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Intralesional Human Recombinant Epidermal Growth Factor for the Treatment of Advanced Diabetic Foot Ulcer: From Proof of Concept to Confirmation of the Efficacy and Safety of the Procedure

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1. Introduction

Foot ulceration is among the most significant complications of diabetes. It is estimated that 15% of the diabetic patients develop ulcers at some point in their lives (Reiber, 1996). The therapeutic management of a diabetic patient carrying a diabetic foot ulcer (DFU) is currently based on: metabolic control, debridement (Brem et al., 2004), moist cures, wound dressing, local pressure off-loading, antimicrobial treatment of infections, and revascularization procedures, when indicated. More recent therapies such as topical growth factors (Tsang et al., 2003; Brem et al., 2004; Eldor et al., 2004; Hong et al., 2006; Viswanathan et al., 2006), skin substitutes (Marston et al., 2003; Veves et al., 2001), and others have shown efficacy in pure neuropathic, non-complicated ulcers. However, these products would still have to be tested in advanced lesions including those with an ischemic etiopathogenic component. Still 10 to 30% of the cases progress to amputation, frequently preceded by gangrene and infection (Lipsky, 2004). After amputation of a lower limb, the five year mortality rate reaches 50-60% (Reiber, 1996). Therefore, despite progress in the diagnosis and treatment of infection and other complications (Lipsky, 2004; Williams et al., 2004), the advanced DFU is still an unmet medical need.

The local (intralesional) instillation of recombinant human epidermal growth factor (rhEGF) to promote granulation and healing of chronic, advanced DFU has been recently introduced in medical practice in some countries. This chapter will review the rationale, experimental background, and clinical development of such procedure.

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2. Why can Epidermal Growth Factor (EGF) be used for the treatment of Diabetic Foot Ulcers (DFU)?

Epidermal growth factor (EGF) is a 53 aminoacid polypeptide that was isolated for the first time by Cohen from mice submaxillary glands (Cohen, 1962). It stimulates the proliferation of fibroblasts, keratinocytes and vascular endothelial cells, which contribute to its scar tissue formation property. Its action is launched by the interaction with specific receptors located on the cellular membrane. The EGF receptor is a glycoprotein with an extracellular binding domain, a transmembrane region and a cytoplasmic portion with tyrosine kinase activity (Bazley & Gullick, 2005). This receptor is expressed on most human cell types including those which play critical roles for wound repair such as fibroblasts, endothelial cells and keratinocytes (undifferentiated, marginal, leading edge, hair follicles, sweat ducts and sebaceous glands). Only hematopoietic cell lineages lack the EGF receptor (Werner & Grose, 2003). The rationale of the use of Epidermal Growth Factor (EGF) for the treatment of (DFU) is based on:

2.1 Impairment of healing in diabetic patients, partially due to a relative deficit of growth factors (EGF among them) in the wound area

Wound healing is an ancestral mechanism evolutionarily designed to ensure the structural and functional restoration of an injured area. The mechanism involves cellular responses from two major classes: (i) repair-committed cells such as fibroblasts, other mesenchymal-derived cells, endothelial / angiogenic precursor cells and epithelial keratinocytes and (ii) inflammatory cells that are transiently recruited and temporarily infiltrate the wound (Eming et al., 2007). Under physiological conditions this process is ensued by inflammatory cells' progressive apoptosis and inflammation cessation. Although in diabetic wounds the cells and the pro-inflammatory cytokines are the same than in non-diabetic, acute counterparts; inflammation is more a condition than a reaction. 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 (Berlanga et al., 2008).

Under these circumstances the local pool of growth factors (GFs) and their corresponding receptors turn detrimental due to a reduced transcriptional expression by the wound bed committed cells and/or to increased enzymatic degradation (Clark, 2008). The role of GFs turns more important in the context of a diabetic wound since high glucose levels and other associated metabolic by-products are toxic for endothelial and fibroblastic cells, which become arrested and senescent. In this environment the granulation tissue promoting cells launch a pro-apoptogenic program which eventually hinders the granulation process (Goren et al., 2006). The observation that diabetic wounds are enriched in proteases supports the premise that impaired GFs availability may act as a rate limiting factor in diabetic wound healing (Burrow et al., 2007), which justifies an appropriate wound bed preparation and a GFs replacement therapy.

2.2 The growth stimulating, healing promoting, and cytoprotective actions of EGF, including angiogenesis

The EGF family of ligands exhibit mitogenic activity upon binding to four different high-affinity receptors: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Upon

ligand binding, the formation of a functionally active EGFR-EGFR homodimer or EGFR-HER2, EGFR-HER3, or EGFR-HER4 heterodimers causes the ATP-dependent phosphorylation of specific tyrosine residues in the EGFR intracellular domain, which triggers a complex program of intracellular signals to the cytoplasm and then to the nucleus (Citri & Yarden, 2006).

Another biological action unleashed by the EGF-EGFR binomium is the locomotion stimulation of epithelial and fibroblastic cells (Barrandon & Green, 1987). This pro-motogenic impulse induced by the EGF-EGFR complex on keratinocytes is important for re-epithelialization. EGF can also control fibroblasts extension, attachment or detachment directly or indirectly via modifications of the injured tissue extracellular matrix composition (Maheshwari et al., 1999).

The EGF-induced mitogenic, motogenic, and cyto-protective actions are instrumental for healing events that may be summarized as: (a) stimulation of productive cells migration toward the injured area, (b) stimulation of granulation tissue outgrowth – including extracellular matrix accumulation, maturation and de novo angiogenesis, (c) stimulation of wound contraction by myofibroblast activation and proliferation, (d) stimulation of the damaged area resurfacing by epithelial cells migration and proliferation (Werner & Grose, 2003).

