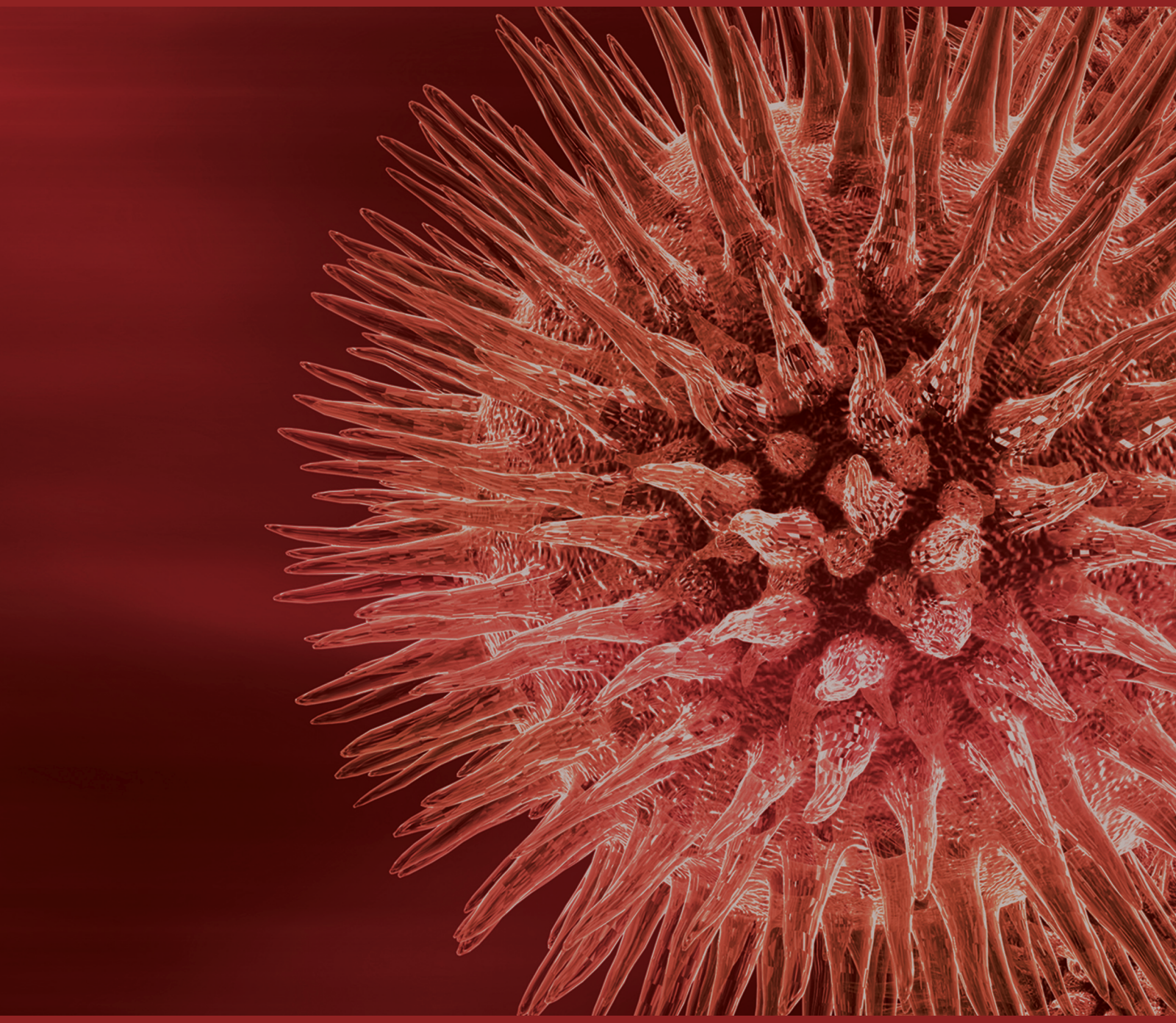
BioMed Research International

Chronic Wounds with Emphasis in Diabetic Foot Ulcers

Guest Editors: Jorge Berlanga-Acosta, David G. Armstrong, Gregory S. Schultz, and Luis Herrera-Martinez



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Contents

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Editorial

Chronic Wounds with Emphasis in Diabetic Foot Ulcers

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Since the seminal contribution of Frederick Banting and Charles Best, medicine, in the field of diabetes therapy was revolutionized. From that moment on, insulin therapy eliminated ketoacidosis as a principal cause of death among diabetics. As a result, diabetic patients enjoyed a longer lifespan and since then, novel pharmacological interventions have definitively contributed to their metabolic homeostasis. However, it is clear that traditional insulin therapy combined with emerging novel approaches did not translate into a significant reduction of major complications that lead to morbidity and mortality in patients with type II diabetes. Thus, these basic disease complications' as neuropathy, macro and microangiopathy, nephropathy, retinopathy and so many others; remain as challenge for scientists and clinicians. About 20 years ago an exciting concept emerged from large and well-controlled clinical trials. Perhaps the foremost message of these trials was the demonstration that once initiated; complications persisted and continued to progress, even in those subjects in which good metabolic control was achieved. It was the birth or the coining of the "Metabolic Memory" concept. To our understanding, only a deep and broad penetration in the pathophysiology of metabolic memory will help in the control, drugging and alleviation of diabetic complications.

Efforts and progresses have been achieved along the last few years. First, it is the elegant Michael Brownlee's unifying mechanistic hypothesis which in essence proposes that intracellular hyperglycemia causes increased mitochondrial reactive oxygen species production, which pulls the trigger to a vast number of downstream toxic events. Furthermore, this basic knowledge has expanded from a damaged and dysfunctional mitochondrial DNA, to the endoplasmic reticulum stress and the imprinting of a gone awry epigenetic control which seems to perpetuate the diabetic phenotype. At the end, all the roads converge to predispose diabetic cells to apoptosis, autophagy, early aging, etc. Diabetic foot ulceration is one of the most frightened diabetic complications, leading to disability, social exclusion and early mortality. The observation that diabetic patients exhibit a failure in their healing mechanism is ancestral. However, today this problem remains an unmet medical need, since diabetic population contribute to 80% of all non-traumatic lower extremities amputation around the world. Studies have shown that even in those patients with normal lower limb perfusion, good metabolic control, and a comprehensive wound care; the processes of wound granulation, contraction and re-epithelialization appear slower that in non-diabetic subjects. This situation is further complicated given the susceptibility of diabetic subjects to control peripheral soft tissues infections.

The challenging truth is that skin cells cannot hide out from the long arm of the biochemical diabetic milieu and an imprinting is left in fibroblasts, vascular cells and keratinocytes. These cells when harvested from diabetic foot

wounds and transferred to proper culture conditions still exhibit an abnormal behavior and short replicative life span. In other words, the same replicative refractoriness remains in their "memory" as if they were still living within the diabetic ulcer environment. Molecular characterization of these cells has remarked precocious cells senescence, an abnormal susceptibility to apoptosis and a tendency to proliferative arrest as major pillars presiding wound chronification.

Multiple mechanisms may be involved in the genesis of the chronic phenotype and healing stubbornness in diabetes. Biomed Research International has gifted us a tremendous example of sensitivity and attachment to the daily medical needs. The Journal has dedicated this special issue to focus in the biology, and clinical and surgical aspects of chronic wounds with special emphasis in diabetic foot ulcers. The issue is varied and rich by including a series of basic and clinical studies, as well as reviews that will certainly furnish and satisfy a broad audience of readers. This number is therefore an additional and significant contribution in the struggle against a mutilating diabetic complication.

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Research Article

Revealing the Mechanism of *In Vitro* Wound Healing Properties of *Citrus tamurana* Extract

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In the present investigation, we examined the effect of Hyuganatsu (*Citrus tamurana*) extract (HE) on skin fibroblast (TIG-119) proliferation and migration during *in vitro* wound healing. HE selectively inhibited proliferation of TIG-119 cells at higher concentration (>1.0 mg/mL); at lower concentrations (0.1, 0.25, 0.5, and 0.75 mg/mL), it exhibited linear and time-dependent cell proliferation. *In vitro* scratch wound healing studies showed that the HE also accelerated the migration of cells towards the wounded region. Cytometric analysis demonstrated that HE extract did not alter G_1/0 and S phases of cell cycle in any concentration studied; however, G_2/M phases of cell cycle were significantly ($P < 0.05$) accelerated at 0.75 mg/mL dose. RT-PCR and Western blotting analysis indicated that HE markedly overexpressed levels of Rac-1, Rho-A, and Cdc-42 mRNA and the respective proteins. Cyclin-dependent kinases (Cdk-1 and -2) gene expression activity was significantly ($P < 0.05$) increased, but protein content decreased during treatment with HE. The induction of Cdk-1 and -2 by HE was abolished by inhibitors, transcription (DRB), and translation (CHX), implying transcriptional regulation that required *de novo* protein synthesis.

1. Introduction

Cell migration and proliferation coupled with controlled cell cycle are beneficial for the repair of sagged and wrinkled skin, dermal, and gastrointestinal wound healing. Cell cycle is a conserved proliferative signaling cascade pathway in mammals and comprises the G_1, S, G_2, and M phases. The G_1/G0 and S transition is a rate-limiting step in the cell cycle and represents the restriction point of the cycle [1]. G_2/M phase is important for cell multiplication. The basic migratory cycle includes extension of a protrusion edge of a cell, formation of stable attachments near the leading edge of the protrusion, translocation of the cell body forward, and the release of adhesion molecule. All these steps require arrangement of actin cytoskeleton. Small GTPases of the Rho family are key regulators of these cytoskeletal dynamics. Rac-1, Rho-A, and Cdc-42 of Rho family GTPases are required for cell lamellipodial protrusions and activation of wave complex which provides force to cell migration and cell polarity establishment [2]. Like GTPase, cyclin-dependent kinases 1 and 2 are important for cell cycle control [3]. Wound healing requires both migration and proliferation of many cell types like neutrophils, fibroblasts, endothelial cells, and keratinocytes. Fibroblasts play important role in the process of wound healing and maintenance of epidermis dynamics with involvement of Rho-GTPase-dependent activation of basic fibroblast growth factor (bFGF) and collagen. This in turn leads to the activation of Rho-A, thereby facilitating both migration and proliferation of fibroblasts during the process of wound healing [4]. An understanding of the mechanisms that regulate the cell migration and proliferation of dermal fibroblasts cells by a natural compound could be beneficial in devising novel therapies to regulate fibrosis and wound contraction to ultimately improve the wound healing process.

Hyuganatsu, *Citrus tamurana* Hort. ex Tanaka, is one of the predominant citrus crops of Miyazaki, Japan. In recent

years, this crop has increased the commercial value especially in food industries. Traditionally the citrus fruit has been used as a supplement to increase digestion and appetite, relieve flatulence and abdominal distension, and help in respiratory difficulties and also in the prevention of cough. Hyuganatsu peel extract (HE) has been reported to inhibit cytochrome P450 3A [5], suppress midazolam 1-hydroxylase activity of human CYP 3A [6] and inhibit hyaluronidase activity [7]. Furthermore, we have tested the efficacy of water soluble extract of Hyuganatsu extract in suppressing bone loss in ovariectomised rats [8]. However, whether it facilitates the process of *in vitro* wound healing and has a beneficial effect on the proliferation and migration of fibroblast cells remains to be explored. Therefore, in the present investigation, we tested the efficacy of HE on human fibroblast cell migration and proliferation and the associated cell cycle pattern and expressions of cell cycle regulatory pathways.

2. Materials and Methods

2.1. Materials. Lyophilised *Citrus tamurana* peel water extract powder was obtained from Ichimaru Pharcos Co., Ltd. (Gifu, Japan). Alpha medium and FBS were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Antibiotic cocktail (2500 U/mL penicillin, 2.5 mg/mL streptomycin sulfate, 2.5 mg/mL neomycin) was obtained from Life Technologies Corporation (Invitrogen, Corp., NY, USA). All other chemicals were of pure and molecular grade.

2.2. Methods

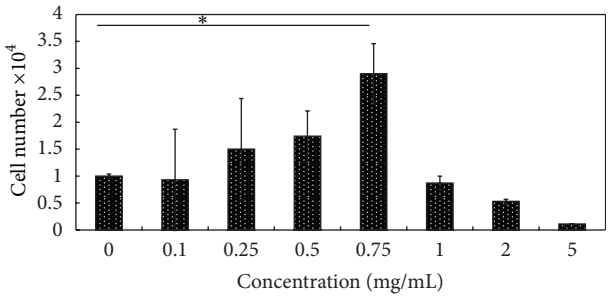
2.2.1. Cell Culture and Treatment. Human fibroblast cells (TIG-119) were purchased from Health Science Research Resources Bank (HRSBB, Osaka, Japan) and cultured in type-1 collagen coated plates (CELLCOAT, Greiner Bio-One, Germany). Cells were maintained in MEM α with glutamine and 5% FBS in 10 cm culture plates. Cells were maintained in antibiotic cocktail at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Cells at passages 2–5 were used for the experiments. All experiments were carried out in FBS deprived MEM +α condition. HE was dissolved in sterilized water, sonicated, filtered, and sterilized through 20 μM filter caps (Millex, Millipore Corp., Billerica, MA, USA), and stored at −20°C till further use.

2.2.2. MTT-Based Cytotoxicity and Cell Proliferation Assay. TIG-119 cells were cultured in 96-well culture plates with or without HE (0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, and 5.0 mg/mL) for 16 h time duration. The viable cell viability was assessed using MTT assay method [9]. Briefly, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, and pH 7.4) at a concentration of 5 mg/mL. MTT was added to each well (10 μL per 100 μL medium), and plates were incubated at 37°C for 1 h. The medium was replaced with 100 μL DMSO, and the absorbance for each well was measured at 570 nm on a microplate reader (Biorad, Corp, USA). For cell proliferation assay, cells were cultured in 24-well culture plate with or without different concentration (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) of HE. Total viable cell count was done as per MTT method at different time periods of 24, 48, 72, and 96 h.

2.2.3. In Vitro Wound Healing Assay. TIG-119 cells were grown in 6-well plates at a density of 3×10^6/mL, and a small linear scratch was created in the confluent monolayer by gently scraping with sterile cell scrapper as per standard methods [10]. Cells were extensively rinsed with medium to remove cellular debris before treating with different concentrations (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) of HE in FBS deprived condition. A positive control, prostaglandin I2 (PG12) analogue, and beraprost sodium (Kaken Pharmaceuticals, Co., Fukuoka, Japan) were used separately to judge the rate of cell migration. Twenty-four hours later, images of the migrated cells were taken using digital camera (Nikon, Tokyo, Japan), connected to the inverted microscope (Nikon, TMS-F, Japan), and analyzed by image analysis software (Image J, National Institutes of Health, Bethesda, MD, USA). Extent of wound healing was determined by the distance traversed by cells migrating into the denuded area. Representative data is cumulative of three independent experiments.

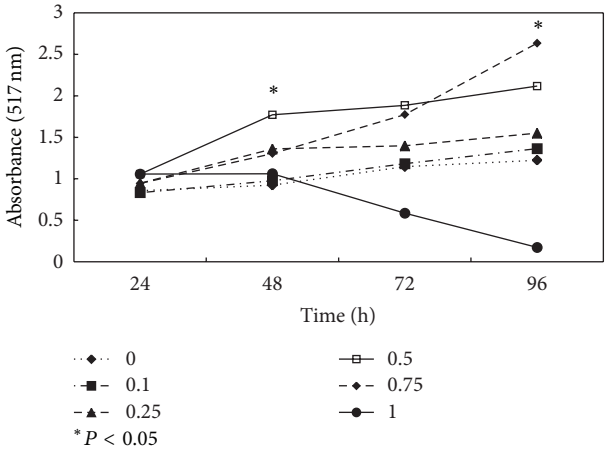
2.2.4. Cell Cycle Analysis by Flow Cytometer. Fibroblasts cells (TIG-119) were treated with different concentrations of HE (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) for 24 h and fixed in cold 70% ethanol at 4°C. Cells were stored in the fixative at −20°C for 1 h. Following fixation, cells were centrifuged at 800 ×g for 5 min, resuspended in phosphate-citrate (PC) buffer at room temperature for 30 min, and again centrifuged at 1000 ×g for 5 min. The cells were resuspended in 800 μL PBS before incubation in 100 μL each of 100 μg/mL RNase A and 0.01% propidium iodide. Flow cytometry analysis was performed after 30 min using Guava Cell Cycle Assay Mini Flow Cytometer (Millipore, Billerica, MA, USA).

2.2.5. Western Blotting. TIG-119 cells were plated at a density of 3×10^6 cells/mL in 80 mm collagen coated tissue culture dishes (CELLCOAT, Greiner Bio-One, Germany) and incubated with or without HE (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) for different time intervals. Cells were washed twice with ice-cold PBS and lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethanesulfonyl fluoride. Proteins from lysates were separated on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with blocking solution (EZ-20, Atto Corp, Tokyo, Japan), the membranes were probed with antibodies against Rac-1, Rho-A, Cdc-42 (Santa Cruz, CA, USA), Cdk-1, and Cdk-2 (Cell signaling, Beverly, MA, USA) followed by anti-rabbit peroxidase-conjugated secondary IgG-2 antibodies (Cell signaling, Beverly, MA, USA). Finally, the protein bands were visualized with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Beta-actin was used as loading control. Relative expression signal intensities were quantified by densitometric analysis.



*$P < 0.05$

Figure 1: MTT-based cytotoxicity assay. Human skin fibroblast (TIG-199) cells were treated with different concentration of HE (0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, and 5.0 mg/mL), and total viable cells were count-based on MTT assay. Each value is the mean ± SD of three independent experiments. Asterisks indicate values which are significantly ($P < 0.05$) different from control.



*$P < 0.05$

Figure 2: Cell proliferation assay. After the treatment with different (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) concentration of HE, increase in total viable cells numbers was counted through MTT dye reduction assay. Cells were counted at different time intervals of 24, 48, 72, and 96 h. Values represent the mean of three independent experiments.

2.2.6. Semiquantitative Real-Time PCR. Total RNA was isolated from HE-treated groups using one step RNA isolation kit (ZyGEM Corp., Hamilton, New Zealand), and cDNA was prepared using Transcription High Fidelity cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN, USA). RT-PCR was carried out as per standard procedures using ReverTra Ace enzyme (Toyobo, Tokyo, Japan).

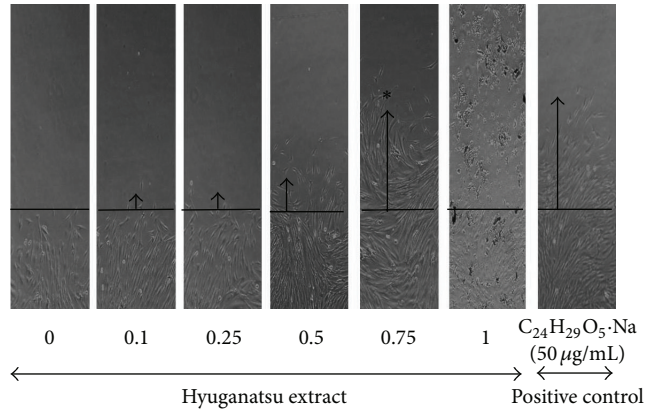
Primer sequences were as follows:

Rac-1: forward: 5′-CCCTATCCTATCCGCAAACA-3′,

reverse: 5′-CGCACCTCAGGATACCACTT-3′;

Rho-A: forward: 5′-CATCCGGAAGAAACTGGT-3′,

reverse: 5′-TCCCACAAAGCCAACTC-3′;



*$P < 0.05$

Figure 3: *In vitro* monolayer scratch cell migration assay. Confluent cells were treated with different concentration of HE (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) and also beraprost (50 μg/mL) for 16 h time duration. Distance travelled from wound edge to the highest cell point was calculated in mm. Black line indicates the wound edge.

Cdc-42: Forward: 5′-CATCCGGAAGAAACTGGT-3′,

reverse: 5′-TCCCACAAAGCCAACTC-3′;

Cdk-1: forward: 5′-GGGTAGAGGAGGTGCGGGC-3′,

reverse: 5′-GCGATGGCCCAGCTCCTC-3′;

Cdk-2: forward: 5′-CGCTTCATGGAGAACTTC-3′;

reverse: 5′-GAAGTTCTCCATGAAGCG-3′.

2.2.7. Translation and Transcription Inhibition Assays. Sub confluent cells were pretreated for 2 h with 10 μg/mL of the translational inhibitor, cycloheximide (CHX) in serum-free medium. The cells were then treated with or without HE (0.75 mg/mL) and incubated further for 6 h. Total RNA was isolated, and expression levels of Cdk-1 and Cdk-2 were measured with β-actin as internal control. DRB, a transcriptional inhibitor, was used to inhibit the rate of transcription. Cells were pretreated with 10 μg/mL of DRB for 2 h in serum-free medium to prevent new DNA synthesis and treated with HE (0.75 mg/mL). Zero h represents the time of HE addition. Total RNA was isolated at various time intervals (0, 2, 4, 6, and 8 h), and Cdk-1 and Cdk-2 mRNA expression levels were measured against β-actin.

2.3. Statistical Analysis. Each experiment was carried out in three independent sets. Mean values and standard deviation were calculated. The Kruskal-Wallis test to compare more than two groups was used to judge the statistical significance. Statistically significant values were set at the level of $P < 0.05$.

3. Results and Discussion

In this study, we used human skin fibroblasts (TIG-119) to investigate the effect of HE on the patterns of cellular

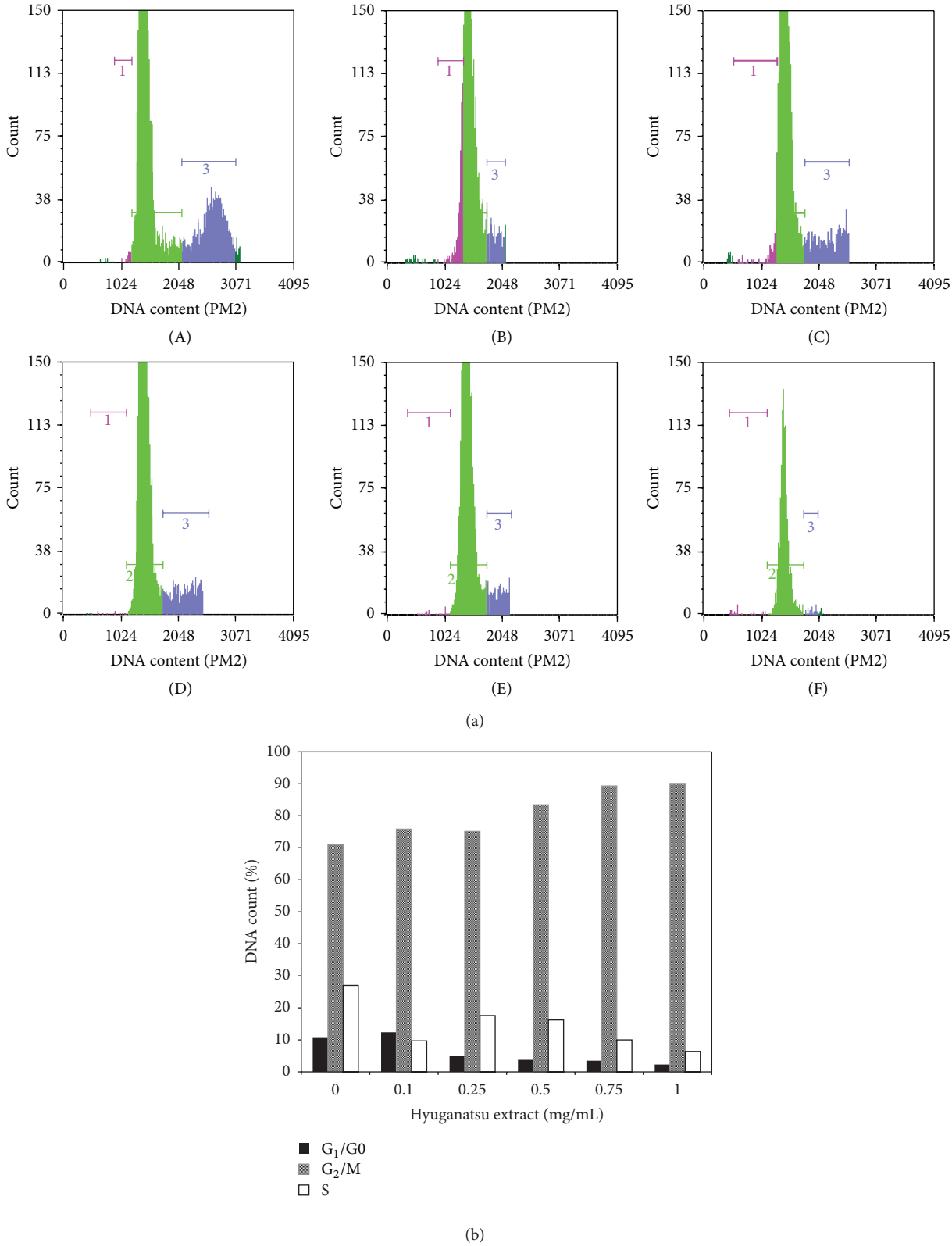


Figure 4: (a) Cell cycle analysis. TIG-119 fibroblast was incubated with different concentrations: (A) 0.0 mg/mL, (B) 0.1 mg/mL, (C) 0.25 mg/mL, (D) 0.5 mg/mL, (E) 0.75 mg/mL, and (F) 1.0 mg/mL of HE for 16. Both floating and adherent cells were collected and analyzed by flow cytometry. The inserts show the proportion of cells in each phase and marked with different colors (pink: $G_1/0$ phase: green, G_2/M phase, and blue: S phase). (b) The data are expressed (% of each phase) as the mean percentage of each phase from independent experiments.

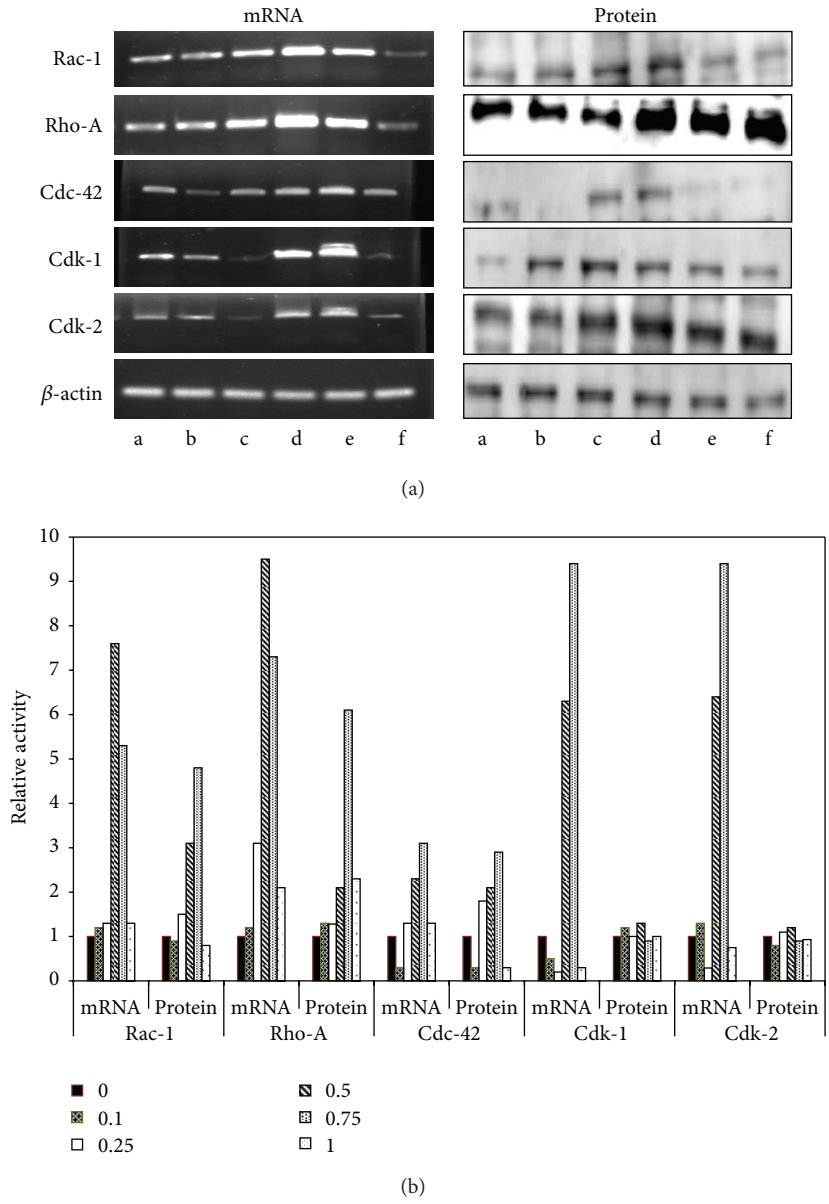


Figure 5: RT-PCR and WB analysis. (a) Western blot analysis and semiquantitative reverse transcription analysis of Rac-1, Rho-A, Cdc-42, Cdk-1, Cdk-2, and β-actin. Cells were treated with different concentration of HE (0, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL) and processed for RT-PCR and W.B analysis for genes (right panel) and proteins (left panel), respectively. Data here represents the picture from one of three independent experiments. (b) Relative values of mRNA and protein expression which are normalised against β-actin.

proliferation, migration, and associated mechanism. The analyzed end points were (i) cell replicative capacity (PDL), (ii) cell cycle changes, (iii) *in vitro* cell migration pattern and (iv) fluctuations of cell cycle target genes and proteins.

3.1. Dose-Dependent Activity of HE on Cytotoxicity. A dose-dependent increase in cell number was noticed during 16 h treatment period with maximum and significant ($P < 0.05$) increase at 0.75 mg/mL concentration level. High concentration of HE (1 mg/mL) inhibited cell proliferation and displayed cytotoxic effect on TIG-119 cells (Figure 1). Lactic dehydrogenase release to the medium during treatment with different concentrations of a compound is the hallmark of cytotoxicity of the compound because of membrane lysis, oxidation reaction of lactate to pyruvate, and subsequent reaction of pyruvate with INT tetrazolium to form formazan. Treatment with 1 mg/mL of HE resulted a 50% decrease in cell number at 72 h. However, at other concentration, there was no difference in cell proliferation between the control groups cells and cells treated with HE (Figure 2). Above results collectively show HE exerts a biphasic action on the fibroblast proliferation. At lower concentrations, HE did not show any cytotoxicity, but at higher concentration of 1 mg/mL, HE displayed cytotoxicity reversed; this could be attributed to the

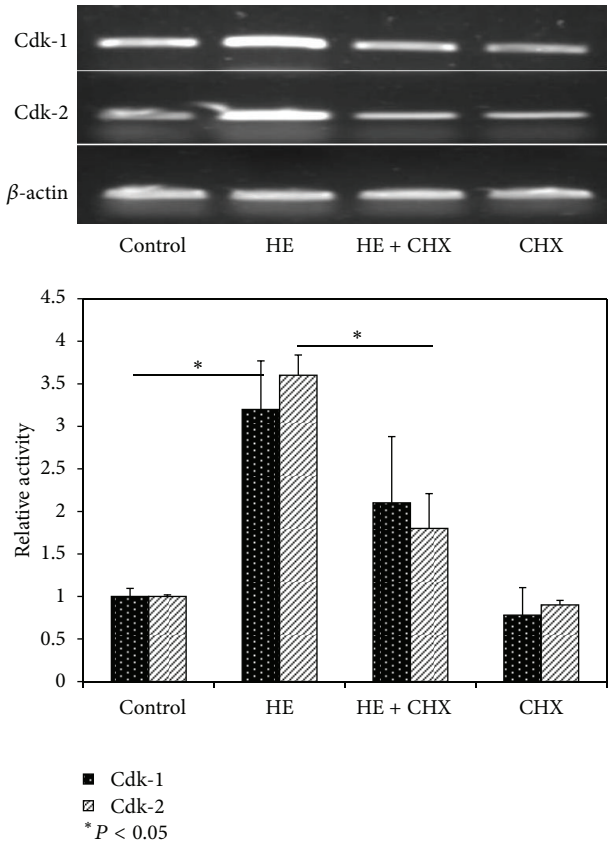


Figure 6: Translation inhibition assay. Effect of cycloheximide (CHX) on HE-induced Cdk-1 and -2 mRNA expression. TIG-119 cells were pretreated for 2 h with 10 μg/mL CHX in serum-free medium and then treated with or without 0.75 mg/mL/mL of HE for 6 h. Total RNA was isolated and analysed for mRNA signals. Relative values of gene expression were depicted. Data represents the values of three independent experiments. Significant values were calculated by Student's t-test ($P < 0.05$).

crude nature of the test sample. This study supports several other investigations that reported that natural antioxidants increased the number of oral fibroblast [11] and endothelial cells [12] and modulated the growth of endometrial stromal cells [13].

3.2. HE Induces G_2/M Stage of Cell Cycle. Functional end point of fibroblast cell division and proliferation is cell migration [3] into wounded area [11]. An established *in vitro* scratch assay model was used to quantitatively define human skin fibroblast migration in a monolayer cell model by using NIH Image J software analysis. As depicted in Figure 3, HE dose dependently increased the rate of migration into wounded area up to the concentration of 0.75 mg/mL. The rate of cell migration was similar to that observed in the presence of beraprost, a potent cell migration inducer. Therefore it is proposed that HE is noncytostatic with proproliferative capacity to induce fibroblast cell migration.

Cell proliferation and migration are hallmarks of cell division. DNA duplication is the key step in cell division and it is controlled by different stages: G_1/0, G_2/M, and S phases of cell cycle [14]. Subsequently, we studied if HE treatment affected different stages of cell cycle. As seen in Figure 4, G_2/M phases of cell cycle were dose dependently increased upon HE treatment with highest value obtained with 0.75 mg/mL treatment. As depicted in Figure 4(b), there were no significant differences in percentage of DNA count at G_2/M phase (21.2, 9.7, 17.6, 16.2, 10.1, and 16.3) and S phase (10.2, 12.4, 3.9, 2.5, 2.3, and 0.7) between different treatment groups, while DNA content in G1/0 phase (50.2, 53.3, 59.7, 82.1, 89.4, and 63.3) varied significantly. This shows that HE does not arrest cells in G_2/M phase but induces the initiation of DNA synthesis. Several lines of evidence support a molecular mechanism in the response to natural compounds stimulation that does arrest the G_1/0 phases, and increases G_2/M phase in fibroblast [15, 16].

3.3. HE Acts Differentially on Rho Family GTPase and Cdk. Fibroblast cell proliferation and migration phenomena are principally governed by Rho family GTPase like Rac-1, Rho-A, and Cdc-42 [4]. The cell cycle phases are coordinated by the expression and/or activation of regulatory proteins, like cyclins (e.g., cyclin A, D, and E), cyclin-dependent kinases (Cdk) mainly Cdk-1 and -2, and Cdk inhibitors. Both cyclins and cyclin-dependent kinases have also been implicated in the formation of actin cytoskeleton in mammalian fibroblast cells [17]. In order to gain an insight into the mechanisms of HE action, we studied the expression of Rac-1, Rho-A, Cdc-42, and Cdk-1 and Cdk-2 mRNA by RT-PCR assay and WB analysis. Cells exposed to 0.1, 0.25, 0.5, and 0.75 mg/mL. HE showed concomitant increase in both mRNA and protein levels of Rac-1, Rho-A, and Cdc-42 (Figure 5(a)). Cyclin-dependent kinases have been identified as key proteins in the G_2/M transition that help maintain steady-state level of M phase through inhibition of PP 2A/B558 [18]. Molecular mechanisms of mammalian cell migration were first revealed in fibroblasts where Rho-A, Rac-1, and Cdc-42 facilitate the multistep process including the establishment and maintenance of polarity, formation of actin-rich protrusions, remodeling of adhesive contacts, and generation of force. Our results revealed that HE (0.75 mg/mL) induces levels of Rho-A, Rac-1, and Cdc-42 (Figure 5(a)) and helps in cell migrations as shown in Figure 3. Results also showed that mRNA levels of Cdk-1 and -2 increased significantly (Figure 5(a)). However HE did not induce Cdk-1 & -2 protein levels (Figures 5(a) and 5(b)). This observation leads us to hypothesie that HE extract differentially acts on mRNA and protein level working either at transcriptional or translation stage.

