Epidermal Growth Factor based Therapy Promotes Intracellular Trafficking and Accumulation of its Receptor in the Nucleus of Fibroblasts from Diabetic Foot Ulcers

Viviana Falcón-Cama 1, Maday Fernández-Mayola 1, Yssel Mendoza-Mari 1, Nelson Acosta-Rivero 1, Ariana García-Ojalvo 1, Ricardo Bringas-Pérez 1, Ivón Menéndez-Valdés 1, Mariuska Matos-Terrero 1, Lilianne López-Noudo 1, Rocío Garateix-Suárez 1, Karla Pereira-Yañez 1, Maritza González 2, Sirenia González-Pozos 1, Juan Kouri-Flores 1, William Savigne-Gutiérrez 2, Ivonne Salgado 2, Alejandro Hernández-Seara 1, David G Armstrong 6 and Jorge Berlanga-Acosta 1*

1 Center for Genetic Engineering and Biotechnology, Havana, Cuba
2 National Center for Scientific Research, Havana, Cuba
3 Latin American School of Medicine, Havana, Cuba
4 Electronic Microscopy Unit, LaNSE, Mexico City, Mexico
5 National Institute of Angiology and Vascular Surgery, Havana, Cuba
6 Southern Arizona Limb Salvage Alliance (SALSA), Arizona Health Science Center: 1501 N. Campbell Ave, Arizona, USA

Abstract

Objective: To gain a better understanding of the Epidermal Growth Factor (EGF) Receptor (EGFR) activation, trafficking and biological response in diabetic foot ulcers (DFU), exposed to recombinant human EGF via intra-ulcer infiltration as a healing alternative.

Methods: We studied by immunoelectron microscopy the intracellular localization of the EGFR and Proliferating Cell Nuclear Antigen (PCNA) in fibroblast-like cells (FLC) from granulation tissue of DFU patients, collected before and at different time points after EGF treatment.

Results: EGF therapy appears to increase EGFR immunolabeling. At early time-points, EGFR labeling is observed predominantly in the nucleus, suggesting a fast EGFR internalization and nuclear translocation. Interestingly EGFR is also detected in the mitochondrial outer membrane. PCNA expression and trafficking were also detected in a time-dependent manner after EGF infiltration.

Conclusion: Differential subcellular distribution of EGFR and PCNA and accumulation in the nucleus, in a time-point specific manner, supports the induction of an EGF-mediated activation program that is sustained for at least 24 hours after the EGF administration. These findings substantiate the therapeutic ability of EGF to restore the healing process in DFU.

Keywords: Diabetes; Diabetes complications; Diabetic foot ulcers; EGF; EGFR; PCNA; Immunoelectron microscopy

Introduction

Diabetic foot ulceration (DFU) is one of the most feared complications of diabetes, remaining as the universal cause of non-traumatic amputations resulting in significant disability, morbidity and mortality [1]. Diabetes-related ulcers and amputations are associated with high 5-year mortality rates that even surpass some aggressive forms of cancers [2]. Abnormal wound repair process of peripheral soft tissues has been proposed as ingredient to sustain and/or amplify diabetic ulcers [3]. Diabetes-associated healing impairment results from amalgamated systemic and local factors that converge to the establishment of a pro-senescent phenotype, along with mitogenic arrest and anticipated programmed cell death of granulation tissue fibroblasts [4]. Concurrently, keratinocytes, fibroblasts, myofibroblasts and endothelial precursor cells’ migration, homing, proliferation and extracellular matrix (ECM) synthetic properties are impaired in diabetes [5].

Diabetic healing functional impairment has also been related to a substantial dysregulation in the availability and activity of growth factors [6]. Mounting evidences suggest that epidermal growth factor (EGF)/epidermal growth factor receptor (EGFR) system becomes deteriorated by diabetes [7] showing downregulation of EGFR tyrosine kinase activity in peripheral tissues [7]. EGF is perhaps the most broadly studied growth factor in relation to wound healing. It has been endowed with the biological competence for reverting the proliferative arrest that characterizes the chronic wounds phenotype [8]. On the other hand, locally prolonged bioavailability and timely receptor stimulation have been shown to be required for a significant EGF-mediated impact in wound closure [9,10]. EGF binding to EGFR can drive receptor conformational changes, trans-auto-phosphorylation reactions, and ultimately the activation of different target genes involved in the most relevant events required for tissue repair [11]. Remarkably, the EGF/EGFR signaling axis has been considered as a particular target for decades in cancer research, developmental biology, and wound healing.