EGF is also endowed with angiogenic activity thus promoting the growth of a vascular mesh within the wound bed. The mechanisms behind this angiogenic effect appears to be related to chemotaxis of endothelial cells and the enhancement of other angiogenic factors expression (van Cruijsen et al., 2005). This EGF-mediated neoangiogenic action is significant for ischemic wounds (Grazul-Bilska et al., 2003).

2.3 The nerve restoration action of EGF in sciatic nerve section experiments, where it prevented distal limb ulcers and toe loss

Several experiments demonstrated that single or repeated EGF systemic injections exerted cyto-protective and proliferative responses, supporting the intrinsic ability of EGF at supra-physiological concentrations to unleash biological events required for an effective tissue repair (Berlanga et al., 1998a, 1999, 2001, 2002a, 2002b).

The effect of an EGF local injection was evaluated for the first time in tissues unrelated to the digestive system (which at the moment was the most common experimental substrate - Curling's ulcers prevention) through the perilesional injection of EGF in rats that had been subjected to a complete sciatic nerve section. Two independent studies demonstrated that the intralesional injection of EGF produced: (i) recovery of the motor nerve impulse conduction; (ii) axonal recovery and remyelination, and (iii) prevented or delayed the onset of trophic changes of the hind limb soft tissues (plantar ulcers and toe necrosis). These sciatic nerve experiments suggested the possibility for a pharmacological management of trophic ulcers derived from a neurogenic ischemia (Prats et al., 1998). This animal model somewhat mirrored the condition of a neurogenic diabetic lower extremity in which both neuropathy and angiopathy concertedly predispose to ulceration (Dyck & Giannini, 1996).

3. Why EGF has to be injected intralesionally?

3.1 The availability of the growth factor on the surface of the wound is limited as it can be degraded by proteases from the biofilm that covers the lesion and/or from its fluid

The need for a prolonged interaction between EGF and its receptor to achieve a significant granulation tissue response in controlled wounds in mice had been reported (Buckley et al., 1985). Proteolysis exerted by the wound-derived exudate was observed incubating the ulcers´ material at neutral pH with a fluorescent-synthetic peptide at room temperature. Pre-incubation with a protease inhibitor prevented substrate's degradation. These observations suggested a possible reduction of EGF bioavailability by proteases derived from controlled wounds (Berlanga et al., 1998b). Other studies had already established proteolysis of growth factors and their receptors in chronic circumstances (Mast & Schultz, 1996; Saarialho-Kere, 1998; Trengove et al., 1999;, Medina et al., 2005).

Furthermore, profiles of ^{125}I-EGF in a rat full-thickness wound model, demonstrated tissue-bound radioactivity 2 h after the administration. Within this period, ^{125}I-EGF degraded subspecies with no diffusion of the peptide to the surrounding skin were identified. The receptor expression was increased 2 h after wounding, followed by a slow decline up to 12 h below baseline. These 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 (MRT) values suggested that more than 60% of the amount administered could have disappeared as early as 2 hour post-administration (Prats et al., 2002). Previous clinical evidences of topically applied EGF had already rendered disappointing results possibly due to local bioavailability limitations (Falanga et al., 1992;, Falanga, 1992).

3.2 Responding granulation tissue develops from the deep layers of the wound

An immuno-histochemical characterization of biopsy cylinders from diabetic wound beds shed more light on the biological sustentation for the infiltrative modality. Granulation tissue biopsy cylinders (approximately 7 millimeters length) from neuropathic patients were collected and paraffin-processed for different histochemical techniques and for incubation with anti-EGF receptor and anti-prohibitin (PHB) antibodies. The monoclonal antibody against EGF receptor EGFR-pY1197 (1:250, DAKO) binds to tyrosine residue 1197 when it is phosphorylated, indicating downstream signaling activation. Prohibitin rabbit monoclonal (Abcam. EP2804Y) was diluted at 1:200. EnVision + Dual Link System-HRP was used to develop the reaction. Slides were hematoxylin counterstained. Pictures were obtained at X 40 constant magnification. The figure shows three strata along the longitudinal axis of the biopsy material were defined: upper, middle, and bottom. Fibroblasts populating the more superficial stratum expressed more prohibitin and less EGF-receptor. Prohibitin is an inhibitor of cell cycle progression and may therefore contribute to the onset of wound's chronic phenotype. (Mishra et al., 2010; Lee et al., 2010; Dong et al. 2010). This expression profile became progressively inverted going through deeper cells layers (Fig. 1). Advanced glycation end products and elastase also appeared more intensely labeled next to the wound surface than in deeper cells strata (not shown).

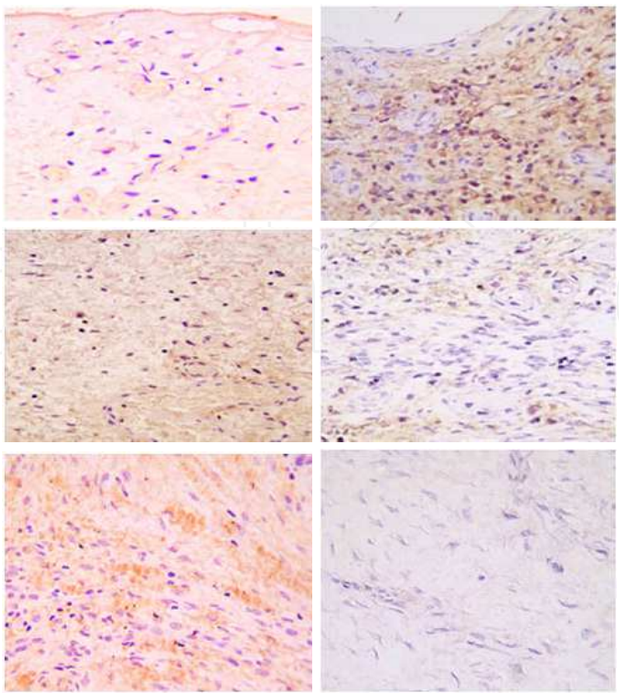

Fig. 1. EGF receptor (left) and prohibitin (right) expressions along the biopsy longitudinal axis.