In order to prove the above hypothesis, we first treated subconfluent cells with HE (0.75 mg/mL) with/or without the translational inhibitor, CHX for 6 h, and analysed Cdk-1 and -2 mRNA expressions. As shown in Figure 6, HE alone (0.75 mg/mL) caused about 3-fold increases in both Cdk-1 and -2 mRNA activities; however, CHX abolished the effect of HE. Interestingly, CHX *per se* did not have any effect on Cdk-1 and -2 mRNAs. It is possible that induction of T Cdk-1 and Cdk-2 by HE could depend on some protein factor,

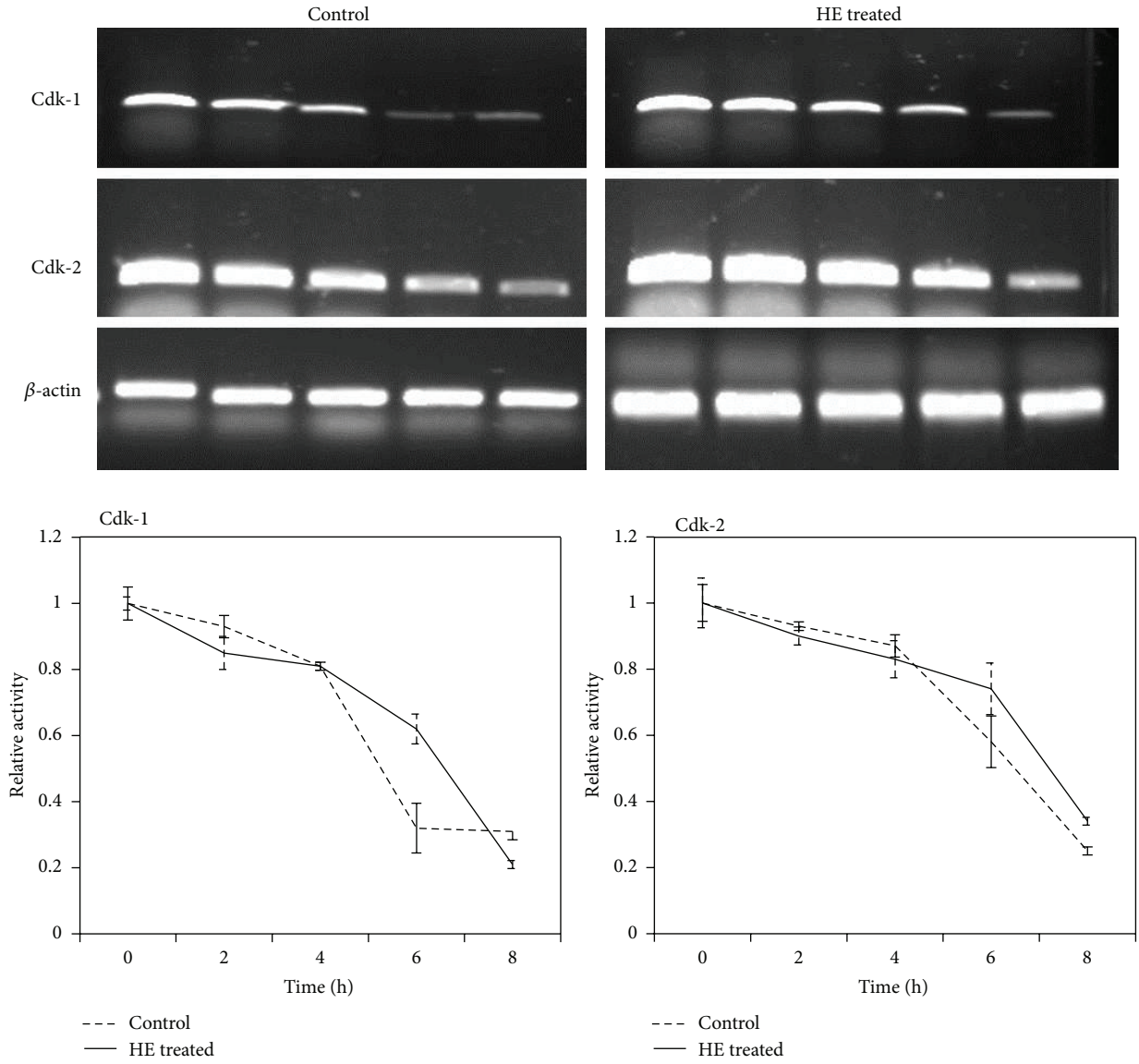


Figure 7: Transcription inhibition assay. Cdk-1 and -2 mRNA expression levels were measured following the treatment of fibroblasts cells with transcription inhibitor, DRB. Cells were pretreated with DRB for 2 h in serum free medium. Time 0 represents the time of HE addition. Total RNA was collected at various times thereafter and analysed by RT-PCR. Relative gene expressions were calculated plotted.

that is, inhibited by CHX treatment. We further checked the status of regulation at the transcription level. Subconfluent cells were conditioned for 2 h with DRB to inhibit ongoing transcription prior to treatment with HE (0.75 mg/mL) for varying time period. Figure 7 reveals the influence of DRB on Cdk-1 & -2 mRNA expression. The mRNA levels in both control as well as HE-treated group diminished with time course. HE failed to overexpress both Cdk-1 and -2 mRNA in the event of transcription inhibition. This implied that ongoing transcription was necessary for the HE action on Cdk-1 and -2 expressions.

The cyclin-E-dependent kinase Cdk-2 plays a crucial role in cell cycle progression, and the activity of Cdk-2 has been shown to be adhesion dependent and also correlates with mitogenic activity [19]. Taking into consideration the translation and transcription inhibition assays, we conclude that Cdk-1 and -2 regulations by HE are governed at transcription stage where *de novo* protein synthesis is required.

4. Summary

The evidence reported in the current study, combined with the relative safety of HE, suggests that HE has the potential to be developed as a new non-toxic nutraceutical agent for treating skin disorders. The results reported here should stimulate further research into identifying the active molecule which induces the coordinated cell migration and proliferation

response in wounded tissues and thereby promotes a better understanding of this fundamental homeostatic process.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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Review Article

Current Aspects in the Pathophysiology and Treatment of Chronic Wounds in Diabetes Mellitus

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Impaired wound healing is a frequent and very severe problem in patients with diabetes mellitus, yet little is known about the underlying pathomechanisms. In this paper we review the biology of wound healing with particular attention to the pathophysiology of chronic wounds in diabetic patients. The standard treatment of diabetic ulcers includes measures to optimize glycemic control as well as extensive debridement, infection elimination by antibiotic therapy based on wound pathogen cultures, the use of moisture dressings, and offloading high pressure from the wound bed. In this paper we discuss novel adjuvant therapies with particular reference to the use of autologous skin transplants for the treatment of diabetic foot ulcers which do not respond to standard care.

1. Introduction

The diabetic foot syndrome is a very severe and common complication in patients with diabetes mellitus with a cumulative lifetime incidence of up to 25 percent [1]. The escalating high rates of diabetes in many parts of the world make diabetic foot ulcers a major and increasing public-health problem. Foot ulcers cause substantial morbidity, impair quality of life, are the most important risk factor for lower-extremity amputation, and result in high treatment costs and enormous economic losses [2]. The factors that delay wound healing in diabetes are multiple and relate both to the impaired glucose metabolism and to the effect of neurovascular complications. Diabetic foot ulcers readily become chronic; all too often these wounds do not heal primarily. Treatment of chronic wounds should be essentially directed against the main etiologic factors responsible for the wound. Management is based on the simple principles of eliminating infection, the use of dressings to maintain a moist wound bed and to absorb exsudate, offloading high pressure from the wound bed, and debridement to accelerate endogenous healing and facilitate the effectiveness of topically applied substances [3]. Nevertheless, there are often cases of persistent diabetic foot ulcers that do not respond to standard care. In such patients, skin replacement therapies either by autologous skin transplantation or by tissue-engineered human skin equivalents are second-line options which could prevent an amputation and should therefore be considered.

2. Physiological Process of Wound Healing

The physiological process of wound healing is traditionally divided into four phases: haemostasis, inflammation, proliferation, and maturation or remodelling. These phases are orchestrated by a subtle interplay of cellular and humoral factors [4]. Haemostasis occurs within an hour after injury and is characterized by vasoconstriction and clotting. Platelets not only initiate the clotting cascade but also secrete growth factors and cytokines which initiate healing. The subsequent inflammation phase takes up to seven days and is mediated through neutrophil granulocytes which prevent bacterial contamination and cleanse the wound from cell debris.

Monocytes are attracted to the wound by chemotactic factors and differentiate into wound macrophages. The latter not only remove bacteria and nonviable tissue by phagocytosis but also release various growth factors required to stimulate fibroplasia and angiogenesis, thereby providing the basis for the formation of the provisional extracellular matrix (ECM). The proliferation phase is initiated at day 2 after injury and takes up to 20 days. This phase is primarily characterized by tissue granulation and formation of new blood vessels (angiogenesis). The angiogenic process involves growth factors such as platelet-derived growth factor (PDGF), macrophage angiogenesis factor, and angiotensin. Concomitant epithelialisation is then initiated to cover the granulation tissue with a cellular barrier. The last phase involving extensive tissue remodelling lasts from one week to six months after injury. During that phase the provisional wound matrix is replaced with proteoglycan and collagen molecules which readily become organised into thicker bundles resulting in stronger but more rigid scar tissue.

3. Pathophysiology of Wound Healing in Diabetes

Wound healing in diabetes is impaired by factors that are both extrinsic and intrinsic to the wound and its biology. Extrinsic factors include repeated trauma or mechanical stress applied to a foot that has been rendered insensitive due to neuropathy as well as ischemia as a result of macro- or microvascular disease [5]. Thickening of the basement membrane of the capillaries and arterioles frequently occurs in individuals with diabetes, resulting in an impaired wound healing and persistent ulcer formation [6]. An important role has been attributed to factors intrinsic to the biology of the chronic wound in diabetes. It has been postulated that hyperglycaemia itself has a deleterious effect on wound healing through the formation of advanced glycation end-products (AGEs) which induce the production of inflammatory molecules (TNF-α, IL-1) and interfere with collagen synthesis [7]. Furthermore, Spravchikov et al. showed that exposure to high glucose is associated with changes in cellular morphology, decreased proliferation, and abnormal differentiation of keratinocytes [8], thus revealing another mechanism by which hyperglycaemia may affect wound healing in diabetes. Interestingly, the healing times of leg and foot ulcers are decreased in diabetic patients with lower HbA1c, thereby emphasizing the clinical correlation between hyperglycaemia and impaired wound healing [9]. An altered immune function may also contribute to poor wound healing in patients with diabetes. Decreased chemotaxis, phagocytosis, bacterial killing [10], and reduced heat shock protein expression [11] have been implicated in the early phase of wound healing in diabetes. Fahey et al. demonstrated that altered leukocyte infiltration and wound fluid IL-6 characterize the late inflammatory phases of wound healing in diabetes [12]. It therefore seems that an altered pattern of cytokine appearance in the wound milieu may contribute to delayed wound healing in diabetes. This is substantiated by the fact that altered bioavailability of cytokines and growth factors have been implicated in the pathogenesis of chronic wounds. These signalling molecules are secreted by various cell types to control cellular proliferation, differentiation, migration, and metabolism. Abnormal expression of growth factors has been observed in diabetic foot ulcers [13]. It has been postulated that trapping of growth factors and cytokines by certain macromolecules such as albumin, fibrinogen, and β2-macroglobulin may disrupt the healing process [14]. Furthermore, increased degradation of growth factors in wound fluid of diabetic subjects has been discussed as a factor contributing to an impaired wound healing process. For example, Duckworth et al. have reported an increased activity of insulin degrading enzyme (IDE) activity in wound fluid from patients with diabetic foot ulcers [15]. Interestingly, insulin degrading activity in the wound fluid was found to be positively correlated with HbA1c levels, thereby supporting the fact that glucose control is an essential prerequisite for wound healing. In addition, normal wound healing requires a balance between the accumulation of collagenous and noncollagenous extracellular matrix components. Their remodelling is determined by matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) [16]. MMPs play essential roles in initial wound debridement as well as in angiogenesis, epithelialization, and remodelling of scar tissue [17]. Several studies reported elevated levels of MMPs and reduced levels of TIMPs in chronic wounds [18] with a similar pattern in wounds of patients with diabetes mellitus [19]. Last but not least, there is also increasing evidence that the resident cells of chronic wounds may undergo phenotypic changes that impair their capacity for proliferation and movement. For example, it has been reported that fibroblasts from venous and pressure ulcers are senescent and have a diminished ability to proliferate with the proliferative capacity being directly correlated to the failure to heal [20].

4. Standard Treatment Methods in Diabetic Foot Ulcers

The standard treatment of diabetic ulcers includes measures to assess vascular status and optimize glycemic control as well as extensive debridement, infection elimination by antibiotic therapy based on wound pathogen cultures, the use of moisture dressings, and offloading high pressure from the wound bed. Vascular assessment should include palpation of all lower-extremity pulses, including femoral, popliteal, posterior tibial, and dorsalis pedis pulses. A surrogative and more accurate method of diagnosing vascular insufficiency in the lower limbs is the use of the ankle branchial pressure index (ABPI), the results of which can be validated through Doppler waveform and pulse oximetry. In case of significant peripheral arterial disease, therapeutic revascularisation should be undertaken, since adequate vascular supply is essential for wound healing. The correlation between normoglycaemia and facilitated wound healing in diabetes has been discussed in the previous section. The pivotal role of surgical debridement in healing of diabetic foot ulcers is widely acknowledged [21]. The rationale lies in removing necrotic, devitalized wound bed and wound edge tissue that

inhibits healing, so that secondary wound healing can be achieved [22]. The determination of organisms responsible for a diabetic foot infection via culture of appropriately collected tissue specimens enables clinicians to make optimal antibiotic choices based on culture and sensitivity results [23]. A recent meta-analysis of randomized controlled trials (RCTs) comparing the effects of different types of wound dressings in the treatment of diabetic foot ulcers found no significant differences between them so that aspects such as the dressing cost and the wound properties should be considered when making a decision [24]. A strong association between the efficacy to offload the foot and clinical outcome is supported through evidence-based guidelines [25].

5. Additional Current Treatment Methods in Persistent Diabetic Foot Ulcers

5.1. Autologous Skin Transplantation in Diabetic Foot Ulcers. Flaps and grafts are the two principal surgical procedures for skin tissue replacement. A flap is a full-thickness portion of skin sectioned and isolated peripherally and in depth from the surrounding skin, except along one side, called the peduncle. A graft is a section of skin of variable thicknesses and sizes completely detached from its original site and used to cover the zone to be repaired. Particular attention should be paid to mesh grafts which are obtained by passing a whole dermoepidermal explant through a special surgical tool (mesher), thereby increasing the initial surface area of the explanted skin [26]. Skin grafts are traditionally used in the treatment of severe burns. However, a number of studies have recently reported successful managing of large tissue defects in patients with diabetic foot ulcers with microsurgical grafts [27–29]. The process of graft adoption is defined as the adhesion of the graft skin to the recipient wound area and its subsequent vascularization. This process is identical to that of wound healing. Following an initial rejection phase after the skin grafting procedure with massive inflammation, revascularization of the graft starts after 24 to 48 hours. Initially the graft is pale and white but subsequently adopts a pinkish colour which indicates successful adoption in association with firm attachment to the bed. Apart from immune compatibility, basic conditions for graft taking encompass the ability for neoangiogenesis, good adherence of the graft to recipient areas, and hence accurate immobilization of the graft. A graft can only be placed to vital exposed dermis capable of producing granulation tissue. The recipient area must not be infected or excessively exudative. In addition well-functioning haemostasis is required. In fact, any accumulation of exudate or blood underneath the graft jeopardizes its survival as it impedes adherence and penetration of new capillaries. The consequent handling of the transplant is of utter importance. In the first weeks after transplantation, complete removal of pressure is essential. Protective footwear with dully formed inserts can secure adequate offloading of the area of high pressure and protect the transplant.

5.2. Tissue-Engineered Human Skin Equivalents in Diabetic Foot Ulcers. In the recent years much attention has been paid to the use of tissue-engineered human skin equivalents in the treatment of diabetic foot ulcers. The first engineered skin substitutes were matrix-based products consisting of cross-linked collagen and glycosaminoglycans. The matrix eventually undergoes degradation, while simultaneously the host's cells invade and proliferate within it. Integra, a product of this category, has shown promising results in deep wounds [30]. The second generation of tissue-engineered skin equivalents consisted of cell-based products, mostly keratinocytes. Marston et al. demonstrated that dermagraft, a cryopreserved human fibroblast-derived dermal substitute, is a safe and effective treatment for diabetic foot ulcers [31]. Veves et al. showed that the application of graft skin (Apligraf)—a human skin equivalent manufactured from cultured living dermis and sequentially cultured epidermis of neonatal foreskins—results in significantly improved healing compared to other available treatments. Moreover, there were no significant side effects [32]. Nevertheless, both products are ultimately rejected, so that their primary task appears to be a transient restoration of the dermis until the patients' keratinocytes can migrate and close the wound.

5.3. Bone Marrow-Derived Cells. Another very promising therapeutic option involves the use of bone marrow-derived cells, and recent evidence indicates that bone marrow contains stem cells with the potential for differentiation into a variety of tissues. For example, patients with diabetes are known to have an impaired mobilization of endothelial progenitor cells (EPCs) in the bone marrow and decreased accumulation of these cells in wounds [33, 34]. Bone marrow-derived cells may thus be a valuable and unlimited source of progenitor and/or stem cells [35]. For example, Badiavas and Falanga described that the local application of autologous bone marrow-derived cells resulted in complete wound closure in 3 patients unresponsive to standard therapies including bioengineered skin application and autologous skin grafting [36].

Furthermore, it is assumed that hyperbaric oxygen results in EPC recruitment but does not improve migration of EPC to the wound site. However, in a murine model of diabetes coadministration of stromal cell-derived factor-1-alpha (SDF-1α) resulted in homing of the activated EPCs to the wound site [37]. These data suggest that combining oxygen therapy with SDF-1α may improve wound healing in patients with diabetes.

Another novel interesting approach consists of lineage commitment of stem cells to the keratinocyte lineage. This can be achieved through exposure of the stem cells to a mixture of cytokines, growth factors, and extracellular matrix components in vitro and has been attempted with only moderate success [38, 39]. Another method is through genetic modulation, in particular transfection of stem cells with recombinant DNA encoding for proteins that regulate the commitment to the keratinocyte lineage [40]. Although this method presents with exciting new potential, one cannot overlook the potential detrimental effects and safety concerns of genetic manipulation of stem cells [41].

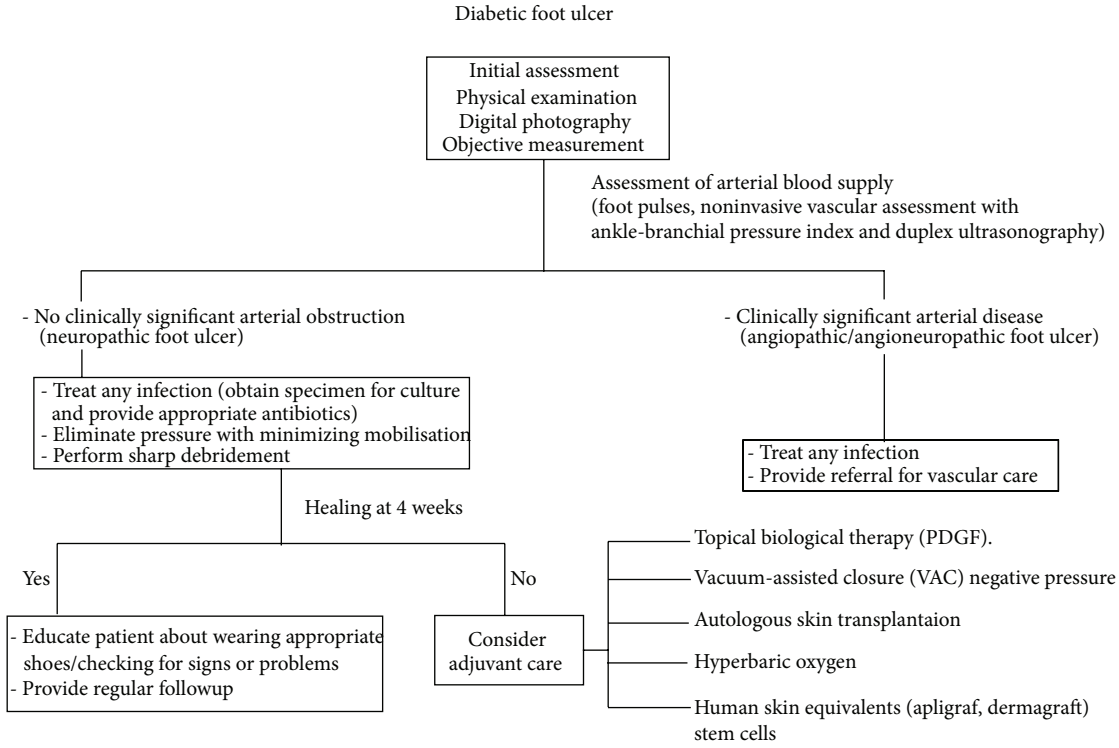

Figure 1: Algorithm for the management of diabetic foot ulcers.

5.4. Growth Factors. Of the known growth factors with a proposed role in wound healing, therapeutic efficacy has been demonstrated only for becaplermin (recombinant human platelet-derived growth factor, Regranex) in several randomized controlled clinical trials [42]. Nevertheless, recent data reported an increased cancer risk in patients treated with more than three tubes of becaplermin so that pending lower follow-up data on the potential risk of malignancy in connection with its use this agent should be used with extreme caution in patients with diagnosed malignancy [43].

5.5. Subatmospheric Pressure Dressings. The use of subatmospheric pressure dressings such as the commercially available vacuum-assisted closure (VAC) device have been shown to be an effective way in accelerating the healing of various wounds. This technique optimizes blood flow, decreases local tissue edema, and removes excessive fluid from the wound bed. Additionally, the cyclical application of subatmospheric pressure alters the cytoskeleton of the cells in the wound bed thereby triggering a cascade of intercellular signals that increases the rate of cell division and formation of granulation tissue. The success rate of skin grafting is significantly increased when VAC is used as bolster covering the freshly skin-grafted wound [44, 45]. A recent review assessing current modalities in the treatment of diabetic foot ulcers [46] concluded that although vacuum compression therapy has been linked to significant reduction in wound area [47] and time to healing [48], this treatment was not shown to be costeffective and should therefore be used only in exceptional circumstances [49].

6. Perspectives and Conclusion

The treatment of diabetic foot ulcers is a constant challenge in diabetes care and requires a multidisciplinary approach involving doctors, physiotherapists, specialised podologists, and orthopedic technicians. Over the recent years, novel and promising therapeutic options have emerged for the treatment of chronic diabetic foot ulcers, as summarized in Figure 1. However, clinical studies are needed in order to develop a well-structured algorithm for the assessment and treatment of diabetic ulcers to prevent lower-extremity amputations due to this complication.

7. Basic Conclusions

(i) The four phases of physiological wound healing are: haemostasis, inflammation, proliferation, and remodelling.

(ii) Wound healing in diabetes is impaired by factors that are both extrinsic and intrinsic to the biology of wound.

(iii) The standard treatment of diabetic ulcers includes optimization of glycemic control, extensive debridement, infection elimination, use of moisture dressings, and offloading high pressure.

(iv) Current treatment methods in persistent diabetic foot ulcers include autologous skin transplantation, tissue-engineered human skin equivalents, bone marrow

derived cells, growth factors, and subatmospheric pressure dressings.

Abbreviations

AGEs: Advanced glycation end-products
ECM: Extracellular matrix
EPCs: Endothelial progenitor cells
HbA1c: Glycosylated haemoglobin
IDE: Insulin degrading enzyme
IL-1: Interleukin 1
IL-6: Interleukin 6
MMPs: Matrix metalloproteinases
PDGF: Platelet-derived growth factor
SDF-1α: Stromal cell-derived factor-1-alpha
TIMPs: Tissue inhibitors of metalloproteins
TNF-α: Tumour necrosis factor-α.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Treatment of Nonhealing Diabetic Lower Extremity Ulcers with Skin Graft and Autologous Platelet Gel: A Case Series

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Lower extremity ulcers in diabetic patients are difficult to treat. Recently, the use of human blood platelet-derived components in this indication has been raising interest. In this study, we have evaluated the safety and efficacy of the combination of autologous platelet gel (PG) and skin graft for treating large size recalcitrant ulcers. Eight consecutive diabetic patients aged 25 to 82 with nine nonhealing lower extremity ulcers (median size of 50 cm^2; range 15–150 cm^2) were treated. Skin ulcer was debrided, and the wound was sprayed after 7 to 10 days with autologous platelet-rich plasma and thrombin. Thin split-thickness skin graft with multiple slits was then applied on the wound bed and fixed with staples or cat-gut sutures. There were no adverse reactions observed during the study. Eight out of 9 skin grafts took well. The interval between skin graft and complete wound healing ranged from 2 to 3 weeks in the 8 successful cases. No ulcer recurrence was noted in those patients during the follow-up period of 2 to 19 months. In this study, the combination of autologous platelet gel and skin grafting has proven beneficial to heal large-size recalcitrant ulcers.

1. Introduction

About 15% of diabetic patients will develop chronic ulcer, and about 25% of those will have to undergo foot amputation [1, 2]. In the nonhealing diabetes mellitus (DM) ulcers, in addition to vascular and neurological disorders, the healing process is impaired in part due to deficiency of growth factors [3]. Becaplermin, a recombinant human platelet-derived growth factor-BB (Regranex, PDGF-BB, Systagenix Wound Management, Gargrave, UK) is the only growth factor preparation approved by the FDA for treating DM ulcers, but it requires daily applications for weeks to months [4, 5]. Live skin equivalents, known as Apligraf and Dermagraft, accelerate wound healing, but also require frequent (weekly) applications, exhibit short shelf-life, and are expensive [6]. The use of an adenovirus encoding human platelet-derived growth factor formulated in bovine collagen gel (GAM501) for treating small nonhealing diabetic foot ulcer has been reported [7, 8]. Despite these advanced researches, a more practical and effective therapy for nonhealing diabetic ulcer is clinically needed.

Platelet-rich plasma (PRP) has been proposed as an adjunct for the treatment of diabetic foot ulcers [9–11]. PRP is most often mixed with thrombin before application in order to generate a fibrin gel, often called platelet gel, and a platelet-growth-factors-rich exudate [12]. Thrombin-activated platelets release numerous growth factors from their α-granules [13] that can modulate cell proliferation and differentiation and accelerate soft tissue repair *in vivo* [14]. A recent systematic review and meta-analysis of the use of PRP therapy in cutaneous wounds does show that it can improve wound healing compared to control wound care in small hard-to-heal acute and chronic wounds [15, 16]. In addition, platelet materials exert antimicrobial activity against some

bacteria of the skin flora [17], and clinical data show that the presence of infection is reduced in PRP-treated wounds [15]. Therefore, platelet materials exhibit a set of advantages that can provide a practical and effective treatment approach for small hard-to-heal ulcers. However, for the large unhealing diabetic ulcers, our experience is that it is imperative to use skin graft as the definite procedure for wound healing.

In a recent study with 17 ulcers of various etiology, we have shown that a skin grafting was improved by a combination of single-donor allogeneic platelet gel and fibrin glue [18]. However, as patients may express safety concerns on the use of allogeneic blood products, the current study evaluates for the first time to our knowledge the safety and efficacy of using autologous platelet gel, without fibrin glue, to enhance skin graft take for nonhealing diabetic lower extremity ulcers.

2. Material and Methods

2.1. Clinical Study Approval and Patients. This clinical study was a prospective pilot trial approved by the Institutional Review Board of Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan (Protocol 098-05-301). Eligible patients were enrolled after informed written consent was obtained. The protocol conformed to ethical guidelines of the 1975 Declaration of Helsinki. From January 2010 to September 2012, eight consecutive diabetic patients with nine nonhealing lower extremity ulcers were treated. The ulcers were not curable for at least 3 months prior to the enrollment using conservative treatments including daily dressing change, topical application of antibiotic ointment, and synthetic dressing coverage using Aquacel and Duo-DERM (ConvaTec, Garenne-Colombes, France). Pregnant women, patients with ischemic change of leg (Transcutaneous oxygen tension $TcPO_2$ < 30 mmHg), severe cardiovascular disorder, and patients refusing to donate blood were excluded. No patient required revascularization surgery of the leg. $TcPO_2$ was measured for vascular perfusion of the leg. All patients have value above 30 mmHg. There were two men and six women, aged 25 to 82. The ulcers had a median size of 57 cm^2 (range 15 to 150 cm^2). The median duration of diabetes and ulcer before study enrollment was 10.6 years (range 5 to 25 years) and 6.5 months (range 3 to 24 months). None of the patients had received conventional skin grafting in the past, and no one was tobacco user. Patient's demography is reported in Table 1.

2.2. Preparation of PRP. PRP was prepared using the SEPAX system (Biosafe SA, Eysins, Switzerland) (Figure 1(a)). An amount of 100 mL venous blood was drawn from the patient into blood bag containing 22 mL of anticoagulant (JMS Singapore Pte Ltd, Singapore). After 20 minutes of processing using the VGR protocol (SEPAX), PRP, PPP (platelet poor plasma), and RBC (red blood cell) were collected individually (Figure 1(b)). The PRP was drawn aseptically into a sterile syringe.

2.3. Preparation of Thrombin. Thrombin was prepared as in our previous studies [18, 19]. Then, 10 mL of PPP and 0.3 mL of a 10% calcium chloride solution were introduced into a sterile thrombin generation device (TGD-001; Merries International Inc., Shin Tien, Taiwan) (Figure 1(c)). The device was shaken gently for 30 seconds and then put aside to let the plasma activation reaction proceed at room temperature. After approximately 15 minutes, a fibrin clot was formed, and the thrombin-rich supernatant was drawn aseptically using a sterile syringe.

2.4. Preparation of Platelet Gel. Autologous platelet gel was obtained by spraying simultaneously equal volumes of PRP and thrombin using a spray applicator (Merries International Inc.) (Figure 1(d)). Within 5 to 10 seconds, a platelet gel was formed on the wound.

2.5. Surgical Procedures. The nonhealing ulcers were first debrided to remove the infected and necrotic tissues. The wounds were covered with moist saline dressing. Daily dressing change without additional treatment was performed. Repeated debridement was necessary in 6 patients because of residual necrotic tissue. The interval between the debridement and skin graft ranged from 7 to 10 days. During skin graft surgery, the wound bed was sprayed evenly with equal volumes (5 to 7 mL) of autologous PRP and autologous thrombin to form the platelet gel, and a split-thickness skin graft with multiple slits was put on the gel-covered bed, fixed with staples or cat-gut sutures, while a short leg P-P splint was used to immobilize the lower extremity. Every patient was placed on antibiotics during the course according to wound cultural results. Bolster dressing with sofa-tulle was used to avoid postgraft hematoma formation. The skin graft was checked 3 days after surgery. Negative pressure wound therapy (VAC) was not used in this study.

3. Results

3.1. Overall Clinical Data. Fibrinogen presents in the PRP polymerized into a fibrin gel, leading to the formation of platelet gel that adhered to the wound bed (Figure 2). No treatment associated adverse reactions were observed during the study. Most (8/9) of the skin grafts took well apart from one (patient no. 7; case 3 described later). The interval between skin graft and complete wound healing in the seven successful cases ranged from 2 to 3 weeks. No recurrence of the ulcer was noted in those patients during the follow-up period, which ranged from 2 to 19 months. Review of treatments is reported in Table 1. Eight of nine ulcers had complete healing corresponding to a healing rate of 88%, the time to healing ranging from 2 to 3 weeks.

3.2. Case Presentations

3.2.1. Case 1. A 65-year-old male, diabetic for 6 years, suffered from two nonhealing ulcers of left lower leg, measuring 15 × 10 cm^2 and 5 × 7 cm^2, respectively, due to stasis dermatitis for 6 months. The surrounding tissue was severely scared (Figure 3(a)). Debridement was performed twice to remove the necrotic tissue. One week after the second debridement,

Figure 1: Biosafe SEPAX system (a). Autologous PRP and plasma to prepare platelet gel and thrombin (b). Thrombin generation device to activate plasma (c). Double-syringe applicator containing PRP and thrombin (d).

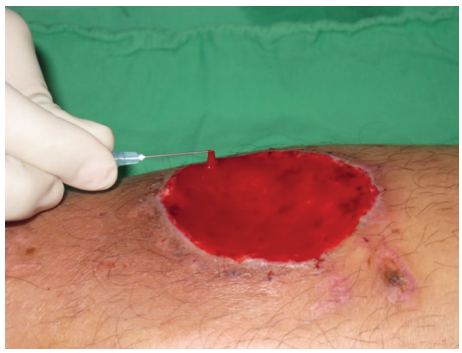

Figure 2: Platelet gel formed on the wound by conversion of fibrinogen into fibrin.

the wound bed was sprayed with autologous platelet gel (Figure 3(b)). A thin split-thickness skin graft was put on gel-covered bed (Figure 3(c)). Compression stocking (30–40 mmHg at the ankle) was used once the wound healed. The postoperative course was uneventful, and the patient has durable wound coverage 10 months after skin graft (Figures 3(d) and 3(e)).

3.2.2. Case 2. A 45-year-old female, diabetic for 13 years, suffered from nonhealing ulcer over right ankle, measuring $6 \times 10\,cm^2$ due to infrared radiation burn for 2 months (Figure 4(a)). One week after debridement, the wound bed was sprayed with autologous platelet gel (Figure 4(b)). A split-thickness skin graft was put on gel-covered bed (Figure 3(c)). The post-operative course was uneventful, and the patient has durable wound coverage 12 months after skin graft (Figure 4(d)).

3.2.3. Case 3. A 72-year-old female, diabetic for 8 years, suffered from nonhealing ulcer over left heel, measuring $10 \times 15\,cm^2$, due to contusion injury for 2 months. The ulcer was deep to the periosteum of calcaneus bone (Figure 5(a)). The patient had no evidence of osteomyelitis with negative bone scan and had normal ESR (erythrocyte sedimentation rate), or CRP (C-reactive protein). Although free tissue transfer would have been required, patient refused the microsurgery, due to the age and medical condition. One week after the third debridement, the wound bed was sprayed with autologous platelet gel (Figure 5(b)). A split-thickness skin graft was put on gel-covered bed (Figure 5(c)). A skin graft

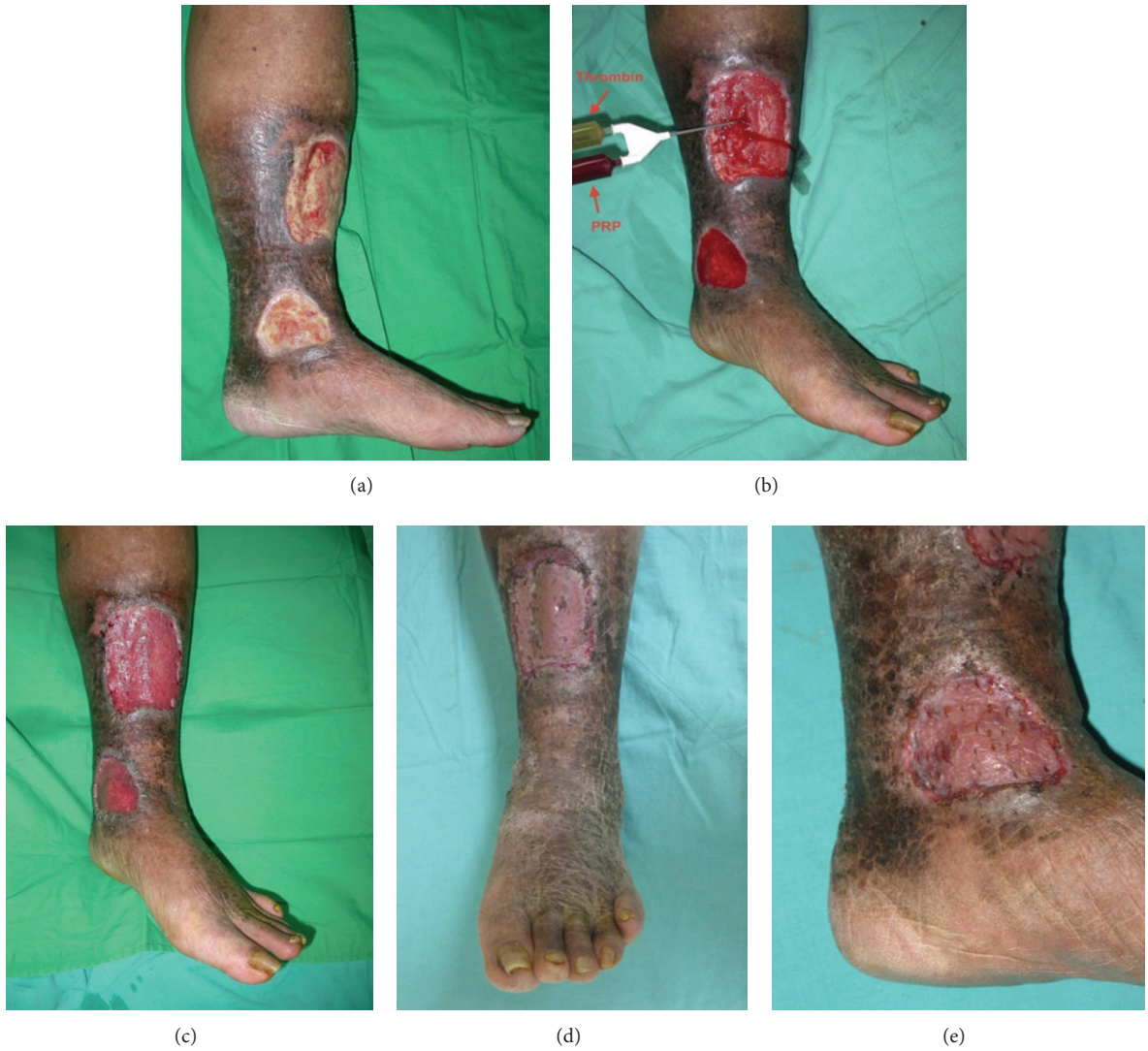

Figure 3: Two chronic ulcers ($15 \times 10\,cm^2$ and $5 \times 7\,cm^2$) with surrounding scar tissues (a). After adequate debridement, the wound was sprayed with PRP and thrombin (b). Skin graft was applied on gel-covered wound bed (c). Durable wound coverage 10 months after skin graft (d, e).

loss of about 3 cm in diameter due to grafting on periosteum of calcaneus bone was noted (Figure 5(d)). The patient died 2 years after surgery because of lethal arrhythmia during dialysis. As we were told by the family, the ulcer did not heal.