As an alternative to circumvent the hostile environment of diabetic wounds and to ensure an adequate EGF availability to its receptor in
responsive cells, our group has conducted the intra-ulcer infiltrations of EGF for 15 years [10,12-15]. Most recent data from about 3800 patients, confirmed treatment success in granulation, re-epithelialization and amputation reduction risk (5%) relapses rates per year for Wagner’s grade 3 to 5 wounds [13,15].

Several studies have shown that, upon EGF stimulation, full-length EGFR translocates to the interior of the cell. Intracellular trafficking of EGFR involves the nucleus where it binds to c-myc (MYC) and cyclin D1 (CNN1D1) promoters, and phosphorylates the proliferating cell nuclear antigen (PCNA), all together committed in cell division. PCNA is important for both DNA synthesis and DNA repair, promoting division cell cycle progression from G1 to S phase [16]. Therefore, owing to its responsiveness to EGF and its commitment in cell proliferation [17]. However, little is known about EGFR cellular compartmentation and its biological response to natural ligands in vivo. Scarce information exists regarding the EGFR intracellular trafficking and the ensued downstream response in a human tissue exposed to EGF as a pharmacological agent in a clinical setting. Therefore, the goal of this study was to conduct an immunoelectron microscopy investigation to monitor, in a temporal sequence, the intracellular trafficking of both EGFR and PCNA in fibroblasts collected from DFU’s samples of patients treated with locally infiltrated EGF.

Methods

Ethics

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. In addition, this protocol was reviewed and approved by the ethics committees at the National Institute of Angiology and Vascular Surgery, and the National Center for Integral Diabetes Care, Havana, Cuba. All the patients approved to be involved in the study and signed an informed consent.

Study population

Twelve diabetic patients (type 1 or 2 diabetes) affected by chronic neuropathic lower extremity wounds and admitted at the National Institute of Angiology and Vascular Surgery in Havana, Cuba, were included in this study. The wounds were classified as grades 3 and 4 according to the Wagner’s scale [18-20]. All the patients were part of the National Program for Integral Diabetes Care. This program involves the intralesional infiltration of recombinant human EGF as instrumental adjunctive pharmacological intervention. Under the in-hospital regime, the patients received the standard wound care including instrumental adjunctive pharmacological intervention. Sampling was performed as described elsewhere [26]. Granulation tissue samples were fixed and analyzed by transmission electron microscopy as previously described [27,28]. Briefly, 2 mm thick fragments of granulation tissue samples were fixed for 1 h at 4°C in 1% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde, rinsed in 0.1 M sodium cacodylate (pH 7.4), post-fixed for 1 h at 4°C in 1% O₃ and dehydrated in increasing concentrations of ethanol. Ultrathin sections (400-500 Å) made with an ultramicrotome (NOVA, LKB), were placed on 400 mesh grids, stained with saturated uranyl acetate and lead citrate and examined with a JEOL/JEM 1400 transmission electron microscope (JEOL, Japan).

Antibodies

EGF immunostaining was performed using a mouse monoclonal IgG that targets the extracellular domain of EGF with high affinity. It was developed by the Center for Molecular Immunology (Havana, Cuba) and marketed by CIMAB S.A., Havana, Cuba [29]. PCNA was detected using a commercially available rabbit polyclonal IgG antibody (ab15497) (Abcam, Cambridge, United Kingdom) that recognizes human PCNA.

