Diabetic lower extremity wounds: the rationale for growth factors-based infiltration treatment

Jorge Berlanga-Acosta

ABSTRACT
Repair machinery and local infection control failure contribute to wound chronicity and lower extremity amputation in diabetic patients. In these wounds, inflammation is a proximal condition which disrupts wound matrix turnover and the local redox balance. Contemporary therapeutic interventions are relatively broad including drugs, devices and surgical procedures. However, clinical efficacy remains modest and recurrences are frequent. Recombinant growth factors advent was followed by their premature and empiric introduction in the clinical practice. Its topical administration is still challenged by local kinetic and pharmacodynamic limitations related to the hostile microenvironment of chronic wounds. The rationale of infiltrating epidermal growth factor (EGF) down inside complex diabetic wounds as an alternative treatment modality is described here. The concept emerged from two experimental evidences: (a) locally infiltrated EGF prevented trophic ulcers and limb necrosis upon denervation, (b) acute, controlled experimental wounds’ exudate exhibited proteolytic activity. Depositing EGF in deep cells’ responsive strata allows for two main pharmacological actions indispensable for chronic wounds healing: cytoprotection and proliferation of fibroblasts and endothelial cells, thus inducing progressive granulation. Ten years of clinical experience have validated laboratory and theoretical concepts, while most importantly have improved quality-of-life to thousands of diabetic patients.

Key words: Diabetes • EGF • Healing • Infiltration • Wounds

INTRODUCTION
Diabetes mellitus (DM) remains as the only endocrine-metabolic disorder that is expanding at a rate that approaches a world-wide pandemic disease. Lower extremity ulceration is one of the many serious, long-term complications associated with DM (1).

Diabetic ulcers are sustained and/or amplified by the underlying failure in the repair process of peripheral soft tissues in this population. One of the distinguishing hallmarks within this process is the incapability to trigger and sustain the growth of a productive granulation tissue with an appropriate extracellular matrix for contraction and re-epithelialisation. Different biochemical disorders discussed here act as the operational forces that lead to the onset of a pro-inflamed, pro-oxidant and pro-degradative phenotype (2–4). Thus, this type of wounds has historically posed a therapeutic challenge and a major cause of amputation in patients with diabetes (1).

Anti-microbial agents, surgical techniques and a broad variety of therapeutic approaches
here, we describe the line of thoughts and fundamentals that encouraged us to fuel the hypothesis that injecting EGF into the lesion’s base and contours could result in healing of poor-prognosis diabetic foot wounds.

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Topical application of recombinant human growth factors (GFs) dates back to almost 30 years ago. Their arrival sparked hopes as tissue healing magic bullets. To our understanding two main factors quenched the initial excitement: (a) the almost simultaneous inputs from basic science that GFs were involved in malignant growth (15) and (b) the setback stemmed from a rigorous clinical trial in which epidermal growth factor (EGF) was topically administered to acute, controlled and experimentally induced wounds in healthy volunteers (16). This clinical failure warned about the need for additional research in GFs pharmacology as in wounds environment biochemistry. The need to precondition the bed of chronic wounds and to ensure GFs local bioavailability for subsequent receptor stimulation and downstream signalling activation emerged as novel paradigmatic concepts (17–19). After all, with peaks and troughs EGF and platelet-derived growth factor (PDGF) stand as major players within the clinical armamentarium for hard-to-heal lower limb wounds (5,20–24).

Here, we describe the line of thoughts and fundamentals that encouraged us to fuel the hypothesis that injecting EGF into the lesion’s base and contours could result in healing of poor-prognosis diabetic foot wounds. Today, after 10 years of experience the basic science has turned validated by the clinical routine.