4. Discussion

Chronic nonhealing diabetic ulcers of lower extremity develop as a result of peripheral neuropathy, ischemia, and trauma [20]. The goal of treatment is to obtain expeditious wound closure. The standard treatments include adequate debridement, control of infection, re-vascularization of ischemic tissue, and avoidance of undue pressure on the wound. Live skin equivalents show some efficacy but have short shelf-life and are expensive [6]. GAM501 was found to help the treatment of nonhealing diabetic foot ulcer in 15 patients [7]. However, the ulcer size at base-line was small (1.2 to 4,86 cm^2), thereby questioning the clinical relevance for the cure of serious ulcer cases [7]. In a more recent study, complete closure incidence observed in GAM501 (ulcer size: $3.1 \pm 1.7\,cm^2$) and formulated collagen alone (ulcer size: $2.9 \pm 1.1\,cm^2$) was not statistically significant [8].

In vivo cellular-therapy-based PRP or growth factors can serve as an adjunct to those treatments. There is increasing evidence of the efficacy of PRP-based materials to enhance wound healing [15], and, in particular, the results of clinical studies using these materials to treat small-size nonhealing diabetic ulcers are definitely encouraging [18, 21, 22]. In the most common size of diabetic foot ulcers ($<7.0\,cm^2$ in area and $<2.0\,cm^3$ in volume), PRP gel-treated wounds are more likely to heal than control wounds [11].

The benefits of PRP in the treatment of severe and large ulcers have not been evaluated in randomized clinical trials [23]. From a clinical point of view, our experience is that skin

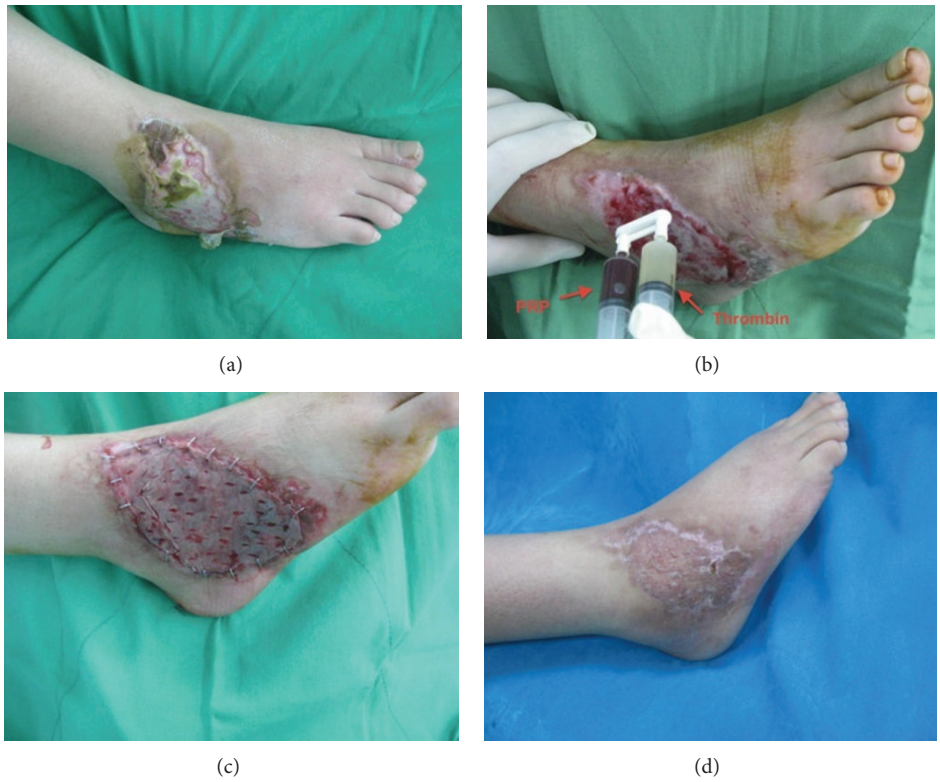

FIGURE 4: Burn injury with chronic ulcer ($6 \times 10\ cm^2$) (a). After adequate debridement, the wound was sprayed with PRP and thrombin (b). Skin graft was applied on gel-covered wound bed (c). Durable wound coverage 12 months after skin graft (d).

grafting is commonly required as a definite surgical procedure for healing large size and deep ulcers. In a recent study, we have presented the benefits of a novel approach for leg ulcer treatment that combines three blood components (PRP, thrombin, and cryoprecipitate) from single-donor allogeneic origin with skin grafting [18]. PRP and human thrombin are first sprayed on the debrided wound to form a platelet gel, and thin spilt-thickness skin graft is then applied on top of the wound. Fibrin glue, obtained by mixing cryoprecipitate with thrombin, was sprayed on the graft to form a fibrin glue that acted as a hemostatic tissue sealant [24] that fixed the graft and avoided the use of staple or sutures [18].

In the current study, patients expressed some concerns about the use of allogeneic blood components due to perceived viral infectious risks. They were able and willing to donate about 100 mL of blood that was centrifuged in the Biosafe SEPAX system to obtain autologous PRP and PPP. PPP was activated by calcium chloride in a specifically designed medical device to generate thrombin. We could not use fibrin glue as it would have required collecting a large volume of blood (typically 450 mL) from the patients to obtain enough plasma for cryoprecipitation. In addition, preparing autologous cryoprecipitate under safe and standardized conditions is not easy within a hospital setting. After debridement of the ulcers, converting a chronic ulcer into acute wound, autologous platelet gel obtained by mixing PRP and thrombin was applied on the wound to form a platelet gel [18, 19]. A thin split-thickness skin graft was applied on top of the platelet gel and fixed with staples or cat-gut sutures. As in our previous study [18], platelet gel was found to enhance the take of the skin graft. Eight out of the 9 skin grafts took without major loss, time to healing ranged from 2 to 3 weeks, and patients achieved durable wound healing in the follow-up period, ranging from 10 to 19 months. One case presented skin graft loss due to exposure of periosteum of calcaneus bone, which would have required a more sophisticated microsurgical free tissue transfer which was denied by the patient.

PRP was prepared using a medical device that concentrates platelets 2.5-to 3.5-fold compared to baseline values in whole blood. Activation by thrombin releases multiple growth factors from the platelet alpha-granules [25]. Those include three isomers of platelet-derived growth factor (PDGF-AA, PDGF-AB, and PDGF-BB), two isomers of transforming growth factor-β (TGF-β1 and TGF-β2), vascular endothelial growth factor (VEGF), and epithelial growth factor (EGF). They are important for neovascularization by mesenchymal cell recruitment and extracellular matrix synthesis [25], resulting in favorable skin graft incorporation. Previous studies using different PRP production devices showed that PDGF-AB and TGF-β1 concentrations in platelet releasates range from 100 to 200 ng/mL [13, 26–28]. PDGF-BB is at about 10 ng/mL [26], EGF and VEGF at 1–5 ng/mL, TGF-β2 at about 0.5 ng/mL [26], and IGF-1 at about 100 ng/mL

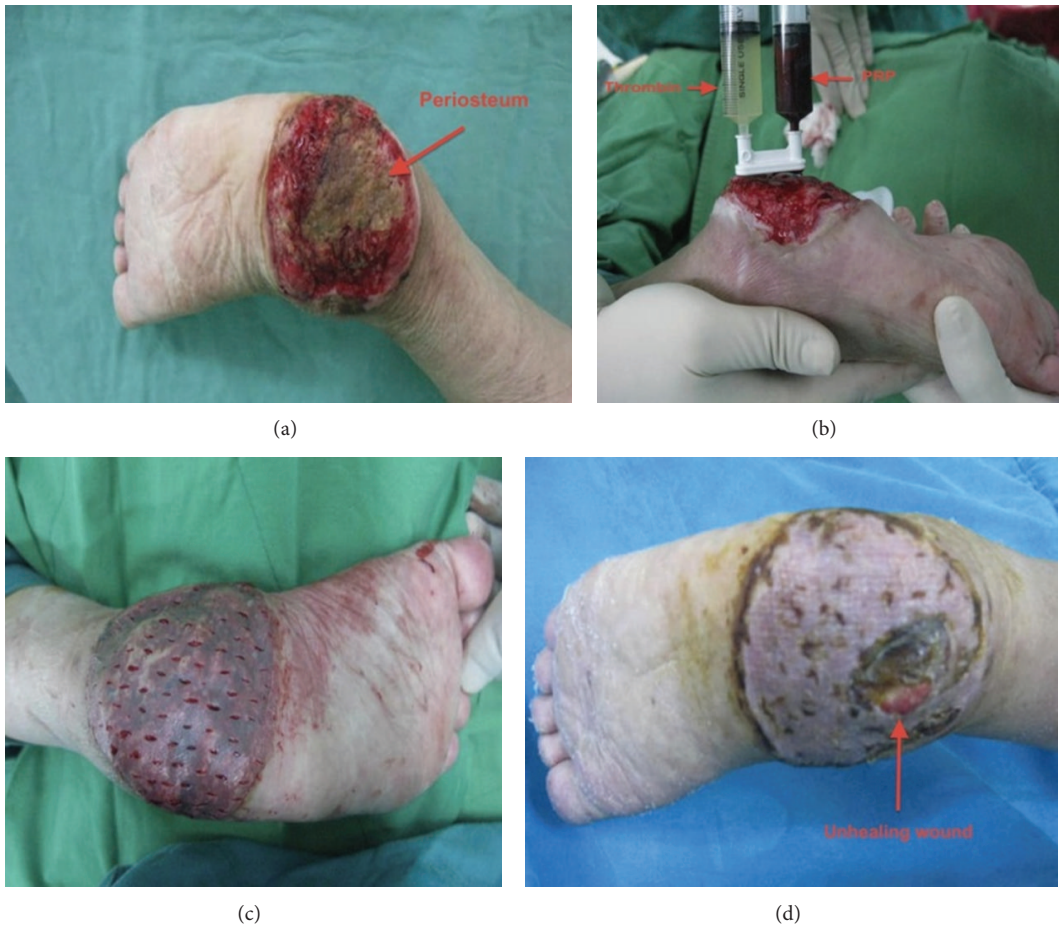

Figure 5: Chronic ulcer ($10 \times 15\,cm^2$) deep to the periosteum of calcaneus bone (Arrow) (a). After adequate debridement, the wound was sprayed with PRP and thrombin (b). Skin graft was applied on gel-covered wound bed (c). Skin graft loss ($3\,cm^2$) over the periosteum, 2 months after skin graft (d).

[29]. Such a physiological mixture of growth factors may be advantageous clinically to achieve wound healing compared to single recombinant growth factor like PDGF-BB [4, 5].

There are pros and cons in the use of autologous versus allogeneic blood materials. In the absence of pathogen inactivation treatment, a major advantage of using autologous platelet gel is avoiding the ethical and legal concerns of exposing the patient to the viral risks of allogeneic products [30], especially in countries with high infectious rates and limited donor screening and donation testing [31]. Using autologous blood leads to better acceptance of the surgical procedure by some patients. Drawbacks of autologous products include potential larger individual variability in the quality of PRP compared to allogeneic products prepared from healthy blood following standardized working procedures of blood establishments [21]. Another limitation relates to the difficulty of preparing autologous cryoprecipitate as a source for fibrin glue. Fibrin glue may be beneficial to stabilize the graft as it comes into direct contact with the wound [18] and to avoid the use of staples or sutures. Finally, preparing autologous thrombin from the patient's plasma avoids relying on bovine thrombin that may carry immunological [32, 33] and infectious risks, most particularly transmissible spongiform encephalopathy agent responsible for Creutzfeldt-Jakob disease [34]. A prospective, randomized, controlled trial of autologous platelet-rich plasma gel for the treatment of diabetic foot ulcers indicated that 13 out 19 patients (68.4%) treated with PRP gel healed in 12 weeks [11]. The effect of split-thickness skin grafts versus a conservative wound dressing on the healing times of diabetic foot ulcers has also been studied. The results showed that a 100% skin graft take was recorded in 84% of the patients on the fifth postoperative day and in 62% on weeks 3 and 8, but 8% had ulcer recurrence and 4% a superficial infection within the following year [35]. Comparing to these previous studies, our results suggest that combining PRP and skin graft enhances the efficacy of treating chronic diabetic wounds by enhancing healing rate and decreasing recurrence rate.

In conclusion, although the clinical safety and effectiveness data is derived from a pilot study rather than from a randomized controlled trial, it provides, together with our previous series [18], a confirmation of the advantages of platelet materials in skin graft procedure to treat large nonhealing diabetic ulcers of lower extremity.

Table 1: Patients demography, clinical situation, ulcer location and size, and time to healing.

Patient	Age	Gender	Diabetes duration (year)	Glycated hemoglobin (%)	Cause of ulcer	Comorbidity	Ulcer location	Ulcer Size (cm)	Duration of ulcer	Take of skin graft	Time to healing	Follow-up months
1	62	F	25	10.2	Pressure sore	Renal failure; hypertension; hyperlipidemia	Right heel	4×7	4 months	Complete	2 weeks	12
2	25	F	5	7.1	Falling down	Rheumatoid arthritis	Right ankle	5×8	2 years	Complete	3 weeks	13
3	82	F	11	6.8	Cellulitis	Hypertension	Right lower leg	3×5	2 months	Complete	2 weeks	13
4	47	M	7	6.5	Traffic accident	Nil	Right lower leg	4×5	3 months	Complete	3 weeks	10
5	65	M	6	6.0	Stasis dermatitis	Varicose vein Hypertension	Left lower leg	15×10 5×7	6 months	Complete	2 weeks	10
6	80	F	10	8.2	Falling down	peripheral arterial occlusive disease Hypertension	Right ankle	3×5	3 months	Complete	2 weeks	19
7	72	F	8	5.5	Contusion injury	Hypertension Cervix Ca.	Left heel	8×10	2 months	$3 \times 3\ cm^2$ skin graft loss	Residual ulcer	Passed away 2 years after surgery
8	45	F	13	7.7	Infrared radiation burn	Spinal cavernous angioma s/p OP with paralysis	Right ankle	6×10	2 months	Complete	3 weeks	18

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Review Article

Antimicrobial Photodynamic Therapy for Methicillin-Resistant *Staphylococcus aureus* Infection

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Nowadays methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common multidrug resistant bacteria both in hospitals and in the community. In the last two decades, there has been growing concern about the increasing resistance to MRSA of the most potent antibiotic glycopeptides. MRSA infection poses a serious problem for physicians and their patients. Photosensitizer-mediated antimicrobial photodynamic therapy (PDT) appears to be a promising and innovative approach for treating multidrug resistant infection. In spite of encouraging reports of the use of antimicrobial PDT to inactivate MRSA in large in vitro studies, there are only few in vivo studies. Therefore, applying PDT in the clinic for MRSA infection is still a long way off.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961 [1], and since then MRSA has undergone rapid evolutionary changes and epidemiologic expansion. The problem of MRSA infection has rapidly grown in these years. Currently, MRSA results in more than one-half of the nosocomial infections with *S. aureus* strains in most countries [2]. MRSA accounts for approximately 60% of clinical *S. aureus* strains isolated from intensive care units in the United States [3]. Most people acquire MRSA in a hospital setting (HA-MRSA). These strains establish an ecological niche in the hospital environment and are easily transmitted between patients and from doctor to patient [4]. In recent years, community-acquired MRSA (CA-MRSA) strains have emerged, where they are rapidly becoming the dominant pathogens in the community [5].

MRSA has altered penicillin-binding proteins (PBPs) with reduced affinity to penicillin and other available β-lactam antibiotics [6]. For a long time, glycopeptide antibiotics, especially Vancomycin, were extensively used in clinical practice. In the last two decades, there has also been growing concern about the increasing glycopeptide minimum inhibitory concentrations (MICs) for MRSA [7, 8]. Therefore, MRSA poses a serious problem for clinicians and patients. Due to the limited therapeutic options, infections caused by these resistant strains are usually difficult to treat. The problem of a relatively rapid acquisition of antibiotic resistance of MRSA is complicated by the relatively long-time period needed for the development of antibiotics with new mechanisms of action. As it can be anticipated that the development of resistance will continue in the coming years, it is just a question of time until the bacterium develops resistance towards newly developed antibiotics. Therefore, the necessity exists for an immediate and continual search for alternative methods against MRSA towards which no resistance can develop. One of the most promising and innovative approaches in this respect is antimicrobial photodynamic therapy (PDT) [9–11]. This therapeutic approach involves the administration of a photosensitizer, usually a porphyrin-based compound, which, upon photoactivation with visible light of appropriate wavelength, generates reactive oxygen species (ROS), such as singlet oxygen and free radicals, which are cytotoxic to bacterial cells.

This paper summarizes the mechanism of antimicrobial PDT and the progress of preclinical studies of antimicrobial PDT towards MRSA and identifies the potential applications to MRSA infection that may become valuable in the clinic.

2. Mechanisms of Antimicrobial PDT

Although the exact mechanism of PDT is not known in detail, there are two possible molecular mechanisms that are believed to play central roles in antimicrobial PDT. Both mechanisms cannot preclude the prerequisites for PDT: the sufficient presence of molecular oxygen, photosensitizer, and light of the appropriate wavelength. In the type I mechanism, free radicals are formed that react with lipids and proteins leading to a chain reaction that produces more oxidation products [12]. In the type II mechanism, energy from the triplet state of the photosensitizer, formed by light excitation, is transferred to the molecular oxygen, resulting in the generation of highly reactive singlet oxygen. The singlet oxygen can directly react with cellular molecules in its immediate vicinity and also creates further oxygen radicals [13]. It is generally accepted that the production of singlet oxygen plays the key role in PDT for infection and other diseases [11]. The ROS from both mechanisms react inside the bacterial cell or in vicinity and induce necrosis or apoptosis of bacteria (Figure 1).

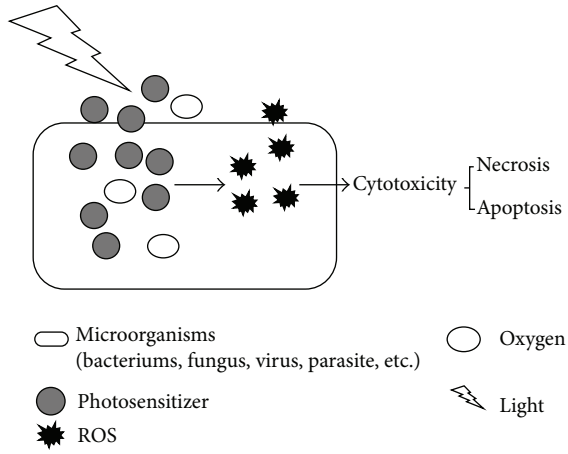

Figure 1: The mechanism of antibacterial PDT. Photosensitizers can be preferentially uptaken by bacteria, accumulating inside the bacteria and in the cytoplasm membranes, or in the vicinity. Upon absorption of a photon by the ground-state photosensitizer after light illumination, the reactive oxygen species (ROS) will be generated from two alternative pathways: type I mechanism and type II mechanism. The generated ROS then react rapidly with their environment depending on the localization of the excited photosensitizer: bacteria cell wall, lipid membranes, proteins and enzymes, and nucleic acids. The reaction of these important cellular components may result in necrosis or apoptosis of the bacteria at last.

ROS from photosensitizer-mediated antibacterial therapy can cause bacterial lethal injury by means of damage to DNA and the cytoplasmic membrane. Treatment of bacteria with various photosensitizers and light leads to both single- and double-stranded DNA break-in and the disappearance of the plasmid supercoiled fraction, which has been detected in both Gram-positive and Gram-negative species after PDT [14, 15]. Some photosensitizers that more easily intercalate into double-stranded DNA can cause more damage [16]. Evidence also shows that guanine residues of DNA are the most susceptible to oxidation by ROS [16]. However, DNA damage might not be the prime reason for bacterial cell death, because the damage may be able to be repaired by various DNA repairing systems [17]. Due to the usually lipophilic nature of many photosensitizers, they tend to locate primarily in membranes consisting of lipid double layers. Therefore, another critical damage site by ROS during PDT is the cytoplasmic membrane, which allows leakage of cellular contents or inactivation of membrane transport systems and enzymes. The alterations of cytoplasmic membrane proteins, disturbance of cell-wall synthesis and the appearance of a multilamellar structure near the septum of dividing cells, and loss of potassium ions from the cells have been reported [18–20].

The photosensitizer is the key component in the photosensitization process because it absorbs light and initiates formation of toxic species. Photosensitizers are mainly from the following classes: porphyrins, chlorines, phthalocyanine, Rose Bengal, phenothiazines, and acridines. The structures of porphyrins, chlorines, and phthalocyanine are based on the tetrapyrrole nucleus, whereas the others have different molecular frameworks [21]. These photosensitizers induce varying photodynamic activities towards Gram-positive and Gram-negative bacteria [21]. Due to structural differences of the outer bacterial cell wall of Gram-positive and Gram-negative bacteria, differences naturally exist with respect to the efficacy of the various photosensitizers. The 40–80 nm thick outer cell wall and up to 100 peptidoglycan layers of Gram-positive bacteria do not represent an effective permeability barrier. In contrast, the outer membrane of Gram-negative bacteria with a bilamellar membrane covering the only 3 nm thick peptidoglycan layer is able to impede photosensitizer diffusion considerably, especially the negatively charged or neutral photosensitizers. Various strategies have been developed to cross this barrier, such as pretreatment with EDTA or polymyxin B, which make the outer wall of bacteria more permeable and allow photosensitizer to penetrate and accumulate on the cytoplasmic membrane [22, 23]. In contrast to the low penetration of negatively charged and neutral photosensitizers, the positively charged photosensitizers are photodynamically active even without the addition of a penetration booster [24–26]. Not only resting or vegetative cells but also Bacillus spores have been shown to be inactivated using photodynamic administration [27]. As a result of the high reactivity of singlet oxygen with proteins, its lifespan in a cellular environment is very short, which results in a very short diffusion distance. Therefore, the effectiveness of a photosensitizer depends not only on the amount taken up, but also on the location of the photosensitizer at the time point of irradiation [28].

3. Inactivating MRSA by PDT

With various photosensitizers and the appropriate wavelength light, MRSA has been observed to be dramatically

inactivated in a serial of in vitro studies. Wilson and Pratten [29] found that cultured MRSA was inactivated significantly by aluminum disulphonated phthalocyanine and light even in the presence of horse serum. Eight isolates of MRSA from patients were demonstrated to be completely eradicated following 15 min exposure to a 632.8 nm HeNe laser in the presence of 50 μg/mL photosensitizer toluidine blue O (TBO) under in vitro conditions [30]. No significant effect was observed on the MRSA isolates exposed to the laser alone. In another study [31], light-activated antimicrobial agent aluminium disulphonated phthalocyanine (AlPcS 2) was used to determine whether 16 epidemic MRSA strains could be inactivated by antimicrobial PDT. The results indicated that all 16 strains were susceptible to inactivating by PDT. The bactericidal effect was dependent on the AlPcS 2 concentration and the light dose, and inactivation was not affected by the growth phase of the organism. Scavengers of singlet oxygen and free radicals protected the bacteria from inactivation [31].

For better simulating in vivo condition, an artificial skin construct was applied to test whether methylene blue (MB) mediating PDT could inactivate MRSA growing on it [32]. The artificial skin was composed of human-derived epidermal keratinocytes and dermal fibroblasts cultured at an air/media interface to form a stratified model of full thickness epithelialized human skin. PDT combined with MB treatment produced a significant reduction (5.1 logs) from control immediately after treatment and the effect was sustained over multiple days, while application of MB alone resulted in small reduction in MRSA viability from nontreated control [32].

In another study, penetration and antibacterial efficacy of a cationic porphyrin photosensitizer XF73 against MRSA was examined on an ex vivo porcine skin model [33]. The researchers performed both preincubation of bacteria in solution with XF73 followed by subsequent application on the ex vivo porcine skin and application of bacteria on the skin followed by an incubation with XF73. The localization of XF73 was restricted to the stratum corneum. Preincubation of *S. aureus* demonstrated a high photoinactivation efficacy (>3 logs reduction) after irradiation, while illumination after XF73 was delivered to the bacteria on the skin resulted in an approximately 1 log growth reduction independently of the antibiotic resistance pattern of the *S. aureus* strains used [33]. Histological evaluations of untreated and treated skin areas upon irradiation within 24 h did not show significant degree of necrosis or apoptosis [33].

Over 40 different virulence factors including a wide range of enzymes and toxins have been identified in *S. aureus*, which are involved in almost all processes from colonization of the host to nutrition and dissemination [34, 35]. However, in general, conventional antibiotics have no effect on inactivating these virulences. The activities of V8 protease, α-haemolysin, and sphingomyelinase expressed by epidemic MRSA16 were identified to be inhibited in a dose-dependent manner (1–20 μM) by exposure to laser light in the presence of MB [36]. Moreover, inactivation of α-haemolysin and sphingomyelinase is not affected by the presence of human serum, indicating that PDT may be effective against these toxins in vivo [36]. The ability of PDT to reduce the virulence of MRSA, as well as effectively inactivating the organism, would represent a significant advantage over conventional antibiotic strategies.

Although there have been encouraging reports of the use of antimicrobial PDT to inactivate MRSA in large in vitro studies, there have been relatively few reports of their use to treat MRSA infection in vivo. And all the current in vivo studies are confined within local MRSA infection on rodent models. A mouse model of skin abrasion wound infected with bioluminescent strain of MRSA Xen31 was developed [37]. This bioluminescent strain allows the real-time monitoring of infection in mouse wounds. PDT was performed with the combination of a series of concentrations of photosensitizer polyethylenimine- (PEI-) ce6 and a series of doses of noncoherent red light 30 minutes after bacterial inoculation. PDT resulted in 2.7 logs of inactivation of MRSA as judged by loss of bioluminescence in mouse skin abrasion wounds and accelerated wound healing by 8.6 days compared with the untreated infected wounds [37]. A tetracationic Zn(II)phthalocyanine derivative was also shown to inactivate MRSA, inhibit regrowth, and accelerate wound healing by using the mouse skin abrasion model [38]. Simonetti et al. [39] established full-thickness wounds with diameter of 0.8 cm, which were then inoculated with 5×10^7 CFU of MRSA in the back subcutaneous tissue of BALB/c and CD1 mice. A strong reduction of bacterial counts (3 logs) was observed in mice treated with RLP068/Cl and illumination in comparison with infected untreated mice 2 days after infection. By day 9, a comparable and significant reduction of bacterium and a complete reepithelialization were found in mice treated with RLP068/Cl or with antibiotic teicoplanin [39]. A 25-fold reduction in the number of epidemic MRSA16 treated with 100 μg/mL of MB and 670 nm laser light (360 J/cm^2) was achieved in another mouse skin wound model [40].

MRSA arthritis is another animal model chosen to test the effectiveness of PDT for MRSA infection in vivo. A murine MRSA arthritis model showed that approximately 30% of intra-articular leukocytes, mainly neutrophils, died immediately after PDT [41]. A further decrease in the number of intra-articular leukocytes and atrophy of the synovial tissue were seen 24 h after PDT. The isolated peripheral neutrophils presented significant affinity to Photofrin and showed significant morphological damage after PDT with Photofrin [41]. These results indicated that PDT might not be highly effective for treating MRSA arthritis, because intra-articular neutrophils and synovial tissue were also injured by PDT. In order to maximize bacterial inactivation and minimize inactivation of host neutrophils, an intra-articular injection of Photofrin instead of intravenous administration was used and the light dosimetry was optimized to treat arthritis induced by MRSA infection [42]. Each animal received a knee injection with MRSA (5×10^7 CFU) followed 3 days later by 1 mg of Photofrin and 635 nm illumination with a range of fluences within 5 minutes. The greatest reduction of MRSA was seen with a fluence of 20 J/cm^2, whereas lower antibacterial efficacy was observed

with fluences that were either lower or higher. Consistent with these results, a significantly higher concentration of macrophage inflammatory protein-2 (a CXC chemokine) and greater accumulation of neutrophils were seen in the infected knee joint after PDT with a fluence of 20 J/cm^2 compared to fluences of 5 or 70 J/cm^2 [42]. These results indicate that PDT for murine MRSA arthritis requires appropriate light dosimetry to simultaneously maximize bacterial inactivation and neutrophil accumulation into the infected site, while too little light inactivates sufficient bacteria and too much light inactivates neutrophils and damages host tissue as well as bacteria and allows bacteria to grow unimpeded by host defense.

4. Modification on Charge and Structure of Photosensitizers

A potential photosensitizer for antimicrobial PDT must have appropriate photophysical properties, such as a large and long wavelength absorption band and a high quantum yield for the generation of both long-lived triplet excited state and cytotoxic ROS species. It also has to be water-soluble and must have a high affinity to microbial cells and a low affinity to host cells. These characteristics are strongly related to the presence of cationic charges in the molecular structure.

Several groups [9, 43, 44] observed that photosensitizer charge and structure might be important factors in determining the success of antimicrobial PDT, especially when applied on negative surface charge of microorganisms like Gram-negative bacteria. Meso-substituted tetrahydroporphyrin tetratosylat (BL1065) was reported to acquire the ability to bind both Gram-positive (MRSA) and Gram-negative bacterial cell envelope more strongly than the dianionic chlorine BLC1013, resulting in better efficiency of photoinactivation [45].

Foley et al. [46] demonstrated that replacement of the oxygen atom in photosensitizer 5-(ethylamino)-9-diethylaminobenzo[a]phenoxazinium chloride (EtNBA) with sulfur and selenium afforded thiazinium (EtNBS) and selenazinium (EtNBSe) analogues that had similar water solubility, lipophilic character, and uptaking rate. But this small change on the molecule gave EtNBS and EtNBSe better antimicrobial efficacy than their chalcogen analogue EtNBA mainly due to higher triplet quantum yield. Replacing the central oxygen atom with a somewhat heavier sulfur atom resulted in a small but significant increase in the triplet yield (0.03) and that as expected the replacement by a much heavier selenium atom resulted in a dramatic improvement in the triplet yield (0.78) [46]. It is well known that incorporating a heavier atom into a molecule with a low intrinsic intersystem crossing rate constant will increase the probability of such transitions roughly in proportion to the square of the spin-orbit coupling constant of the atom where the transition occurs [47].

In another report [48], two EtNBS derivatives were synthesized, each functionalized with a different side-chain end-group, alcohol or carboxylic acid. There were no significant changes in absolute quantum yield of singlet oxygen formation, and both derivatives were phototoxic to *S. aureus* 29213, but the carboxylic acid derivative was nontoxic to *E. coli* 25922. This suggests that small functional groups of photosensitizer could achieve Gram-type-specific phototoxicity through altering the photodynamic activity of photosensitizer and deserve further exploration in a larger number of representative strains of each Gram type including MRSA.

5. New Drug Delivery Strategies Design

For antimicrobial PDT to be of clinical use, effective delivery methods for both light and photosensitizers to the site of action are necessary. Due to limited light penetration through tissue, clinical antimicrobial PDT will be necessarily limited to areas of the body where light can be delivered relatively easily, such as the skin and body cavities, as opposed to systemic infections such as bacteremia [49]. In contrast to conventional high irradiance treatments, recent preclinical and clinical photodynamic studies have focused on low irradiance schemes [50–52], which consume less oxygen than high irradiance. Compared with light and oxygen delivery, photosensitizer delivery system seems much more complicated. Researchers focused on drug delivery strategies for efficient but specific therapy.

Currently, photosensitizers under investigation at either a preclinical or clinical level are systemically administered after incorporation into lipophilic delivery systems, such as liposomes, oil emulsions, or cyclodextrin inclusion complexes in order to minimize precipitation in the bloodstream or aggregation in a polar milieu, which decreases PDT therapeutic efficiency [53, 54]. As for MRSA, an enhanced inactivation of MRSA by a liposome-delivered photosensitizer was demonstrated compared with the free dye [55]. Hematoporphyrin was embedded in fluid cationic vesicles composed of the monocationic lipid N-[1-(2.3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, which yields an endocellular concentration of photosensitiser much higher, yet promotes a tighter binding and a more efficient photoinactivation of MRSA.

The use of polymeric micelles as vehicles of photosensitizers is another very promising approach for photodynamic therapy [56–58]. The polymeric micelle delivery system may improve drug solubility and prevent the formation of aggregates in the aqueous medium. Compared to the use of liposomes, preparation of polymeric micelles can be much less expensive and simpler. In a recent study, photosensitizer hematoporphyrin was encapsulated with liposomes and micelles by the reversed-phase evaporation method, and both micelle and liposome delivered hematoporphyrin induced complete eradication of the Gram-positive pathogens including both MSSA and MRSA [59]. The hematoporphyrin dose completely eradicating pathogens using micelle and liposome was significantly lower than the dose required when using the nonencapsulated hematoporphyrin. The photodynamic inactivation effect of the hematoporphyrin encapsulated in polymeric micelles was superior to the hematoporphyrin

encapsulated in liposomes at lower hematoporphyrin doses [59].

In a different approach, an optimised formulation (8.0% w/w poly(vinyl alcohol), 2.0% w/w borax) of hydrogel was synthesized with 1.0 mg/mL of the photosensitizers MB and meso-tetra(N-methyl-4-pyridyl)porphine tetra tosylate (TMP), both of which were found to be phototoxic to planktonic and biofilm-grown MRSA [60]. Furthermore, newborn calf serum, which was used to simulate the conditions prevalent in an exuding wound, did not adversely affect the properties of the hydrogels and had no significant effect on TMP-mediated photodynamic inactivation of MRSA, despite appreciably reducing the fluence rate of incident light. Topically applied to treat wound infection, hydrogels loaded with photosensitizers possess the ability to flow into and produce intimate contact with wounds even heavily exuding wounds, whilst their dilated structure allows for intact removal once the treatment is completed. These characteristics may facilitate clinical use of photodynamic therapy.

6. Targeted Antimicrobial PDT of MRSA

One possible problem with the use of light activated antimicrobial agents is that the ROS produced during the process have the potential to damage neighboring host cells. There is, therefore, great interest in developing methods of targeting the photosensitizer of the infecting organism. The challenge in antimicrobial PDT is to find a therapeutic window, in which hazardous bacteria are efficiently inactivated without harming the surrounding tissue and disturbing the local microenvironment at a given concentration and light dose. The ability to confine activation of the photosensitizer by restricting illumination to the bacteria allows for a certain degree of selectivity towards these cells. Improved selectivity with preferential bacterial uptake of photosensitizer through modification of photosensitizer is another promising approach. To date, methods of targeting photosensitizers specifically to a certain type of microorganism include antibody conjugation [61, 62], attachment of peptides [63], employing bacteriophages [64], and taking advantage of the resistance mechanism of microorganisms [65].