4. Clinical development of intralesional recombinant human EGF in advanced DFU. Efficacy results

A formulation of recombinant human Epidermal Growth Factor (rhEGF) for intralesional administration in DFU has been developed. The growth factor is purified from a transformed *Saccharomyces cerevisiae* strain and presented as a lyophilized preparation containing 25 or 75 µg of rhEGF per vial. The efficacy and safety of the intervention has been tested in five exploratory and one confirmatory randomized, double-blind, placebo control clinical trials (Berlanga et al., 2006; Fernandez-Montequin et al., 2007, 2009a, 2009b). A total of 344 patients took part in these studies (Table 1).

This clinical development has dealt with a type of patient not usually recruited in DFU clinical trials. The characteristics of the patients included have been quite homogeneous across the trials, given essentially by (i) long-lasting Diabetes Mellitus, mostly type 2; (ii) median age >60 years; (iii) chronic ulcers (more than one month of evolution); (iv) deep ulcers (exposes cellular subcutaneous tissue or tendons, or joint capsule); (v) advanced, given by large size (median area always > 20 cm^2), baseline infection (that had to treated before the use of rhEGF), necrotic tissue (that had to be removed surgically); (vi) approximately one half of the patients with deficient irrigation of the corresponding leg (ischemia). The three latter features are usually exclusion criteria in DFU clinical trials. Such ulcers corresponded to grades 3 or 4 of the Wagner´s classification (Armstrong et al., 1998).These patients, which can represent 50% of the whole DFU population, lead to most of the amputations.

Patients with hemoglobin <100 g/l, uncontrolled chronic diseases, psychiatric disorders, malignancies, pregnant or breastfeeding, were excluded. Signed, informed consent was an inclusion requisite in all trials, which were approved by the corresponding Ethics Committees and by the National Regulatory Authority.

Intralesional rhEGF was administered adjuvant to the conventional good wound care measures: metabolic control, thorough debridement of necrotic and infected tissue, pressure off-loading, and antimicrobials if needed. The product was dissolved in 5 ml of water for injection or saline and distributed evenly throughout the lesion in 5 to 10 punctures, starting from the cleanest zones. The needle was changed between injections in order to prevent infection dissemination. Treatment was given three times per week on alternate days, until complete granulation response or a maximum of 8 weeks. Only in trial No. 0705 treatment was continued until wound closure. Subjects were hospitalized during treatment in all trials and then followed-up, generally for one year, as outpatients.

The main outcome variable in most of the studies was the development of granulation tissue, classified, according to the lesion area covered, in: (i) no response: less than 25% granulation; (ii) minimal response: 25 to 50%; partial response: 50 to 75%; complete response: more than 75% of the lesion area, that could support a skin graft to complete ulcer closure. A 100% granulation response was nonetheless achieved in most complete responders.

In these, more advanced ulcers, granulation is a valid outcome since it is part of the healing process and should necessarily precede the final healing. Besides, granulation over the ulcer area permits skin grafting to attain final healing, so a group of patients does not reach complete spontaneous healing to be evaluated. Additionally, a significant correlation was found between granulation and healing in the confirmatory study No. 0503 (Fernandez-Montequin et al., 2009b). Previous studies had identified partial wound closure as predictive of complete healing for Wagner's grade 1 or 2 DFU (Sheehan et al., 2006), and other ulcers (van Rijswijk & Polansky, 1994;, Kantor & Margolis, 2000), but this was the first report of an early surrogate endpoint in Wagner's grade 3 or 4 DFU.

4.1 Pilot, exploratory trial in 29 patients who were bound to amputation

Patients bore advanced DFU, Wagner´s grade 3 (10 cases) or 4 (19 cases); 23 of them were ischemic and had no other therapeutic alternative. Some granulation response was obtained in 25 cases (86%) and the complete response and lesion closure in 17 (59%; one of them through skin graft), thus preventing amputation. Recurrence appeared in only one patient after a one-year follow-up (Berlanga et al., 2006). An immuno-histochemical study suggested that TGF β could be the main effector mediating the EGF-stimulated granulation process. Biopsies taken from neuropathic and ischemic patients showed the stimulation of granulation tissue growth and organization as well as the formation of new blood vessels after the treatment with rhEGF, illustrating its angiogenic effect (Figure 2 in Berlanga et al., 2006).

4.2 Dose exploratory trial

A controlled, double-blinded, randomized and multicenter clinical trial was later carried out in five institutions to explore a possible dose-dependent response (Fernandez-Montequín et al., 2007).

Forty-one patients bearing Wagner's grade 3 and 4 lesions were included. They were randomly distributed in two groups receiving rhEGF at 75 µg or 25 µg. A tendency to better results with the higher dose was observed: higher granulation response rate and faster, but groups behaved similar concerning wound closure during follow-up. Only one patient relapsed in the follow-up. In some of the cases wound contraction could be evidenced as part of the healing process (Figure 2). This property is usually abolished in diabetic patients.