**BRIEF RECAPITULATION OF DIABETIC WOUNDS BIOLOGY**

Wound healing is an ancestral mechanism evolutionarily designed to conduct the structural and functional restoration of an injured area. The mechanism involves cellular responses from two major classes: (a) Repair-committed cells as fibroblasts, other mesenchymal-derived cells, endothelial/angiogenic precursor cells and epithelial keratinocytes. (b) Inflammatory cells which are transiently recruited and temporarily infiltrate the wound (25–27). Under physiological conditions, inflammation is down-regulated in a temporary manner because of inflammatory cells apoptosis. The cells and the pro-inflammatory cytokines are the same for both diabetic and non diabetic wounds; however, in diabetics, inflammation is more a condition than a reaction. Thus, it has sustained the concept that chronic diabetic wounds may be considered a pro-inflammatory organ superimposed into a metabolically deregulated host. Cytokines (TNF-α, IL-6 and IL-1β) produced by wound-homed neutrophils and macrophages enrich their pool in the central circulation and disrupt insulin action via nuclear factor-kappaB (NFκB) and c-Jun N-terminal kinases systems pathways (26–28). Insulin axis disruption is associated to reduced cells’ anabolism among other factors, because forkhead box remains unphosphorylated thus allowing for the blockade of anabolic gene activation (29).

The perpetuation of neutrophils, macrophages and their related pro-inflammatory cytokines in diabetic wounds contribute to the onset of a pro-degradative microenvironment, which results from the imbalance between matrix synthesis and degradation. The synthesis of matrix metalloproteinases (MMPs) prevails as a consequence of local TNF-α secretion (30). High levels of TNF-α have been identified as a molecular predictive factor for wound closure failure (31,32). In general, there is a sharp antagonism between the pro-synthetic role played by transforming growth factor-beta 1 and the catabolic effect mediated by TNF-α. Within the wound environment, molecular targets of MMPs are myriad, and include not only structural components of the extracellular matrix but also include ‘off-targets’ molecules such as locally secreted GFs and their receptors. The aberrant profile of diabetic wounds in most of the healing cascade is described in Table 1.

The pool of locally secreted cytokines creates a self-perpetuating loop. These cytokines may endow inflammatory cells with apoptosis refractoriness, while those essential cells for granulation tissue formation are prone to commit apoptotic suicide (2,33,34). It is also likely that insulin resistance on the wound’s cells could act as a pro-apoptogenic factor for fibroblasts and endothelial cells (35).
Table 1 General characteristics of diabetic chronic wounds

<table>
<thead>
<tr>
<th>Wound phase</th>
<th>Characteristics in diabetic wounds</th>
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<tbody>
<tr>
<td>Haemostasis</td>
<td>Ordinarily shifted towards a pro-coagulant state. Fibrinolysis is impaired leading to microcapillary plugging.</td>
</tr>
<tr>
<td>Inflammation</td>
<td>These wounds become arrested in the inflammatory phase. Intense infiltration of neutrophils or mononuclear cells turns chronic. Inflammatory cells evade apoptosis, disrupting wound’s sequential trajectory. The local secretion of cytokines hinders the transcription of growth factors genes for extracellular matrix synthesis and angiogenesis.</td>
</tr>
<tr>
<td>Granulation tissue formation and angiogenesis</td>
<td>Chemotaxis of fibroblasts and fibrocytes to the wound is reduced. Replication of fibroblasts and vascular endothelial cells is delayed or arrested and apoptosis prevails. Secretion of extracellular matrix is reduced leading to lower tensile strength and impaired cellular anchoring. Myofibroblast differentiation is reduced; α-smooth muscle acting production is impaired and wound contraction very limited. Angiogenic response is impaired contributing to tissue perfusion deficit. Hyperglycaemia disturbs angiogenesis and endothelial nitric oxide synthesis. Microcapillary tone regulation is aborted.</td>
</tr>
<tr>
<td>Re-epithelialisation</td>
<td>Epithelial migration and normal keratinocyte differentiation is impaired, delaying wound closure. Re-epithelialisation is torpid, slow and may turn failed for months or years.</td>
</tr>
<tr>
<td>Remodelling</td>
<td>Removal of scar matrix and replacement with more normal matrix is slow, leading to increased risk of re-ulceration.</td>
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An unbalanced pro-oxidant microenvironment which complements and amplifies the pro-inflammatory arm distinguishes diabetic wounds (36). Neutrophils are a rich source of reactive oxygen species (ROS) released into the wound environment. Endothelial cells and fibroblasts are preferential targets of ROS. Fibroblasts become senescent upon ROS intoxication and afterwards they turn a prominent potential source of ROS. Thus, the disturbed oxidant/antioxidant balance within the chronic wound is considered a significant factor for the amplification of the pro-inflammatory and pro-degradative phenotypes (36,37).

