Diabetic healing failure is the clinical expression of countless molecular and cellular disorders having hyperglycemia as the proximal trigger. The fibroblast is a critical building-block cell type for the healing process. Under high glucose concentrations fibroblasts physiology is perturbed. The epidermal growth factor receptor (EGFR) signaling system is crucial for different healing events. We examined the effect of high glucose burden on healthy-donor cutaneous fibroblasts proliferation, so as the EGFR autophosphorylation on a critical tyrosine residue, along with the activation of downstream signaling pathways proximal to cyclin D1 expression. Fibroblasts were cultured in 15 % FBS at either 5 (normal glucose) or 35 (high glucose) mM under standard culture conditions. Concurrent osmotic control was included. After 6 days of incubation under high glucose, doubling time was calculated. Cells suspensions were plated, fixed and immunolabelled with antibodies directed to phosphorylated forms of EGFR (Y1197), AKT1 (S473) and mTOR (S2448), and native forms of PI3K p85 alpha subunit and cyclin D1. The ratio of cells positive to the diaminobenzidine/peroxidase reaction was calculated and its intensity estimated according to published methodologies. High glucose concentration significantly increased doubling time 5-fold, as compared to cells grown in physiological conditions. Hyperglycemia reduced the constitutive EGFR autophosphorylation. Accordingly, PI3K was also significantly attenuated. Downstream switches AKT1 and mTOR were also affected and very significantly on the signal intensity. Cyclin D1 expression was completely abrogated due to high glucose burden. Collectively, these data suggest that high glucose exposure hinders fibroblasts proliferation by disrupting the EGFR/PI3K/AKT1/mTOR/Cyclin D1 axis.

**Keywords:** fibroblasts, hyperglycemia, diabetic ulcer, signaling pathways, EGFR
main functions is to secrete growth factors as vital messengers for mesenchymal and epithelial communication, especially for establishing the emerging basement membrane and the subsequent epithelial migration [9]. Consequently, any impediment to fibroblast functioning is detrimental for normal wound healing and may lead to wound chronication.

Under the high glucose burden imposed by DM, fibroblasts appear perturbed and for many years, in vitro models recreating "clinical hyperglycemia" have proved to disrupt normal fibroblasts physiology and derange the secretion of extracellular matrix ingredients. These experiments have suggested that acute and chronic exposure to high glucose concentration is the proximal toxic trigger for cutaneous fibroblasts’ demise [10-15]. Nevertheless, the molecular mechanisms underlying the glucotoxicity-associated fibroblasts’ proliferation reluctance and premature senescence have not been enlightened. Additionally, interfering epidermal growth factor receptor (EGFR) cascade acts as a restrictive factor for normal tissue repair by reducing cell proliferation among other factors [16]. Porto and co-workers successfully demonstrated that one of the advanced glycation end-products (AGEs) precursors, and vastly represented in diabetic patients circulation, abrogates EGFR tyrosine autophosphorylation and the subsequent activation of diverse downstream signaling kinases [17]. These findings encouraged us to examine the potential impact of a high glucose burden on the EGFR and downstream-related kinases phosphorylation, using primarily cultured human cutaneous fibroblasts.

Here we provide the first evidences that the exposure to a high glucose burden reduces the autophosphorylation ability of a catalytic tyrosine residue of the EGFR in dermal fibroblasts. Accordingly, subsequent underphosphorylation on downstream signaling substrates is detected along with an eventual suppression of cyclin D1 expression. These evidences provide a theoretical frame to explain, at least in part, the cytotoxic effects of high glucose on granulation tissue-producing cells. The EGFR in dermal fibroblasts. Subsequently, low glucose (1 g/L, equivalent to 5.5 mM) DMEM supplemented with 30 % fetal bovine serum (FBS), 1 × antibiotic - antimycotic solution, 2 mM glutamine, was added slowly to avoid tissue fragments detachment. Once fibroblasts sprouts started to emerge from each explant, cells were fed every 4-5 days and maintained in a humidified, 5 % CO2 atmosphere at 37 °C. The experiments described here were conducted with cells from healthy infants of non-diabetic parental background and under passages 3-5.