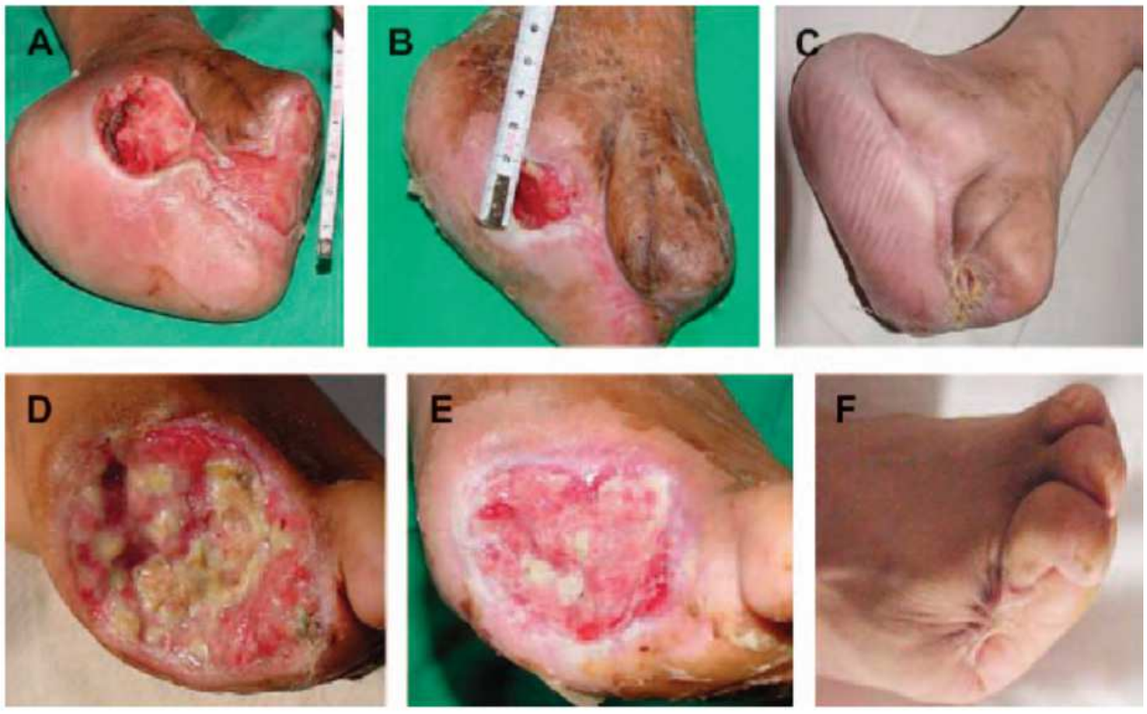

Fig. 2. Evidence of granulation and healing in two patients from study No. 0202. Upper line: 65 years-old female. An ischemic 38 cm² transmetatarsal amputation base was treated with rhEGF. A: before the beginning of treatment; B: after 5 weeks (15 infiltrations) complete granulation was achieved; C: healing occurred at 29 weeks. Lower line: 46 years-old female with ischemic and infected DFU. After toe disarticulation and antibiotics, she was treated with rhEGF. D: before treatment; E: after 4 weeks (12 infiltrations) complete granulation was achieved; F: Healing was reported at 17 weeks. Wound contraction is evident as part of the healing mechanism. (Picture taken from Fernandez-Montequin et al. 2007)

4.3 Series of 12 patients treated with the 75 µg dose (not published)

The results obtained agree with those obtained in previous trials. Most of the patients responded satisfactorily. The observed total response rate was 75%. Major amputations were carried out in 3 patients (25%), all ischemic, associated to the occurrence of local infection in two of them.

4.4 Monitoring of patients treated after initial conditional marketing approval of the product (not published)

A multicenter, non-controlled, phase IV-type study was based on the reports of all treated cases after an initial conditional marketing approval in Cuba. The rhEGF began to be used throughout the country and was administered at 25 µg, three times per week until complete granulation or up to 8 weeks. Results were consistent with the previous ones. Relapse was present in only one patient after one year follow-up.

4.5 Series of patients treated with 75 µg up to wound closure

Twenty patients were included and treated with intralesional rhEGF, three times per week, until the lesion closure was attained (Fernandez-Montequín et al, 2009a). According to Wagner's classification, 16 patients (80%) were grade 3 and 4 (20%) were grade 4. The mean initial area of the lesions was 16.3 ± 21.3 cm^2. There was evidence of ischemia in 5 patients (25%), whose limb occlusion pattern was at a distal level. A complete granulation response was obtained in all cases, complete lesion closure in 17 (85%) and none of them required amputation. The mean healing time was only 6 weeks. There was one wound recurrence 6 months after healing. This study indicated that rhEGF can be used after total granulation until the lesion is completely closed or until its area is significantly reduced (≤ 1 cm^2).

4.6 Multicenter, double-blind, placebo-controlled confirmatory trial

A confirmatory, multicenter, randomized, double-blind trial was carried out (Fernandez-Montequín et al, 2009b). Besides, it was compared to placebo. The latter was required to provide unquestionable evidence on the efficacy of intralesional rhEGF administration; to rule out that the results obtained were explained by good wound care that improves under clinical trial settings or due to the endogenous local production of growth factors induced by the debridement and injection procedures.

The study was carried out at 20 institutions in all Cuban provinces. The treatment consisted of three applications of 75 µg EGF, 25 µg, or placebo for 8 weeks or until complete granulation, adjuvant to general good wound care.

Due to ethical reasons, to avoid giving placebo to a non-responder, if less than 25% of the ulcer area was covered by granulation tissue after two weeks of treatment, the patient´s code was opened and the patient switched to 25 µg rhEGF, when on the placebo, or 75 µg, when on 25 µg. The main evaluation variable was the existence of at least a partial response (50% of the lesion area covered by granulation tissue) after two weeks of treatment, since this was the period when all patients were randomized and double-blinded in their original treatment groups. The hypothesis of the trial was to obtain at least 30% advantage in the proportion of individuals with an objective response at that moment in the groups treated with rhEGF as compared to the placebo. The secondary variables taken into account were the existence of a complete granulation response at the end of treatment, the time to complete response, lesion closure during the 12 months follow-up, time to closure, the occurrence of relapses and the need for amputation. Safety was evaluated by adverse events monitoring and laboratory variables. All the analyses were made under the principle of "intention to treat".