Antibody conjugated with various photosensitizers was reported as a very promising targeting PDT [66–68]. As for antibacterial PDT, a lethal photosensitization of MRSA using an immunoglobulin G-tin(IV)chlorine e6 conjugate as the respective photosensitizer was reported [62]. A number of isotypes of immunoglobulin G bind through the Fc region to protein A, which is expressed and localized as a typical cell wall protein by quite few MRSA strains. The amount of protein A embedded in the cell wall areas can vary among these strains [69]. A close relationship between protein A amount and inactivation efficacy was observed in the use of the immunoglobulin G-tin(IV)chlorine e6 conjugate [62]. Despite many promising in vitro results, antibody targeting antibacterial therapy has only had little real success in either antibacterial PDT or cancer therapy. There are a number of problems associated with antibody-based photodynamic therapies, including difficulty to achieve specific antibodies that also display high affinity, inconsistent expression of target antigens, and difficulty to internalize antibodies by the same cells [70].

The possibility of using a bacteriophage to deliver the photosensitizer tin(IV)chlorine e6 (SnCe6) to a serial strain of *S. aureus* was also investigated [64]. Substantial inactivations of both MRSA and vancomycin-intermediate strains were achieved with low concentrations of the conjugate (1.5 μg/mL SnCe6) and low light doses (21 J/cm^2). Under these conditions, the viability of human epithelial cells in the absence of bacteria was largely unaffected. On the molar equivalent basis, the conjugate was more effective than the unconjugated SnCe6, and bacterial inactivation was not growth phase dependent. Furthermore, the conjugate was effective against vancomycin-intermediate strains even after growth in vancomycin [64]. These results indicated that a bacteriophage might be used to deliver a photosensitizer to a target organism, resulting in improving efficiency and specificity in inactivation of the MRSA and other organisms, which are desirable in the photodynamic therapy of infectious diseases.

Another method was demonstrated to target MRSA by taking advantage of its most common resistance mechanism [65]. A specific enzyme-activated structure (β-LEAP) was developed, for which two phenothiazinium photosensitizers (EtNBS-COOH) were combined to the side chains of cephalosporin. The two photosensitizers were quenched in the uncleaved construct due to close proximity to each other, but were activated through cleavage of the lactam ring by beta-lactamase, which was synthesized only by resistant strains. The selectivity of β-LEAP was demonstrated through coculture experiments with human foreskin fibroblasts (HFF-1) and MRSA strain. There was only little nonspecific uptake of β-LEAP by the HFF-1 cells in the presence of MRSA, while the MRSA stain had far greater β-LEAP uptake [65]. This novel targeting strategy of the resistance mechanism itself has, besides the specificity for enzyme-mediated resistant microbia, the potential advantage to distinguish between human and microbial cells.

7. Microorganism Strain Selective and Antimicrobial PDT Resistant

Compared with traditional antibiotic therapy, microbes, including MRSA, rarely develop resistance to antimicrobial PDT. However, Grinholc et al. [71] recently demonstrated that biofilm not producing *S. aureus* strains was much more sensitive to PDT than to their slime-producing isolates. In addition, neither correlation between antibacterial PDT effectiveness and the antibiotic resistance pattern of the different strains, nor correlation between photodynamic inactivation efficacy and differences within proteins profiles could be demonstrated [71]. Possibly biofilm produced by bacterium that obstructs the photosensitizer penetrating into bacterial cells plays a role in resistance to PDT. The effect of extracellular slime on photodynamic inactivation of bacteria was also analyzed by another group [72], who

reported that extracellular slime significantly influenced the sensitizer uptake by the *S. aureus* cells. However, biofilm nonproducing strains could also be found with elevated resistance to PDT, and strains with a similar uptake possess significantly different susceptibility to PDT [73]. The different uptake due to extracellular slime did not determine the strain dependence of PDT solely.

Efflux mechanisms have been recognized as important components of microbial resistance of MRSA to various classes of antibiotics. NorA efflux pump as one of the multidrug resistance pumps (MDRs) has the ability to expel a variety of structurally diverse compounds [74]. The uptake levels of phenothiazinium-based sensitizers MB, TBO, and 1,9-dimethylmethylene blue (DMMB) by various strains of *S. aureus* were showed to be proportional to levels of NorA expression [75]. This suggested that MDRs were able to pump the photosensitizer out of the cells and thereby lessen the photoinactivity. However, the uptake level of non-phenothiazinium-based photosensitizer protoporphyrin diarginate was observed not to be affected by NorA expression levels [73]. And the MDR inhibitor reserpine did not affect the bactericidal activity either [73]. Therefore, the efflux mechanism might not influence the uptake level of all photosensitizers or the efficiency of MRSA inactivation.

Despite numerous reports demonstrating that a variety of photosensitizers can be used to inactivate *S. aureus* strains including MRSA, some sensitizers show little or no bactericidal effect towards several strains [71]. The mechanism responsible for strain-dependent inactivation and photodynamic resistance has not yet been definitively identified.

8. Present Problems and Future Works

Due to the requirement that light should be delivered to the microorganism, indications for antimicrobial PDT for MRSA are the treatment of local, superficial skin and soft tissue infections and arthritis. Topically applied photosensitizer with subsequent irradiation has locally limited action of the PDT and side effects such as allergic contact sensitization and disturbance of the resident flora. Therefore, the severe side effects of systemic administration of conventional antibiotic for local MRSA infection are avoidable.

In order to attain high antibacterial activity with topical antibacterial PDT, sufficient concentration of photosensitizer at site (within bacterial cells or attached to the cell membrane) is needed. A basic prerequisite for the effective use of antimicrobial PDT is the uptake and/or binding of the photosensitizer on the bacterial cell wall or plasma membrane. Thus the design of the molecular structure and the functional side chains of the photosensitizer [46, 48] and the charge [44], as well as the manner in which the photosensitizer is transported [20, 53–55, 59, 60], could influence the efficiency of antimicrobial therapy. Cationic photosensitizers with positive charge are usually more efficient than their neutral and negative charged analogues when they are used to inactivate Gram-positive and Gram-negative microorganisms. Significant alteration of the efficiency for inactivating Gram-positive and Gram-negative bacteria can be achieved through modifying benzo[a]phenothiazinium dyes with one atom and one side chain, respectively [46, 48]. Systemic administration of photosensitizers after incorporation into lipophilic delivery systems, such as liposomes, oil emulsions, or cyclodextrin inclusion complexes, can minimize precipitation in the bloodstream or aggregation in a polar circumstance, which reduces PDT therapeutic efficiency. Photosensitizers encapsulated in liposomes and micelles or loaded into hydrogel achieved better inactivation of MRSA for local application of PDT to inactivate MRSA in vitro. Additionally, combining cationic modification and delivery system of polymer was believed to increase the efficacy of inactivation [76].

Moreover, in order to improve specificity, targeting photosensitizers specifically to a certain type of microorganisms was tested. Those targeting systems which have shown promise in laboratory included chemical modification of the photosensitizer itself, drug delivery strategy optimization, the usage of antibodies and bacteriophage, and conjugation with traditional antibiotic [61–65]. As well as achieving better selectivity, another advantage of using a targeted photosensitizer is the increased antimicrobial efficiency. That is because, following binding of the targeted photosensitizer to the organism, subsequent irradiation results in the generation of ROS only in the vicinity of the pathogen and not at extraneous sites. Consequently, less photosensitizer needs to be applied, and because there is less attenuation of the incident light by unbound photosensitizer, a lower light dose can be used. However, a variety of disadvantages can hamper effective photodynamic inactivation. For instance, the very high molecular weight of such photosensitizer complexes may inhibit penetration of the upper layers of the epidermis needed for effective treatment of superficial skin MRSA infections. Also alterations of the binding epitopes on the protein surface of MRSA could result in a loss of antibody recognition and thus in a loss of photodynamic activity.

At present, it is still unknown whether resistance to PDT will be developed by MRSA. The number of photosensitizer molecules binding to the surface of MRSA cells is limited by biofilm formation and tunnel protein-deficient mutation, and active outward transport of photosensitizer can reduce photosensitizing efficiency toward MRSA [72, 74]. But in the studies from Grinholc et al. [71, 73], biofilm nonproducing strains could also be found among *S. aureus* strains with elevated resistance to PDT, and no association between photodynamic inactivation efficacy and the antibiotic resistance pattern (MDRs) of the different MRSA strains or the antibiotic-sensitive MSSA strains could be demonstrated. In addition to membrane structure and extracellular biofilm, cellular repair systems or concentration of antioxidant enzymes might also contribute to resistance. ROS inducing cellular necrosis and apoptosis play pivotal role in photodynamic bacterial inactivation. However, the production of ROS, particularly singlet oxygen, during irradiation occurs only precisely at the location of the photosensitizer. Singlet oxygen is only short lived in biological systems and in parallel possesses only a very limited diffusion distance (in pure water about 1 μm, while no more than 50 nm in the vicinity of protein-rich lipid milieu) [77]. To date, it is

uncertain whether MRSA is capable of developing resistance towards ROS through antioxidant enzymes activation or other possible mechanisms. Nevertheless, the mechanism responsible for resistance of certain MRSA strains and MSSA strains towards PDT thus needs to be definitively clarified in the future.

9. Conclusion

It can be said that the optimized physicochemical properties of photosensitizers as well as specific delivery systems will decide whether antimicrobial PDT for MRSA infection could be accepted as an alternative way to traditional antibiotic therapy. After further well-designed preclinical and clinical studies, this novel therapeutic approach for MRSA infection treatment may be established in clinical practices.

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Review Article

Vasculogenic Cytokines in Wound Healing

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Chronic wounds represent a growing healthcare burden that particularly afflicts aged, diabetic, vasculopathic, and obese patients. Studies have shown that nonhealing wounds are characterized by dysregulated cytokine networks that impair blood vessel formation. Two distinct forms of neovascularization have been described: vasculogenesis (driven by bone-marrow-derived circulating endothelial progenitor cells) and angiogenesis (local endothelial cell sprouting from existing vasculature). Researchers have traditionally focused on angiogenesis but defects in vasculogenesis are increasingly recognized to impact diseases including wound healing. A more comprehensive understanding of vasculogenic cytokine networks may facilitate the development of novel strategies to treat recalcitrant wounds. Further, the clinical success of endothelial progenitor cell-based therapies will depend not only on the delivery of the cells themselves but also on the appropriate cytokine milieu to promote tissue regeneration. This paper will highlight major cytokines involved in vasculogenesis within the context of cutaneous wound healing.

1. Introduction

It is estimated that diabetic and cardiovascular complications will account for $9 trillion in US healthcare costs over the next thirty years [1]. These complications are often associated with impaired blood vessel growth in response to tissue hypoxia and ischemia. Chronic nonhealing wounds represent an important public health problem as populations prone to impaired wound healing continue to grow (e.g., diabetics, elderly, and obese) [2]. The estimated healthcare cost of diabetic foot ulcers alone has been estimated at $45,000 per patient [3, 4]. Thus, strategies to augment the neovascularization response to injury may dramatically improve the quality of life for these patients and significantly reduce the global biomedical burden [1, 5, 6].

Regulation of blood vessel development in response to tissue injury or ischemia is critical for maintenance of healthy tissues [7]. A robust vascular response to deliver immune cells and metabolic substrates is important for cutaneous wound healing [8]. In addition, coordinated neovascularization programs are essential for normal organ development during embryogenesis [9]. Conversely, dysregulated signaling can promote tumor growth and metastasis [10, 11]. A better understanding of blood vessel formation in both health and disease states may result in more effective therapies for a wide range of diseases.

During embryogenesis, mesoderm-derived angioblasts organize to form blood vessels via *vasculogenesis* [12]. It was initially believed that all subsequent blood vessel growth occurred through sprouting of preexisting endothelial cells via *angiogenesis* [13]. However, it is now known that the vascular programming present during embryonic development is recapitulated in various postnatal states during a process known as adult *vasculogenesis* [14] (Figure 1). Vasculogenesis plays a critical role in maintaining tissue homeostasis throughout the body [15]. Disruption of these pathways can sustain pathogenic processes (e.g., in skin, heart, kidney, and brain) that are only starting to be appreciated on a molecular level. The remainder of this paper refers to postnatal vasculogenesis and focuses on major vasculogenic cytokines in the clinical context of wound healing.

2. Endothelial Precursor and Other Provasculogenic Cells

Endothelial precursor cells (EPCs) are bone-marrow-derived progenitor cells that participate in vasculogenesis and were

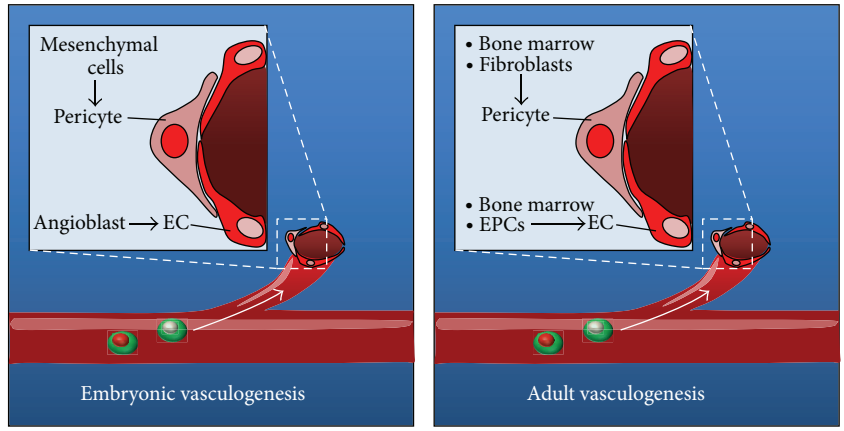

Figure 1: Embryonic versus adult vasculogenesis. During embryonic vascular development, endothelial cells (EC) derived from angioblast precursors migrate to regions of neovessel formation. Additionally, mesenchymal stem cells (MSCs) differentiate into pericytes which support and guide the development of endothelial cells. In adult tissues, vasculogenesis proceeds via recruitment of endothelial progenitor cells (EPCs) to neovessels. Supporting pericytes are thought to be derived from local fibroblasts or bone-marrow-derived mesenchymal cells. These complex interactions are mediated by cytokine networks responsible for creating functional three-dimensional vascular systems during development and throughout life.

first identified by Asahara et al. [16]. These cells are recruited to sites of ischemia and divide to form syncytial masses which tubularize and canalize to form a patent vascular network [17]. Although the molecular identification of EPCs remains a topic of debate, studies suggest that two functionally distinct subpopulations exist based on *in vitro* isolation techniques: early outgrowth EPCs and late outgrowth EPCs [18, 19]. Specifically, early outgrowth EPCs appear to function in a paracrine role in promoting neovascularization whereas late outgrowth EPCs directly differentiate into endothelial tubules [19]. Transcriptional and proteomic profiling of these populations suggests that early outgrowth EPCs may be of monocytic origin and restricted in their ability to promote neovascularization clinically [20].

EPCs have also been characterized based on their surface expression profiles [21]. In human studies, combinations of surface markers used to identify EPCs often include CD34+, CD133+, and VEGFR-2+. In mice, common EPC surface markers include Sca-1+, Lin-, Flk-1+, and cKit+. It is important to note that none of the markers used are specific for EPCs. Regardless of how they are classified, a common feature of EPCs is their ability to mobilize and home to injured areas and promote vessel formation [22]. Various signaling molecules are highly implicated in this process and include transforming growth factor beta (TGFβ) and matrix metalloproteinase-9 (MMP-9) [23].

EPCs are thought to mobilize from the bone-marrow or other tissues and home to areas of endothelial damage via adhesion molecules. The secreted proteases cathepsin L and MMP2 regulate the transmigration of EPCs, which subsequently mature and differentiate towards the endothelial lineage [24, 25]. Nitric oxide signaling and reactive oxygen species have also been implicated in EPC activity, potentially affecting their colony-forming potential and ability to counteract ischemic stress [26]. Nitric oxide pathways have even been linked to the ability of hormonal estrogens to promote EPC proliferation and mobilization [27].

Another cell population intimately involved in vascular morphogenesis is the pericyte, a supportive stromal-like cell that retains the pluripotency of mesenchymal stem cells (MSCs) [28]. They reside at the interface between endothelial cells and the surrounding tissue, producing proangiogenic signals that regulate endothelial cell differentiation and growth [29]. Through both direct physical interaction and paracrine signaling, endothelial cells and pericytes engage in complex crosstalk that is essential for normal adult vasculogenesis [30] (Figure 2). Fibroblasts have also been shown to facilitate EPC migration, branching, and sprouting in collagen matrices *in vitro*, potentially via cytokine signaling [31]. Finally, platelets are synergistically involved in vasculogenesis, elaborating potent cytokines that regulate the recruitment and differentiation of EPCs [32]. Diverse cell types are clearly involved in the formation of new blood vessels and the cytokine networks through which they communicate play a critical role in the tissue response to injury.

3. Major Vasculogenic Cytokines (Table 1)

3.1. VEGF. Vascular endothelial growth factors (VEGF) are a family of cytokines important in both embryonic and postnatal vascular development [33]. They play a crucial role in endothelial cell motility, proliferation, and survival [34]. This wide range of effects is mediated in part by the multiple VEGF subtypes and the associated family of VEGF receptor (VEGFR) protein tyrosine kinases. Five human VEGF isoforms (A, B, C, D, and placental growth factor-PlGF) are produced by differential splicing of VEGF mRNA. VEGF-A is involved in vascular growth, lymphatic development, and

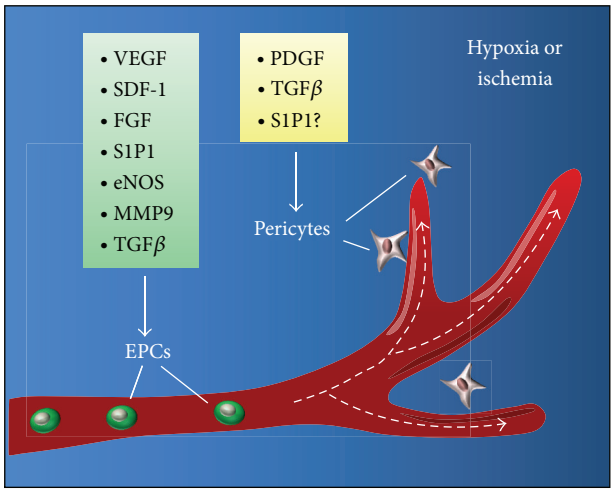

Figure 2: Critical cytokines implicated in vasculogenesis. Signaling molecules such as vascular endothelial growth factor (VEGF), stromal derived factor-1 (SDF-1), fibroblast growth factor (FGF), sphingosine-1-phosphate (S1P), endothelial nitric oxide synthase (eNOS), matrix metalloproteinase-9 (MMP9), and transforming growth factor β (TGFβ) regulate the function of EPCs during vasculogenesis. Pericyte activity during vasculogenesis appears to be modulated by platelet-derived growth factor (PDGF), TGFβ, and possibly S1P.

Table 1: Cytokines important in adult vasculogenesis.

Cytokine	Proposed vasculogenic mechanism
VEGF	Endothelial cell motility, proliferation, and survival
	EPC mobilization and homing
	Upregulation of other vasculogenic cytokines
	Promotes integrin expression
SDF-1	Trafficking of EPCs and HSCs
	Hypoxia-responsive EPC recruitment
PDGF	Pericyte recruitment and vessel maturation
	EPC migration and expansion
	Closely associated with VEGF pathways
FGF	VEGF-dependent neovascularization
	Bone-marrow-derived perivascular cell recruitment
	Vascular remodeling
GM-CSF	EPC recruitment and mobilization
	Monocyte/macrophage recruitment and activation
	Modulation of immune and inflammatory pathways
S1P	Promotes migration of embryonic angioblasts and endothelial cells
	Blood vessel maturation
	May augment vasculogenic effects of PDGF
MMP-9	EPC recruitment and mobilization
	Induces release of vasculogenic growth factors from the extracellular matrix
TGFβ	Promotes VEGF pathways
	Enhances MSC differentiation into pericytes
	Activates EPC transdifferentiation into smooth muscle

vascular malformations [35]. The role of VEGF-B in vascular development is poorly understood but may be associated with blocking apoptosis [36]. VEGF-C and VEGF-D are involved in lymphangiogenesis, and PIGF appears to regulate angiogenesis, wound repair, and inflammation [35].

VEGF-A has been shown to promote adult vasculogenesis via bone-marrow-derived EPC mobilization [37], a process that acts via VEGFR1 and VEGFR2 in a tumor model [38]. In a small animal model of soft tissue ischemia, VEGF levels and circulating VEGFR2+ cells were increased following injury, findings that correlated with migration of EPC populations to ischemic tissue [14]. Furthermore, it has been shown that topical VEGF delivery can improve diabetic wound healing in a murine model through local upregulation of angiogenic cytokines and recruitment of bone-marrow-derived vasculogenic cells [39].

VEGF has also been shown to regulate the expression of endothelial cell surface proteins known as integrins that link cells with the extracellular matrix. Integrins, which comprise a family of transmembrane heterodimeric proteins, play a major role in controlling EPC mobilization and homing to areas of tissue injury and ischemia [40]. Specifically, integrin $\alpha5\beta1$ has been shown to promote VEGF-induced differentiation of EPCs *in vitro*, highlighting the importance of both structural and cytokine signals in regulating EPC activity following injury.

A key mechanism that regulates VEGF expression is the hypoxia-inducible factor-1 (HIF-1) pathway. HIF-1 is a transcription factor that exists as a dimeric complex consisting of a cytoplasmic α subunit and a nuclear β subunit [41]. In the setting of hypoxia, HIF-1α (which is degraded under normoxic conditions) translocates into the nucleus to complex with HIF-1β, initiating the transcription of neovascularization genes including VEGF. Studies have demonstrated that impaired HIF-1α binding to its coactivator p300 may underlie diabetic impairments in wound healing [42]. Thus, strategies to stabilize HIF-1α may enhance EPC mobilization and function [43] and have been shown to improve cutaneous wound healing in diabetic mice [42, 44].

3.2. SDF-1. Stromal cell-derived factor-1 (SDF-1) is a chemokine which plays a crucial role in EPC and hematopoietic stem cell (HSC) trafficking through the circulation [41]. SDF-1 binds exclusively to the chemokine receptor CXCR4, which is expressed by circulating cells and regulates their recruitment from bone-marrow [45]. In addition, SDF-1 mediates the activation of circulating stem cells during embryonic organogenesis and vascular development [46], suggesting that it may serve similar functions in post-natal neovascularization. Dysfunctional SDF-1 pathways have been highly implicated in aged and diabetic wound healing in preclinical models [47], underscoring the importance of chemokine-mediated signaling networks in normal wound healing.

Researchers have examined the role of SDF-1 in peripheral vasculogenesis and tissue repair. It has been demonstrated that SDF-1 gene expression in EPCs is regulated by the transcription factor HIF-1α and that cutaneous tissues express SDF-1 in response to hypoxia [48]. In addition, blockage of either SDF-1 or its receptor CXCR4 can prevent stem cell recruitment to ischemic tissues. Local delivery of SDF-1 into ischemic muscle has been shown to enhance vasculogenesis via EPC recruitment [49], highlighting its potential as a therapeutic chemokine. Additionally, plasmid gene transfer of SDF-1 has been demonstrated to augment neovascularization through VEGF [50]. In the setting of diabetic wound healing, administration of SDF-1 is capable of reversing the impairment in EPC homing to injured tissue [51]. Other forms of tissue injury have also been shown to activate SDF-1. In a mouse burn wound model, researchers have characterized SDF-1 expression in the healing margin of burn wounds [52]. Ionizing radiation injury also appears to stimulate vessel formation via SDF-1, however in a HIF-independent manner [53]. Together, these studies collectively highlight the importance of SDF-1 in regulating wound vasculogenesis and suggest a role for chemokines in the treatment of chronic wounds.

3.3. PDGF. The platelet-derived growth factor (PDGF) family of ligands and receptors is closely related to VEGF and may have evolved from a common gene [54]. The family of four ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) assemble intracellularly and undergo transcriptional and posttranslational modifications. Specifically, the homodimer PDGF-BB recruits perivascular cells during vasculogenesis, possibly through the generation of reactive oxygen species and subsequent activation of extracellular-regulated kinase 1, 2 (ERK 1, 2) [55]. Endothelial-derived PDGF-BB also induces progenitor cell migration and expansion during vascular development [56] and is critical during vascular bed formation by mesangial progenitor cells [57]. VEGF ligands can also bind and activate PDGF pathways, a process important during MSC-associated vasculogenesis [58].

Researchers have exploited PDGF pathways to control neovascularization in various animal models. For example, nanofibrous scaffolds incorporated with PDGF have been shown to activate cytokine signaling and improve angiogenesis during wound repair in rats [59]. Additionally, a constitutively activating mutation of the PDGF receptor was introduced into embryonic stem cells and shown to enhance vascular development both *in vivo* and *in vitro*, potentially through VEGF pathways [60]. Furthermore, a PDGF-receptor antagonist has been shown to inhibit human tumor growth in a rat model, an effect that was augmented using anti-VEGF antibody [61].

In human studies, neovessels in revascularized wounds exhibit strong PDGF receptor staining [62]. These findings are consistent with data demonstrating that PDGF is a primary mediator of vessel maturation [63]. PDGF-dependent pathways are thought to drive angiogenic sprouting and vessel enlargement via vascular cell migration and proliferation [56]. In fact, PDGF was the first growth factor to be approved by the United States Food and Drug Administration for the clinical treatment of ulcers [64]. Taken together, these studies indicate that PDGF signaling is closely associated with VEGF pathways and is important during both developmental and adult vasculogenesis.

3.4. FGF. The fibroblast growth factor (FGF) family of cytokines displays diverse functional properties that are important in multiple aspects of wound repair including vasculogenesis [65]. Although FGF and VEGF differentially activate genes and stimulate the development of different vessel types, FGF appears to induce a vasculogenic response that is highly dependent on VEGF [66]. In myocardial tissues, FGF-2 has been shown to augment angiogenesis and vascular remodeling in response to ischemic injury [67]. FGF-1 has been used to induce neovascularization in both an omentum model and a vascular pedicle model in rats [68, 69], suggesting that FGF-based strategies may be effective in promoting blood vessel formation in complex tissue constructs. Recently, researchers demonstrated improved neovascularization in a murine hindlimb ischemia model using an FGF-based hydrogel delivery system [70].

EPCs express receptors for FGF and a subpopulation of CD34-expressing HSCs that specifically expresses FGFR-1 has been shown to differentiate into endothelial cells *in vitro* [71]. FGF-1 has been shown to regulate the proliferation and differentiation of EPC-like mesenchymal cells [72], suggesting it may control neovascularization mediated by endothelial-stromal cell interactions. FGF also appears to function via autocrine and paracrine mechanisms in endothelial cells [73] and may play a role in tumor angiogenesis and invasiveness [74]. Recently, researchers topically applied EPCs to diabetic wounds in mice and detected increased local expression of FGF and VEGF which corresponded with improved wound healing and vascularization [75], supporting a key role for these cytokines in vasculogenesis during soft tissue repair.

3.5. GM-CSF. Granulocyte macrophage colony stimulating factor (GM-CSF) is a potent cytokine that stimulates the mobilization of hematopoietic progenitor/myeloid cells and nonhematopoietic cells (e.g., bone-marrow MSCs) [76]. During wound healing, multiple cell types including keratinocytes, fibroblasts, macrophages, endothelial cells, dendritic cells, and lymphocytes secrete GM-CSF. It has been shown to directly promote reepithelialization and induce secondary cytokine secretion from various wound healing cells [77]. Clinical trials have demonstrated the efficacy of topically applied recombinant human GM-CSF for deep partial thickness burn wounds, highlighting the importance of this cytokine in human wound repair [78].

GM-CSF is related to interleukins (IL)-3 and IL-5 and plays diverse roles in homeostasis and disease [79]. Its role in angiogenesis is partly mediated by monocytes and VEGF-associated pathways [80]. In human endothelial cells, GM-CSF activates intracellular phosphatidyl-inositol-3-kinase and Jak/Stat signaling during vascular tubule formation *in vitro*. Interestingly, immune defense pathways have been

associated with GM-CSF-stimulated angiogenesis and may represent an integrated mechanism for tissue defense and regeneration following injury [81].

GM-CSF has also been closely linked to vasculogenic processes. It has been shown to stimulate EPC tubule formation, proliferation, migration, and viability in a dose- and time-dependent manner, effects which were mediated in part by ERK signaling and upregulation of VEGF and integrin $\beta 2$ [82]. GM-CSF has also been shown to enhance EPC recruitment and vasculogenesis in murine and rabbit hindlimb ischemia models [76], potentially via direct activation of endothelial cells during neovascularization [83]. GM-CSF pathways have also been implicated in tumor vasculogenesis [84], indicating that it regulates blood vessel formation in both health and disease states.

3.6. S1P. Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite found in high concentrations in blood and implicated in vascular development. It is secreted most prominently by platelets, suggesting that it may have an important role in tissue repair. Further, it has been shown to act via distinct receptor pathways to regulate keratinocyte and fibroblast chemotaxis, processes that are critical for normal wound healing [85, 86]. As proof of concept, subcutaneous injections of S1P were able to significantly improve diabetic wound healing and neovascularization in rodent models [87].

Gradients in S1P levels are known to mediate the migration of endothelial cells, potentially through a recently identified S1P transporter (SPNS2) [88]. S1P is thought to stabilize vasculature in part through regulation of VEGF pathways and cadherins junctions, processes potentially altered by blood flow mechanotransduction signaling [89]. Cadherin and S1P pathways have also been linked to vascular development in a zebrafish model [90], suggesting a key role for S1P in maintaining vascular integrity.

During embryonic vasculogenesis in mice, S1P has been demonstrated to promote migration of angioblasts and endothelial cells [91]. S1P pathways have also been implicated in blood vessel development. For example, mice lacking the receptor for S1P displayed immature vessels that lacked pericytes and smooth muscle elements [92]. Furthermore, other growth factors such as PDGF may act through sphingolipid signaling to promote cellular motility during blood vessel development [93], highlighting the functional diversity of this signaling pathway in vascular biology.

3.7. MMP-9 and Other Proteases. MMP-9 is a soluble extracellular protease that plays diverse roles in wound repair. Paradoxically, high levels of MMP-9 have been implicated in chronic nonhealing wounds as well as scarless wound repair in athymic mice [94, 95]. However, mice that lack MMP-9 also exhibit delayed wound healing with disordered collagen remodeling, suggesting that tight regulation of this protease is critical for normal cutaneous repair and remodeling [96]. Recent studies suggest that keratinocyte secretion of MMP-9 may be crucial to maintain normal basement membrane and matrix integrity [97].

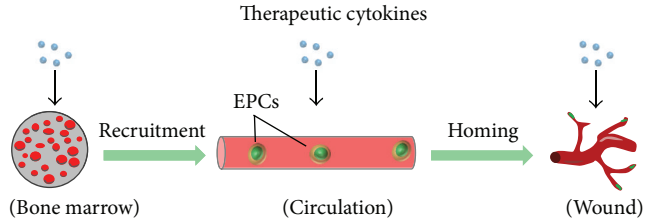

Figure 3: Cytokine-based approaches to augment vasculogenesis. Soluble molecules can be used to promote EPC production or activation from quiescent states in the bone-marrow during recruitment. Circulating EPCs can be targeted to injury sites via chemokines or modified at the cell surface level to promote egress from the circulation to the injury site during cell homing. Within the wound, EPC motility, proliferation, survival, and differentiation can be enhanced with cytokine therapies. Ultimately, a combination of cytokine cocktails, precise control of biochemical gradients, and modification of EPCs themselves may be needed to optimize vasculogenic therapies for clinical use.

The formation of new blood vessels involves not only cellular motility, growth, and sprouting, but also dynamic interactions with the endothelial basement membrane. Integrin-laminin interactions have been shown to regulate vessel branching [98] while recruited pericytes play an active role in vascular morphogenesis [99]. Matrix remodeling pathways are also highly involved in neovascularization, controlling neovessel growth, maturation, and regression during tissue repair [100]. Specific proteases such as MMP-9 and their inhibitors regulate major aspects of extracellular matrix turnover and degradation during vascular remodeling [101].

In addition to local effects at the injury site, MMP-9 has been shown to recruit EPCs from the bone-marrow [102] and can induce matrix release of vasculogenic cytokines including VEGF and TGFβ [103, 104]. Studies using MMP-9 knockout mice have demonstrated that MMP-9 is essential for tumor vascularization [105] and augments EPC mobilization and migration in a hindlimb ischemia model [106]. Additionally, stem-cell-activating cytokines may be released from the extracellular matrix by MMPs [107], further potentiating the neovascularization process.

Other proteases implicated in vascular formation include membrane-type MMPs (MT-MMPs) that act on the matrix directly surrounding new vascular cells [108]. Other soluble proteases include MMPs-1, 2, 8, and 13 that are only activated in the extracellular matrix and degrade matrix components to enable neovessel growth [108]. MMPs are inhibited by mediators known as tissue inhibitors of MMPs (TIMPs) that highly regulate the breakdown of matrix. Additionally, cysteine proteases known as cathepsins and serine proteases have been shown to control blood vessel formation. These complex interactions between cells and their matrix help facilitate neovascularization from the initial mobilization of EPCs to their ultimate fate as neovessels [109, 110]. Together, these studies indicate that remodeling enzymes such as MMP-9 and others play a crucial role in vasculogenesis at both the injury site and in the bone-marrow where quiescent EPCs reside.

3.8. TGFβ. The TGFβ superfamily consists of over 30 growth and differentiation factors that play vital roles in development and regulation of stem cell fate [111]. During wound healing, specific TGFβ isoforms (β1, β2, and β3) are secreted as a complex with latent precursors that are modified in the extracellular space. TGFβ is amongst the most well-studied signaling molecule in wound healing and is particularly linked to matrix and collagen production during wound healing. The ratio of expression of TGFβ1 and TGFβ3 is thought to regulate the ability of certain species and early gestation human fetuses to heal without scar [112].

In addition to its established role in fibrotic processes, TGFβ has been linked to neovascularization pathways through multiple receptor and intracellular signaling mechanisms. For example, TGFβ modulates vascular development by augmenting VEGF synthesis through Akt and ERK pathways [113]. TGFβ has also been shown to activate the recruitment of VEGF-expressing hematopoietic effector cells, establishing a potent signaling network in the inflammatory wound environment that simultaneously stimulates neovascularization [114].

TGFβ pathways can also act independently of VEGF. In an embryonic stem cell vasculogenesis model, TGFβ was shown to stimulate neovessel growth via activin receptor-like kinase (ALK) receptors [115]. Moreover, TGFβ can regulate non-endothelial cells during blood vessel maturation, specifically promoting vessel muscularization by stimulating MSC differentiation into pericytes [116]. TGFβ can also activate the transdifferentiation of EPCs into myocytes (a process linked to pathologic intimal hyperplasia), highlighting the importance of tightly controlled cytokine pathways in vascular homeostasis [117].

Dysregulated TGFβ signaling has been linked to vascular pathology in humans. Mutations in the human endoglin gene, a TGFβ co-receptor, result in a vascular dysplasia known as hereditary hemorrhagic telangiectasia [118]. EPCs from these patients exhibit aberrant ALK signaling and impaired vascular tubule formation *in vitro*, suggesting that TGFβ pathways are relevant to EPC function and vascular morphogenesis in humans. Collectively, these studies underscore the complex roles played by cytokines such as TGFβ in activating EPCs and mesenchymal precursors to produce functional neovasculature.

4. Challenges for Translation

A cascade of cytokines, growth factors, and other soluble mediators is released immediately following injury to orchestrate the repair of complex tissues [119]. Numerous *in vitro* and preclinical studies have demonstrated that cytokine-based therapies can have a profound and multifaceted effect on neovascularization and chronic wound healing [120] (Figure 3). Although most of these therapies remain unproven in controlled clinical trials, several recombinant cytokines have been shown to have a positive impact on nonhealing wounds.

Recombinant human PDGF (becaplermin) is approved by the US Food and Drug Administration for the topical treatment of lower extremity diabetic neuropathic ulcers. Although several randomized controlled studies have validated its efficacy for nonhealing wounds, it remains expensive and not widely utilized [121, 122]. Granulocyte-CSF (G-CSF) is another cytokine that has demonstrated clinical benefit for diabetic patients with foot infections. G-CSF limits the duration of antibiotic treatment, hospital length of stay, and rate of amputation [123, 124]. Despite small case reports suggesting its effectiveness for chronic ulcers [125–129], larger clinical studies are needed to determine its ability to enhance wound healing.