Hyperglycemia stress

Three independent and extemporaneous experiments were conducted under the same stressor conditions with cells on passages 3-5. When reaching approximately 90 % confluence, cultures were trypsinized and an homogeneous number of cells was plated in 96 plates with DMEM devoid of FBS for 48 h to synchronize cells’ cycle. Afterwards, medium was replaced and replenished with 15 % FBS under the following culture conditions: D-glucose at either 5 mM (normal glucose) or 35 mM (high glucose). Concurrent osmotic control consisted in fibroblasts exposed to 35 mM glucose as largely used [18]. After 6 days of “chronic” exposure to this high glucose burden, cells were quantified according to Trypan blue dyeing exclusion method and doubling time was calculated as described [19]. Cell suspensions adjusted to 3000 cells/25 μL were plated onto sterile siliconized slides (Dako, USA) in the same culture medium and incubated overnight for another 24 h in moist chamber to ensure attachment. Afterwards, the slides were washed in cold phosphate-buffered saline solution (pH 7.2; PBS), immersed in ice cold acetone/methanol 1:1 (v/v) for 10 min and air-dried at room temperature.

Immunocytochemistry

As the main goal of this experiment was to learn on the effect of high glucose concentration exposure on the EGFR and key signaling substrates, five antibodies were selected to study their posttranslational modifications (Table). They were purchased from Abcam (USA) and used at a 1:100 dilution, in a commercial antibody diluent solution (Abcam 64211).

The immunocytochemistry reaction was developed following the manufacturer’s instructions of a commercial anti-mouse & anti-rabbit HRP/DAB detection kit (Abcam 64264). Briefly, slides were washed in PBS for 15 min and cells permeabilized by immersion in PBS-Tween (0.1 % Tween 20) for 2 min at room temperature, and washed three times for 5 min with PBS. Endogenous peroxidase was quenched with the Dako Cytochemical peroxidase block solution for 15 min, and the slides were washed again for 15 min in PBS. Unspecific binding of the antibodies was neutralized using Abcam protein block for 25 min. Excessive blocking solution was decanted prior to the incubation for 40 min with the primary antibody, and the Di-aminobenzidine/peroxidase reaction was followed under the microscope. The slides were washed in running tap water for 5 min and counterstained with Mayer’s Hematoxylin.

Slides evaluation

Photographs of four to five non-overlapping areas of the plated cells were taken using a 40× magnification, with 30 % fetal bovine serum (FBS), 1 × antibiotic - antimycotic solution, 2 mM glutamine, was added slowly to avoid tissue fragments detachment. Once fibroblasts sprouts started to emerge from each explant, cells were fed every 4-5 days and maintained in a humidified, 5 % CO2 atmosphere at 37 °C. The experiments described here were conducted with cells from healthy infants of non-diabetic parental background and under passages 3-5.

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Photographs of four to five non-overlapping areas of the plated cells were taken using a 40× magnification,
to provide a representative picture of the immunolabeling scenario. Given the cell homogeneity of the experimental system, the absolute fibroblasts count in one specific area was considered an objective denominator to normalize the number of peroxidase immuno-stained cells. Labeled percentage of total cells per 40× fields were averaged from 4-5 fields as described [20]. Furthermore, cell immunoreactivity was qualitatively graded using the scale of Galkowska et al. [21] considering the following score system: 0, no expression; 1-2, low; 3-4, mild; 5-6, high. The microphotographs were taken and scored by two researchers (JBA and YMM) unaware of the treatment group, through an artificial code introduced by an external investigator.

Statistical analysis

Data were processed using GraphPad Prism, version 6.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Results were expressed as mean ± standard deviation. Normal distribution (D’Agostino-Pearson omnibus) and variance homogeneity (Brown-Forsythe) tests were performed. Once demonstrated the normality of the data, comparisons between experimental groups were carried out using the two-tailed unpaired Student’s t test. In all cases, p-values lower than 0.05 were considered statistically significant.

Results and discussion

We calculated doubling time as an expression of the proliferation rate. Under the present experimental conditions, normal cutaneous fibroblasts exposed to 5 mM glucose exhibited a doubling time of 5.71 ± 0.55 days. However, the high-glucose environment increased doubling time to 32.15 ± 3.07 days (p < 0.0001), regardless the 15 % FBS supplementation (Figure 1). In contrast, the presence of 35 mM glucose did not affect proliferation (not shown), indicating that osmotic damage could be ruled out.