Immunoelectron microscopy (IEM)

Samples of granulation tissue were fixed with 4% (v/v) paraformaldehyde containing 0.1% (v/v) glutaraldehyde in 0.1

<table>
<thead>
<tr>
<th>Age (mean ± SD)</th>
<th>58.7 ± 5.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes classification</td>
<td>T1-DM: N=3  T2-DM: N=9</td>
</tr>
<tr>
<td>DM evolution (years) (mean ± SD)</td>
<td>16.5 ± 7.1</td>
</tr>
<tr>
<td>Wound classification (Wagner’s scale (from 0 to 5)</td>
<td>Grade 3: N=6  Grade 4: N=7</td>
</tr>
<tr>
<td>Wound evolution (days) (mean ± SD)</td>
<td>51 ± 15.8</td>
</tr>
<tr>
<td>Fasting glycaemia at time zero (mmol/L)</td>
<td>8.2 ± 0.65</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.3 ± 0.82</td>
</tr>
</tbody>
</table>

Table 1: Demographic characteristic of the study population.
M phosphate buffer (pH 7.3) at 4°C for 3 h and washed with 0.1 M phosphate buffer, pH 7.3. Fixed samples were dehydrated as described above, embedded in Lowicryl, and polymerized by exposure to ultraviolet light at room temperature (RT) for 72 h. Ultrathin sections of biopsies were incubated with either anti-EGFR or anti-PCNA antibodies in phosphate buffer, for 45 min at RT. The sections were rinsed three times for 30 min at RT with 0.1% bovine serum albumin in phosphate-buffered saline, pH 7.3 (BSA–PBS), and incubated for 1 h at RT with gold-labeled (15 nm) anti-mouse IgG or anti-rabbit IgG (GE Healthcare Life Sciences, Mississauga, Ontario, Canada) diluted 1:100 in BSA–PBS. As internal immunolabeling control, the primary antibody was replaced by either normal mouse or rabbit serum. All sections were stained and analyzed with a transmission electron microscope, as mentioned above. At least ten photographs were obtained for each time-point and patient. The images were blindly and independently analyzed by two investigators (VFC and SG), focusing on the granulation tissue fibroblast-like cells (FLCs) as the principal granulation tissue cell.

Comparing distributions of gold particles between different compartments within a cell was done as described [30]. The objective was to test whether the observed distribution of gold particles between compartments within a cell is a random event. If not, then some compartments must be preferentially labeled. Gold particles representing the ‘observed gold particles’ (No) and a superimposed lattice of test points (‘Test points’, P), were counted in each cell compartment (endoplasmic reticulum (ER) and Golgi complex (GC), mitochondria, plasma membrane, rest of cytoplasm (RC) (included all residual compartments in cytoplasm not of individual interest) and nucleus) using the ImageJ 1.38 software (http://imagej.nih.gov/ij/). Then, the ‘expected gold particles’ (Ne) (i.e. random distribution), the relative labelling index: RLI (No/Ne), and a two-sample Chi squared (X2) analysis were calculated as previously described distribution), the relative labelling index: RLI (No/Ne), and a two-sample Chi squared (X2) analysis were calculated as previously described

Analysis of the regulatory relationship between EGFR and PCNA from data of the Encyclopedia of DNA elements

Information on ChIP-seq experiments from ENCODE projects was obtained from the UCSC Genome Browser ftp site (hgdownload.cse.ucsc.edu). The file wgEncodeRegTfbsClustered.bed, containing data on transcription factor (TF) binding sites (TFBS) was downloaded. This file contains information on 161 TFs. The data on TFBSs includes chromosomal start and end positions of clusters of sequenced reads as results of ChIP set experiments. From the same site was downloaded thereGene.txt.gz file containing information on gene chromosomal positions. The TFBSfile was scanned and those binding sites located on promoters regions were assigned to the corresponding genes. Promoters were chosen as the DNA segments (-2000, -1) relative to the transcription start site.

Results

Study population

As illustrated in Table 1, most of our patients (75%) suffered for more than 15 years of type 2 diabetes. The targeted lesions included clean, debrided ulcers and amputation residual bases which, given their slow trajectory and torpid granulation process, were clinically entitled as chronic wounds. Most of them were classified as complex wounds by involving not only soft tissues, but bone, capsules and tendons. Given the in-hospital regime of metabolic control and other medical interventions, the patients achieved an acceptable compensation state.