Chemokine therapies are a promising strategy to promote neovascularization via modulation of the inflammatory response. Studies indicate that altered chemokine pathways may play a role in perpetuating the nonhealing nature of venous stasis ulcers [130]. These wounds may also have ineffective angiogenic drives, suggesting that molecular strategies capable of augmenting blood vessel formation may prove clinically successful [131]. For example, gene transfer of SDF-1 significantly enhanced EPC mobilization and vascularization in a hindlimb ischemia model, effects mediated through VEGF and nitric oxide synthase (NOS) [132]. In a similar model, VEGF-transduced EPCs significantly improved wound vascularity compared to control EPCs [133], suggesting that EPC-targeted approaches may be a feasible option for clinical therapy.

In addition to gene-based therapies, biomaterial delivery of vasculogenic cytokines has been shown to improve vasculogenesis during wound healing [134, 135]. Matrix components and spatial patterning can precisely regulate vasculogenic programs and have the potential to promote a richly vascularized repair environment [136, 137]. As these biomaterial and molecular technologies continue to advance, combination cytokine-EPC impregnated scaffolds may become a clinical reality. Currently, wound therapies targeting vasculogenic pathways are largely in the preclinical stage but we believe these evolving strategies will continue to represent a promising approach to chronic wound healing.

Conflict of Interests

The authors have no financial conflict of interests to disclose.

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Research Article

The Influence of Flightless I on Toll-Like-Receptor-Mediated Inflammation in a Murine Model of Diabetic Wound Healing

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Impaired wound healing and ulceration represent a serious complication of both type 1 and type 2 diabetes. Cytoskeletal protein Flightless I (Flii) is an important inhibitor of wound repair, and reduced Flii gene expression in fibroblasts increased migration, proliferation, and adhesion. As such it has the ability to influence all phases of wound healing including inflammation, remodelling and angiogenesis. Flii has the potential to modulate inflammation through its interaction with MyD88 which it an adaptor protein for TLR4. To assess the effect of Flii on the inflammatory response of diabetic wounds, we used a murine model of streptozocin-induced diabetes and Flii genetic mice. Increased levels of Flii were detected in Flii transgenic murine wounds resulting in impaired healing which was exacerbated when diabetes was induced. When Flii levels were reduced in diabetic wounds of Flii-deficient mice, healing was improved and decreased levels of TLR4 were observed. In contrast, increasing the level of Flii in diabetic mouse wounds led to increased TLR4 and NF-κB production. Treatment of murine diabetic wounds with neutralising antibodies to Flii led to an improvement in healing with decreased expression of TLR4. Decreasing the level of Flii in diabetic wounds may therefore reduce the inflammatory response and improve healing.

1. Introduction

Up to 25% of people with diabetes can expect to develop a foot ulcer at some point in their lives [1]. Due to poor outcomes of existing therapies, lower extremity amputation is a common complication, affecting 15% of diabetics with foot ulcers, with one major amputation occurring every 30 seconds worldwide and over 2500 limbs lost a day [2]. The effectiveness of current treatments for diabetic foot ulcers is limited, and many patients with chronic, unhealed wounds need continual care. Understanding the processes involved in impaired wound healing will help to develop new therapeutic targets and tools for improving wound repair.

One area of research which has been shown to be integral to the wound repair process is that of the actin cytoskeleton which is a filamentous network found in all cells and facilitates processes such as cellular adhesion, migration and contraction [3, 4]. One member of the actin cytoskeleton is Flightless I (Flii) which is a member of the gelsolin family of actin remodelling proteins [5]. Flii colocalizes with actin and microtubule-based structures, is required for normal actin distribution, and possesses Ca^{2+}-independent G-actin binding activity as well as F-actin binding and severing activities [3, 6–8]. In addition to its role as a regulator of the cytoskeleton, the LRR domain allows Flii to bind a number of other proteins unrelated to actin including LRR Flii interacting proteins 1 and 2 (LLRFIP1 and LRRFIP2) and the double-stranded RNA binding protein TRIP [4, 9]. Flii is involved in numerous cellular activities including regulating transcription via coactivation of nuclear hormone

receptors [10, 11] and regulation of beta-catenin-dependent transcription [12], important signalling pathways including the TLR pathway [13, 14], cellular polarity, asymmetric cell division [15], proliferation via interactions with calmodulin-dependent protein kinase type II [16], and inflammation and cytokine production via caspase activation and IL-1β maturation [17]. Flii-deficient mice have improved reepithelialisation after wounding while Flii overexpressing mice have impaired healing with larger wounds with reduced contraction, cellular proliferation and delayed reepithelialisation [18].

Increased inflammation is an important contributing factor in the failure to heal of diabetic foot ulcers [19]. Inflammation is an integral part of the wound healing process and is regulated by toll-like receptors (TLRs). TLRs are key innate immune receptors that alert the immune system to tissue damage and mediate the inflammatory response. The human TLR family consists of 10 members structurally characterised by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and toll/interleukin-(IL-) 1 receptor (TIR) domain, in their intracellular domain. Through their intracellular TIR domain TLRs activate or deactivate signalling pathways that generate cytokine and chemokine production and thereby regulate inflammatory responses. TLR signalling is tightly regulated to control the intensity and duration of inflammation [12]. TLRs are expressed on a wide variety of cells including macrophages and neutrophils, and they respond to an array of viral, bacterial, and fungal ligands as well as cellular debris [13]. The receptors convey their specificity through the utilization of different adaptor proteins such as myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP), TIR domain containing adaptor protein-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) [13–15]. Upon activation, the adaptor proteins promote signalling to result in the expression of proinflammatory cytokines, growth factors, and interferons. MyD88 has been shown to play an important role during wound healing as MyD88 knockout mice have impaired wound healing [16] with wounds showing reduced contraction, decreased and delayed granulation tissue formation, and reduced blood vessel density [16].

The LRR region of Flii shares 29% sequence identity and 42% similarity to TLR4 [20]. Through its interaction with MyD88, it has been suggested that Flii can modulate inflammation by suppressing TLR4-MyD88-mediated activation of NF-κB [21]. Conversely, a reduction in the Flii level may enhance activation of NF-κB and increase cytokine secretion [20]. Several studies investigating the effect of Flii on TLR signalling in murine macrophages suggest that Flii can sequester activator proteins such as LPS and adaptor proteins such as MyD88 resulting in reduced cytokine expression [10, 11, 16, 17]. In this study we used mice with low ($Flii^{+/-}$), normal (WT), and high ($Flii^{Tg/Tg}$) Flii gene expression levels to investigate the function of Flii in a murine wound healing model of streptozocin-induced type 1 diabetes. We also investigated whether modulation of Flii by exogenous application of Flii neutralising antibodies improved diabetic wound healing via effects on TLR-mediated inflammation.

2. Materials and Methods

2.1. Antibodies. Mouse monoclonal anti-Flii antibodies raised to the LRR domain of the human Flii protein and the rabbit anti-human MyD88 antibody were obtained from Santa Cruz Biotechnology (VIC, Australia). Rabbit anti-human TLR4 and TLR9 antibodies were obtained from Imgenex (SA, Australia), mouse anti-human CD14 antibody and mouse anti-human CD16 antibody from BD Biosciences (NSW, Australia), and rabbit anti-human NF-κB antibody from Abcam (NSW, Australia). All antibodies were used at a 1 : 100 dilution. The appropriate secondary antibodies were used depending on the fluorescence required—goat anti-mouse Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 were obtained from Life Technologies (VIC, Australia) and also used at a 1 : 100 dilution. Flii is a highly conserved protein with 95% homology between mice and humans [19]. The Flii neutralising antibodies (FnAbs) used in *in vivo* diabetic mouse trials were mouse monoclonal anti-Flii antibodies raised against the N-terminus of the LRR domain of the human Flii protein [22] and obtained from Monoclonal Antibody SA Technologies (SA, Australia). Mouse IgG antibody used as a control *in vivo* diabetic mouse was obtained from Sigma (MO, USA)

2.2. Animal Studies. All experiments were approved by the Women's and Children's Health Network Animal Ethics Committee following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. Studies were performed using mice with a BALB/c background. Three strains of mice were used in this study with low ($Flii^{+/-}$), normal (WT), and high ($Flii^{Tg/Tg}$) Flii gene expression levels. Mice lacking one copy of the Flii gene, the double knockout being embryonically lethal [23], were made as described previously [23] and will be written as $Flii^{+/-}$. Transgenic mice carrying two extra copies of human Flii gene incorporated into mouse genome will be written as $Flii^{Tg/Tg}$.

2.3. Murine Model of Diabetic Wound Healing. Six female $Flii^{+/-}$, WT, and $Flii^{Tg/Tg}$ mice of 12–16 weeks of age and weighing 20–35 g were used for the induction of diabetes. Streptozocin (STZ) was used to induce type 1 diabetes (Sigma-Aldrich, MO, USA). STZ is toxic to the pancreatic beta-islet cells, rendering the mouse unable to produce adequate amount of insulin. Mice were given one intraperitoneal (IP) injection of STZ for 5 consecutive days (STZ: 50 mg/kg) in citrate buffer of pH 6.5. This dose was chosen based on previously reported studies [24]. Age-matched nondiabetic control animals were treated with an equivalent dose of vehicle (citrate buffer alone). Diabetic symptoms were observed closely, and nonfasting blood glucose levels (BGLs) were tested weekly by tail vein sampling. To maintain body weight and prevent ketoacidosis, animals with confirmed diabetes were maintained with subcutaneous injection of insulin (1 IU, Mixtard 30/70, Novo-Nordisk, NSW, Australia). Mice were tested for sufficient levels of hyperglycaemia at 6 weeks after

the last STZ injection, and only those with blood glucose levels greater than 15 mmol/L were wounded. Diabetic animals were wounded using a 6 mm biopsy punch (Stiefel Laboratories, NSW, Australia). Anaesthesia was induced by inhalation of isoflurane (5% induction at 2 L/min and 2% maintenance at 500 mL/min). To expose the skin, hair was removed by shaving and then application of hair removal cream (Veet, Reckitt Benckiser, NSW, Australia). Two 6 mm full thickness wounds, one on each side of the midline, were created on the dorsum of the mouse. Temgesic (buprenorphine 0.05 mg/kg) was administered post-operatively to provide analgesia for up to 8 hours. The mice were euthanized at 7 days following wounding. Digital photographs of the wounds were taken at 0 and 7 days after wounding. A ruler was aligned next to the wound to allow direct wound area measurements to be made. Wounds were fixed in formalin and processed for histology and immunohistochemistry.

An additional cohort of female WT diabetic mice were injected intradermally around the wound margins with 200 μL of FnAb (50 μg/mL; $n = 10$) or mouse IgG control (50 μg/mL; $n = 10$) immediately after surgery and at 1 and 2 days after wounding. Digital photographs of wounds were taken at days 0 and 7 after wounding. All animals were euthanized at day 7 post-wounding with the wounds harvested, fixed in formalin, and processed for histological analysis.

2.4. Histology, Immunohistochemistry, and Image Analysis. Histological sections (4 μm) of mouse wounds were cut and stained with haematoxylin and eosin or subjected to immunohistochemistry following antigen retrieval as described previously [25]. Briefly, following antigen retrieval, sections were blocked in 3% normal goat serum, primary antibodies against Flii (1 : 100), TLR9 (1 : 100), TLR4 (1 : 100), MyD88 (1 : 100) and NF-κB (1 : 100) were applied and incubated overnight at 4°C. Appropriate Alexa Fluor 488-conjugated secondary antibodies (1 : 100) were used and incubated for 1 hour. Fluorescence intensity was determined using AnalySIS software package (Soft Imaging System GmbH, Munster, Germany), and optical fluorescence in the epidermis and dermis of the wounds was analysed as previously described [25]. Negative controls included replacing primary antibodies with normal mouse or normal rabbit IgG. Primary or secondary antibodies were omitted to verify the staining and detect nonspecific binding. All control sections had negligible immunofluorescence.

2.5. Histological Image Analysis. Histological image analysis was performed using the Digital Microscope Camera ProgRes C5 (JENOPTIK Laser, Jena, Germany). Wound size was determined by manually drawing below the clot or the portions of the wound that were not covered by epidermis. Dermal wound gape was determined by measuring between the dermal wound margins. Fluorescent images were taken using an Olympus IX81 (Olympus Australia, Melbourne, VIC, Australia) at a magnification of ×20. The fluorescent intensity of the staining was calculated using Image Pro-Plus software (Media Cybernetics, MD, USA).

2.6. Statistical Analysis. Statistical significance was calculated using a paired Students t-test or analysis of variance. A P value of 0.05 or less was considered significant.

3. Results

3.1. Diabetic Wounds Heal Faster in Mice with Low Levels of Flii Gene Expression. To assess the biological function of Flii and determine the effect of Flii gene modulation on diabetic wound healing, three lines of mice were used expressing low ($Flii^{+/-}$), normal ($Flii^{+/+}$), and high ($Flii^{Tg/Tg}$) levels of Flii. Representative digital images of wounds at 7 days subsequent to wounding are shown in Figure 1(A). Overexpression of Flii in diabetic and nondiabetic wounds resulted in delayed wound closure at day 7 following wounding (Figure 1(A)). In contrast, wound area was decreased significantly when Flii levels were reduced ($Flii^{+/-}$; $P = 0.01$) compared to $Flii^{Tg/Tg}$ mice at day 7 following wounding (Figure 1(B)(a)). Representative microscopic images of day 7 wounds are shown in Figure 1(A). Histological assessment of these diabetic mouse wounds showed that at day 7 the dermal wound gape was significantly smaller in Flii-deficient mice ($Flii^{+/-}$) compared with Flii overexpressing ($Flii^{Tg/Tg}$) mice (Figure 1(B)(b); $P = 0.05$).

3.2. Treatment of Diabetic Wounds with Flii Neutralising Antibodies (FnAbs) Improves Wound Healing. Previous studies have shown that intradermal injection of Flii neutralising antibodies reduces the level of Flii in wounds and improves healing [26]. FnAbs were injected intradermally at days 0, 1, and 2, and representative images of macroscopic (at days 0 and 7) and microscopic (day 7) appearances of diabetic wounds are shown in Figure 2(A). Intradermal administration of FnAb to diabetic wounds resulted in a 1.9-fold decrease in average wound area (Figure 2 (B)(a)) and histological wound gape (Figure 2(B)(b)) compared to IgG-treated WT diabetic controls (Figure 2(B) (a) and (b); $P \leq 0.05$; IgG versus FnAb).

3.3. Elevating Flii Gene Expression Increases TLR4 in Diabetic Mouse Wounds. Immunofluorescence staining of day 7 diabetic wounds in $Flii^{+/-}$, WT, and $Flii^{Tg/Tg}$ mice shows increased Flii in $Flii^{Tg/Tg}$ diabetic wounds > WT > $Flii^{+/-}$ wounds (Figure 3(A)) with significantly less Flii staining being observed in day 7 diabetic $Flii^{+/-}$ and WT wounds ($P = 0.04$; $Flii^{+/-}$ versus $Flii^{Tg/Tg}$; $P = 0.05$; WT versus $Flii^{Tg/Tg}$; Figure 3(B)(a)). The presence of foreign molecules and pathogens was detected by a family of receptors known as toll-like receptors (TLRs), which contribute to prolonged inflammation [27]. To assess if altered levels of Flii affected TLR4 expression, diabetic wounds were stained for TLR4. Increasing Flii leads to a concomitant increase in TLR4 expression (Figure 3(A)(d)–(f)). Flii deficiency caused a significant reduction in TLR4 expression (Figure 3(B)(b))

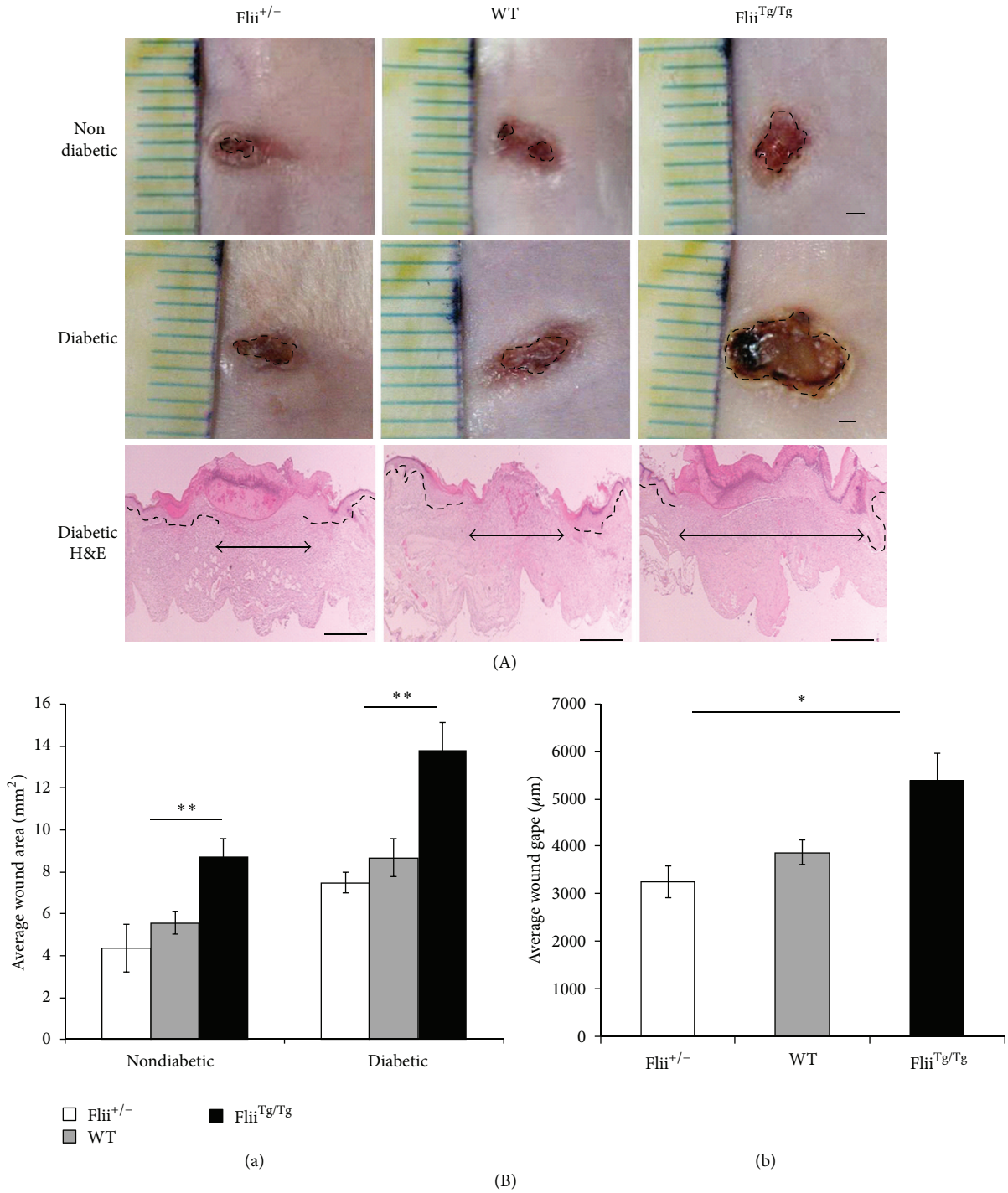

FIGURE 1: Flii delays wound healing in diabetic murine wounds after 7 days.(A) Macroscopic images of day 7 nondiabetic and diabetic mouse wounds in the Flii $^{+/-}$, WT, and Flii$^{Tg/Tg}$ mice and representative histological H&E staining of day 7 diabetic wounds. Scale bars = 1 mm for macroscopic pictures, and for the histology magnification was ×4 and scale bars = 100 μm. (B) shows graphical representations of the wound areas of the day 7 (a) wound areas of the three Flii genotype diabetic wounds ($n = 6$) and (b) the microscopic dermal wound gape of the day 7 diabetic wounds where $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ ($n = 6$).

at day 7 following wounding which was significantly lower than WT and Flii$^{Tg/Tg}$ wounds ($P \leq 0.01$ WT versus Flii$^{+/-}$; $P \leq 0.001$ WT versus Flii$^{Tg/Tg}$). Given that the inflammatory response in diabetic wounds was associated with increased TLR4 expression, we next proceeded to test if the downstream molecule NF-κB production was also affected. Figure 3(A) shows that NF-κB expression was also increased in Flii over-expressing wounds (5-fold higher than WT diabetic wounds)

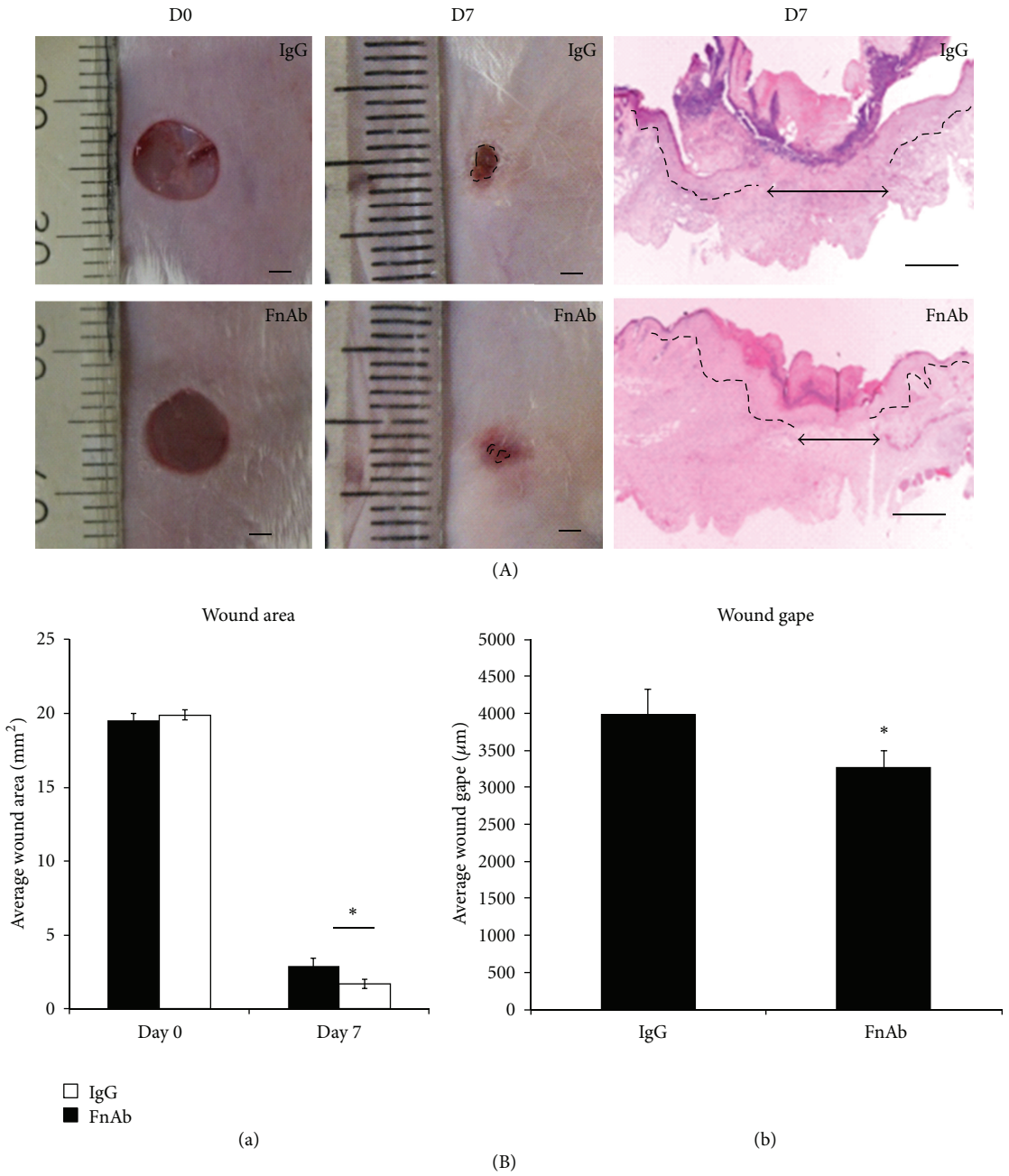

FIGURE 2: Healing can be improved by the application of Flii neutralizing antibodies. (A) shows macroscopic images of day 0 and day 7 excisional WT diabetic wounds treated with IgG isotype control ($n = 10$) and Flii neutralizing antibody (FnAb) ($n = 10$). Representative pictures of H&E staining of day 7 diabetic wounds treated with IgG and FnAb. (B) Graphical representation of the (a) wound areas and (b) histological wound gapes of the days 0 and 7 IgG control-treated and FnAb-treated WT diabetic wounds where $^*P \leq 0.05$ ($n = 10$).

(Figure 3(B)(c); $P \leq 0.001$ Flii$^{Tg/Tg}$ versus WT). A reduction in NF-κB was observed between Flii$^{+/-}$ wounds and WT, but this was not statistically significant (Figure 3(B)(c)).

3.4. Flii Neutralising Antibodies Reduce TLR4 Expression in Diabetic Wounds. Given that intradermal application of FnAb resulted in improved healing of diabetic wounds (Figure 2(A)), we proceeded to test whether this was in part due to modulated levels of TLR4-mediated inflammation. TLR4 and NF-κB expression was quantified, and representative images are shown in Figure 4(A). Treatment with FnAbs resulted in a significant decrease in TLR4 expression (Figure 4(B); $P \leq 0.05$ IgG versus FnAb) whereas NF-κB expression in day 7 FnAb-treated wounds remained unchanged compared to IgG controls (Figure 4(B)(b)).

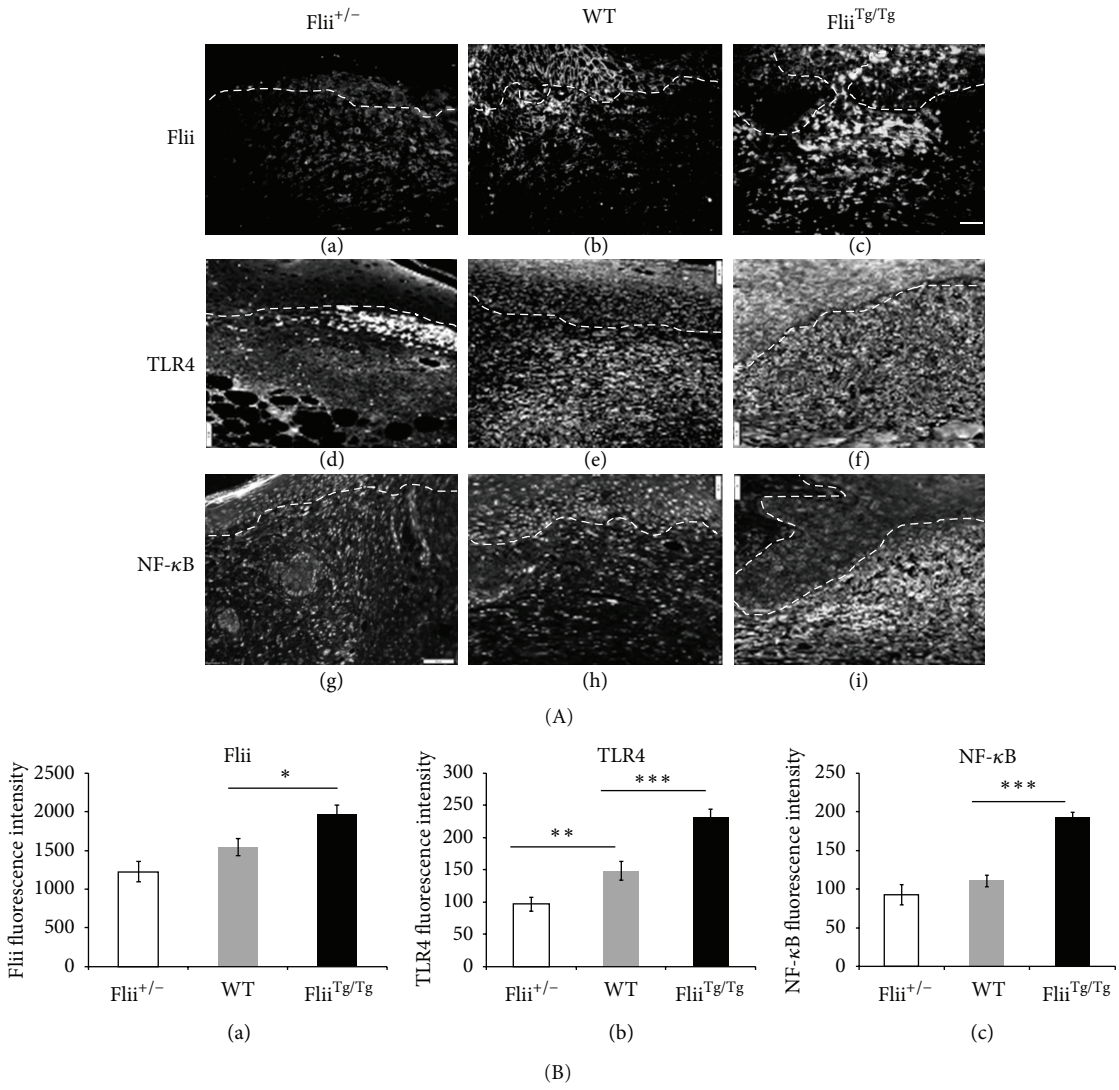

Figure 3: Concomitant increase in TLR4 and NF-κB staining occurs with increasing levels of Flii. (A) Representative images for Flii (a)–(c), TLR4 (d)–(f), and NF-κB (g)–(i) immunostaining of the three Flii genotypes (Flii$^{+/-}$, WT, and Flii$^{Tg/Tg}$). (B) Graphical representation of (a) Flii, (b) TLR4, and (c) NF-κB in Flii$^{+/-}$, WT, and Flii$^{Tg/Tg}$ day 7 diabetic wounds. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ ($n = 6$) and scale bar = 100 μm.

4. Discussion

Flii has been identified as a protein that can inhibit the rate of healing by reducing the migration of keratinocytes and fibroblasts and limiting the degree of wound contraction [18, 22, 28]. Flii deficiency is associated with improved reepithelialisation in acute wounds [18] while Flii overexpressing mice have impaired wound healing with delayed reepithelialisation. Here we have investigated if Flii is involved in the impaired healing associated with diabetic wounds. Wound healing was impaired as Flii levels increased, and this impairment was exacerbated when diabetes was induced. Flii is upregulated during the wound repair process [18, 25] and is constitutively secreted by two of the major cell types found in wounds: fibroblasts and macrophages in response to wounding both *in vitro* [29] and *in vivo* [22, 30]. Recent studies show that Flii is also secreted through a nonclassical late endocytic/lysosomal pathway of secretion by fibroblasts and macrophages [29]. Addition of Flii monoclonal neutralising antibodies as a means of reducing the Flii protein in the wound environment was able to counteract the negative effect of Flii on wound healing [18], and treatment of murine diabetic wounds with neutralising antibodies to Flii led to an improvement in healing suggesting that high levels of Flii in diabetic wounds contribute to wound chronicity.

Inflammation is an essential component of the normal wound healing process; however excessive inflammation is detrimental to this process [31]. Disproportionate inflammation is one of the major contributing factors to the formation of diabetic ulcers as these chronic wounds often have an unregulated and excessive inflammatory reaction [31, 32]. The innate immune system detects foreign particles such as

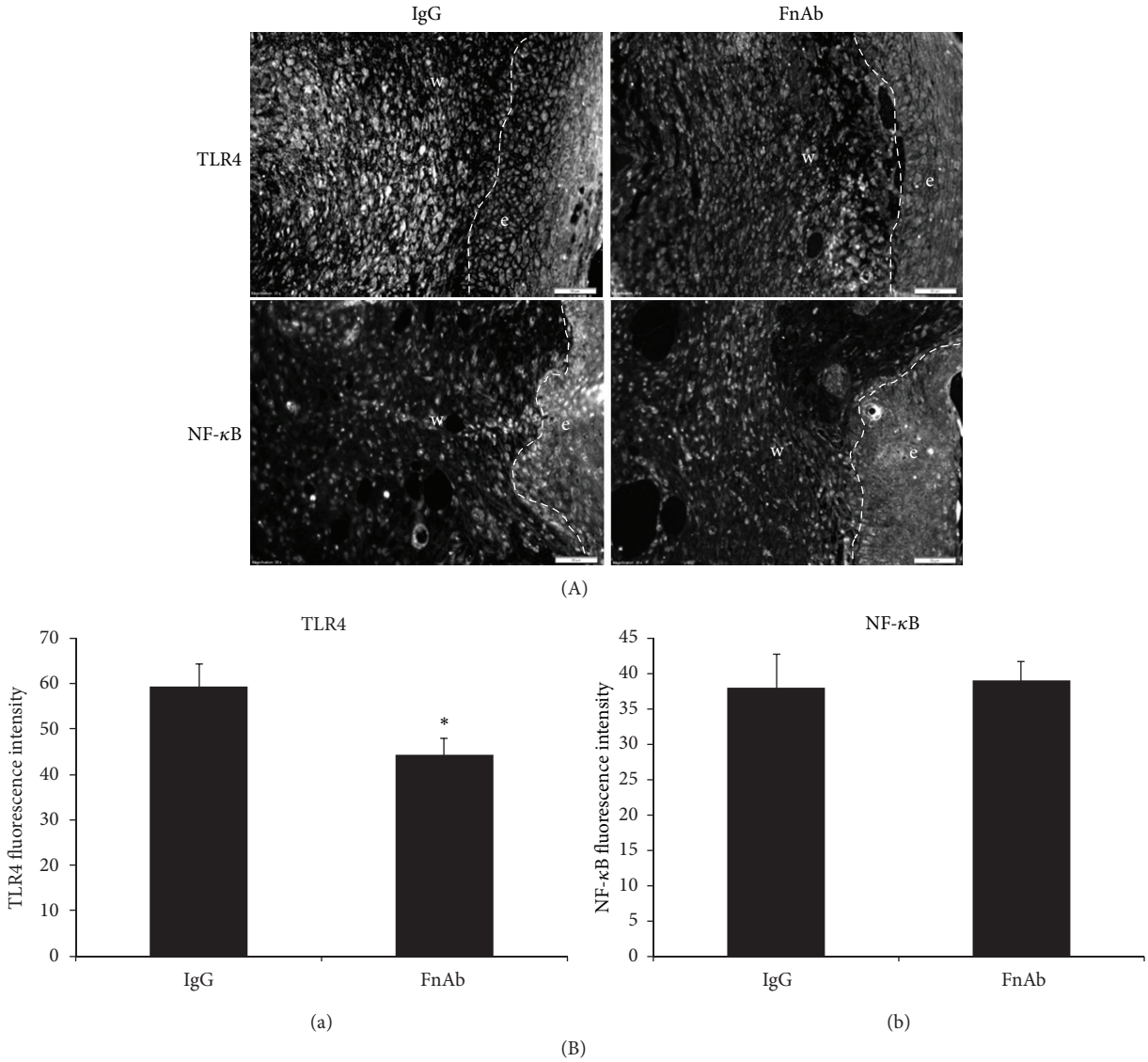

Figure 4: Modulation of Flii by exogenous application of Flii neutralising antibodies (FnAbs) reduces TLR4 expression in diabetic wounds. (A) Representative images of TLR4 and NF-κB staining of day 7 diabetic wounds treated with IgG and FnAb. (B) Graphical representation of (a) TLR4 and (b) NF-κB staining of day 7 diabetic wounds treated with IgG and FnAb. $^{*}P \leq 0.05$.

bacteria, fungi, and viruses via pathogen-associated molecular pattern (PAMP) molecules leading to the activation of the inflammatory response via toll-like receptors (TLRs) which recognise PAMPs [33, 34]. TLRs have also been linked to diabetes with studies showing that TLR-immune activation can result in activation of proinflammatory pathways leading to autoimmunity which may cause the onset of diabetes [27]. Previous reports suggested that stimulation of TLR4 leads to activation of the downstream transcriptional regulator NF-κB resulting in cytokine secretion [21], and both TLR4 expression and pro-inflammatory cytokine NF-κB were elevated in the murine diabetic wounds which would further contribute to inflammation and chronicity. In Flii-deficient diabetic mouse wounds, decreased TLR4 and NF-κB were observed and when Flii neutralising antibodies were administered to diabetic wounds, a decrease in TLR4 expression was seen suggesting a dampening of the inflammatory response when Flii levels were reduced. Interestingly, the effect of FnAb was specific to TLR4 and did not appear to affect the production of the proinflammatory cytokine NF-κB suggesting that alternative pathways may still be active in these wounds.