One hundred and forty-nine patients were included. Groups were equivalent in regard to demographic and baseline variables. Both dosage groups fulfilled the study hypothesis of more than 30% advantage over the placebo group, in the proportion of patients with more than 50% of the lesion area covered by granulation tissue. Differences (and their 95% CI) with respect to the placebo are shown in Table 1. Superiority in relative terms (OR) was 7.5 (2.9 – 18.9) and 3.7 (1.6 – 8.7) for the high and the low doses, respectively.

After eight weeks, the proportion of patients with complete granulation response was significantly higher only for the 75 µg treatment compared to placebo. The benefits of the treatment were more evident for the neuroinfectious origin cases than in those with an ischemic component. The time required for a complete response was 3 weeks for each group receiving EGF and 5 weeks for the placebo. This difference was statistically significant for both dosage levels.

The evaluation of the complete closure of the ulcers was performed during the 12-month follow-up. The statistical multivariate analysis of lesion healing showed that the factors that favored ulcer closure were rhEGF 75 µg vs. control (OR: 3.6; 95% CI: 1.4 – 9.5), the "pure" neuropathic vs. ischemic character (5.5; 2.3 – 13.5), and an initial smaller ulcer size (0.98; 0.96 – 0.99). Relapses only occurred in the placebo group. Although amputations were too few to support any statistical analysis, it is remarkable that there was only one amputation among the neuropathic patients treated with rhEGF at any of the dosages, while 15% of the neuropathic cases required amputation in the placebo group. There was only one failure after two weeks in the group receiving rhEGF at 75 µg.

This trial confirmed the efficacy of intralesional rhEGF in advanced DFUs. The results were better in the group using the high dose, although both dosage levels fulfilled the efficacy hypothesis for the main variable (partial granulation response after two weeks). Besides, only the high dose showed significant differences compared to the control group for some secondary variables, suggesting a dose-dependent effect. This is particularly important for patients with ischemia, which is an adverse condition for granulation and healing

Study details	Variables evaluated	Treatment		
		75 µg rhEGF	25 µg rhEGF	Placebo
Code: 0102	N		29	
Pilot; exploratory; grades 3 and 4 ulcers, ischemic and neuropathic; high risk of amputation	Complete granulation at the end of treatment		17 (59%)	
	Wound closure during follow-up		17 (59%)	
Treatment: 25 µg, 3 times per week until complete granulation or 8 weeks maximum. Follow-up for one year.	Time to complete granulation in weeks (mean ± SD)		4.7 ± 1.5	
	Time to wound closure in weeks (95% CI)		7 (1; 13)	
Ref: Berlanga et al. 2006	Amputations		12 (41%)	
Code: 0202	N	23	18	
Dose exploratory study; grades 3 and 4 ulcers,	Complete granulation at the end of treatment	19 (83%)	11 (61%)	

Study details	Variables evaluated	Treatment		
		75 µg rhEGF	25 µg rhEGF	Placebo
ischemic and neuropathic; Design: multicenter, randomized, double blind. Treatment: 25 µg or 75 µg, 3 times per week until complete granulation or 8 weeks maximum. Follow-up for one year Ref.: Fernandez et al., 2007	Wound closure during follow-up	13 (57%)	9 (50%)	
	Time to complete granulation in weeks (mean ± SD)	3.8 ± 2.2	4.9 ± 2.2	
	Time to wound closure in weeks (95% CI)	21 (17; 24)	20 (16; 23)	
	Amputations	8 (35%)	6 (33%)	
Code: 0504 Lineal, patient series. Ulcers: grade 4, ischemic and neuropathic; Treatment: 75 µg, 3 times per week until granulation or 8 weeks maximum Not published	N	12		
	Complete granulation at the end of treatment	9 (75%)		
	Amputations	3 (25%)		
Code: 0604; Lineal, phase IV – type study after conditional approval; Ulcers: grade 3 and 4, ischemic and neuropathic. Treatment: 25 µg, 3 times per week until granulation or 8 weeks maximum. 3- year follow-up. Not published	N		93	
	Complete granulation at the end of treatment		78 (84%)	
	Wound closure during follow-up (16 patients lost)		46 (49%)	
	Time to wound closure in weeks (95% CI)		10 (9; 11)	
	Amputations		26 (28%)	
Code: 0705; Lineal, patient series. Ulcers: grade 3 and 4, ischemic and neuropathic; Treatment: 75 µg, 3 times per week until healing or 12 weeks maximum Ref.: Fernandez et al., 2009a	N	20		
	Complete granulation at the end of treatment	20 (100%)		
	Wound closure	17 (85%)		
	Time to complete granulation in weeks (mean ± SD)	3.4 ± 0.5		
	Time to wound closure in weeks (95% CI)	6.3 (4; 9)		
	Amputations	0		
Code: 0503 Confirmatory multicenter, randomized, double blind, placebo-controlled trial; 20 sites; Ulcers: grade 3 and 4,	N	53	48	48
	No response at 2 weeks; code opened	1 (2%)	5 (10%)	8 (17%)
	≥50% granulation at 2 weeks (95% CI of the difference with placebo)	44 (83%) (24; 63)	34 (71%) (10; 52)	19 (40%)