EGF therapy increases EGFR immunolabeling and its subcellular trafficking

Immunolabeling of EGFR in Fibroblast-like cells (FLC) of biopsies obtained from patients with diabetic foot ulcer. Samples were incubated with an anti-EGFR mouse monoclonal antibody. Figures depict a time sequence of activation and trafficking events upon local infiltration of EGF (Heberprot-P) within the ulcers (Figure 1). As shown in Figure 1A, EGFR was barely recognized on FLCs in samples obtained prior to the initial EGF treatment (T0). Early after EGF therapy (from T15 to T60), increased anti-EGFR immunostaining was observed in the rough endoplasmic reticulum (RER), in nucleus and mitochondria of FLCs (Figure 1B, Table 2). Immunolabeling was also shown in various intracellular vesicles near the plasma membrane, RER and GC (Figure 1B). It is interesting to note that following EGF treatment, immunolabeling was seen in the extracellular matrix (ECM), in exosome-like structures (ELS) and on collagen-like fibers (CF).

At a later time points after EGF treatment (T6), EGFR was specially immunolabelled in RER, GC and mitochondria (Table 2). Immunostaining was also detected in nucleus, various vesicles, multivesicular bodies (MVBs) and phagosomes (Figure 1C). Besides, gold particles were seen in the ECM, in ELS and on CF (Figure 1C). EGFR immunolabeling was observed on plasma membrane in contact with CF (Figure 1Ca). A similar pattern of EGFR detection was observed in FLCs at T24 after EGF infiltration (Figure 1D). A
preferential immunolabeling was shown in RER, and also in nucleus, mitochondria and vesicles. Interestingly, EGFR was seen on RER membranes adjacent to mitochondria.

To test whether the overall distribution of labeling of compartments was consistent with a random pattern within FLCs, we compared the RLI values for different compartments. As shown in Table 2, the distribution pattern of gold particles in different cell compartments was significantly different from random. In FLCs analyzed at early times after EGF treatment, a preferential labeling was shown in mitochondria (RLI=1.91 and 58.40% of the total X²). At later time points after EGF treatment (T6-T24), preferential gold labeling of ER+GC (RLI=1.24 and 30.07% of the total X²) and mitochondria (RLI=1.60 and 42.86% of the total X²) were observed (Table 2).

Gold labeling over the same compartments of FLCs obtained either at early or at late time points after treatment with EGF were also compared (Table 3). This analysis indicated that the distribution of EGFR labeling in FLCs analyzed at different times is significantly different. Data indicated that the major contributors to the difference were the nucleus (40.73% of the total X²), mitochondria (40.07% of the total X²) and ER+GC (15.25% of the total X²).

<table>
<thead>
<tr>
<th>Compartments</th>
<th>EGFR (T15-T60)</th>
<th>EGFR (T6-T24)</th>
<th>PCNA (T15-T60)</th>
<th>PCNA (T6-T24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLI</td>
<td>X²</td>
<td>X² %</td>
<td>RLI</td>
<td>X²</td>
</tr>
<tr>
<td>ER+GC</td>
<td>1.09</td>
<td>1.44</td>
<td>7.44</td>
<td>1.24</td>
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<td>M</td>
<td>1.91</td>
<td>11.29</td>
<td>58.40</td>
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</tr>
<tr>
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<td>0.89</td>
<td>5.00</td>
<td>25.86</td>
<td>0.91</td>
</tr>
<tr>
<td>PM</td>
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</tr>
<tr>
<td>Total</td>
<td>19.33</td>
<td>100</td>
<td>50.02</td>
<td>100</td>
</tr>
</tbody>
</table>

For degrees of freedom (df)=4 (2-1 columns by 5-1 rows), and total chi-squared values of: (EGFR) T15-T60: X²=19.33, P<0.001; T6-T24: X²=50.02, P<0.001; (PCNA) T15-T60: X²=26.39, P<0.001; T45-T60: X²=27.44, P<0.001; so the distribution patterns of gold particles is significantly different from random. For EGFR, at T15-T60, mitochondria (RLI=1.91 and 58.40% of the total) meet the two criteria for preferential labelling: 1) RLI is greater than 1, and 2) partial X² values make substantial contributions to the total, >10%. At T6-T24, there is preferential labelling of ER+GC (RLI=1.24 and 30.07% of the total) and mitochondria (RLI=1.60 and 42.86% of the total). For PCNA, at T15-T60, there is preferential labeling of ER+GC (RLI=1.35 and 63.71% of the total), at T6-T24, there is preferential labeling of ER+GC (RLI=1.16 and 12.42% of the total) and mitochondria (RLI=1.36 and 27.00% of the total). RLI, Relative Labeling Index; X², chi-squared values; X² %, chi-squared values expressed as percent; ER: Endoplasmic Reticulum; GC: Golgi Complex; M: Mitochondria; RC: Rest Of Cytoplasm; PM: Plasma Membrane; N: Nucleus.