In vitro studies have previously shown that the LRR region of Flii shares 29% sequence identity and 42% similarity to TLR4 [20] suggesting that Flii may influence TLR signalling. However, while *in vitro* studies showed that through its interaction with MyD88, Flii was able to negatively regulate the TLR4-MyD88-mediated activation of NF-κB and the subsequent cytokine secretion in macrophages [20, 21], our studies show that *in vivo*, in mouse diabetic wounds the

increased levels of Flii appear to correlate with an increase in the expression of TLR4 and its signalling protein NF-κB. This is clearly in opposition to the findings in these cell-based studies but may be accounted for by the differing *in vivo* and *in vitro* environments. The time points investigated also differ with the *in vitro* studies looking at responses from 3 to 6 hours whereas this wounding study investigated time points which were measured in days rather than hours. It is, however, not inconceivable that Flii may have a dual role in wounding and in the inflammatory response which depends on the time point investigated.

In conclusion, inflammation is an integral component of the normal wound healing process and occurs even in the absence of infection; however excessive and prolonged inflammation impairs healing. Flii is a multifunctional protein and is currently emerging as a regulator of inflammation; however, whether it is pro-or anti-inflammatory is still to be determined. Our *in vivo* studies show that reducing the expression of Flii in diabetic murine wounds improves healing and reduces the proinflammatory response. Being able to manipulate the level of inflammation in a wound would greatly improve the wound healing outcomes of patients with diabetes, and it remains to be elucidated whether neutralisation of Flii in human diabetic wounds could help improve healing of these chronic nonhealing ulcers.

Conflict of Interests

The authors know of no potential conflict of interests.

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Review Article

Abnormal Cell Responses and Role of TNF-α in Impaired Diabetic Wound Healing

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Impaired diabetic wound healing constitutes a major health problem. The impaired healing is caused by complex factors such as abnormal keratinocyte and fibroblast migration, proliferation, differentiation, and apoptosis, abnormal macrophage polarization, impaired recruitment of mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs), and decreased vascularization. Diabetes-enhanced and prolonged expression of TNF-α also contributes to impaired healing. In this paper, we discuss the abnormal cell responses in diabetic wound healing and the contribution of TNF-α.

1. Introduction

Diabetes mellitus is one of the most prevalent and costly chronic diseases in the United States [1]. Impaired wound healing and diabetic foot ulcers constitute a major health problem in patients with diabetes. Diabetic foot ulceration is estimated to occur in 15% of diabetic patients, often requires prolonged hospitalization for its management, and is a major cause of disease-associated amputations in the western world [2].

Wound healing is a complex process involving a number of interdependent and overlapping stages including hemostasis, inflammation, proliferation, vascularization, and production of matrix and remodeling [3]. Many types of cells are involved in each phase of wound healing including immune cells, endothelial cells, keratinocytes, and fibroblasts which undergo marked changes in gene expression and phenotype [4, 5]. The delayed wound healing in diabetes is caused by complex factors such as diminished keratinocyte and fibroblast migration, proliferation, differentiation, apoptosis, and vascularization. Several of these cellular deficits have been linked to greater inflammation and proinflammatory cytokine production [6] (Figure 1).

Diabetic foot ulcers result from the simultaneous action of multiple contributing causes. A critical triad of neuropathy, minor foot trauma, and foot deformity is responsible for over 50% of diabetic foot ulcers [7]. Inflammation, immunodeficiency, peripheral neuropathy and ischemia from peripheral vascular disease, and subsequent infection are underlying factors that contribute to unhealed chronic wounds in diabetic foot ulcers [8].

One aspect of diabetic healing that has recently received considerable attention is the enhanced and prolonged expression of TNF-α, a potent proinflammatory cytokine [9].This review focuses on factors that are affected by diabetes-enhanced inflammation, particularly elevated or prolonged expression of TNF-α.

2. Cells Affected by Diabetes in Wound Healing

The inflammatory stage of wound repair occurs shortly after tissue damage. After acute injury, platelets and neutrophils are released passively from disrupted blood vessels. The formation of a fibrin clot provides a temporary scaffold for infiltration of inflammatory cells. A large number of growth factors are important in stimulating and coordinating

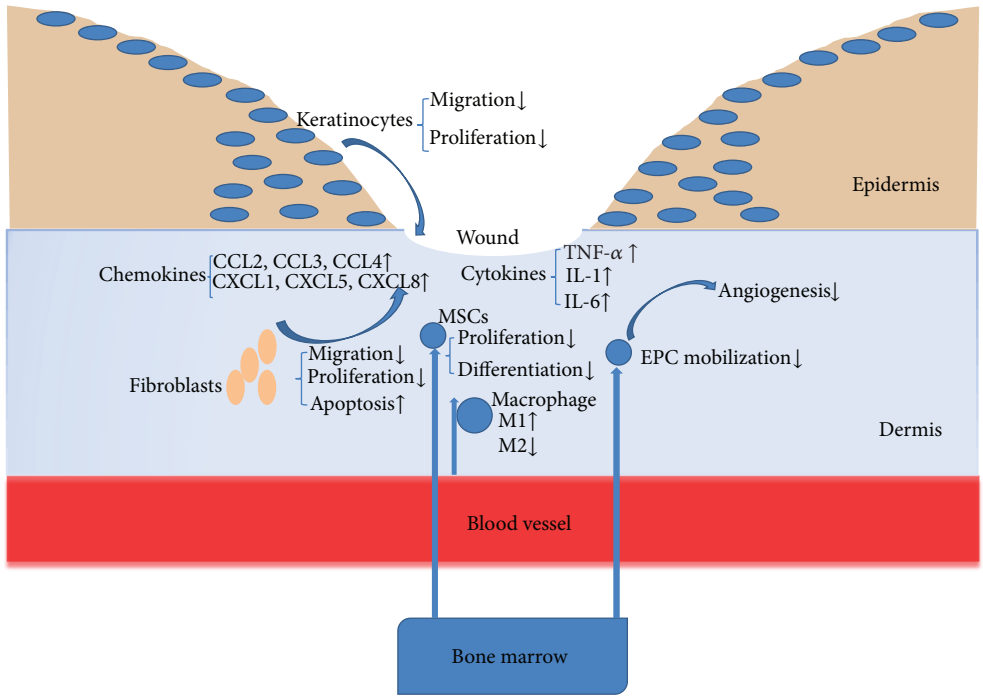

Figure 1: Mechanisms of impaired diabetic wound healing. The normal wound-healing process is initiated by the integration of multiple intercellular signals (cytokines and chemokines) released by keratinocytes, fibroblasts, endothelial cells, macrophages, platelets, etc. In diabetes, inflammatory cytokines and chemokines are elevated, such as TNF-α, IL-1, IL-6, CCL2, CCL3, CCL4, CXCL1, CXCL5, and CXCL8. Cellular processes affected by diabetes include abnormal keratinocyte and fibroblast migration, proliferation, and enhanced apoptosis; abnormal macrophage polarization (increased proinflammatory M1 macrophages and decreased anti-inflammatory M2 macrophages); impaired recruitment of mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs), and decreased vascularization.

cellular events that occur during normal wound healing [10]. Among them, cytokines and chemokines are especially noted because of their roles in promoting inflammation, angiogenesis, leukocyte recruitment, recruitment of stem cells, and epithelialization. Proinflammatory cytokines that are elevated shortly after wounding both in human wounds, and animal wound models include IL-1α, IL-1β, IL-6, IL-12, and TNF-α [11, 12]. Some proinflammatory cytokines and chemokines are essential for normal skin wound-healing process. Delayed wound healing is observed in IL-6-deficient mice [13]. It has been shown that deletion of IL-1 receptor signaling impairs oral wound healing due to its importance in upregulating an antibacterial defense but has relatively little impact on dermal healing [14]. The lack of ICAM-1 in mice results in prolonged wound healing because of the decreased recruitment of macrophages and other leukocytes [15, 16].

The CXC chemokine family of chemotactic cytokines CXCL1, CXCL5, and CXCL8 is expressed in keratinocytes and upregulated in wounding by stimulation of proinflammatory cytokines such as IL-1 and TNF-α, bacterial products, and hypoxia [17]. The induced expression of chemokines stimulates recruitment of leukocytes and monocytes, neutrophils, and macrophages to the wound site to remove foreign material, bacteria, dead cells, and damaged matrix [3]. Chemokine CX3CL1 and its receptor CX3CR1 were both highly induced at wound sites mediating recruitment of bone marrow-derived monocytes/macrophages in a mouse model of excisional skin wound healing [18]. CXCR3 chemokine receptor and its ligands CXCL11, CXCL10, and CXCL4 are also crucial for dermal maturation. Disruption of CXCR3 signaling in mice results in delayed reepithelialization [19]. Chemokines also induce recruitment of stem cells to sites of injury and include epithelial stem cells from hair follicles or sweat glands, endothelial progenitor cells, and mesenchymal stem cells [20–22].

Impaired wound healing in diabetic patients is accompanied by decreased early inflammatory cell infiltration but increased numbers of neutrophils and macrophages in late stages. These changes in inflammatory cell recruitment occur in conjunction with alterations in chemokine and growth factor expression [23]. An increase in inflammatory cytokines is observed in wounds of type-1 diabetic patients including CD40, IL-1α, IL-2, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL3, and CCL4 [24]. In diabetic models, increased levels of the proinflammatory cytokines such as TNF-α and IL-6 and decreased levels of anti-inflammatory IL-10 are observed in diabetic wound tissue compared to nondiabetic healing wound [25, 26]. This leads to sustained expression of chemokines CXCL2 and CCL2 that cause prolonged infiltration of leukocytes during impaired healing in diabetic mice [27].

2.1. Macrophages. Wound-site macrophages represent a key player that drives wound inflammation. Macrophages are

important in clearance of dead cells and debris within the wound. Depletion of macrophages during the inflammatory phase results in significant delay of wound repair in a mouse model [28]. Diabetes is known to compromise macrophage function including phagocytosis activity [29]. Macrophages isolated from wounds of diabetic mice and diabetic patients showed significant impairment in efferocytosis, leading to a higher burden of apoptotic cells in wound tissue as well as increased proinflammatory cytokine expression [25].

High glucose levels stimulate macrophages to enhance the production of proinflammatory cytokines such as IL-1β, IL-6, IL-12, IL-18, TNF-α, and IFN-γ both *in vivo* and *in vitro* [12]. Macrophages may polarize along two lines that have functional differences, proinflammatory macrophages (M1), and anti-inflammatory macrophages (M2), which can be further subdivided in M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1β or LPS), and M2c (IL-10, TGF-β or glucocorticoids) [30]. M1 macrophages are polarized by the stimulation of IFN-γ, GM-CSF and in the presence of bacterial products such as LPS [30]. M1 macrophages have a proinflammatory phenotype exhibiting increased phagocytic activity and secretion of proinflammatory cytokines that aid in the removal of pathogens and damaged tissues [31, 32]. M2 macrophages have a polar opposite phenotype exhibiting high levels of anti-inflammatory cytokines and fibrogenic and angiogenic factors that serve to resolve inflammation and promote wound healing [30, 32]. Recently, an additional M2 subtype (M2d) which involves "switching" from an inflammatory M1 into an angiogenic M2 phenotype was discovered [33]. M2d macrophages express high levels of IL-10 and VEGF and low levels of TNF-α and IL-12 [33]. Macrophage polarization may play an important role in the pathogenesis of obesity-induced insulin resistance and type 2 diabetes mellitus [34]. Macrophages isolated from diabetic mice exhibit greater infiltration by inflammatory M1 macrophages and may contribute to impaired diabetic wound healing [35].

Wound macrophages in the early stage of repair are more M1-like when the generation of inflammatory signals is important while M2 macrophages predominate in later stages of repair in response to the need for new tissue formation [36]. In the normal wounds, the M1 macrophage phase is relatively short and the phase with M2 macrophages is longer [37]. M2 macrophages are a prominent source of TGF-β, which promotes many aspects of wound repair including chemotaxis, wound contraction, angiogenesis, reepithelialization, and connective tissue regeneration [5]. Diabetes may prolong the phase of M1 macrophage polarization. In addition infection in chronic wounds leads to prolonged M1 macrophage activation, which in turn can delay healing [38, 39].

2.2. Mesenchymal Stem Cells. Adult mesenchymal stem cells (MSCs) have the capacity for self-renewal and differentiating into a variety of mesenchymal cell lineages such as fibroblasts, osteoblasts, adipocytes, and chondrocytes. Increasing evidence shows that MSCs participate in the regeneration of skin in cutaneous wounds [40]. Hypoxia-inducible factor-1α (HIF-1α) and chemokines such as CCL2 facilitate MSC mobilization into the peripheral blood and to sites of wound healing [41, 42]. In addition to forming fibroblasts and myofibroblasts, MSCs also enhance wound healing through the secretion of mediators such as VEGF-α, IGF-1, EGF, keratinocyte growth factor, angiopoietin-1, stromal-derived factor-1, CCL3, CCL4, and erythropoietin [43, 44]. MSCs also play an important role in immunomodulation and are anti-inflammatory. MSCs inhibit the proliferation and activation of effector T cells, natural killer (NK) cells, dendritic cells (DCs), and macrophages by promoting the formation of anti-inflammatory regulatory T cells [45]. Thus there are multiple mechanisms through which MSCs can promote wound healing.

Diabetes has detrimental effects on MSCs. Bone marrow-derived MSCs from diabetic rats have reduced proliferation and reduced myogenic differentiation [46]. The application of autologous MSCs improves healing of chronic diabetic foot ulcers [47]. Local application of MSCs to the wound sites improves wound healing in normal and diabetic mice, with increased reepithelialization, cellularity, and angiogenesis [43]. MSCs enhance diabetic wound healing by reducing inflammation, upregulating the expression of growth factors, and promoting the proliferation of fibroblasts and basal keratinocytes in diabetic rats [48].

2.3. Keratinocytes. Wound healing requires the transition of basal and suprabasal keratinocytes from a sedentary phenotype to a migratory and hyperproliferative phenotype. The reepithelialization process involves local keratinocytes at the wound edges and epithelial stem cells from hair follicles or sweat glands [49, 50]. Keratinocytes are a major source of growth factors such as TGF-β, VEGF, EGF, KGF, and TGF-α that stimulate fibrogenesis and angiogenesis in adjacent tissue [4, 51, 52]. Although there is no direct evidence that the proliferative activity of keratinocytes is affected in diabetes, migration is impaired [53, 54]. Keratinocytes at the chronic ulcer edge from diabetic patients have a reduced expression of migration markers [53, 55]. *In vitro* keratinocytes have reduced migration and proliferation capacities in high-glucose conditions [56].

2.4. Fibroblasts and Myofibroblasts. Fibroblasts are the primary source of extracellular matrix proteins such as collagen and fibronectin [57]. In diabetic oral and dermal wounds fibroblasts have decreased migration, proliferation, and increased apoptosis [58–60]. The proliferation and migration of diabetic rat fibroblasts are suppressed when the cells are cultured in high-glucose containing media [60, 61]. Myofibroblasts are specialized fibroblasts that contribute to wound healing by producing extracellular matrix and by generating a contractile force to bring the edges of a wound together. The transition from fibroblasts to myofibroblasts is influenced by mechanical stress, endothelin-1, TGF-β, and cellular fibronectin (ED-A splice variant) [62, 63]. During acute wound healing in nondiabetic mice, mRNA levels for both TGF-β RI and TGF-β RII in wound tissue are elevated

[64]. TGF-β receptor elevation is reduced in chronic diabetic ulcers [65]. Diabetics have reduced levels of TGF-β and reduced formation of myofibroblasts which may contribute to impaired wound contraction [66].

2.5. Endothelial Cells. Angiogenesis is a complex cascade of cellular, humoral, and molecular events, which initiates at the binding of growth factors to their receptors on the endothelial cells of existing vessels, such as VEGF. The stimulated endothelial cells proliferate and migrate into the wounded tissue to form small tubular canals which then mature [3]. Impaired angiogenesis is considered a major contributing factor to nonhealing wounds. Wound-induced hypoxia stimulates vascular regeneration by activating hypoxia-inducible transcription factors (HIF-1α), which increase the production of angiogenic growth factors such as VEGF and expression of the chemokine receptor CXCR4 [67]. The number and function of endothelial progenitors are reduced in diabetes mellitus [68, 69]. The importance of angiogenesis in contributing to impaired diabetic healing is demonstrated by improvement when diabetic wounds are treated with endothelial progenitors or VEGF. Injection of CD 34+ endothelial cell progenitors to the wounds of diabetic mice accelerates vascularization and healing of diabetic mouse skin wounds [70]. Topical application of VEGF also improves diabetic wound healing by locally upregulating growth factors PDGF and FGF-2 and promoting angiogenesis [71].

2.6. MMPs/TIMPs Imbalance in Diabetes. The balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) is crucial for normal wound healing processes. A low MMP/TIMP ratio is a good predictor of successful wound-healing in diabetic foot ulcers [72]. Diabetes creates an unfavorable ratio. It increases the activity and expression of MMP-9, MMP-2, and MMP-8 while reducing TIMP-2 [73, 74]. The abnormally elevated level of MMPs may impair cell migration and result in sustained inflammation with net increased tissue destruction. In the chronic diabetic foot lesions, local administration of protease inhibitors reduces the ratio of MMP/TIMP and improves wound healing [68].

3. Role of TNF-α in Diabetic Wounds

In normal wound healing the highest levels of TNF-α are seen from 12 to 24 h after wounding [75]. After the completion of the proliferative phase of wound healing, TNF-α returns to basal levels. During the early phase of wound repair, it is predominantly expressed in polymorphonuclear leukocytes, and later by macrophages. It is also expressed in the hyperproliferative epithelium at the wound edge. TNF-α contributes to the stimulation of fibroblasts and keratinocytes the expression of growth factors and upregulation of antimicrobial defenses [76]. TNF-α levels are elevated in diabetes in part through increased oxidative stress that promotes inflammation [77]. Other factors may contribute to this elevation including the downregulation of CD33 that inhibits cytokine production [78]. TNF-α is found threefold higher in diabetic mouse wounds than wounds in normal mice [59] and threefold higher found in wound fluid from nonhealing venous leg ulcers than in healing ulcers [79]. Chronic gastric ulcers are also associated with increased TNF-α [80].

3.1. Cellular Events Affected by TNF-α. In diabetic wound healing impaired fibroblast proliferation has been linked to increased levels of TNF-α [81]. Inhibiting TNF *in vivo* significantly increases the number of proliferating fibroblasts but it has a little effect on fibroblast proliferation in normoglycemic mice [59]. Apoptosis of fibroblasts in diabetic mice is significantly higher than in normoglycemic counterparts [59, 82], and apoptosis is high in skin biopsies from diabetic foot ulcers [83, 84]. TNF stimulates apoptosis of fibroblasts, keratinocytes, and endothelial cells *in vitro* [85, 86]. A cause-and-effect relationship has been established between the treatment of TNF blocker and reduced apoptosis which was elevated in diabetic healing [59]. Diabetes also impairs the migration of fibroblasts and keratinocytes [55, 87]. High levels of TNF-α inhibit cell migration [88]. This may occur by increasing the level of Smad 7 [89] and inhibiting the activation of the Smad 2/3 [90] (Figure 2).

The neutralization of TNF in the diabetic wounds improves wound angiogenesis and closure. Blocking TNF reduces the overproduction of small noncoding RNAs such as miR-200b in the diabetic wounds, which improves the expression of globin transcription factor-binding protein 2 (GATA2) and vascular endothelial growth factor receptor 2 (VEGFR2), both of which promote angiogenesis [91].

The ability of cells at the wound site to respond to insulin is reduced in diabetic wounds. Insulin insensitivity occurs when the response to insulin is reduced. Long-term treatment of cells with TNF-α contributes to reduced insulin sensitivity [92]. Insulin receptor expression in proliferating keratinocytes at the wound margins and in granulation tissue is reduced in diabetic mice but enhanced with anti-TNF-α antibody treatment [93]. The effect of neutralization of TNF-α on insulin sensitivity may be involved in inhibiting the effects of TNF-α on the downregulation of GLUT4 genes that are required for normal insulin action, the downregulation of PPARγ which is an important insulin-sensitizing nuclear receptor, and the upregulation of Ser phosphorylation of IRS-1 that results in a net decrease in insulin receptor-mediated signaling [94]. Thus, an important component of impaired diabetic wound healing may be due to the reduced sensitivity of cells that participate in the wound healing process to insulin stimulation, which is mediated in part by high levels of TNF.

3.2. Effect of TNF-Induced FOXO1 on Diabetic Wound Healing. Some of the negative effects of diabetes-enhanced TNF on wound healing may be due to the impact of the FOXO1 transcription factor [77, 95]. FOXO1 activity is increased in a number of different diabetic conditions and may be detrimental because it induces cell cycle arrest and apoptosis and increases the production of proinflammatory cytokines

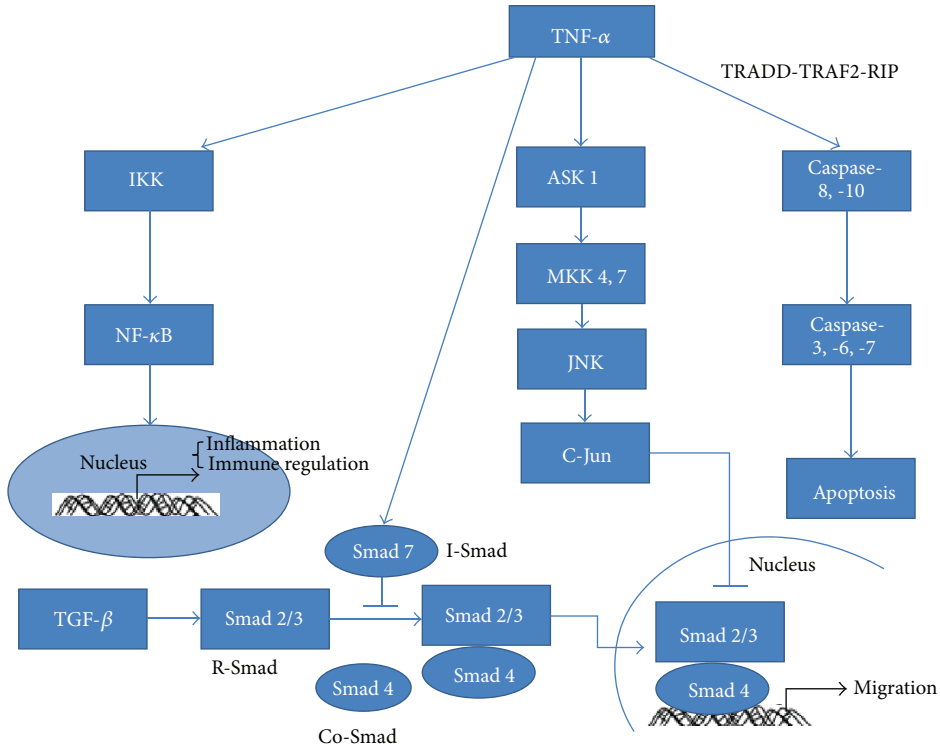

Figure 2: Model of TNF-α on regulation of inflammation, immue response, migration, and differentiation. TNF-α induces cell apoptosis via caspase pathway, and it negatively regulates cell migration by increasing the level of Smad 7 and inhibiting the activation of the Smad 2/3. TNF-α also induces NF-κB activation to enhance inflammatory responses.

[77]. TNF-α-induced apoptosis of endothelial cells and pericytes is FOXO1 dependent *in vivo* and *in vitro* [96, 97]. *In vivo*, FOXO1 DNA activity is increased twofold in diabetic wounds, and the increase is driven by diabetes-enhanced TNF levels [59]. FOXO1 activity is also increased *in vivo* in fracture healing and linked to greater inflammation [98, 99]. In normal wound healing FOXO1 may play a positive role in endothelial migration and tube formation [95].

3.3. Advanced Glycation Endproducts. AGEs are proteins or lipids that become glycated after exposure to sugars. Enhanced formation and accumulation of advanced glycation end-products (AGEs) and receptors for AGEs have been reported to occur in diabetes mellitus [100–102]. The activation of one of the AGE receptors, (receptor for AGEs), RAGE causes the upregulation of the transcription factor nuclear factor-kappa B (NF-kappa B) and its target genes such as intercellular adhesion molecule-1 (ICAM-1), VEGF, IL-1α, IL-6, and TNF-α. Mice fed with high levels of AGE display impaired wound closure [103]. Blockade of RAGE restores effective wound healing in diabetic mice by accelerating reepithelialization and angiogenesis, limiting inflammatory cell infiltration, and reducing the expression of TNF-α, IL-6, MMP-2, -3, and -9 [100]. AGEs cause the production of reactive oxygen species at least in part, through the activation of NADPH oxidase [77, 104]. In mononuclear phagocytes, AGEs increases the generation of cytokines such as TNF-α, IL-1, and IL-6 and enhanced the production of O^{2-} [101, 105, 106].

4. Conclusion

The impaired diabetic wound healing and diabetic ulcer impair the quality of life of millions of people and burden the healthcare systems globally. The etiological factors involve a high level of TNF-α, which inhibits angiogenesis and cell proliferation and migration in diabetic wounds and increases apoptosis levels. TNF inhibition attenuates the impact of diabetes-enhanced TNF-α, which offers potentially new therapeutic avenue for treatment of abnormally diabetic wounds healing.

Authors' Contribution

F. Xu and C. Zhang contributed equally to this work.

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Research Article

Low Circulating Protein C Levels Are Associated with Lower Leg Ulcers in Patients with Diabetes

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Activated protein C (APC) promotes angiogenesis and reepithelialisation and accelerates healing of diabetic ulcers. The aim of this study was to determine the relationship between the incidence of lower leg ulcers and plasma levels of APC's precursor, protein C (PC), in diabetic patients. Patients with diabetes who had a lower leg ulcer(s) for >6 months ($n = 36$) were compared with age-, type of diabetes-, and sex-matched subjects with diabetes but without an ulcer ($n = 36$, controls). Total PC was assessed using a routine PC colorimetric assay. There was a significantly ($P < 0.001$) lower level of plasma PC in patients with ulcers (103.3 ± 22.7, mean ± SD) compared with control (127.1 ± 34.0) subjects, when corrected for age and matched for gender and type of diabetes. Ulcer type (neuropathic, ischaemic, or mixed) was not a significant covariate for plasma PC levels ($P = 0.35$). There was no correlation between PC levels and gender, type of diabetes, HbA_{1c}, or C-reactive protein in either group. In summary, decreased circulating PC levels are associated with, and may predispose to, lower leg ulceration in patients with diabetes.

1. Introduction

Activated protein C (APC) is a plasma protease derived from its precursor, protein C (PC), which circulates in plasma at 3–5 μg/mL. APC was originally described as an anticoagulant but has recently been found to exert potent cytoprotective properties including the inhibition of inflammation and apoptosis and maintenance of the endothelial and epithelial barriers [1–4]. APC exerts its cytoprotective effect through its receptor, endothelial protein C receptor (EPCR), which binds to both PC and APC with high affinity [5]. A soluble form of EPCR (sEPCR), circulating in normal human plasma [6], has similar affinity for binding PC as that of intact membrane-bound EPCR.

In humans, recombinant APC reduces the mortality rate in severe sepsis [7], and we have recently shown its potential application in the healing of chronic wounds [8, 9]. Interestingly, biopsies taken immediately adjacent to chronic wounds in patients with diabetes exhibit very low total PC (PC plus APC) levels compared to normal skin [9]. In animal models, APC is neuroprotective after stroke onset [10], protects diabetic nephropathy [11], significantly inhibits the development of lung fibrosis in bleomycin-induced lung injury [12], reduces intestinal injury in necrotizing enterocolitis [13], and accelerates healing in streptozotocin-induced diabetic rats [14]. In vitro, APC modulates keratinocyte and endothelial function towards a phenotype necessary to promote wound healing by enhancing reepithelialisation and angiogenesis

[15–17]. Notably, total PC expression in skin surrounding lower leg ulcers in diabetic patients is lower than normal skin [9].

Taken together, these findings triggered our hypothesis that low total PC levels may predispose to lower leg ulcers in diabetes. The aim of the present study was to determine if an association exists between circulating levels of total PC and lower leg ulcers in patients with diabetes.

2. Methods

A total of 72 outpatients with diabetes mellitus participated. This study was approved by the Northern Sydney Health Human Research Ethics Committee, and written informed consent was obtained from each subject. The diagnosis of either type 1 or 2 diabetes mellitus was made according to the criteria of the American Diabetes Association [18]. Thirty-six patients had at least one lower leg ulcer, and these patients were matched for age, gender, and type of diabetes with 36 patients with diabetes with no history of previous or current lower leg ulcer. Ulcer types were classified as neuropathic ($n = 14$), ischemic ($n = 10$), mixed neuropathic/ischemic ($n = 11$) or venous ($n = 1$). Peripheral ischemia was determined by absence of both dorsalis pedis and posterior tibial pulses on clinical palpation. Peripheral neuropathy was assessed by clinical insensitivity to a 10-gram monofilament. All ulcers were located at or below the malleolus, except the venous ulcer which was located on the lower leg. Our control group consisted of matched patients with diabetes because patients with type 1 or type 2 diabetes have altered levels of circulating PC levels compared to normals [19, 20]. Patients on warfarin or any anticoagulant therapy were excluded from the study.

Blood sampling was carried out in all subjects from an antecubital vein, and plasma was separated. All assays were performed in a routine diagnostic laboratory. Total PC was assessed using the Stachrom PC colorimetric assay after activation of plasma PC with Agkistrodon contortrix venom (Diagnostica Stago, Asniers, France). Protein S and fibrinogen were measured by an immunoturbidimetric and clot-based test, respectively, performed on a fully automated coagulation analyser (STAR by Diagnostica Stago). The prothrombin time (PT), activated partial thromboplastin time (APTT), and International Normalised Ratio (INR) were also performed on the STAR analyser. Soluble (s)EPCR was measured by ELISA (R & D Systems, Minneapolis, USA).

Data was tested for normality and, if not normally distributed, transformed to a normal distribution before modelling by linear regression using Stata 11.0. Age was included in the regression as a confounding covariate. Differences in proportions were by Fisher's Exact tests. To quantify any significant associations within the data, pairwise Pearson correlation coefficients were calculated.

3. Results

Of the 72 patients with diabetes in this study, 36 had chronic (>6 month duration) lower leg ulcers, and 36 patients did not have any ulcers (control group). All dependent variables were normally distributed, except for sEPCR and HbA_{1c} which required log transformation. Results are shown in Table 1. Between the 2 groups, there was no difference in age, sex, duration of diabetes, or HbA_{1c}. There was no correlation between PC and gender, type of diabetes, HbA_{1c}, or CRP. There was a negative correlation between PC levels and age ($r = -0.38$, $P = 0.03$) that remained when groups were analysed separately (Figure 1). The most striking difference was the significantly lower levels of plasma total PC in patients with lower leg ulcers compared to control subjects ($P < 0.001$). Of the 36 patients with lower leg ulcers, 8 had total PC levels that were below the normal range (70%–180%), whereas only 1 of 36 control patients was lower than normal (Fisher's Exact $P = 0.028$). Ulcer type was not a significant covariate for plasma total PC ($P = 0.35$). Levels were 105 ± 26% for neuropathic ($n = 14$) ulcers, 84 ± 29% for ischaemic ulcers ($n = 10$), and 91 ± 29% for mixed neuropathic/ischaemic ($n = 11$). The protein C level of the only patient with a venous ulcer was 116%.

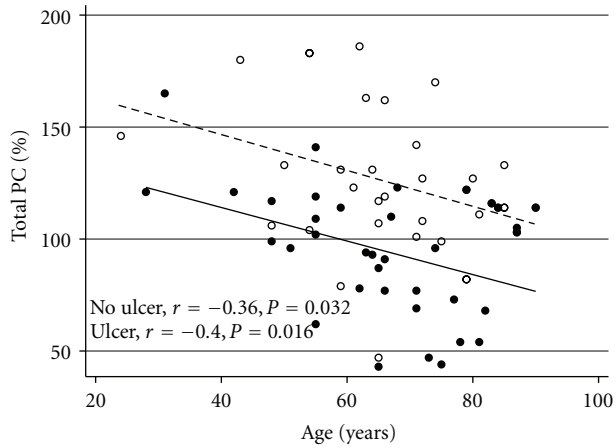

Figure 1: Correlation between total PC and age for patients with no ulcer (open circles, upper dotted line) and patients with lower leg ulcers (closed circles, lower full line).

There was no difference in protein S, APTT, or fibrinogen levels between the 2 groups; however, there was significantly higher INR, C reactive protein, and prothrombin time in patients with ulcers. INR was negatively correlated with PC in the ulcer group only ($r = -0.57$, $P = 0.001$). Plasma sEPCR levels did not statistically differ in patients with diabetes who had lower leg ulcers compared with matched controls.

4. Discussion

This is the first study to examine circulating PC levels in patients with diabetes who have lower leg ulcers compared to those without ulcers. Our results show that patients with diabetes who have lower leg ulcers have lower levels of plasma total PC than their counterparts without ulcers. There was no difference in Protein S, APTT, fibrinogen, or sEPCR between the two patient groups, suggesting that the low total PC levels in these patients are not a direct result of

Table 1: Demographics and laboratory data of patients with diabetes with and without lower leg ulcers. *P* values for differences between patients with and without ulcers were performed by linear regression, corrected for age.

	n	Normal range	Diabetes without ulcers (mean ± s.d)	Diabetes with ulcers (mean ± s.d)	*P* value by LR (age as covariate)
Age	72	—	59.82 ± 16.31	65.56 ± 15.15	0.46
Gender	72	—	64% male	64% male	—
Type of diabetes	72	—	82% Type 2	83% Type 2	—
Total PC (%)	72	70–180	127.1 ± 34.0	103.3 ± 22.7	<0.001
Free PS (%)	60	60–160	124.7 ± 22.4	108.4 ± 37.8	0.056
Fibrinogen (g/L)	60	5.9–11.8	11.6 ± 2.23	12.3 ± 2.6	0.76
INR	60	0.8–1.2	0.97 ± 0.08	1.02 ± 0.08	0.003
Prothrombin time (sec)	60	13–15	13.33 ± 0.83	13.90 ± 0.82	0.026
APTT (sec)	60	26–36	27.28 ± 2.512	28.75 ± 2.75	0.12
CRP (mg/L)	32	<47	61.0 ± 62.5	211.4 ± 262.0	0.022
HbA_{1c} (%)	54	4–6	6.96 ± 1.42	7.15 ± 1.79	0.61
sEPCR (ng/mL)	54	—	25.0 ± 15.9	28.0 ± 20.1	0.77

an altered coagulation profile. Blood glucose control appears to be unrelated to low PC levels in patients with lower leg ulcers, as there was no difference in HbA_{1c} between the two groups.

Test results were not available for all patients, and thus patient numbers are reduced for some tests, particularly CRP. Nonetheless, when corrected for age, there was a significant increase in CRP in patients with lower leg ulcers compared to those without ulcers, suggesting increased inflammation, which is a feature of diabetic skin ulceration. A further limitation to this study is that data on other diabetes complications, such as denervation or arteriopathy which may contribute to failed wound healing, were not available and may confound the observed relationships.

Liu et al. [21] have recently shown that high wound fluid concentrations of matrix metalloproteinase-(MMP-9) predict poor wound healing in diabetic foot ulcers. Considering that APC inhibits MMP-9 production by monocytes [17], it would be interesting to determine whether reduced APC in wound fluid results in increased MMP-9. Regardless of the mechanism, our results show an association between low total PC levels and lower leg ulcers in patients with diabetes. Whilst ~20% of patients with diabetes and lower leg ulcers had total PC levels lower than the normal range, the mean total PC level (96%) of this group fell within the broad normal range (70%–180%). This demonstrates that it may not be necessary for total PC levels to be lower than the normal range for lower leg ulcers to occur. Further work is required to delineate what PC level constitutes "low" in terms of failed wound healing in diabetes.

Treatment of diabetic lower leg ulcers frequently presents a management challenge as they often respond poorly to conventional wound management therapy, often due to complications such as neuropathy and peripheral vascular disease. We found no significant difference between these different ulcer types in this study. Previous evidence indicates that exogenous APC promotes healing of recalcitrant ulcers in patients with diabetes, acting via numerous different mechanisms including inhibition of inflammation, stimulation of angiogenesis, and reepithelialisation [8, 14, 15]. APC is emerging as a potential therapeutic agent not only for lower leg ulcers but also for a number of other disorders including lung injury, spinal cord injury, and kidney injury [9]. Interestingly, plasma total PC levels are substantially decreased in patients who develop severe sepsis, and the level of total PC correlates inversely with morbidity and mortality [22]. After showing positive results in a clinical trial [7] and obtaining FDA approval for APC to treat sepsis in 10 years previously, Eli Lilly, the company who marketed the drug, controversially withdrew APC from the market in 2011.