Study details	Variables evaluated	Treatment		
		75 µg rhEGF	25 µg rhEGF	Placebo
ischemic and neuropathic; Treatment: 25 µg, 75 µg, or placebo 3 times per week until complete granulation or 8 weeks maximum. Follow-up for one year. If no response at 2 weeks, code opened and if placebo, switched to 25 µg; if 25 µg, switched to 75 µg. Ref.: Fernandez et al., 2009b	Complete granulation at the end of treatment (95% CI of the difference with placebo)	46 (87%) (6; 39)	35 (73%) (-8; 29)	28 (58%)
	Time to complete granulation in weeks (95% CI)	3 (2.6; 3.4)	3 (2.2; 3.8)	5 (3.4; 6.6)
	Wound closure during follow-up (95% CI of the difference with placebo)	41 (77%) (1; 41)	25 (52%)	27 (56%)
	Time to wound closure in weeks (95% CI)	15.7 (10; 22)	17.3 (11; 24)	21.4 (8; 35)
	Amputations	7 (13%)	10 (21%)	12 (25%)
Pooled analysis	N	108	188	48
	Complete granulation at the end of treatment	94 (87%)	141 (75%)	28 (58%)
	Wound closure during follow-up	71/96 (74%)	97 (52%)	27 (56%)
	Time to wound closure in weeks (95% CI)	14 (12; 16)	12 (10; 14)	21.4 (8; 35)
	Amputations	18 (17%)	54 (29%)	12 (25%)

Table 1. Summary of characteristics and results of clinical trials with rhEGF in DFU. SD: standard deviation; CI: confidence interval

4.7 Long-term follow-up of the patients included in all clinical trials

Overall, 323 individuals were enrolled in clinical trials since 2001 (one patient took part in studies 0604 and 0503). They were visited in 2010 looking for information on survival, recurrences, new foot ulcers, amputations, adverse events and co-morbidities, including neoplasia. The median follow-up period was 3 years (maximum, 8 years). The results are summarized in Table 2. Patients from study No. 0705 were not followed-up since they live abroad. Patients from study No. 0503 were considered "per protocol" for this evaluation, since some had switched treatment at 2 weeks.

No evidence was gathered indicating that intralesional rhEGF administration could stimulate cancer growth. Two patients had developed malignancies: one who had received rhEGF at 25 µg; the other was from the placebo group of the phase III trial.

Thirty nine percent of the patients had deceased. The more frequent causes of death were heart infarct, chronic renal failure, and stroke. Median survival was longer in patients that had attained ulcer-healing: 5.7 ± 0.8 years vs. 4.2 ± 0.7 years in those who did not heal ($p=0.004$).

The frequency of relapses at any moment was significantly lower ($p<0.001$) in patients that received rhEGF (10% and 4% for the 75 µg and 25 µg doses, respectively) as compared to the

control group of the confirmatory, No. 0503, trial (23%). This effect was obtained for both neuropathic and ischemic patients. On the contrary, no effect was seen on the appearance of DFU on other locations (mainly on the contralateral limb). The rates were 26%, 24%, and 32% for patients treated with 75 μg rhEGF, 25 μg, and placebo, respectively. It seems as if the tissue keeps a sort of "memory" of the treatment received, which is not transferable to non-treated zones. Molecular and immunohistochemistry studies are in course to elucidate these mechanisms.

Trial	Dose	Enrolled	Followed-up	Recurrences	New ulcers	Amputations	Cancer
0102	25 μg	29	29	1	7	13	
0202	25 μg	18	18	0	6	9	
	75 μg	23	22	1	11	12	
0504	75 μg	12	11	1	0	4	
0604	25 μg	93	66	3	20	29	1
0503	25 μg	49	43	1	12	15	
	75 μg	57	51	5	11	16	
	Placebo	43	42	8	13	15	1

Table 2. Long term follow-up of patients from all clinical trials with rhEGF in DFU

Amputations were necessary in 114 patients at some moment after rhEGF treatment. The amputation rates were 35% for both rhEGF dose levels, the same as for the control group of the confirmatory trial. However, if only neuropathic and mild ischemic patients are taken into account amputations were less among patients treated with rhEGF 75 μg (14%) or 25 μg (20%) than among those who only received the good standard care (33%). Thus, severe and critic ischemia is still an adverse condition, difficult to overcome. Probably rhEGF should be used in those cases after appropriate revascularization procedures that improve tissue blood supply.

4.8 Consistency of the efficacy results from the different trials

Efficacy measurements across the different studies, summarized in Table 1, are consistent. More than 80% granulation was obtained globally for both dose levels used. They are much better than what was obtained for a group of patients that received only standard care (the placebo group of study No. 0503). The fact than only one study had a control group is a limitation to this comparison, but there is homogeneity among the different studies with respect to the results obtained and the 95% confidence interval of the result in the placebo group falls below those of the treated groups. There seems to be a dose-dependent effect, since results were always better for the higher dose. Subgroup analyses, limited by the fact that they were not previewed in the protocols, suggest that the difference between doses is given by patients with more complicated ulcers (larger, with more severe ischemia).

Results on healing are clinically relevant as well, for the 75 μg dose. Treatment-dependency was also found for complete closure, despite being reached during follow-up, as

outpatients, only under general wound care measures (except for study No. 0705, where treatment was continued until healing). This apparent "EGF-memory" effect on wound closure can be explained by the granulation tissue stimulation, which was predictive of closure. Granulation can also reduce the probability of infection progression, since the fresh tissue is better prepared to "fight" against invading micro-organisms and better irrigation can increase the local bioavailability of systemic antibiotic treatment. As mentioned above, some persistent action of rhEGF treatment should take place, leading to less relapses as well. Time-to-closure was also shortened in approximately 4 weeks, which is a clinically significant feature.

With respect to amputations, the number of events has been very small in each study in order to make a proper statistical analysis. For that reason this outcome has been secondary in all trials. The pooled analysis after long-term follow-up, mentioned above is interesting for the 75 μg dose.

5. Overview of safety

Since all trials included very similar populations and the same therapeutic schedule (only the dose varied), a pooled analysis was done. Intralesional rhEGF was well tolerated. About half of the included patients (58.5%) reported some adverse event. Those occurring in more than 1% of the patients are summarized in Table 3**.** Pain and burning sensation at the administration site were the most frequent. A dose-effect relation associated to the appearance of shivering and chills was systematically obtained in all trials that used both doses and in the pooled analysis.

