a common complication in diabetes (10). As a result of metabolic abnormalities, these patients may also present endothelial dysfunction and alterations in the vascular supply to organs, including the skin (8,52). Both decreased innervation and vascularisation were exhibited on the regenerated human skin of diabetic mice exposed to hyperglycaemia for 6 weeks, proving the suitability of our protocol. Moreover, the significantly delayed wound closure in STZ treated mice reproduces the healing impairment associated with diabetes in patients (4,11) and in other diabetic animal models (24,26).

Defects associated with granulation tissue formation and their subsequent resolution seem to be responsible for the delayed heal ing process in the present model. Specifically, poorly cellularised 7 day diabetic wound beds with reduced collagen deposition resulted in an inadequate matrix for cell migration. These altera tions correlate with the defective fibrinolysis and loose extracellu lar matrix deposition described in diabetic patients (44,53). It has been proposed that glycation of proteins, such as fibrinogen or enzymes related to fibrin plug degradation, delays its resolution and thus adversely affects migration of epithelial tongues in dia betic wounds (54). In addition, alterations of the subepidermal vascular plexus of diabetic mice observed prior to wound creation were further evidenced during the angiogenic response triggered by the healing stimulus. Thus, inadequately vascularised granula tion tissue might not provide the metabolic support required for cell recruitment, hampering the tissue repair process as described in patients (45) and in other diabetic animal models (26).

In the new diabetic model herein described, a prolonged inflam matory response, mainly consisting of neutrophils, could be gener ating a proteolytic and pro oxidant environment in the wound bed, thus contributing to the delayed healing progress (55). Indeed, a sustained presence of polymorphonuclear granulocytes has been reported in patients and animal models of diabetes (56,57).

Controversial data concerning keratinocyte proliferation and hyperglycaemia have been reported. Although prolonged exposure to high glucose inhibits *in vitro* human keratinocyte proliferation (58,59), other evidences support that keratinocytes at the chronic ulcer edge are hyperproliferative (60). In the model described here, a postinjury keratinocyte proliferation burst without significant differ ences associated with diabetes was observed. We thus hypothesise that epithelial cells of the diabetic wound edges were capable of responding to proliferative signals but epidermal migration might be compromised because of the immaturity of the wound bed. As a result, keratinocytes still expressing markers of differentiation accu mulated at the edge of the wound, forming an acanthotic epithelium.

Long term analysis revealed that defects responsible for delayed healing were attenuated in completely re epithelialised 14 day wounds, thus wound chronification was not attained in the dia betic skin humanised mouse model. Limiting factors, such as, the acute toxicity exerted by STZ and the use of cells from healthy donors employed for assembling the bioengineered human skin equivalents, could explain the lack of chronicity.

As diabetic ulcers respond poorly to conventional treatments (12), new approaches are needed. Advanced cell therapies using healthy allogenic fibroblasts emerge as attractive strategies to over come the senescence of fibroblasts present in chronic wound beds (61,62). Intracellular signalling activated via cytokines, growth fac tors and proteolytic enzymes secreted by allogenic healthy fibro blasts seems to stimulate the recipient's own wound bed derived skin cells and therefore crucial processes frequently altered in diabetic wounds, such as re epithelialisation and angiogenesis, are activated (16 18). In addition, human fibroblasts are an easy to handle, accessible cell source with acceptable cell yields obtained from a relatively small skin biopsy. These properties, together with their low immunogenicity (63,64) make fibroblasts a suitable tar get in tissue engineering for the treatment of chronic wounds of different aetiology (65).

Furthermore, the use of fibrin, unlike other biomaterials such as collagen, preserves the viability and functionality of epidermal stem cells (29,30,32,66,67), because it is a reservoir of cytokines, clotting, cell adhesion and growth factors. Plasma derived fibrin, unlike commercial purified fibrinogen preparations, contains addi tional factors such as fibronectin or thrombospondin that may contribute to keratinocyte adherence and survival (39,68). More over, fibrin provides a suitable three dimensional scaffold to pro mote migration, proliferation and differentiation of the cells in the wound bed (69).

In the present work, the skin humanised mouse model has been exploited as an ideal preclinical platform to evaluate the effective ness of fibroblast containing fibrin based dermal scaffolds on wound healing in a diabetic context. After 7 days of treatment, a noteworthy enhancement of wound closure was achieved in dia betic wounds, showing re epithelialisation rates comparable to untreated control wounds. Interestingly, treatment with bioengi neered dermis improved diabetic wounds by promoting wound bed maturation. As these improvements were not achieved when fibroblasts were not included in the fibrin matrix and these cells are a well known source of soluble mediators, we suggest fibroblasts induced maturation of diabetic wounds in terms of collagen deposition and wound bed vascularisation. Consequently, the granulation tissue of improved quality would provide a suit able matrix for keratinocyte migration, explaining the differences observed in wound closure after treatment.

Clinical benefits of tissue engineered products have been exten sively demonstrated (19), although their use is considerably lim ited due to the scarce availability and high cost associated with its manufacture and development (70). In this context, the self made, non commercial, bioengineered dermal setting we report herein appears to be an attractive therapeutic alternative for diabetic wounds not only in terms of efficacy, but also because of the sub stantial cost savings.

In conclusion, the proposed model of delayed wound healing emerges as a suitable preclinical tool to evaluate clinically mean ingful innovative therapeutic approaches in the field of dermatol ogy and also to provide a better understanding of the biological mechanisms involved in wound healing improvement after a treat ment. Furthermore, we have demonstrated that bioengineered fibroblast containing dermal substitutes are a powerful and trust worthy tool for the treatment of diabetic wounds in a preclinical context. The bioengineered dermis may represent a realistic, effi cient and inexpensive strategy to overcome the granulation tissue defects frequently associated not only with diabetic wounds but also with ulcers of different aetiology.

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LMS and MJE performed the experiments, and together with MDR and CJC designed the research study, analysed the data and wrote the manuscript. SL, EG, LC, JML and AM manufactured the bioengineered skin equivalents. LR, AH, NI and BD performed mice grafting and tissue processing. FL and JLJ contributed to data interpretation and experimental design. All the authors read and approved the final manuscript. We especially thank our technicians I. Santos and F. Sánchez for histology assistance, and J. Martínez and E. Almeida for animal care. This work was supported by grants from the Science and Innovation Ministry of Spain (SAF2010-16976), from the European VI Framework Programme (LSHB-CT-512102), from Comunidad de Madrid (S2010/BMD-2420; CELLCAM) and from Fundación Ramón Areces (CIVP16A1864).

Conflict of interests

The authors have no conflicting financial interests.

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Supporting Information

Figure S1. Establishment of a diabetic skin-humanised mouse model.

Figure S2. Delayed wound healing in the diabetic skin-humanised mouse model.

Figure S3. Species-specific antibodies confirmed the human origin of keratinocytes and fibroblasts in the regenerated human skin and in wounds in the skin-humanised mouse model.

Figure S4. Treatment of diabetic wounds with bioengineered dermal equivalents in the skin-humanised mouse model.