Low circulating PC levels may either predict or be a consequence of lower leg ulcers. However, when combined with our previous findings that (i) APC treatment stimulates healing of lower leg ulcers [8, 9] and (ii) total PC expression in skin surrounding lower leg ulcers is low [9], the current findings provide supportive evidence that low PC levels predispose to lower leg ulcers which do not heal in patients with diabetes. It is feasible that a blood test to measure PC may assist clinicians in the difficult judgement of whether a diabetic ulcer will heal or not. Further longitudinal clinical studies will help confirm the value of such a test.

Abbreviations

APC: Activated protein C
APPT: Activated partial thromboplastin time
CRP: C-reactive protein
EPCR: Endothelial protein C receptor
INR: International Normalised Ratio
MMP: Matrix metalloproteinase
PC: Protein C
PT: Prothrombin time
sEPCR: Soluble endothelial protein C receptor.

Conflict of Interests

C. J. Jackson has commercial interest and patents for the use of APC in wound healing. The rest of the authors have no other conflict of interests.

Authors' Contribution

K. Whitmont, I. Reid, and C. J. Jackson contributed to conception and design, or analysis and interpretation of data, drafting the paper, or revising it critically for important intellectual content. G. Fulcher, Y. Xie, M. Aboud, C. Ward, M. M. Smith, and A. Cooper contributed to analysis and interpretation of data, and revising paper critically for important intellectual content. All authors gave final approval of the version to be published.

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Review Article

Glucose Toxic Effects on Granulation Tissue Productive Cells: The Diabetics' Impaired Healing

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Type 2 diabetes mellitus is a metabolic noncommunicable disease with an expanding pandemic magnitude. Diabetes predisposes to lower extremities ulceration and impairs the healing process leading to wound chronification. Diabetes also dismantles innate immunity favoring wound infection. Amputation is therefore acknowledged as one of the disease's complications. Hyperglycemia is the proximal detonator of systemic and local toxic effectors including proinflammation, acute-phase proteins elevation, and spillover of reactive oxygen and nitrogen species. Insulin axis deficiency weakens wounds' anabolism and predisposes to inflammation. The systemic accumulation of advanced glycation end-products irreversibly impairs the entire physiology from cells-to-organs. These factors in concert hamper fibroblasts and endothelial cells proliferation, migration, homing, secretion, and organization of a productive granulation tissue. Diabetic wound bed may turn chronically inflammed, procatabolic, and an additional source of circulating pro-inflammatory cytokines, establishing a self-perpetuating loop. Diabetic fibroblasts and endothelial cells may bear mitochondrial damages becoming prone to apoptosis, which impairs granulation tissue cellularity and perfusion. Endothelial progenitor cells recruitment and tubulogenesis are also impaired. Failure of wound reepithelialization remains a clinical challenge while it appears to be biologically multifactorial. Ulcer prevention by primary care surveillance, education, and attention programs is of outmost importance to reduce worldwide amputation figures.

1. Introduction

What represents today a worldwide pandemic of a noncommunicable disease, diabetes mellitus, has two principal clinical forms identified as types 1 and 2. The former is a condition in which by autoimmune mechanisms pancreatic β-cells are eventually destroyed with an absolute insulin deficiency [1]. Type 2 diabetes mellitus (T2DM) is the most prevalent form of the disease and recently acknowledged not as a single clinical condition, but importantly, as a group of metabolic disorders. Diabetes, in general, causes chronic hyperglycemia and a wide range of downstream metabolic disturbances and multiorgan complications [2]. It is notorious, however, that although insulin secretion collapse, peripheral insulin resistance, and/or receptors' activity failure play a definitive role for the onset of sustained hyperglycemia in T2DM, a large portion of body glucose is cleared by insulin-independent mechanisms, derived from the ability of plasma glucose to influence its own clearance by a mass action effect [3]. T2DM usually most common in adult subjects exhibits a slow, silent, and insidious evolution. Hyperglycemia and its adjoined biochemical consequences undermine the whole tissues being sufficient to orchestrate irreversible systemic complications, from which the cells comprised in soft peripheral tissues and vascular structures do not escape. Lower extremities ulcerations and the potential for amputation are currently acknowledged as members of the list of diabetes complications [4].

Surgeon Davies Pryce put forward as early as 1887 the link between diabetes and foot ulceration by writing in The Lancet

that "diabetes itself may play an active part in the causation of perforating ulcers" [5]. However, despite the years of efforts and research, the pathogenesis of impaired wound healing in diabetes remains incompletely elucidated [6]. This poor-healing condition appears to be a multifactorial process, which includes the amalgamation of systemic and local factors that ensure a perpetual forward loop up to chronification. Along this path, the cells seem to progressively wipe out their ability to trigger evolutionarily imprinted mechanisms as migration, proliferation, and transdifferentiation becoming increasingly statics. Thus, diabetic wounds do not only become chronic by a concept of aberrant healing trajectory within a physiological time frame, but also by the asynchrony on the sequence of overlapping events that make up the tissue repair megaprocess. Broadly speaking, diabetes impairs most if not all these events. Thus, the challenge that represents the diabetic wound healing failure is the clinical gross expression of an outstanding array of biochemical and cellular disorders [7]. These ideas are supported in the clinical arena by the alarming statistics of amputations around the world every year [8].

The healing process in diabetes is also jeopardized by the patient's susceptibility to infection due to deficiencies on the innate immunity. Although the diabetic wound bed may be adversely overwhelmed by inflammatory cells, it does not represent an overt antibacterial protection. By the contrary, the diversion of glucose to the polyol pathway affects bacterial killing by reducing neutrophil opsonophagocytosis. Furthermore, hyperglycemia-induced reactive oxygen species (ROS) deregulated the innate immunity via an overactivation of NF-kappaB (NF-κB), thus amplifying the absurd inflammation and intoxicating the wound milieu [9, 10]. Peripheral arterial disease, leading to ischemia or lower limb hypoperfusion is associated with the most severe outcomes, including lower probability of healing, longer healing times, higher probability of ulcer recurrence, greater risk of amputations, and potentially higher mortality [4]. Cells harvested and cultured from hypoperfused granulation tissues orchestrate a molecular program of arrest and senescence (Jorge Berlanga-Acosta. Manuscript accepted. Int Wound Journal). The outcome of the combination "healing failure" and "infection susceptibility" untowardly contributes to amputation. Here we review the current lines of evidences on the toxic resonance of acute and long-term exposure to high glucose on the two main cells for the granulation tissue organization: fibroblasts and endothelial cells. We have included a characterization of the diabetic granulation tissue organizational disorders and the challenge that represents its ultimate process, wound reepithelialization. The literature search was based on English language articles downloaded from Pubmed and Bioline International (http://www.bioline.org.br/) data sources.

2. Consequences of Glucose Overload Toxicity on Fibroblasts and Endothelial Cells

2.1. Fibroblasts. The fibroblast is central to the wound healing process by secreting, contracting, and remodeling the extracellular matrix (ECM). They also secrete growth factors as important messengers for mesenchymal-to-mesenchymal and epithelial-mesenchymal communication, especially for establishing the emerging basement membrane and subsequent reepithelialization. Therefore, any impediment to fibroblast function is deterrent for normal wound healing and may result in chronic, nonhealing wounds. The fibroblast, when engaged in fibrogenesis, displays the highly activated phenotype characteristic of myofibroblasts. Although their origin has not yet been definitely elucidated; proliferation of preexisting adjacent dermal fibroblasts and, probably, recruited from the bone marrow has been documented [11]. Under the high glucose burden imposed by diabetes, cutaneous and extra cutaneous fibroblasts appear perturbed; and for many years, *in vitro* models recreating "clinical hyperglycemia" have proved to disrupt normal fibroblasts physiology and derange the secretion of extracellular matrix ingredients. These experiments have suggested that high glucose concentration is the proximal detonator of a downstream cascade of molecular disturbances for the skin fibroblast [12].

Rowe and coworkers pioneered the *in vitro* models that demonstrated that in diabetics' cutaneous fibroblasts; synthetic, proliferative, and secreting capabilities are reduced [13]. Other parallel studies, in which high glucose concentrations were introduced, proved to inhibit fibroblast proliferation, while the cells turned resistant to proliferate to growth factors such as insulin-like growth factor type-I (IGF-I) and epidermal growth factor (EGF) [14]. Following this attractive targets, Goldstein's findings allowed for establishing the hypothesis that diabetics fibroblasts replicative life span did proportionally decline with diabetics' predisposition under normal glucose concentrations, concluding that a persistent, heritable abnormality is present in mesenchymal tissues of overt diabetics and genetically predisposed subjects [15]. Years later, Goldstein also announced that cells obtained from insulin-dependent or insulin-independent diabetics not only exhibit abnormal replicative capacity *in vitro*, but that the aging process appeared more precociously than in nondiabetic counterparts [16]. Other studies showed that the addition of conditioned media from non-insulin-dependent diabetes mellitus wound fibroblasts induced a dose-dependent inhibition in normal fibroblast proliferation which appeared related to elevated L-lactate levels [17]. This replicative refractoriness of diabetic fibroblasts has been reproduced by different groups in subsequent years [18], thus confirming the need for additional external supplements to ensure cell cycle progression [19]. Accordingly, Loots and coworkers demonstrated the need of the simultaneous rather than the sequential addition of different growth factors combinations for diabetic ulcer fibroblasts in order to induce a proliferative response [20]. In addition to the onset of a quiescent and senescent phenotype of diabetic wound fibroblasts, their ability for horizontal and vertical migration is also dramatically impaired when compared to normal donor cells in different migration assay as in the modified Boyden chamber haptotaxis assay [21]. Most of these attributes are reproduced under acute exposures to high glucose concentrations so that migration speed is reduced by ~40% associated to a decrease in cell directionality and to nonproductive

protrusive events, as loss of cell polarization, consistent with the increased activity of Rac1 and the projection of multiple lamellipodia. This experiment concluded that the generation of reactive oxygen species (ROS) may lie behind these abnormalities as they were partially or completely rescued by treatment with N-Acetyl-Cysteine (NAC) [2]. In contrast to the cellular reactions when exposed to high glucose *in vitro*, full-thickness wounds induced in nondiabetic pigs exposed to a local hyperglycemic environment exhibited no difference in wound closure when compared with normoglycemic controls, suggesting that delayed wound healing by diabetes is a far more complex phenomenon than circumscribed high-glucose concentration itself [22]. As a consequence of the cutaneous accumulation of advanced glycation-end products (AGEs), the skin increases its chronological age. One of the AGEs precursors is 3-deoxyglucosone (3DG). Fibroblasts cultured on 3DG-treated collagen reduce the ability to migrate efficiently since 3DG increases its adherence to the matrix. Additionally, the authors describe a higher level of misfolded proteins [23]. Using the same experimental system, this group demonstrated two years later that the inhibition in fibroblast migration, proliferation, and collagen expression by exposure to 3DG-collagen was mediated via extracellular regulated kinase 1/2 (ERK1/2) and Akt downregulation through activation of p38 MAPK (Mitogen-Activated Protein Kinase), indicating that p38 is a key signaling molecule that plays an opposite role during times of cellular growth and cellular stress [24]. Enriching the above findings, this group also demonstrated that 3DG-modified collagen induces oxidative stress, endoplasmic reticulum stress, and apoptosis via caspase-3 activation.

Oxidative stress appeared dependent on the upregulation of NAD(P)H oxidase 4 (Nox4), a reactive oxygen species Nox homologue, which appeared activated by p38 MAPK. Proximal to this cascade is the effect caused by the interaction of the modified collagen with 3DG, which signals to the fibroblast by interacting with integrins alpha-1/beta-1 ($\alpha 1\beta 1$) and not through the canonical receptor for advanced glycation end-products (RAGE) [25]. Other groups have also demonstrated the induction of cutaneous fibroblasts apoptosis through cytoplasmic and mitochondrial pathways by plating the cells in an AGE-enriched environment made up by Ne-(carboxymethyl)lysine (CML)-collagen, which primarily activated the classic AGE receptor (RAGE) [26]. A subsequent study elegantly demonstrated that after AGEs-RAGE interaction, oxygen species generation is increased, activating both NOS and ceramides, which in turn activates p38 and c-Jun N-terminal protein kinase (JNK). Activated p38 and JNK triggers a cascade that leads to amplify caspase-3 activity, whereas activation of Forkhead box O class 1 (FOXO1) increases the likelihood of apoptosis through enhanced expression of proapoptotic genes [27]. Under a number of circumstances, FOXO transcription factors induce BIM and other proapoptotic genes expression.

In addition to the deleterious effects of glucose and its derivatives, diabetic fibroblasts exhibit particular features. Literature documents that diabetic mice fibroblasts show a severe impairment in VEGF production under normoxic and hypoxic conditions in addition to an increased prodegradative activity due to the high expression of matrix metalloprotease type 9 (MMP-9) [28]. Similarly, diabetic pigs exhibit an impaired healing that is accompanied by a reduction of IGF-1 in the wound milieu [22]. Studies with human fibroblasts have confirmed the prodegradative phenotype by the increased MMP-2 and MMP-3 production and reduced collagens gene expression [29]. Human diabetic fibroblasts also exhibit a failure in nitric oxide (NO) production, which is concomitant to elevations in MMP-8 and -9 [30]. The fact that these fibroblasts fail in secreting NO is particularly negative given its role for wound healing. Conversely, NO donors' administration has shown to stimulate cell proliferation and restore the balance of MMPs [31].

It seems that amplification of oxidative stress acts as a primary culprit in harming fibroblasts biology in diabetes, involving electron transport in mitochondria. High intracellular glucose levels increase the electron transport chain in mitochondria during oxidative respiration, leading to formation of O2- and the generation of various reactive oxygen species derivatives in the mitochondria. Other sources of oxidative stress in diabetes include glucose autooxidation, the polyol pathway with ensued depletion of antioxidant reserves and the formation of AGEs [32]. Chronic hyperglycemia-induced mitochondrial ROS stimulate various signaling pathways that amplify inflammation and cell death. They include protein kinase C (PKC), JNK, and p38/MAPK [31]. According to an excellent review by Ponugoti et al. [33]; ROS leads to the activation of members of the FOXO family. This is a family of transcription factors with apparently opposing roles that may defend cells against oxidative stress but also promote cell-cycle arrest in G1 by inducing p27kip1 [34]. FOXO1 activation appears elevated in diabetic connective tissue cells and mediates AGEs and tumor necrosis factor-alpha- (TNF-α-) induced apoptosis both of which are abundant in diabetic connective tissue [35]. FOXO1 limits wound healing by inhibiting fibroblasts proliferation and enhancing their apoptosis [35, 36]. Interestingly, insulin inactivates FOXO1 via Akt leading to its nuclear export and degradation. Defective insulin action in the skin has been proposed as an important mechanism contributing to wound healing defects in diabetes. Perhaps the assorted constellation of the hormone's pharmacological bounties (increased expression of endothelial nitric oxide synthase, vascular endothelial growth factor, and stromal-derived factor-1α/SDF-1α) observed in experimental and clinical wounds when insulin is topically administered may be attributable to FOXO1 neutralization. Curiously, the acceleration of wound healing occurs in parallel to a local recovery in the expression of proteins involved in insulin signaling pathways [37]. Aside from the above arguments, these preclinical and clinical findings are not surprising in light of the potent anti-inflammatory, proanabolic, and cytoprotective actions of insulin [38], which extend beyond the exclusive regulation of glucose homeostasis [3]. Figure 1 depicts the main pathogenic players and their interconnection as an attempt to summarize the high glucose-triggered fibroblasts damage process.

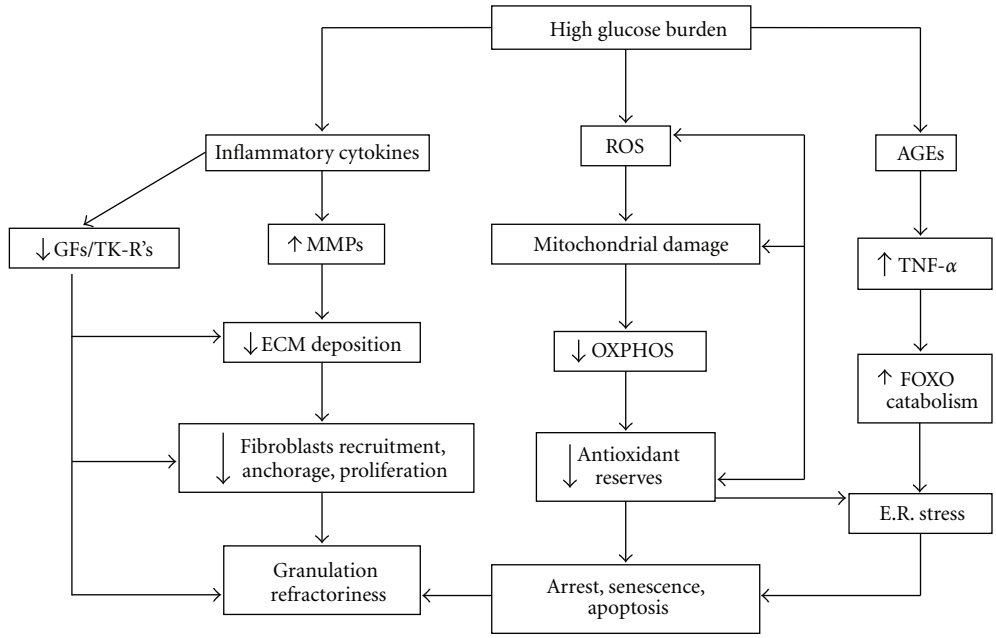

Figure 1: Negative impact of high glucose levels on cutaneous fibroblasts biology. Short- or long-term exposure to high glucose concentrations is toxic for cutaneous fibroblasts suppressing the cells' biological activities. The fibroblasts become reactive but not active. The high glucose burden engenders and uncontrolled production of ROS within the mitochondria with three major consequences: detriment on the OXPHOS reactions, depletion of the cells antioxidant reserves, and amplification of the mitochondrial dysfunction due to ROS-mediated attack to its DNA. Under this scenario apoptosis may prevail. ROS also may lead to cell cycle arrest due to p53 and p21 upregulation and nuclear compartmentalization. Alternatively, high glucose concentrations may impose a proinflammatory program within the wound by perpetuating a special population of macrophages (M1) so that fibroblasts become intoxicated and suppress the secretion of ECM ingredients. Conversely, the inflammation mediators fuel the secretion of MMPs. The negative balance of ECM inhibits fibroblasts chemotaxis, homing, anchoring, and proliferation. The proinflammatory environment inhibits the secretion of numerous growth factors with fibro angiogenic potential as TGF-β, PDF, EGF, and so forth and interferes with the signaling pathways of the TK-R's. Inhibition of the TK-R's downstream networking entails the suppression of positive forces for a balanced control of granulation tissue repopulation with productive cells. The accumulation of AGEs activates the AGE/RAGE axis, which further amplifies local inflammation and reactivity by increasing the secretion of TNF-alpha and adhesion molecules. This cytokine interferes with insulin and growth factors signaling, TGF-β1 for instance, which further amplifies the obstruction of the PI3K/Akt/mTOR/Cyclin D axis. The balance against this vital axis promotes the nuclear compartmentalization of representative of the FOXO family members, which contributes to catabolism, senescence, arrest, and apoptosis. In this prooxidative environment, it is common to activate cells autophagy in response to the accumulation of missfolded proteins. Thus, all these factors converge to slow down granulation tissue outgrowth.

Despite the prolific investigation conducted during all these years, still questions remain to be answered in relation to *ex vivo* diabetics' fibroblasts behavior.

(1) Why diabetics' fibroblasts evoke behavioral traits in culture mirroring the donor's tissue, even when grown under optimized oxygenation, nutrient, growth factors, and glucose supply?

(2) Is there any sort of "behavioral imprinting" so that they are reminiscent from a diabetic donor?

(3) Why cultured fibroblasts from both ischemic and neuropathic ulcers exhibit different ultrastructural morphology and organize the monolayer in a privative manner?

(4) Is there any epiphenomenon beyond the irreversible glycation sustaining the "impersonation" of the *in vivo* traits?

2.2. Endothelial Cells. Angiogenesis is a comprehensive term that indicates the physiological process involving the growth of new blood vessels or neovascularization. This is a vital process for embryological growth, tissue development, and wound healing. Different growth factors families as vascular endothelial growth factors (VEGF), fibroblast growth factor (FGF), angiopoietins, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), in collaboration with other proteins as integrins, cadherins, and ephrins, regulate angiogenesis by promoting endothelial cells recruitment, proliferation, migration, coopting, and collar stabilization. There is an enormous and ever-growing body of evidence indicating the close correlation between hyperglycemia and the abnormalities in endothelial function and morphology [39]. The UK Prospective Diabetes Study (UKPDS) and Diabetes Control of Complications Trial (DCCT) found microvascular disease and hyperglycaemia to be intrinsically related. Thus, anomalous angiogenesis is a hallmark of both

type forms of diabetes, which is clearly and early observable during the process of granulation tissue growth, a condition that has been successfully reproduced in animal models [40]. For subjects with macrovascular disease, the defective angiogenesis prolongs and disturbs the healing process. The concept of abnormal angiogenesis extends beyond the wound, given the inability of these patients to create collateral circuits due to VEGF-dependent monocytes dysfunction [41]. Furthermore, insulin has a dramatic impact on the endothelial homeostasis by its ability to stimulate NO release via a cascade that involves activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling and endothelial nitric oxide synthase (e-NOS) phosphorylation. The later being of paramount importance in angiogenesis and wound healing as described below [42].

As depicted for fibroblasts, high glucose and the glycated by-products exert a toxic effect on endothelial cells and the vascular wall in general. In parallel, the endothelial cells *per se* seem to be a very sensitive target to high glucose. Endothelial dysfunction is intricately related to insulin resistance through the stimulatory effects of insulin on glucose disposal and NO production in the endothelium. Today, vascular dysfunction remains as a major cause of morbidity, amputation/disability, and mortality in diabetic patients. Even after achieving the successful reperfusion of an ulcerated lower extremity, the healing process is slow and torpid. Therapeutic angiogenesis has been pursued for years, but the clinical results have shown relatively limited outcomes [43–45]. High glucose concentrations have been associated with endothelial metabolic dysfunction *in vitro* and *in vivo* and as for multiple physiological processes; insulin and its downstream signaling regulate most of the endothelial cell functions [46]. High glucose ambient has been shown to disturb endothelial cells cycle, increase DNA damage, and delay endothelial cells replication, as inducing excessive cell death [47]. In addition, high glucose also prevents NO-induced inhibition of vascular smooth muscle cells (VSMC) migration [27] thus contributing to Monckeberg's media thickening. *In vitro* models simulating "normoglycemia" and "hyperglycemia" have demonstrated that under high glucose ambient, proliferation and tube formation of dermal microvascular endothelial cells appear impaired [48]. Furthermore, high glucose levels selectively trigger apoptosis in cultured endothelial cells as has been demonstrated by different laboratories [49]. High glucose induces the upregulation of TNF-α level concomitant to the death receptors TNF-R1 and Fas in a variety of cultured endothelial cells [50]. Under this ambient, Bax protein expression increases, cytochrome c is released, subsequently conjugating to Apaf-1 and triggering a caspase cascade-induced death [51].

Hyperglycemia-induced oxidative stress promotes inflammation through increased endothelial cells damage, microvascular permeability, and uncontrolled release of proinflammatory cytokines, including TNF-α, interleukin-1β (IL-1β), and interleukin-6 (IL-6), ultimately leading to decreased insulin sensitivity and diabetic vascular complications. Moreover, hyperglycemia-induced FOXO also plays an important role in the induction and amplification of proinflammatory cytokines production. FOXO1 directly binds to IL-1β promoter and increases its expression in macrophages [52].

Hyperglycemia and the accumulation of AGEs disturb the role of angiogenic growth factors as VEGF, and its receptor, its signaling pathway, thus disrupting endothelial proliferation, migration, and endothelial progenitor cells (EPCs) recruitment and release from bone marrow [53]. Insulin resistance disrupts the NO-mediated angiogenic positive regulation over angiogenic growth factors such as VEGF, FGF, and TGF-β [53]. Studies using streptozotocin-induced diabetic mice with simultaneous hind-limb ischemia have suggested that the angiogenic responses remain preserved even under the diabetic state, and that 40 to 50% reduction of platelet-derived growth factor-BB (PDGF-BB) expression is responsible for the induction of functional and morphological vascular abnormalities and pericytes apoptosis. Conversely, PDGF-BB external supplementation was sufficient to prevent limb autoamputation, an event also reproduced with a PKC inhibitor that restored the expression of endogenous PDGF-BB [54].

The glycation of collagen and other proteins within the wound extracellular matrix and AGEs accumulation bring catastrophic consequences for the angiogenic reaction with inhibition of angiogenesis *in vivo*. The fact that angiogenesis is restored by aminoguanidine treatment reinforces the antiangiogenic role of AGEs [55]. Angiogenesis is a multifaceted process demanding an appropriate, nonglycated extracellular substrate. This is clearly illustrated by the fact that PDGF-BB anchors to different components of the ECM under physiological conditions acting as a natural depot and slow release system for the growth factor. Local PDGF unavailability has proved to impair the coverage of newly formed vessels with mural cells and local pericytes [56]. This evidence reinforces the pathophysiological impact of high glucose toxicity, the release of proinflammatory cytokines, and the activation of the intrinsic mitochondrial-mediated apoptotic signaling pathway on endothelial cells. In summary, endothelial cells exposed to excess glucose trigger the onset of a pro-inflammatory profile turning these cells into a cytokines and ROS manufacturing plant. The agonistic stimulation of the AGEs receptor is able to mount the same response leading to apoptosis and vascular ruin. The pathogenic effects of hyperglucose associated to insulin axis failure on endothelial cells are summarized in Figure 2.

Compelling evidence indicates that at least a portion of the hyperglycemia-mediated endothelial damages and dysfunctions are associated with an impaired mitochondrial activity resulting in mutations of mitochondrial DNA, due to a disproportionate reactive oxygen radicals production, leading to an inflammatory reaction and apoptosis [57]. As a matter of fact mitochondrial DNA has a much higher mutation rate than nuclear DNA because it lacks histones and is exposed to the direct action of oxygen radicals while its repair system is limited. Therefore, ROS appear to play a pivotal role in systemic endothelial deterioration and biological aging [58]. As described, ROS generation enhances FOXO1 activation and induction of several classes of genes that regulate endothelial cell behavior, including pro-inflammatory factors and eventually the execution of apoptosis of endothelial and

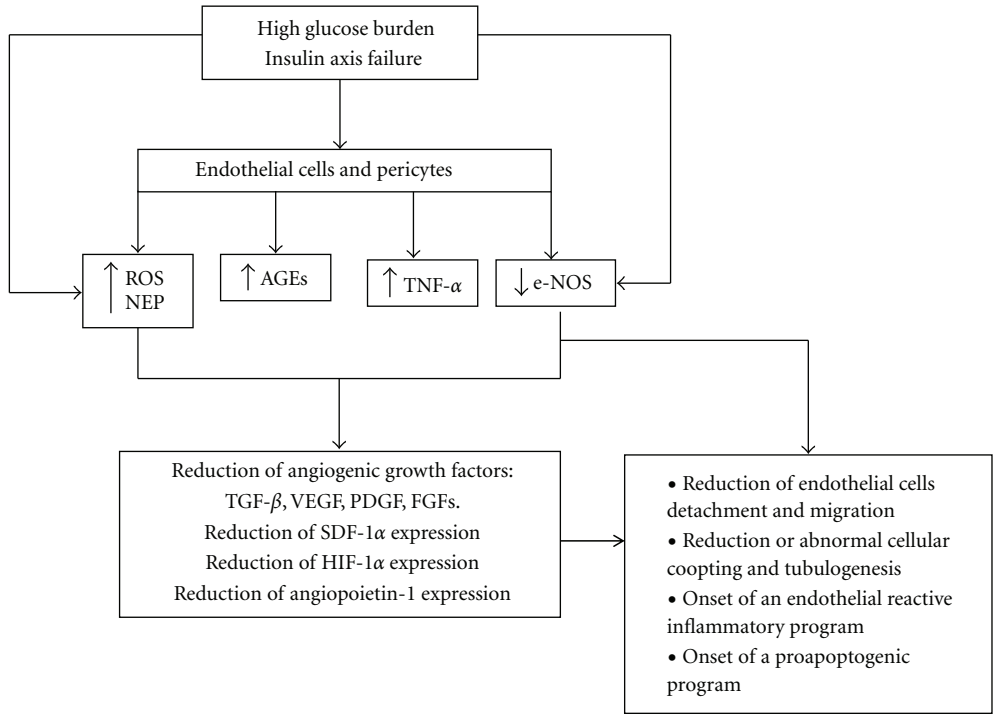

Figure 2: Negative impact of high glucose levels and failure of the insulin system on vascular cells. Endothelial cells are a sensitive target for high glucose concentration and especially for the insulin receptor downstream signaling attenuation. Similarly, pericytes, which are key cells for the angiogenic process to succeed, are targeted by these factors. The endothelial cells metabolic response to the above proximal triggers engenders the accumulation of superoxide and hydroxyl reactive groups. These prooxidative elements disrupt the physiological pathways for NO metabolism, accumulating toxic nitrosylation end-products. In this context, AGEs are precipitately formed and accumulated within the vascular wall. Different pathways converge to induce TNF-α overproduction. The proximal triggers and all these factors also contribute to disrupt e-NOS activity having as net result a deficit in endothelial NO, the inability for vasodilation, and the suppression of endothelial cells proliferative reserves. This circle is further amplified due to the concomitant reduction in the pool of critical angiogenic factors involved in the regulation of vascular regeneration. Eventually endothelial cells and pericytes may onset a pro-apoptogenic program, which will hinder granulation tissue perfusion and wound healing.

adjacent cells [33]. ROS-mediated lipid peroxidation appears to impair most healing events, contributing to growth factors reduction, keratinocytes migration failure, slow or torpid fibroplasia, delayed contraction, and matrix remodeling, not to mention abnormal angiogenesis [59]. Under experimental conditions, the pharmacological intervention with a chemical inhibitor of lipid peroxidation proved to reduce the local edema and to stimulate reepithelialization, neovascularization, proliferation of fibroblasts, and synthesis and maturation of the extracellular matrix. A parallel finding was the normalization of VEGF mRNA expression and secretion in those diabetic mice. This further supports the view that lipid peroxidation perturbs VEGF production [60]. An extraordinary background has accumulated about the role of NO in vascular biology in diverse horizons as ischemia, inflammation, and neovascularization. Impaired endothelium-dependent NO-mediated relaxation occurs in both cellular and *in vivo* models [61]. Many of the metabolic conditions associated with diabetes are conditioned by failure in NO synthesis or its degradation. In this respect, the integrity of the Akt/e-NOS coupling pathway for a normal endothelial function appears compulsory [62]. Hyperglycemia is also associated to a deficit in tetrahydrobiopterin (BH4) and to an increase in arginase expression, which attempt against NO synthesis and normal endothelial functions such as vascular remodeling responses [63]. The increased generation of peroxynitrite levels under high glucose conditions contributed to deplete cellular antioxidant reserves as to activate NF-κB and consequently the expression of the inducible form of nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), and other inflammatory mediators [64].

Endothelial Progenitor Cells (EPCs) are active players for the maintenance and repair of endothelial cells. They participate in angiogenesis as they proliferate, migrate, and differentiate and are a source for proangiogenic factors and cytokines [65]. Multiple lines of evidence indicate that the number of circulating EPCs is decreased under both clinical forms of diabetes, which is likely to be involved in the pathogenesis of vascular complications [66]. Under experimental diabetic conditions, the EPCs number appears significantly, decreased in the bone marrow as in the peripheral blood, which was reverted by treating the mice with insulin [67]. In general, the bone-marrow-derived EPCs in the diabetic patients are considered as dysfunctional, producing fewer endothelial cells with reduced replicative and migratory potential [68]. Tamarat and coworkers have described

a limited capacity of diabetic animals-derived bone marrow mononuclear cell to differentiate into endothelial progenitor cells *in vitro* as to organize tubulogenic structures when subcutaneously implanted in a matrigel plug, thus hindering the revascularization of damaged areas [66]. Over again, the activation of p38 MAPK mediated by an excessive ROS generation has been aimed as responsible for the EPCs impaired proangiogenic potential *in vivo* by limiting cells proliferation and differentiation [69]. As to fully divert the physiological role of EPCs in tissue repair and angiogenesis, the duet hyperglycemia-ROS stimulate the EPCs to produce pro-inflammatory cytokines and to shift NO production by elevating i-NOS and decreasing e-NOS [70]. As described for other cells, AGE treatment disrupts EPCs physiology thus leading to a downregulation of e-NOS and Bcl-2 expression, as well as an elevation in cyclooxygenase-2, Bax, NF-κB, and caspase-3 in a MAPK- (ERK/P38/JNK-) dependent manner [69].

The diabetes-mediated vascular damage is perhaps the most outspoken and ancestrally identified emblem of diabetes. It is varied and broad as it is the concept of systemic endothelial dysfunction. Diabetes distorts the angiogenic program to ironically culminate with a maldistribution of soluble angiogenic factors: shortage where and when required (lower extremities skin) but overproduced where and when not needed (retina). It is also challenging, to understand *how* and *why* microvascular morphological changes that recreate chronic, life-time processes are readily identified in a 7-day-old granulation tissue fragment, even in compensated patients. This incites to investigate which are the diabetes' operational local and/or systemic forces that can disrupt vascular morphogenesis.

3. Failure of Granulation Tissue Onset and Progression

Once we have described the main consequences of high glucose/hyperglycemia on the two principal architects of the granulation tissue: fibroblasts and endothelial cells, we are intended to recapitulate the most distinguishing features on the onset of the granulation process in diabetic cutaneous wound healing.

Tissues' regenerative capabilities have been neglected along the species evolution; thus, scarring process has emerged as an urgent alternative to favor the structural and functional restoration of a wounded zone. Within these events, the process of granulation tissue formation is pivotal as it constitutes a sort of living, temporary aggregate of cells and proteins, acting as a welding material until the tissue's continuity is restored. However, the reluctance to trigger and sustain the out-growth of a productive granulation tissue with an appropriate extracellular matrix is typical in diabetic patients, and particularly if ischemia concurs. As mentioned, these wounds are characterized by a proliferative arrest, proinflammed, prooxidant, and prodegradative phenotype [71].

This stubbornness and slowness to heal in diabetes is conditioned by systemic and local factors that in complicity counteract intrinsic reparative mechanisms. In a broad systemic context, inflammation and the anabolic deficit can be conceptually mentioned. Diabetic patients with foot ulceration bear a specific and nonrandom alteration of the immune status with an active upregulation of circulating levels of acute-phase proteins, cytokines, and chemokines that impose a chronic systemic inflammatory profile and amplify local wound inflammatory networks [72]. The systemically elevated levels of pro-inflammatory response markers and the wound's expression of cytokines and chemokines are among the culprits of the abnormal repair mechanism [73]. Another factor to be considered is that diabetes *per se* is a metabolic disease in which fuels metabolism is perturbed given the rupture of one of the most important anabolic axis of the organism: insulin/insulin-like growth factor type-I. The role of insulin in wound healing is well known by its anabolic effect on wound protein balance favoring synthesis and preventing degradation [74, 75]. IGF-1 has a similar effect on stimulating wound tissue anabolism. Both insulin and IGF-1 appear to act in part by the induction of ATF4 (CREB2), essential for the activation of mammalian target of rapamycin complex 1 (m-TORC1), which in turn is required for protein synthesis via FOXO-dependent genes repression [76]. We do not rule out that the diabetes-concomitant deficit of incretins could participate in the negative anabolic balance observed in such wounds. Glucagon-like peptide-1 (GLP-1) in addition to its antihyperglycemic actions is endowed with a vast number of multi-organ cytoprotective, trophic and antiinflammatory effects [77]. In support to the GLP-1 action is the study by Ta and coworkers with alogliptin, a specific inhibitor of dipeptidyl peptidase-4 (DPP-4) which showed to inhibit macrophage-mediated inflammation response and to speculatively promote tissue remodeling by inhibiting the expression of different matrix metalloproteases [78].