Concerning intensity, the adverse events reported were 65.6 % mild, 28.6% moderate, and only 3.7% severe. Serious adverse events were reported in 13% of the patients from the clinical studies. Local infection was the more frequent serious adverse event. It caused hospitalization and/or amputation. It was the adverse event that most frequently lead to treatment interruption. It is difficult to establish a causal relationship between its appearance and rhEGF administration since this event is also a frequent complication of Wagner´s grade 3 and 4 DFU and a significant risk factor for amputation. It has been reported that lower extremities infection is the most frequent reason for the hospitalization of patients with diabetes (Edmonds & Foster, 2004). Likewise, it appeared in the placebo group of the controlled study 0503. Some of the factors that leave patients predisposed to the progress of local infection include the presence of an entryway for bacteria and the fact that the immune response of diabetic patients is often compromised.

The analysis of the relationship of adverse events with demographic or baseline characteristics only yielded significant results for the age and ulcer etiopathogeny. A multivariate logistic regression model yield a significant more frequent appearance of any adverse event in patients ≥ 65 years old vs. younger (OR: 1.86; 95% CI: 1.09 – 3.15), and less frequent if the ulcer was neuropathic (OR: 0.25; 95% CI: 0.14 – 0.45). Local infection appeared significantly more frequently in patients with Diabetes type II than in type I: 45/222 (16.9%) vs. 2/62 (3.1%).

Three of the patients in the clinical trials died: one with placebo and one with each of the doses. Other 8 deaths occurred during follow-up (up to one year). None of the deaths can be

considered as related to treatment but to the underlying diabetes and/or the age of the patients. The death causes were: acute myocardial infarct (4), bronchopneumonia (2), mesenteric thrombosis, acute renal failure, acute pulmonary edema, and infection. At the time of follow-up, 12 months after the conclusion of the treatment, no long term adverse effects related to the product were reported.

Adverse events	75 µg N=116		25 µg N=181		Placebo N=48	
	N	%	N	%	N	%
Pain at the administration site	21	18.1%	52	28.7%	20	41.7%
Burning sensation at the administration site	26	22.4%	25	13.8%	14	29.2%
Shivering	34	29.3%	18	9.9%	2	4.2%
Local infection	20	17.2%	18	9.9%	8	16.7%
Chills	30	25.9%	15	8.3%	1	2.1%
Fever	9	7.8%	18	9.9%	6	12.5%
Anemia	5	4.3%	5	2.8%	5	10.4%
Vomiting	7	6.0%	4	2.2%	1	2.1%
Pain on the lesion	9	7.8%	2	1.1%	0	
Nauseas	5	4.3%	3	1.7%	2	4.2%
Chest pain	3	2.6%	4	2.2%	0	

Table 3. Frequency of patients with each adverse event in Clinical Trials. Events that appeared in more than 1% of the patients (pooled).

The presence of anti-EGF autoantibodies has not been considered detrimental for adult animals (Raaberg et al., 1995a, 1995b) or in the healing process (Casaco et al., 2004). Nevertheless, immunogenicity of rhEGF was evaluated in three studies since, as a recombinant protein, it could exert antibody production and this is an important safety issue for all agencies. Anti-EGF antibodies were detected in some patients (4 in study 0202 and 5 in study 0503). Some other patients had these antibodies naturally, before treatment. All the titers found were very low and there was no clear relationship with efficacy or safety results.

The adverse event profile from the first report of the postmarketing pharmacovigilance in Cuba (from 1851 patients) is very similar to that from clinical trials. The most frequent events are shivering, pain and burning sensation at the administration site, and chills. Local infection is less frequent, but the most frequent among the serious events found (manuscript in preparation).

A particular concern on the therapeutic use of growth factors is the possibility of development or stimulation of a pre-existing malignancy in the patients. The concern is justified by the growth stimulating property of these active principles. Practically, anti-growth factor therapy is approved or extensively experimented for several neoplasia (Gonzalez et al., 2011; Caraglia et al., 2006; Geva et al., 2010). Additionally an increase in any-site cancer incidence was observed in DFU patients treated with platelet-derived-growth factor (PDGF; becaplermin), which generated a warning from regulatory agencies (FDA-USA, 2008).

EGF is not an exception to this concern. In fact, the presence or history of neoplasia has been an exclusion criteria in all clinical trials with intralesional rhEGF and is a precaution for the

use of the procedure in medical practice. However, there are several theoretical and experimental considerations that do not support the idea that EGF treatment, at the doses and schedules used, could stimulate any tumor growth. These aspects are thoroughly discussed by Berlanga et al., 2009. Briefly:

EGF, although growth stimulating and thus a possible tumor promoter, does not induce malignant transformation.

EGF stimulation of cell lines and tumor grafts growth is not a consistent result. There are cases of growth inhibition by exogenously administered EGF. (Knowles et al., 1985; Barnes, 1982)

EGF overexpression in transgenic animals not always lead to increase in tumor development (Chan & Wing-Chuen, 2000).

No increase in tumors in patients treated with topical EGF for burns, in a controlled trial done in Cuba in 1993-94. These patients were visited in 2008 – 2009 and their incidence of cancer was not above that expected for that age group in Cuba for that long period of time (not published result, reported by Berlanga et al., 2009)

The pharmacokinetics study in DFU patients showed short residence in blood of intralesionally administered rhEGF. After 2 hours all EGF was cleared and no accumulation was detected after repeated injections (not published result; manuscript in preparation). These data are inconsistent with long-term systemic actions.

Treatments with rhEGF are short-term: not more than 8-12 weeks, contrary to a tumor promoting action that would require a longer exposure.

Lack of treatment-related cancer development in the long-term follow up of patients from clinical trials with rhEGF (mentioned above). This argument is nonetheless still weak since the result comes from few individuals.