So far, hyperglycaemia and its acute or chronic downstream consequences remain as the most proximal factor for the above described disturbances. Accordingly, one of the most pathogenically relevant effects of prolonged and non controlled glycaemia is the non enzymatic generation of heterogeneous group of advanced glycation end-products (AGE). AGE(s) acting via cell surface receptor and transducing through the NFκB, enhances the inflammatory response and the pro-catabolic phenotype within the wound bed (38). This is particularly relevant for skin wound healing as AGE tends to accumulate in non labile dermal cutaneous proteins such as collagen and elastin. All these pathogenic ingredients converge to disrupt GFs homeostasis within the granulation tissue cells because GFs receptors are glycated (3,4). Portero (39) showed the inhibition of EGF and PDGF receptors phosphorylation and signalling because of the chemical modification introduced upon the interaction of these receptors with AGE precursors’. These evidences support the notion that diabetic wounds may not only represent a biochemically hostile environment for GFs local availability but also for their biological action.

**Key Points**
- the disturbed oxidant/antioxidant balance within the chronic wound is considered a significant factor for the amplification of the pro-inflammatory and pro-degradative phenotypes
- EGF, PDGF-BB and FGF-2 amplify their mitogenic activity if aggregated with fibronectin or other RGD-containing peptide ligand

**CHRONIC ULCERS AND TOPICALLY ADMINISTERED GROWTH FACTORS**
About two decades ago, wound’s extracellular matrix was recognised as a key and active player in wound healing, rather than a passive and an inoperative substrate. One of the most significant events is its ability to act as a reservoir for GFs. The observation that diabetic wounds are enriched in proteases provides support for the premise that impaired GFs availability may act as a rate limiting factor in diabetic wound healing (26,40–42). Thus, wound’s proteolytic imbalance can also reduce extracellular matrix proteins which are able to entrap and subsequently release GFs. Extracellular matrix and GFs complexes have showed an underappreciated phenomenon: they can either negate GFs activity or generate synergistic signals for cell function, in particular mitogenesis (43). EGF, PDGF-BB and fibroblast growth factor-2 amplify their mitogenic activity if aggregated with...
fibronectin or other Arginine-Glycine-Aspartic Acid-containing peptide ligand (44).

Diabetic wounds exhibit other distinctive elements among the variety of chronic wounds. The first to mention is the existence of a particular protease with insulin-degrading activity, which has been shown to correlate with the level of glycated haemoglobin, suggesting a direct relationship between glycaemia and the wound proteolytic profile (45). Next is the susceptibility of the diabetic wounds to host an abnormal bacterial burden that reduce GFs availability, amplify the inflammatory response and prevent the storage of GFs within the extracellular matrix (17,18). Finally, they host a peculiar biofilm (46,47) that is able to rapidly regenerate following sharp debridement, may contain glycation-derived toxins, and cover a group of genotypically distinct bacteria that symbiotically produce a polimicrobial community difficult to diagnose and eradicate (46–48). The understanding of the pathogenic significance of the biofilm in chronic wounds rendered explanation on why topically administered GFs may have failed in healing some of these lesions.