Table 2: Relative labeling index (RLI) for EGFR and PCNA labeled by gold particles in different compartments of fibroblast-like cells (FLCs), analyzed at early (from T15 to T60) or later time-points (from T6 to T24) after EGF treatment.
EGF therapy increases PCNA immunolabeling and its subcellular trafficking

Immunolabeling of PCNA, as a downstream target of nuclear EGFR kinase activity as well as a DNA replication and cell proliferation marker. Samples were incubated with anti-PCNA rabbit polyclonal antibodies. Figures depict a time sequence of activation and localization events upon local infiltration of EGF (Heberprot-P) in Fibroblast-like cells (FLCs) within the ulcers (Figure 2). Prior to EGF injection (T0), PCNA expression was scarcely detected in FLCs (Figure 2A). Remarkably, at early times following exposure to EGF, increased immunolabeling was observed in nucleus, ER+GC and mitochondria (Figure 2B, Table 2). PCNA was also detected on RER membranes adjacent to mitochondria (arrowhead) (Bar=0.2 um).

Figure 1D: This image corresponds to EGFR immunolabeling (arrows) in a Fibroblast-like cell (FLC) from a diabetic foot ulcer sample, 24 hours (T24) after treatment with EGF. Preferential immunolabeling was observed in RER. In addition, EGFR was detected in nucleus (N), mitochondria (M) and vesicles (V). Note immunolabeling on membranes of RER in contact with mitochondria (filled arrowhead) and in a caveosome-like structure (C) (arrowhead) (Bar=0.2 um).

Figure 2A: The image corresponding to T0, prior to the first EGF infiltration, shows scarce PCNA immunostaining in the nucleus (N) region. (Bar=2 um).

Figure 2B: Immunolabeling of PCNA in a Fibroblast-like cell (FLC) from a biopsy of samples infiltrated with EGF at T60. PCNA labeling mostly accumulated in RER and Golgi complex (GC). Immunolabeling was also detected on the plasma membrane, in the nuclear region (N), mitochondria (M), and multivesicular bodies (MVBs). Note that PCNA was detected on RER membranes adjacent to mitochondria (arrowhead) (Bar=2 um).

Figure 2C: Immunolabeling of PCNA (arrows) in part of a fibroblast-like cell (FLC) from a biopsy taken at T6. PCNA immunostaining was shown in mitochondria (M), RER and nucleus (N). Also note immunolabeling in vesicles (V) and in the ECM on collagen-like fibers (CF) and exosome-like structures (ELS). (Bar=0.2 um).

Analysis of the regulatory relationship between EGFR and PCNA from data of the Encyclopedia of DNA Elements

With the aim of gaining further information on the regulatory relationship between EGFR and PCNA, we analyzed data from the Encyclopedia of DNA Elements.
Encyclopedia of DNA Elements (ENCODE project). TF-interactions were obtained as described in Materials and Methods section. Processed data included information for a total of 161 TFs. We identified a total of 11 TFs (ATF3, E2F1, MAX, MYC, NR3C1, POLR2A, RAD21, RBBP5, SIN3A, TAF1 and ZNF263) that bind to both, EGFR and PCNA promoter regions. Additionally we found three transcription factors, MYC, ELK1 and STAT5 that are activated in the EGF/EGFR signaling pathway and were found to bind PCNA gene promoter according to ChIP Seed data (Figure 3).

Discussion

Despite the present study has the merit to provide the first evidences on the EGFR and PCNA intracellular kinetic in response to a therapeutic intervention with EGFR ligand in a clinical scenario; the findings impact is shadowed by not having a parallel, concurrent control group with a similar morbidity pattern in which EGF infiltration would have been replaced with a placebo solution. This was due to ethical reasons. Therefore, all the data presented here are based on a comparison of the different and progressive time-point samplings versus T0 in the same patient.