Rapid formation and deposition of an appropriate extracellular matrix, in particular by fibroblasts, is required for an efficient cellular anchoring and homing at the wound bed. As mentioned above, the cutaneous fibroblast is a sensitive cell to high glucose, AGE-precursors, AGEs, ROS, and TNF-α, rapidly undergoing premature senesce, arrest or apoptosis. Fibroblasts are the main source of collagen, and the number of fibroblasts can be taken for a measure of repair by their collagen synthesis ability. It is very likely that the deficit of growth factors such as TGF-β1, IGF-I and PDGF could translate into a DFU with scarce extracellular matrix acumulation and impoverished cellularity. Numerous growth factors (TGF-β1, IGF-I, PDGF) are able to regulate the balanced expression of matrix metalloproteases and tissue inhibitors of metalloproteases (MMPs/TIMPs), while most of them exhibit an altered expression in DFU [79]. Moreover, the imbalance in the DFU milieu between TGF-β1 and TGF-β3, in which the former appears downregulated, may explain fibroblasts quiescence in terms of proliferation and secretion [80]. This phenomenon represents the deficit of one of the most potent profibrogenic and fibroblasts-mitogenic growth factors, which at the same time is able to downregulate macrophage activation [81]. The extracellular matrix represents the granulation tissue dynamic stroma that provides support for inflammatory cells, fibroblasts, and endothelial cells and allows for the chemotaxis of epithelial

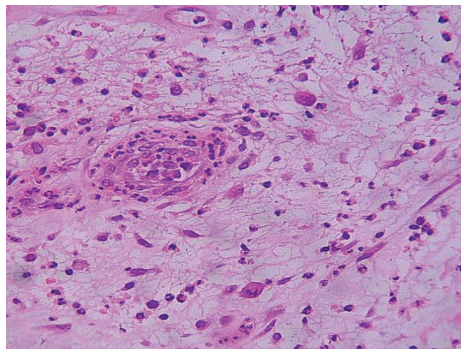

Figure 3: Common histological aspect of a neuropathic granulation tissue. Neuropathic granulation tissue exhibiting a scarce deposition of extracellular matrix. Note a central blood vessel with abundant surrounding heterogeneous cellularity and abundant fibrin accumulation, suggesting hyperpermeability. Hematoxylin/eosin staining, ×10 magnification.

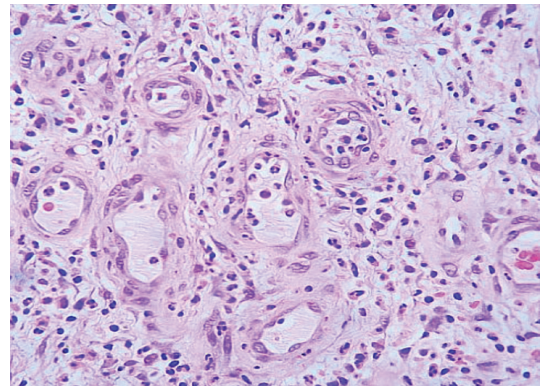

Figure 4: Common histological aspects of ischemic granulation tissue. Note the presence of an intense inflammatory infiltrate of round cells spread all over the tissue area. The emerging vessels appear unfunctional with thickened walls of fibrohyaline material, "hardened aspect", and endothelial nuclei hypertrophy. Hematoxylin/eosin staining, ×10 magnification.

cells, thus hosting the reepithelialization process [82]. One of the main challenges for the diabetic wound healing is the structuring of a normal matrix in quantity and quality. In general, a poor extracellular matrix formation distinguishes DFUs, which can result from (a) diminished synthesis, (b) increased rate of degradation by proteolytic enzymes, (c) toxicity due to glycated by-products accumulation, and (d) toxicity by biofilm bacterial contaminants diffusion [83]. We deem that an important cause of the clinical dilemma of the high rate of reulcerations and ipsilateral amputations in DFU patients' shortly after reepithelialization [84] may be inherent to the qualitative composition of the scar matrix to tolerate tensile forces and mechanical stress.

The diabetic granulation process does not generally exhibit the orderly cascade of events that characterize normal wound healing. This has been confirmed through the histopathological analysis of granulation tissue biopsies by Loots and coworkers who described the lesions as "frozen" in a chronic low-grade inflammatory state associated to a scarce provisional extracellular matrix [85]. Our group's serial biopsies from both neuropathic and ischemic ulcers-derived granulation tissue have identified histological differences for both types of wounds in the absence of clinical infection. Polymorphonuclear cells (PMN) infiltration is intense and prolonged particularly in neuropathic wounds, co-existing with a scarce extracellular matrix accumulation in which collagen deposit is impoverished (Figure 3).

Under more mature stages, the neuropathics may also show an abnormal sprout of new small vessels and capillaries that may derive not from a normal angiogenic response but due to arteriovenous shunts. Our observations remind us with those of Black and coworkers who demonstrated that in neuropathic patients there exists a decrease in fibroblast proliferation and a scarce amount of collagen accumulation within the wound bed [86]. On the contrary, a broadly spread infiltration of round cells predominate in those patients suffering from wound bed ischemia, associated to a fibrohyaline matrix of "hardened" aspect and abnormal angiogenesis in which vascular wall cellular mosaicism, precocious media thickening, endothelial nuclei hypertrophy and many other defects can be identified (Figure 4). It is likely that the combination of arterial hypoperfusion and glucose toxic derivatives imprints a particular pattern of damage to the morphogenesis of vessels in the wound [87]. These observations incite to speculate that the biochemical microenvironment in ischemic and neuropathic diabetic wounds is different and that the inflammatory "badge" is in correspondence with the wound's most prevalent pathogenic component [88]. In contrast to acute wounds in nondiabetic subjects, the inflammatory reaction in diabetics appears prolonged [89] which sharply delays granulation tissue formation and maturation [90]. Data derived from murine diabetic models indicate that the exaggerated inflammatory reaction is related to the prolonged expression of macrophage inflammatory protein-2 (MIP-2) and macrophage chemoattractant protein-1 (MCP-1) [91]. Furthermore, the downregulation of the anti-inflammatory cytokine IL-10 in DFUs environment represents the collapse of an important inflammatory restrainer [73]. Another evidence indicates that PMNs are critical cells toward the acquisition and perpetuation of inflammation and a degradative phenotype. The granulocytes secrete TNF-α and IL-1β, which act as a triggering signal for MMPs expression via the common NF-κB signaling pathway. Within the wound context, TNF-α stimulates its own secretion and that of IL-1β, which contributes to a persistent inflammatory status [92]. TNF-α has proved to negatively impact the repair process as it is early secreted since the inflammatory phase. Its deregulation is not only associated with persistent inflammation but also to connective tissue degradation [93]. Concomitantly, TNF-α mediates its antagonistic effects on TGF-β1 through the JNK pathway via inhibition of Smad phosphorylation, consequently reducing the expression of TGF-β1, and that of several downstream matrix proteins [94]. In this highly proteolytic milieu, fibronectin, collagens, growth factors, and their receptors are degraded while the wound is way down to a catabolic state [95].

Importantly, the perpetuated homing of PMN within the wound bed is associated to high local levels of elastase secretion, ROS, and reactive nitrogen species [96]. High circulating and PMNs-associated elastase levels are attributable to a poor glycemia control and are currently considered as a risk marker for the development of diabetic angiopathy [97]. Fibronectin degradation, for instance, is referred as one amongst the several causes of diabetic reepithelialization failure. Epidermal keratinocytes require of the interaction between fibronectin and its surface receptor integrin $\alpha 5\beta 1$ to effectively migrate [98]. Curiously, insulin-degrading activity has also been demonstrated in the fluid of diabetic experimental and human wounds, which have been shown to correlate with the glycated hemoglobin levels [99]. The connection between NO metabolism and foot ulcer proteases profile has been described. In contrast to elevated MMP-8 and 9 displayed by the nonhealing diabetic foot wound, the concentration of NO appears significantly reduced. Diabetic skin fibroblasts treated with NO donor compounds, selectively raised NO production, increased cell proliferation, and decreased the expression of MMP-8 and -9 in a dose-dependent manner. Thus, that NO resumes the cell proliferation program and promotes the reestablishment of an antiproteases effect has emerged as argument in favor of the NO salutary effect in wound healing [30].

The link between wound cells and apoptosis was described above; we just wish to comment that in sharp contrast to wound-infiltrated inflammatory cells, which become refractory to apoptosis, granulation tissue-producing cells are sensitive to commit suicide where TNF-α stands as a major driving force. The negative impact of TNF-α level on the sensitivity of tissues to insulin has been consistently documented. Skin cells are not excluded from this effect [100]. Conclusively, any therapeutic approach aimed to neutralize TNF-α or to increase the wound local availability of active TGF-$\beta 1$ would be similarly effective for stimulating granulation tissue and wound closure [101].

Chronic wounds and especially diabetic foot ulcers exhibit a highly pro-oxidant microenvironment that amplifies the cytotoxic cascade. Endothelial cells and fibroblasts, in particular senescent fibroblasts, are a prominent source for oxygen radicals, but at the same time they turn into these radicals targets which by converging mechanisms arrest cell proliferation and induce apoptosis [102]. Thus, the disturbed oxidant/antioxidant balance as the AGEs accumulation within the chronic wound microenvironment is considered a major factor, which amplifies the unrestrained and persistent inflammatory, toxic, and catabolic state of nonhealing wounds [96].

The failure of wound contraction is a clinical hallmark of diabetic granulation tissue. Fibroblast-to-myofibroblast transdifferentiation represents a key event during wound healing and tissue repair. The contractile force, generated by myofibroblasts as a highly specialized cell, speeds the healing process of dermal wounds in healthy humans, accounting for an 80–90% of scar tissue reduction [103]. In addition, the contraction process reduces the area to be resurfaced by reepithelialization, which represents a sort of ergonomic response. In diabetic subjects, however, contraction is impaired and deep ulcers heal by the combination of granulation and reepithelialization. The classical view on dermal wound healing implies recruitment of local fibroblasts [104] followed by a subsequent process of transdifferentiation in which the fibroblasts gain a definitive phenotype of differentiated myofibroblasts by neo-expressing a-smooth muscle actin (α-SMA). Nevertheless, α-SMA expression is precisely controlled by the joint action of growth factors like TGF-$\beta 1$ and extracellular matrix proteins like the fibronectin (FN) splice variant ED-A, as by the local mechanical microenvironment [104]. It should be noted, however, that indwelling fibroblasts in diabetic wounds are refractory to proliferate and adopt a senescent phenotype, and that TGF-$\beta 1$, fibronectin, and other matrix proteins may appear in deficit. Hence, all these factors may contribute to the poor contractile activity. Furthermore, Goldberg and coworkers have shown that among the deleterious activities of TNF-α within the wound is to suppress α-SMA expression in human dermal fibroblasts [94]. Figure 5 integrates the cascade of deleterious factors that impact on diabetic granulation tissue onset.

If the animals-derived evidence that a high fraction of the wound myofibroblasts potentially derives from bone marrow fibrocytes is valid for humans [105]; we have already learned that diabetes negatively impacts on the general bone marrow physiology [106] and that beyond this, stromal-derived factor-1alpha (SDF-1α), which acts as a recruiting factor and its CXCR4 chemokine receptor are also impaired by diabetes [107]. Finally, it has been documented that the circulating acute inflammatory reactants involved in insulin resistance inhibit fibrocytes differentiation [108].

There are numerous cellular and molecular aspects unknown and that remain to be answered on the granulation tissue biology.

(1) What are the molecular and cellular driving forces supporting the microscopic structural differences between neuropathic and ischemic ulcers beds?

(2) What is the explanation for the "inheritance" of vascular changes as a dramatic Monckeberg media thickening in nascent arteries within an early hatching granulation tissue?

(3) Why granulation tissue is histomorphologically abnormal even in metabolically compensated patients?

Reepithelialization at the clinical level, it is not a lesser important problem as most of the diabetic wounds may granulate in time, while reepithelialization is even far slower, arrhythmic, and torpid. Reepithelialization is accomplished through the combined actions of keratinocytes' dedifferentiation, proliferation, and migration requiring a complex basement membrane, emerged of the mutual interaction between mesenchymal and epithelial cells. Reepithelialization failure is therefore one of the landmarks of diabetic and other chronic wounds. The epidermal edge of a chronic wound is thick and hyperproliferative with mitotically active keratinocytes unable to migrate along the surface, and by the contrary, moving down deep into the neodermis. Therefore,

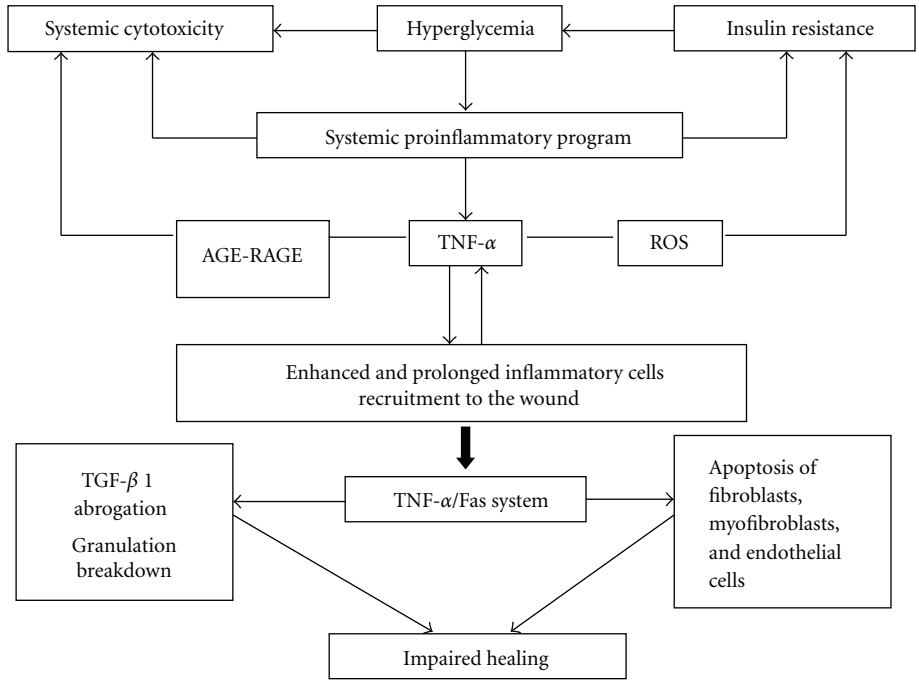

Figure 5: Impact of hyperglycemia on granulation tissue biology. The onset of a systemic pro-inflammatory program due to sustained hyperglycemia is associated with the elevation of circulating levels of TNF-α. The cytokine release is further amplified by the agonistic interaction AGE/RAGE and the generation of ROS. This preliminary triad amplifies insulin receptor resistance and a multiorgan toxicity. Excess TNF-α perpetuates the inflammatory infiltrate into the wound bed hindering the onset of the fibroangiogenic phase in part by abrogating TGF-β1 release. This TNF-α related growth factors deficit within the wound bed may act as a vulnerability factor for granulation tissue productive cells apoptosis.

it has been speculated that the nonhealing edge keratinocytes do not successfully complete either of two possible pathways: activation or differentiation. In consonance with this, one of the major issues in chronic wounds treatments is how to revert the chronic wound keratinocytes' phenotype to a proper differentiating and migratory program [109].

Glucose has shown to exhibit a direct toxic effect on keratinocytes. As for other cells grown in the presence of high glucose concentrations, human epidermal keratinocytes significantly reduce their proliferation rate and replicative life span and were rendered more susceptible to commit apoptosis [110]. Other studies also confirmed that hyperglycemic conditions abort keratinocytes' proliferative ability and their migratory response [111]. Aside from the glucose-mediated direct cytotoxic effect on the keratinocytes, AGE modification of type-I collagen and other ECM proteins impairs the integrin-mediated adhesion of keratinocytes to the basement matrix and could thus contribute to the pathogenesis of diabetic reepithelialization failure [112]. In this context, epithelial-mesenchymal interaction plays a role in establishing the profile and order of released factors regulating keratinocytes proliferation and differentiation [113].

The fact that insulin is biologically relevant for skin cells derives from the observation that insulin is an essential component for cultured human keratinocytes, demonstrating its involvement in the regulation of proliferation, survival, and metabolism [114]. Recent studies in this field document that among other roles, insulin contributes to VEGF release in skin wound cells through an A*kt*1-mediated posttranscriptional mechanism [115]. Glucose is known to affect insulin action by regulating the expression of several genes including insulin receptor at both the transcriptional and translational levels [116]. Lack of insulin receptor expression derives in reduced skin proliferation and abnormal differentiation *in vivo* [117]. Furthermore, TNF-α has also been implicated in epithelial cells arrest by deeply perturbing critical elements of keratinocytes' physiology, including insulin sensitivity [118].

A notorious study has provided evidence aiming at the roles of c-myc and β-catenin in impairing epithelial edges migration. Nuclear beta-catenin stabilization inhibits keratinocytes migration by blocking epidermal growth factor response via c-myc induction, and repressing keratins 6 and 16 expression, depleting at the end the pool of epidermal stem cells at the nonhealing edge [119]. It is therefore evident that keratinocytes migration incapability plays an important role in reepithelialization failure since cytoskeletal keratins K2, K6, and K10 have been observed diminished in DFUs [120]. Moreover, the observation that EGF response appeared blocked may have further deleterious impact. Many peptide growth factors, including members of the EGF family, accelerate wound reepithelialization *in vitro* and *in vivo* [121]. Among them, the activation of the EGF family of ligands and the receptor is of physiological significance. Furthermore, EGF receptor (EGFR) expression is transiently increased at wound margins, suggesting an active role for this receptor in wound repair. EGF stimulates both cell proliferation and

motility [122], with the later being dependent on EGFR autophosphorylation and the subsequent activation of phospholipase Cγ-1 (PLC-γ1). On the other hand, EGFR activation also leads to membrane ruffling and focal adhesions through activation of members of the Rho subfamily of GTP-binding proteins [123]. Recent experiments document the negative effect of the Slug null mutation as a downstream EGFR catalytic mediator for wound reepithelialization. Thus, any interference with the EGFR cascade will hamper epithelial resurfacing [124]. Classic experiments provide illustrative examples on the relevance of the epithelial-mesenchymal cross-talk and on the irreplaceable role of growth factor as a networking bridge [125] for reepithelialization. Skin-reconstitution studies have shown that bone marrow stromal cells (BMSCs), in addition to dermis-localized preadipocytes and fibroblasts distinctively promote epidermal regeneration [126]. As diabetes courses with a deficient secretion of growth factors and other chemotactic mediators in areas of tissue repair, recruitment of circulating stromal cells appears reduced; which may turn into an additional hit to that of high glucose-associated toxicity [127]. At the end, there are so many factors which may interact to obstruct chronic wounds reepithelialization that it may turn into a puzzle.

Above all, questions from the clinical practice remain and pose as a challenge for basic researchers: (1) why after wound contours surgical debridement keratinocytes migration resumes, for soon after become stunted and arrested again? (2) Why the biopsies invariably show a hypertrophic lip of epithelial cells in vertical, downward growth in spite of a horizontal polarization?

4. Concluding Remarks

Although diabetes *per se* is a complex disease, our contemporary understanding on the molecular mechanisms impairing wound healing in diabetes has indefectibly expanded over the last 20 years. The last few years have witnessed the birth of the notion that type 2 diabetes is not a single, unique process, whereas the concept of group of diseases has flourishing. Type 2 diabetes seems to be ethiopathogenically multifactorial and behaves as individual as the affected subject is. So is the pattern of the clinical complications, including the wound itself.

To date, all the evidence aims to high glucose burden as the proximal trigger to unleash acute and chronic self-perpetuating loops, which include but are not limited to ROS-lipid peroxidation, hyperinflammation/disimmunity, AGE-RAGE toxicity, mitochondrial dysfunction, and nytrosilation end-products accumulation. The concerted action of these factors enforces fibroblasts, pericytes, and endothelial cells to a precocious senescence, arrest, and apoptosis. Indeed, the failure of the agonistic stimulation of the insulin axis deeply impacts on the biology of diabetics' cells. This is a seminal axis that connects the anabolic role of insulin via aminoacids transporters translocation and protein synthesis with cell survival and proliferation mechanisms, thus preventing apoptosis, autophagy (cells-self catabolism), and arrest. Fatal for the cells is silencing the agonistic stimulation of tyrosine kinase-growth factors receptors which would ensure cell cycle progression. At both experimental and clinical levels, the diabetic wound phenotype is the expression of countless molecular factors that operate through a complex biochemistry and lead to an aberrant cellular behavior. The pathway to chronification has not been fully elucidated but by all means it represents a form of cells' biological disobedience and entails the need of continuous surgical "cuttings" in order to transiently restore an acute behavior by "refreshing" the cellular environment. It is likely that the scarcity of insulin and growth factors-induced tyrosine kinase receptors downstream signaling may lie, at least in part, behind chronification. Diabetes takes away the resources that ensure wound cells perpetuation and turn-over.

Although type 2 diabetes worldwide expansion is undeniable, primary care ulcer prevention plans together with the emergence of first-line pharmaceuticals and smart devices like engineered skin equivalents will certainly prevent and reduce contemporary amputation figures.

Abbreviations

GFs/TK-R's:	Growth factors/tyrosine kinase receptors
MMPs:	Matrix metalloproteases
ECM:	Extracellular matrix
ROS:	reactive oxygen species
OXPHOS:	oxidative phosphorylation
TNF-α:	Tumor necrosis factor
FOXO:	Forkhead box O family of transcription factors
E.R. Stress:	Endoplasmic reticulum stress
AGE:	Advanced glycation end-products
RAGE:	receptor for the AGE.

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Research Article

Hyperglycemia Increases Susceptibility to Ischemic Necrosis

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Diabetic patients are at risk for spontaneous foot ulcers, chronic wounds, infections, and tissue necrosis. Current theories suggest that the development and progression of diabetic foot ulcers are mainly caused by arteriosclerosis and peripheral neuropathy. Tissue necrosis plays a primordial role in the progression of diabetic foot ulcers but the underlying mechanisms are poorly understood. The aim of the present study was to investigate the effects of hyperglycemia *per se* on the susceptibility of ischemic tissue to necrosis, using a critical ischemic hind limb animal model. We inflicted the same degree of ischemia in both euglycemic and streptozotocin-induced hyperglycemic rats by resecting the external iliac, the femoral, and the saphenous arteries. Postoperative laser Doppler flowmetry of the ischemic feet showed the same degree of reduction in skin perfusion in both hyperglycemic and euglycemic animals. Nevertheless, we found a significantly higher rate of limb necrosis in hyperglycemic rats compared to euglycemic rats (71% versus 29%, resp.). In this study, we revealed that hyperglycemia *per se* increases the susceptibility to limb necrosis in ischemic conditions. Our results may help to better understand the physiopathology of progressive diabetic wounds and underline the importance of strict glycemic control in patients with critical limb ischemia.

1. Introduction

Diabetes mellitus (DM) favors the development of spontaneous foot and leg ulcers, chronic wounds, infections, and gangrene [1, 2]. Approximately 85% of amputations in diabetic patients are preceded by a foot ulcer, which subsequently deteriorates into a severe infection or gangrene [3]. These amputations significantly contribute to the high morbidity and mortality rates as well as the high health care costs in patients with diabetic foot ulcers [4, 5]. The most important factors related to the development of diabetic foot ulcers are peripheral neuropathy and arteriosclerosis. Furthermore, diabetic peripheral neuropathy not only leads to the loss of protective sensation, increasing the risk of unnoticed minor traumas, but also results in the degeneration of the sympathetic nerves innervating thermoregulatory arteriovenous shunt vessels of the skin [6]. These denervated shunt vessels lose their normal capacity of contraction and the nutritional skin capillaries are bypassed leading to the development of chronic capillary ischemia [7]. At the same time, DM is associated with an acceleration of arteriosclerotic changes of the greater arteries resulting in decreased blood flow, further contributing to a limb threatening ischemic condition [8].

However, it remains elusive whether hyperglycemia itself has a direct influence on the development of foot ulcers and its complications, especially in ischemic conditions. The aim of the present study was to evaluate the role of hyperglycemia *per se* in the physiopathology of spontaneous ischemic ulcers and on the susceptibility for ischemic necrosis, using a critical ischemic hind limb animal model with streptozotocin-induced hyperglycemic rats.

2. Methods

2.1. Animals. Male Wistar rats ($n = 14$) aged 90 days and weighing 300–350 g (Charles River Laboratories, L'Arbresle Cedex, France) were fed a standard diet and given water ad libitum. The local ethics committee and veterinary authority approved all procedures according to Swiss guidelines.

Rats were randomly assigned to two groups of equal size ($n = 7$): control group and hyperglycemic group. Hyperglycemia was induced by single i.p. injection of streptozotocin

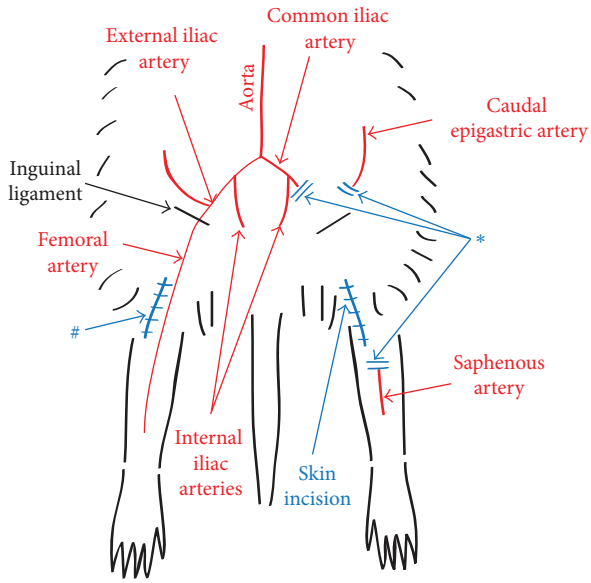

Figure 1: Ischemic foot model. *Ischemic limb: Resection of the external iliac, the femoral, and the saphenous arteries to the level of the knee. #Nonischemic limb: dissection without resection of the above cited arteries.

(STZ) (Sigma-Aldrich, St.Louis, MO, USA) three weeks before surgery. STZ was dissolved in sterile 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally (50 mg/kg body weight) within 10 minutes of the preparation. Blood glucose levels were measured from tail venous blood by blood glucose test strips and just before sacrificing from carotid blood using glucose oxidase method (Glu; Roche Diagnostics, Rotkreuz, Switzerland). Rats with glucose levels >11.1 mM were included in the hyperglycemic group.

2.2. Ischemic Foot Model. As previously described [9], through a longitudinal incision in the inguinal region that was shaved, the external iliac and femoral arteries were dissected from the common iliac to the saphenous arteries. To provoke an ischemic condition, the dissected arteries were resected from the common iliac in the left limb (ischemic limb, Figure 1) while in the right limb arteries were conserved and limbs considered being nonischemic (sham surgery). All surgical procedures were performed under an operating microscope (Carl Zeiss, Jena, Germany), and animals were anesthetized by inhalation of isoflurane 5% for induction and 3% for maintenance of anesthesia.

2.3. Experimental Groups. We compared streptozotocin-induced hyperglycemic rats with euglycemic rats ($n = 7$ per group). By infliction of unilateral ischemia in both animal groups, we created four conditions: (1) hyperglycemic rat with nonischemic limb, (2) hyperglycemic rat with ischemic limb, (3) euglycemic rat with nonischemic limb, and (4) euglycemic rat with ischemic limb.

2.4. Macroscopic Limb Evaluation. Limbs were assessed macroscopically every two days during the first week and thereafter twice a week to evaluate necrosis status. For ethical reasons the animals were sacrificed as soon as limb necrosis was observed.

Table 1: Skin blood perfusion (PU) before (baseline) and after resection of the arteries or sham surgery (days 1 and 7), in euglycemic and hyperglycemic animals.

	Euglycemic animals		Hyperglycemic animals	
	Sham surgery	Resection	Sham surgery	Resection
Baseline	6.1 ± 0.4	5.8 ± 0.4	5.8 ± 0.3	6.5 ± 0.6
Day 1	6.0 ± 0.8	3.3 ± 0.1*	5.0 ± 0.5	3.4 ± 0.2*
Day 7	6.3 ± 0.6	3.6 ± 0.2*	6.8 ± 0.6	3.5 ± 0.3

($n = 7$/group, except at day 7: $n = 5$ euglycemic and 2 hyperglycemic animals). *$P < 0.05$ versus sham surgery.

2.5. Percutaneous Laser Doppler Measurements. Skin blood flow was measured using a percutaneous laser Doppler perfusion monitor (PIM II Laser Doppler Perfusion Imager, LDPIwin 2.0.6 software, Lisca AB Berzelius Science Park, Linköping, Sweden). Measurements were carried out on the dorsal skin of the hind feet, immediately before and after surgery and during the observation period until sacrifice.

2.6. Statistical Methods. Data was analyzed with the use of Stata software, version 11.0. Statistical analysis consisted in a comparison of data from hyperglycemic versus euglycemic animals, in both ischemic and nonischemic conditions. Comparison of values of perfusion was performed using the nonparametric Kruskall-Wallis test followed by the measures to correct the α-error according to Bonferroni probabilities. Comparison of the proportions of limb necrosis was performed using Fisher's test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Blood Flow Measurement. Blood perfusion levels, measured by laser Doppler flowmetry, were similar in euglycemic and hyperglycemic animals in both ischemic and nonischemic conditions. In ischemic limbs the blood flow significantly decreased immediately after resection of the arteries, and in animals which did not present necrosis the blood flow remained low during the first week (Table 1).

3.2. Limb Necrosis. In nonischemic conditions, no foot necrosis was observed neither in euglycemic nor in hyperglycemic animals. In ischemic conditions, a significantly higher occurrence of limb necrosis was observed in hyperglycemic animals compared to euglycemic animals.

In hyperglycemic animals, five out of seven ischemic limbs showed total necrosis (71%), affecting the feet and lower limbs up to the level of the knee. This rate of limb necrosis was significantly higher than in ischemic limbs of euglycemic rats (2 out of 7, 29%, $P < 0.025$, Table 2).

In limbs that would eventually become necrotic, we observed signs of critical ischemia starting from day three

Table 2: Number of foot necrosis after resection of the main limb arteries in euglycemic compared to hyperglycemic animals.

	Euglycemic animals		Hyperglycemic animals	
	Sham surgery	Resection	Sham surgery	Resection
n° of necrotic limbs (% of total n° of limbs)	0	2 (29)	0	**5 (71)***

($n = 7$/group). $^{*}P < 0.05$ versus euglycemic animals.

(cyanotic aspect, stiffness, and coldness) that rapidly progressed in necrosis from proximal to distal, motivating the immediate sacrifice of the animal.

4. Discussion

Every 30 seconds, somewhere in the world, a lower limb is amputated due to DM [10]. The progression of critical ischemic limbs to necrosis and gangrene is reportedly 40% in diabetic patients compared to 9% in nondiabetic patients [11]. Since current therapeutic approaches of diabetic wounds often are unsatisfactory, it is of crucial importance to better understand the pathophysiology of the progressive necrosis at the wound margins that leads to the characteristic extension of diabetic wounds.

In one of our precedent studies, we developed a new model of ischemic foot ulcers. We aimed at creating a severe ischemic condition without provoking limb necrosis to study wound healing in ischemic conditions [9]. We assessed different degrees of ischemic conditions by resecting arteries on different levels. We concluded that in euglycemic rats, resection of the external iliac artery, the femoral artery, and the saphenous artery down to the level of the knee resulted in a severe ischemic condition with a delayed wound healing process, but with low occurrence of limb necrosis.

In the present study, we aimed at assessing the effects of hyperglycemia on tissue susceptibility for ischemic necrosis. We used the same model as described above in euglycemic and streptozotocin-induced hyperglycemic rats. The same degree of ischemia was inflicted to euglycemic and hyperglycemic rats by resecting the external iliac, the femoral and the saphenous artery. To avoid the effects of long-term complications of DM, in particular of vasculopathy and neuropathy, we induced ischemia only three weeks after injection of streptozotocin. By laser Doppler flowmetry we confirmed that the skin perfusion at the dorsal surface of the feet was indeed comparable in hyperglycemic and euglycemic animals. Nevertheless, we found a significantly higher rate of limb necrosis in hyperglycemic rats compared to euglycemic rats (71% versus 29%, resp.).

Our results demonstrate a direct relation between hyperglycemia and acute ischemic necrosis. We hypothesize that the decrease of tissue tolerance to ischemia induced by hyperglycemia could be responsible for the initial skin lesions, which would lead to the development of diabetic ulcers. This could explain the specific evolution of diabetic wounds toward peripheral progressive necrosis.

The phenomenon of decreased tolerance to ischemia caused by hyperglycemia has already been investigated in other ischemic tissues, notably in heart and brain [12–15]. Hyperglycemia has been associated with impaired outcome and expanded infarct volume in patients with ischemic strokes and myocardial infarctions [15, 16]. A recent meta-analysis of the association between hyperglycemia and stroke size in animal models revealed that hyperglycemic animals presented 94% larger infarct areas (140% in streptozotocin induced hyperglycemia and 48% after dextrose infusion) compared to euglycemic animals [17]. Several mechanisms have been identified through which hyperglycemia could aggravate cerebral and cardiac injury after ischemia, whereas a mechanistic link between hyperglycemia and susceptibility to necrosis is yet to be established. We propose the following hypotheses to explain how hyperglycemia *per se* could sensitize to ischemic tissue necrosis: (1) increased blood viscosity and hypercoagulability worsening progressively the level of ischemia, and (2) direct cellular toxicity due to the development of increased oxidative stress and lactic acidosis.

Hyperglycemia increases plasma fibrinogen levels and thereby blood viscosity and coagulability [18–20]. In nondiabetic patients with acute ischemic stroke, high glucose levels are related to a hypercoagulant state and a worsened outcome [21]. Furthermore, it has been shown that increased blood viscosity is associated with decreased tcPO2 levels in diabetic feet [22]. The increased blood viscosity in hyperglycemic conditions could therefore further aggravate the oxygen deprivation caused by the initial ischemia. In fact, it has been shown that dalteparin, a low molecule weight heparin, improves local tissue oxygenation in patients with diabetes, severe vascular disease, and foot ulcers [22].

Oxidative stress induced by hyperglycemia has been suggested as another possible mechanism of tissue injury exacerbation after strokes and myocardial infarctions [23, 24]. Indeed, both ischemia and hyperglycemia leads to excessive production of reactive oxygen species (ROS), and it is possible that the two factors accelerate each other. On the one hand, ischemia leads to oxidative stress through the generation of ROS through NADPH oxidase and other oxidases [25]. On the other hand, glucose has been shown to be the requisite electron donor for superoxide production by NADPH oxidase [23]. Additionally, hyperglycemia leads to the generation of free oxygen radicals also through the accumulation of advanced glycosylation end products (AGEs) [26, 27]. Further research is needed in order to evaluate whether the oxidative stress provoked by ischemia and exacerbated by hyperglycemia is a major player in the pathophysiology of ischemic cell necrosis.

Lactic acidosis could be another important contributor to ischemic cell death in hyperglycemic conditions [28]. When blood flow and oxygen supply decrease to critical levels, cells generate energy predominantly through anaerobic glycolysis. As a result, blood concentration of lactic acid rises and the

pH level drops [29, 30]. Under hyperglycemic conditions, there is more substrate available for anaerobic glycolysis and consequently lactic acid production rises [31]. In fact, in an ischemic stroke animal model, it was shown that bolus infusion of glucose resulted in a severe intracellular acidosis and increased tissue death [32]. We suggest that when combined with elevated glucose levels, the ischemia-induced shift to anaerobic glycolysis leads to elevated tissue acidosis, which may accentuate cellular death related to ischemia.

In hyperglycemic conditions, the combination of oxidative stress, acidosis, and hypercoagulability creates a nocuous cellular environment that favors progressive ischemic necrosis. As the mechanisms through which both ischemia and hyperglycemia induce cellular toxicity are similar, it is possible that they amplify each other.

5. Conclusions

The unfavorable evolution of many diabetic feet is not only a result of neurogenic and arteriosclerotic long-term effects of diabetes, but could also be, as we observed, a direct effect of hyperglycemia itself. In this study, we demonstrated for the first time that at similar levels of ischemia, limb necrosis appears significantly more often in hyperglycemic conditions compared to euglycemic conditions. This finding suggests that hyperglycemia sensitizes to the effects of ischemia, revealing a direct involvement of hyperglycemia in the events leading to ischemic necrosis. Our results may help to better understand the physiopathology of progressive diabetic wounds and underline the importance of strict glycemic control in patients with critical limb ischemia.

Authors' Contribution

D. Lévigne and M. Tobalem have equally contributed to this work.

Conflict of Interests

The authors have no financial interests to declare in relation to the content of this paper. The Swiss National Science Foundation provided the only financial support.

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