This proliferative delay appeared concomitant to a 50 % reduction in the percentage of EGFR positive cells phosphorylated on tyrosine residue 1197 (Figure 2), as compared to cells incubated under normal glucose concentrations (p = 0.004). The immunolabelling intensity was also significantly reduced (p < 0.0001) as a consequence of high glucose exposure. As demonstrated in prior studies, high glucose levels are toxic for healthy donor-cutaneous fibroblasts, by depressing critical pathways controlling cells’ survival, metabolism and proliferation. The novelty of this study lies in the demonstration that, in this type of cells, EGFR is likely a proximal target of high glucose concentrations, reducing the autophosphorylation of its catalytic tyrosine residue tyrosine-1197, which cause the attenuation of a signaling cascade that drives changes in gene expression, especially in cell proliferation [22].

In line with this, high glucose stress significantly reduced PI3K (p85 alpha subunit) expression in terms of percentage of positively labeled cells (p = 0.02) and the associated immunoreaction intensity (p < 0.001) as referred to fibroblasts grown under 5 mM glucose (Figure 2). This finding may be interpreted as resulting from the EGFR reduced autophosphorylation, since the PI3K p85 alpha subunit is an adaptor molecule that regulates the activity of the PI3K catalytic p110 subunit by binding to phosphorylated receptor tyrosine kinases (RTKs) through its SH2 domain and mediating the interaction between p110 and the plasma membrane [23].

Downstream, the master switch kinase AKT1 was also affected under a high glucose environment in relation to normogluco-luxated fibroblasts. Although no statistical differences were detected (p = 0.11),...
Figure 2. Impact of a high glucose burden on the EGFR and downstream-related kinases on the proliferation of primary cultured, human healthy-donor’ cutaneous fibroblasts. Fibroblasts were cultured under normal (5 mM) or high (35 mM) glucose concentrations. Monoclonal antibodies (Abcam, USA) were used to detect the epidermal growth factor receptor (EGFR) phosphorylated on the catalytic tyrosine 1197, and the downstream substrates phosphatidyl inositol 3-kinase (PI3K), AKT1 phosphorylated on serine 473, and the mammalian target of rapamycin (mTOR) phosphorylated on serine 2448. A) Percentage of cells positive to each target. B) Qualitative grading of immunolabeling in normalized peroxidase immunostained human cutaneous fibroblasts, according to the scoring system by Galkowska et al., 2006. C and D) Immunocytochemistry images representative of fibroblasts cultured under 5 mM or 35 mM glucose, respectively (40×). Expression was far evident in the cellular cytoplasm for all the products, and in the nuclei for EGFR-Tyr 1197 and AKT1-Ser 473. Conversely, no expression was detected for all the products under hyperglycemic conditions. * ** *** p < 0.05, 0.01 or 0.001, respectively. Bars stand for 100 µm.
there was a 20 % reduction in the percentage of cells positive to the active isoform which is phosphorylated on serine 473. However, the immunointensity reaction appeared significantly decreased to less than 50 % (p < 0.001; Figure 2). The serine/threonine protein kinaseAkt1 is a major signal transducer of the PI3K pathway in all cells and tissues, and plays a pivotal role in the maintenance of cellular processes [24]. Akt1 is involved in controlling cell proliferation and survival, particularly by preventing apoptosis [25]. Although we found no statistical significance for the percentage of cells expressing phosphorylation on AKT1-serine 473 residue, it was quantitatively and qualitatively reduced in fibroblasts exposed to high glucose. This may stem from the upstream PI3K p85 expression level. Akt1 full activation requires two phosphatidylinositol-3,4,5-triphosphate-dependent phosphorylation events, while one of them relay on serine 473 [26, 27] for which the complex of the mammalian target of rapamycin (mTOR-Rictor complex) is critical [28].