Activation of the EGFR has been shown to be critical in wound healing [31,32]. Within the context of diabetic chronic wounds, a sort of “tyrosine kinase paralysis” and EGFR functional deficiency has been demonstrated in fibroblasts exposed to a hostile glycation environment [7]. Thus, in diabetes-associated healing impairment, the EGFR/EGFR system has been shown to be affected because of low levels of EGF and EGFR cell signaling downregulation [7,33]. According to our data, EGFR is also scarcely detected in the fibroblasts derived from diabetic granulation tissue before receiving EGF treatment. This, in theory, may represent an additional factor contributing to the deterioration of signaling through this receptor pathway.

Although one of the limitations of this work is that T0 is the only reference control, the study suggests that intralesional EGF therapy induced the activation of EGF in FLCs early after EGF administration (from T15 to T60). First, there was an increase of EGF/EGFR immunolabeling upon EGF exposure. Second, early after EGF treatment, accumulation of EGF labeling was observed in the nucleus and to a lesser extent in other cytoplasmic compartments, suggesting EGF internalization and nuclear translocation. Moreover, EGF differential subcellular distribution and accumulation in the ECM, in a time-point manner, support the induction of an EGF-mediated activation program that is sustained for at least 24 h. Finally, detection of EGFR was related to a parallel immunolabeling increase and differential subcellular distribution of its downstream signaling target, PCNA. Accordingly, both EGFR and PCNA were particularly immunolabelled in the nucleus early after EGF treatment. These results converge with previous reports in cell culture systems showing that EGF increases EGFR gene and protein expression, and inhibits both EGF mRNA decay and degradation of internalized EGFR by lysosomes and proteasome [34].

Comparison of different cellular compartments at several time points following EGF therapy indicates a shift in gold labeling of EGFR early after EGF therapy, with fewer-than-expected tags in ER+GC and mitochondria, but more-than-expected in nucleus (Table 3). This suggests internalization and nuclear translocation of EGF. Interestingly, at later time points after EGF treatment (T6-T24), a shift in labeling showed more-than-expected gold particles in ER+GC.

<table>
<thead>
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<th>Compartment</th>
<th>EGFR (T15-T60)</th>
<th>EGFR (T6-T24)</th>
<th>Row total</th>
<th>EGFR (T15-T60)</th>
<th>EGFR (T6-T24)</th>
<th>PCNA (T15-T60)</th>
<th>PCNA (T6-T24)</th>
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<tbody>
<tr>
<td>No</td>
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<td>No</td>
<td>Expected Golds</td>
<td>X²</td>
<td>X²</td>
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<td>Expected Golds</td>
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<tr>
<td>ER+GC</td>
<td>178</td>
<td>209.2</td>
<td>315</td>
<td>283.8</td>
<td>493</td>
<td>4.64</td>
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<tr>
<td>M</td>
<td>26</td>
<td>50.9</td>
<td>94</td>
<td>69.1</td>
<td>120</td>
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<tr>
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<td>9.1</td>
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<td>1947</td>
<td>52.8</td>
<td>792</td>
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<td>714</td>
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For degrees of freedom (df)=4 (2-1 columns by 5-1 rows) and total chi-squared values for EGFR, (X²)=52.82, P<0.0001, and PCNA, (X²)=60.15, P<0.0001 (contingency table analysis). So, the null hypothesis (no difference between labelling distributions obtained at different time points) must be rejected. Compartmental X² values for EGFR indicate that the major contributors to the difference were the nucleus (40.73% of the total X²), mitochondria (40.07% of the total X²) and ER+GC (15.25% of the total X²). Note that FLCs analyzed at T15-T60 have fewer-than-expected gold particles in both ER+GC and mitochondria while FLCs analyzed at T6-T24 have more-than-expected gold particles in both ER+GC and mitochondria. Also note that FLCs at T15-T60 showed more-than-expected gold particles in nucleus while FLCs analyzed at T6-T24 showed fewer-than-expected gold particles in nucleus. Compartmental X² values for PCNA indicate that the major contributors to the difference are nucleus (47.13% of the total), mitochondria (35.2% of the total) and (14.81%). Note that FLCs analyzed at T15-T60 have fewer-than-expected gold particles in mitochondria and RC while FLCs analyzed at T6-T24 have more-than-expected gold particles in mitochondria and RC. Also note that FLCs at T15-T60 showed more-than-expected gold particles in nucleus while FLCs analyzed at T6-T24 showed fewer-than-expected gold particles in nucleus. No: Observed gold particles; P: Test Points; LD: Labelling Density; ER: Endoplasmic Reticulum; GC: Golgi complex; M: Mitochondria; RC: Rest of Cytoplasm; PM: Plasma membrane; N: Nucleus.