Almost 20 years ago, Mast and Schultz (19) established the bases of an adequate wound bed preparation as a requisite to prevent local GFs degradation and improve healing trajectory. Irrespective to the undeniable benefits of wound debridement, decades of efforts towards GFs pharmaceutical development may be considered a failure. Controversies on the efficacy of topically administered GFs have existed (49). Most of the clinical doses and the treatment regimens used so far appear to be empiric. GFs absorption kinetic into the treated wounds does not appear to be investigated. Not surprisingly, the results of many of these studies have been inconclusive, with non uniform dosing to different types of lesions and at various stages of healing, making comparison between studies almost impossible (49).

According to Smiell’s analysis of four randomised studies, combining surgical debridement with the daily topical application of PDGF-BB a modest 15% improvement in the rate of fully healed diabetic ulcers was showed as compared to placebo (13,50). Other GFs which at a moment were clinically investigated in the USA vanished along the way because of lack of efficacy even when topically applied to controlled wounds (16,51). All these pieces of knowledge have encouraged the search of more effective treatment and delivery strategies to ensure GFs activity. So far, these have included (a) GFs administration to the wound bed through more sophisticated delivery tool (b) use of agents that proximally inhibit inflammation and distally wound bed proteolysis by inhibiting TNF-α converting enzyme (4).

**Rationale for GF Infiltration into the Wounds**

We examined the epithelial response to daily topical administration of three different EGF concentrations formulated in a semisolid cream (52). The experimental system consisted on 6-mm diameter partial-thickness wounds in Yorkshire pigs. EGF doses were 50, 10 and 2 μg/g, and representing 7-day total concentration of 124.6, 24.9, and 5 μg/cm². The fact that only the largest dose stimulated wound closure; incited us to examine the potential proteolysis by the wound-derived exudate. Proteolysis was observed following incubating dilutions of the ulcers’ material at neutral pH with a fluorescent-synthetic peptide at room temperature. Subsequently, the preincubation with a Kunitz-type protease inhibitor prevented substrate’s degradation. These observations suggested a possible reduction of EGF bioavailability by proteases derived from non infected, acute, controlled wounds. It was somewhat surprising as other studies had already established proteolysis on GFs and their receptors in chronicity circumstances (19,53). It is worthy to highlight that previous studies documented the need for a prolonged interaction between EGF and its receptor to achieve a significant granulation tissue response in controlled wounds in mice (54). Further evaluations of cumulative profiles of 10, 5 and 1 μg of (125)I-EGF in a rat full-thickness wound model, showed a peak of tissue-bound radioactivity 2 hours after the administration of all doses. Within this period, (125)I-EGF degraded sub-species with no diffusion of the peptide to the surrounding skin were identified. Irrespective to the EGF dose size, the receptor expression was increased within 2 hours after wounding, followed by a slow decline up to 12 hours below baseline. Our results point out that (125)I-EGF is rapidly cleared from the
application site probably by protease-driven cleavage and receptor-mediated endocytosis. The mean residence time values suggested that more than 60% of the amount administered could have disappeared as early as 2-hour post-administration (55). The message of these studies indicated that even those acute, clean and controlled wounds may not represent a ‘comfortable’ substrate for GFs physical and chemical integrity. Previous clinical evidences had already rendered disappointing results possibly because of local bioavailability limitations (56,57).

These pieces of knowledge contributed to shape the idea that injecting EGF deep into the wound base and contours would allow for a larger pharmacodynamic response in terms of granulation tissue growth and wound closure. We had the experience of injecting EGF locally into rats’ hind limbs denervated upon sciatic nerve full-thickness cut. In addition to significantly assist in neurological restoration, the treatment enhanced limbs peripheral soft tissues survival by delaying or preventing the onset of plantar ulcers and toes necrosis (58). These experiments offered an important lesson: Locally injected EGF could stimulate the survival and repair of cutaneous and adjacent soft tissues in a context of circulatory neurogenic deterioration. Trophic ulcers appeared to be prevented. We subsequently showed that single or repeated EGF systemic or local injections exerted ‘clear-cut’ cyto-protective and proliferative responses, supporting the intrinsic ability of EGF at supra-physiological concentrations to unleash biological events required for tissue repair (25,58–62).