We observed a similar behavior in the immunoreaction identifying one of the active isoforms of mTOR (specific phosphorylation on serine 2448). High glucose reduced the percentage of positive cells in about 23 %, with no statistical significance (p = 0.21). Moreover, the immunolabelling intensity score decreased to less than 50 % (p = 0.001) as compared to normal glucose environment fibroblasts (Figure 2). mTOR is a serine/threonine kinase which plays a key role as regulator of protein translation and sensor of nutrient status, thus controlling the balance between cell growth and autophagy [29-32]. The fact that signalization via mTOR and phosphorylation on serine 2448 is stimulated by growth factors and attenuated following amino acids starvation [33, 34], suggests the full disruption of this transduction axis from its proximal trigger; in this case, the EGFR. Conclusively, these findings highlight how intricate, yet finely regulated, the EGFR/PI3K/Akt/mTOR axis continuity is.

In addition, the high glucose-mediated cytotoxicity ultimately led to a complete inhibition of cyclin D1 expression (Figure 3A). This finding may theoretically explain the proliferative arrest detected in such cells. In contrast, under normal culture conditions, fibroblasts' cyclin D1 was found intensively and massively expressed in both nuclear and cytoplasmic compartments (Figure 3B). Cyclin D1 responds to tyrosine kinase-activated receptors by acting as a growth factor sensor [35-37]. In chronic wounds, where growth factors are scarce or their titers are below a threshold level, signals from the wound environment are not able to activate the cell cycle machinery and no cyclin D1 is synthesized [20, 38].

It has been previously shown that high glucose exposure attenuates tyrosine-phosphorylation of EGFR in embryonic rat fibroblasts expressing human insulin receptors [39]. In that experimental system, the authors also detected an attenuation of PI3K kinase activity, with no significant MAPK kinase activity variation between normal and high glucose exposed cells. However, glucose burden did not affect proliferation rate, since DNA content remained the same in all the experimental conditions.

In our study, we detected a clear cut effect of glucose burden on EGFR phosphorylation status, specifically on Y1197 residue, and on PI3K expression levels. This significant reduction was accompanied with a 5-fold lengthening in doubling time, distinctive of a negative effect on the cell proliferation rate. Since our main goal was to understand diabetic wound healing failure from a molecular point of view, other key effectors were studied downstream in the EGFR signaling pathway. We additionally observed that the exposure to high glucose affected the expression of phosphorylated active intermediates, such as AKT1 (S473) and mTOR (S2448) and the key cell cycle promoter cyclin D1.

There was demonstrated in in vitro models that synthesis, proliferative and secretory capabilities are reduced in diabetics’ cutaneous fibroblasts [40-44]. High glucose concentration in the culture medium negatively impacted on the physiology of normal fibroblasts, thus inhibiting proliferation and turning the cells resistant to growth factors-stimulated mitogenesis [45-48]. Other groups reported that high glucose reduced collagen synthesis in a dose-dependent manner [12, 49]; and that cell migration speed is reduced by nearly 40 %, associated to a decrease in cell directionality and to non-productive protractive events due to cells’ polarization failure [50].

The mechanisms whereby high glucose concentrations can impair fibroblasts physiology, including proliferative capabilities, are not fully elucidated but had been associated to high L-lactate production [10, 44, 51] and the generation of cytotoxic reactive oxygen species [13, 14, 52]. We deem, however, that adds formation between EGFR and AGEs precursors could be relevant to explain our findings. Previous experiments have proved significant AGEs accumulation in endothelial cells cultured for 7 days under glucose overload [53, 54] and that AGE modification of growth factors drastically reduced the cells mitogenic activity by 70 % [55, 56]. Obviously, this hypothesis requires intracellular AGEs concentration measurements and their chemical interaction with the EGFR in a time-window manner.

Taken together, our data show that high glucose concentration can hinder fibroblasts' EGFR autophosphorylation and, consequently, deteriorate the activation of critical downstream pathways that may lead to fibroblasts proliferation delay and wound chronification. In a translational manner for clinical medicine, 23. Arcaro A, Guerreiro AS. The phosphoinositide-3 kinase pathway in human cancer: genetic alterations and therapeutic implications. Curr Genomics. 2007;8(5):271-306.
these findings emphasize on the need of a strict metabolic control of the diabetic patient affected by diabetic foot ulcers in order to facilitate the healing mechanism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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