6. Benefit-risk analysis

The clinical studies in patients with advanced DFU (Wagner´s grade 3 or 4, median size >20 cm^2, ischemic not excluded) have shown that intralesionally injected rhEGF, adjuvant to standard good wound care, has the potential to promote granulation, complete wound healing, even in subjects unresponsive to other treatments, faster than subjects treated with standard good wound care alone. The relapse rate is reduced as well. The procedure has the potential to reduce amputation rates, particularly in neuropathic or mild ischemic patients, with a considerable personal and public health improvement.

Potential risks of the use of intralesional rhEGF in DFU can be evaluated by the safety profile obtained from the clinical trials and the possibility of cancer stimulation, discussed above. No particular treatment-related serious adverse event was observed. All can be explained by the underlying disease. No increase in cardiovascular, respiratory or renal complications was reported. Adverse events attributed to treatment have been generally limited to shivering, chills, and injection site pain. These have been mild and self-limiting. No increase in cancer development in treated patients has been observed. Local infection progression is the more frequent serious adverse event. Since the injection procedure can

potentially contribute to infection spreading, caution is recommended in this sense. Clinical signs of infection should be cleared before treatment with rhEGF.

This benefit-risk balance seems quite favorable. This was also suggested by the analysis done using a Bayesian approach (Spiegelhalter et al., 2004) comparing the probability of benefit (given by complete healing) with the probability of risk (given by the occurrence of serious adverse events, including amputation), taking into account data from all clinical trials. These results are shown in Fig. 3.

Assuming that there are two hypotheses H_1 (benefit, given by ulcer healing) and H_2 (risk, given by the occurrence of serious adverse events or amputation) proposed for the patients outcome data set and under H_k the data are related to the parameter vector ψ_k by a distribution with probability density $p(X \mid \psi_{k,} H_k)$. Given the prior probabilities p(*benefit*) and p(*risk*) = 1 – p(*benefit*), the data produce the posterior probabilities $p(benefit \mid X)$ and $p(risk|X) = 1 - p(benefit|X)$.

The Bayes Factor (B_{br}) is then:

$$B_{br} = \frac{p(x|benefit)}{p(x|risk)}$$

representing a summary of the evidence provided by the data in favor of benefit, as opposed to risk. A value larger than 1 means a favorable benefit-risk ratio.

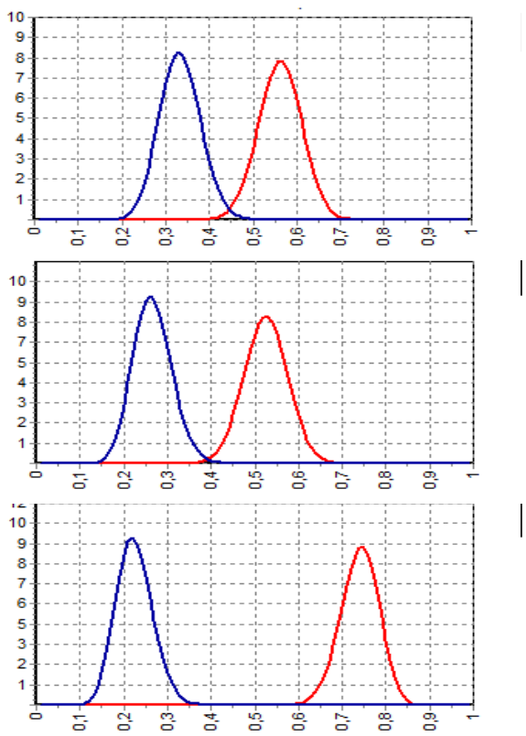

Fig. 3. Benefit and risk probability distributions of the outcome of DFU patients treated with intralesional rhEGF.

The results shown are:

Upper graph: placebo (study No. 0503): B_{br}= 1.69; difference between the probabilities: 23% (95% CI: 11% – 36%).

Middle graph: treatment with 25 µg rhEGF; B_{br}= 1.92; difference between probabilities: 26% (95% CI: 13% – 40%).

Lower graph: treatment with 75 µg rhEGF. B_{br}= 3.36; difference between probabilities: 51% (95% CI: 40% – 63%).

In all cases the differences between benefit and risk probabilities favor the former; the Bayes factors and differences are larger for the rhEGF treated groups than for the controls. The balance of the higher dose is much favorable.

7. Conclusion

The intralesional administration of rhEGF offers a new alternative for the treatment of advanced DFU, reluctant to other treatments, which frequently lead to a lower limb amputation. Further extension of the procedure should yield impact data (pharmacoeconomic, limb salvage).

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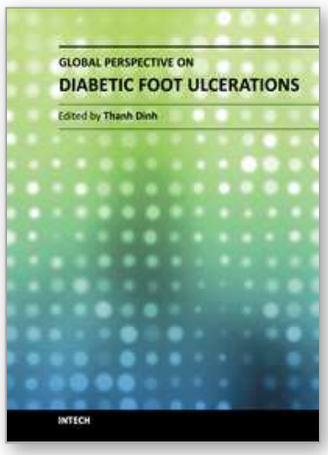

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Over the last decade, it is becoming increasingly clear that diabetes mellitus is a global epidemic. The influence of diabetes is most readily apparent in its manifestation in foot complications across cultures and continents. In this unique collaboration of global specialists, we examine the explosion of foot disease in locations that must quickly grapple with both mobilizing medical expertise and shaping public policy to best prevent and treat these serious complications. In other areas of the world where diabetic foot complications have unfortunately been all too common, diagnostic testing and advanced treatments have been developed in response. The bulk of this book is devoted to examining the newest developments in basic and clinical research on the diabetic foot. It is hoped that as our understanding of the pathophysiologic process expands, the devastating impact of diabetic foot complications can be minimized on a global scale.

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