Table 3: Observed and expected distributions of gold particles for EGFR and PCNA in organelle compartments of fibroblast-like cells (FLCs) obtained either at early (from T15 to T60) or later time-points (from T6 to T24) after treatment with EGF and calculation of chi-squared (X²) values in contingency table analysis.
and mitochondria, but fewer-than-expected in nucleus, suggesting recycling of EGFR from nucleus to cytoplasm compartments and an increased trafficking of EGFR through the secretory pathway. In addition, detection of EGFR in various vesicles, endocytic vesicles, caveosomes-like structures, MVBs as well as phagosomes, supports the intracellular trafficking of the EGFR. These observations are in line with those derived from in vitro studies documenting an EGFR retrograde trafficking [35].

Previously, the EGF infiltration treatment has been proved to increase local EGF concentration in compromised tissues, promoting cytoprotection and cells proliferation [10]. Thus, EGF pharmacological concentration and EGFR levels in fibroblasts might possibly sustain increased receptor signaling (at least for 24 h after EGF treatment), cellular activation and the wound healing-related response observed in treated populations.

Results from this work showed EGFR immunolabeling as an exosome-related product. Several biological functions have been described for exosomes as mediators in intercellular signal communication by delivering proteins, lipids, mRNAs, microRNAs, and DNAs [36]. Thus, our finding presupposes that the accumulation of EGFR and EGFR-containing exosomes-like vesicles in the ECM might be potentially relevant. It would be interesting to study the function of these structures, as exosomes derived from mesenchymal stem cells have been shown to facilitate cutaneous wound healing [37,38]. Identification of EGFR in mitochondria of FLCs is another valuable finding of this work. Previous in vitro studies have found that EGFR localization to mitochondria is related to cell survival [39,40].

It is relevant to note that EGFR preferentially accumulated in the nucleus of FLCs early after EGF therapy. These results are in line with previous evidences from in vitro models showing that full length EGFR translocates to the cell nucleus after ligand binding [16,41]. Several functions of nuclear EGFR have been described. Firstly, it has been shown to operate as a co-transcription factor regulating the expression of various genes, including cyclin D1 [42]. Moreover, EGFR has been demonstrated to interact with DNA-dependent protein kinase (DNA-PK), leading to repair DNA double strand break [43]. Furthermore, nuclear EGFR has been described to phosphorylate chromatin-bound PCNA, thus increasing PCNA stability and enhancing cellular proliferation [41,42]. Nuclear localization of EGFR is consistent with the findings of this study, derived from the PCNA expression and intracellular trafficking in fibroblasts upon EGF infiltration.

The analysis of TF-gene regulatory interactions from the ENCODE project identified three of the transcription factors activated by the early EGF-EGFR signaling (STAT5, ELK1 and MYC) that bind the PCNA promoter to induce early PCNA transcription. Later on, EGFR also accumulates in nucleus and phosphorylates PCNA promoting increased cell proliferation.

A change in intracellular localization, from nucleus to cytoplasm, has also been observed for PCNA but not for other nuclear proteins in neutrophils and fibroblasts after serum starvation [45]. Interestingly, this intracellular PCNA localization was related to the anti-apoptotic activity of PCNA in neutrophils. Hence, the therapeutic effect of EGF might also be related to increased expression and the functions of cytoplasmic PCNA in fibroblasts from DFU.

Finally, activation of both EGFR and PCNA in our fibroblasts was detected up to 24 hours following EGF treatment, suggesting a sustained effect of this therapy. These data lend consistency to the current ulcer-infiltration protocol, based on every 48 hours scheme so as to its ensued therapeutic impact [13,15]. Conclusively, EGF infiltrative intervention appeared to increase the levels of EGFR in “dormant” wounds fibroblasts, so as its intracellular trafficking in a time-sequential manner. Furthermore, its activation and nuclear translocation suggest a role in activating PCNA and its ensued proliferative effect. Globally speaking, all these findings may theoretically account for the therapeutic ability of EGF to restore the healing process in DFU.

References