The first clinical evidences on EGF infiltrative treatment involved diabetic foot ulcers and amputation residual bases. All the lesions were chronic, complex and recalcitrant to heal; staged as III and IV of the Wagner’s scale. The efficacy showed in this type of wounds (20) paved the way for a solid clinical development (21–23), which culminated with a nation-wide, double-blinded, placebo-controlled phase III clinical trial. The EGF infiltrative intervention jerked outsized, poor-prognosis wounds towards a rapid and sustained healing response (20–23).

Injecting EGF into the tissue, millimetres down and inside the base and contours (including the dermo-epidermal junction), possibly reduced its otherwise degradation following topical application upon confrontation with the wound’s exudate. An ulterior immunohistochemical characterisation of biopsy cylinders (~7 mm in-depth) from diabetic wounds bed, shed more light on the biological sustentation for the infiltrative modality. According to the immunolabel intensity, three layers of ‘cellular responsiveness potential’ along the longitudinal axis of the granulation tissue were identified. Fibroblasts populating the more superficial stratum expressed far more prohibitin and far less EGF receptor. This expression profile became progressively inverted going through deeper cells layers (Jorge Berlanga, unpublished data Figure 1). AGE and elastase also appeared far more intensely labelled next to the wound surface than in deeper cells strata (not shown). It is likely that topographic positioning along the
wound bed axis dictates fibroblasts’ intrinsic ability to respond to a mitogenic signal. Note-worthy, prohibitin is a renowned inhibitor of cell cycle progression (63–67). Contemporary evidences support that EGF injected into the ulcer matrix may result in association complex with extracellular matrix proteins, thus enhancing cells proliferation and migration (68).

**OTHER RELEVANT ASPECTS OF THE LOCAL EGF INFILTRATION**

The clinical intervention with EGF for diabetics’ wound healing may be considered as a replacement therapy. Classic studies have shown that GFs exposure is an effective mean to remove the senescent phenotype and the proliferative reluctance of chronic ulcers-derived fibroblasts (69). This concept includes diabetics’ ulcers fibroblasts (70). This infiltrative treatment modality is incorporated into the comprehensive wound care and to the medical interventions to correct patient’s glycaemia and creatinine; thus it does not replace or displace any of the standard procedures. Rather, an appropriate wound bed preparation based on sharp debridement and infection elimination appears as a requisite to proceed with the infiltration modality. The treatment is not methodologically challenging and is very well-tolerated (20–23). EGF doses, treatment regimen and other data have been detailed elsewhere (20–23) and are not the focus of this work. EGF local injections of complex diabetic wounds for 10 years document a favourable risk–benefit balance by speeding healing, reducing recurrences and attenuating the risks of amputations.

**CONCLUDING REMARKS**

The early hypothesis on the infiltrative procedure as a justifiable alternative to ensure GF’s availability within the extracellular matrix of diabetic complex wounds has been validated by the clinical routine in diabetic foot services.

In the most practical arena, oversized and hard-to-heal wounds have been pushed towards full re-epithelialisation; the treatment is also characterised by reducing in situ recurrences (23). During these 10 years, neuropathic and mild-to-moderate ischaemic patients have successfully evolved. These therapeutic achievements appear to be mediated by the combination of two main factors: (a) the biodisponibility of an intact EGF molecule in a zone of responsive cells via EGF receptor stimulation and (b) the particular pharmacological effects induced by EGF at pharmacological concentrations in terms of cyto-protection and proliferation. The infiltration procedure raises an academic question: are the EGF receptors somewhat ‘polarised’ in granulation tissue cells like in mucosal epithelial cells? If the receptors are actually oriented to a more baso-lateral distribution, this would represent an additional argument to explain the limited biological impact of topically administered EGF. Novel GFs-based pharmaceutical forms are in development to satisfy local cells biokinetics requirements, while reducing invasiveness frequency.
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