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Induction by innate defence regulator peptide 1018 of pro-angiogenic molecules and endothelial cell migration in a high glucose environment

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ABSTRACT

Synthetic innate defence regulator (IDR) peptides such as IDR-1018 modulate immunity to promote key protective functions including chemotaxis, wound healing, and anti-infective activity, while suppressing pro-inflammatory responses to non-pathological levels. Here we demonstrated that IDR-1018 induced, by up to 75-fold, pro-angiogenic VEGF-165 in keratinocytes but suppressed this isoform in endothelial cells. It also induced early angiogenin and prolonged anti-inflammatory TGFβ expression on endothelial cells, while suppressing early pro-inflammatory IL-1β expression levels. IDR-1018 also down-regulated the hypoxia induced transcription factor HIF-1α in both keratinocytes and endothelial cells. Consistent with these data, in an in vitro wound healing scratch assay, IDR-1018 induced migration of endothelial cells under conditions of hypoxia while in epithelial cells migration increased only under conditions of normoxia.

1. Introduction

Impaired wound healing is a common complication in patients with diabetes and is associated with increased morbidity and mortality. Poor wound healing may result in the development of chronic diabetic foot ulcers (DFUs), which are clinically challenging to manage and may lead to amputations and in some cases to death [2]. Many factors have been shown to be involved in the poor wound-healing ability of diabetic patients, including the hyperglycemic environment, chronic inflammation, wound infection, hypoxia, sensory neuropathy, abnormal neuropeptide signaling and impaired angiogenesis [15,17].

Angiogenesis is the formation of new blood vessels from a pre-existing vascular bed. In quiescent blood vessels, there is a fine balance between angiogenic growth factors and inhibitors. When pro-angiogenic factors are up-regulated and anti-angiogenic factors are down-regulated, angiogenesis is induced. Various angiogenic factors are produced by both endothelial cells and keratinocytes, including vascular endothelial growth factor (VEGF), in both its pro-angiogenic (VEGF165) and anti-angiogenic (VEGF165b) isoforms, and angiogenin (RNase 5) [14,37]. The defective angiogenesis seen in diabetes has been associated in part with defective recruitment and migration of endothelial cells. Based on this issue several research groups have studied new therapies to promote angiogenesis in these patients.

In recent years, several synthetic innate defence regulator (IDR) peptides have been generated that promote key protective functions such as chemotaxis, wound healing, and anti-infective activity mediated by the immune system, while suppressing pro-inflammatory responses to non-pathological levels [29,32]. One of most studied of these peptides is IDR-1018 (VRLIVAVRIWRR-NH2) which is a promising candidate for clinical use based on its minimal cytotoxic activity, ability to significantly reduce pro-inflammatory cytokines, enhanced resolution of infection and inflammation in animal models and importantly, promotion of wound healing [32,36] and protection from traumatic brain injury [25]. Intriguingly this wound healing property did not occur in diabetic mice [32,36].

Here we determined whether IDR-1018 induced pro- or anti-angiogenic molecules as well as pro-inflammatory cytokines in human keratinocytes and endothelial cells and whether this production promotes cell migration in glucose rich, hypoxic and normoxic atmospheres.

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Table 1
PCR primers and probes utilized.

Protein and Main Function	F-Primer	R-Primer	Probe sequence
Interleukin-1β (IL-1β); Pro-inflammatory cytokine; Component of inflammasome	TCTTTGGGTAATTTTTGGGATCT	TACCTGTCCTGCGTGTTGAA	TTCCTGGC
Tumor necrosis factor-α (TNF-α); Pro-inflammatory cytokine.	GCCAGAGGGCTGATTAGAGA	CAGCCTCTTCTCCTTCCTGAT	CTTCTGCC
Transforming Growth Factor-β (TGF-β); Anti-inflammatory cytokine	CAGCCGGTTGCTGAGGTA	GCAGCACGTGGAGCTGTA	AGCTGGAG
Vascular endothelial growth factor A, variant 4 (VEGF-A165); Potent pro angiogenic factor	GCAGCTTGAGTTAAACGAACG	GGTTCCCGAAACCCTGAG	GGAAGGAG
Vascular endothelial growth factor A, variant 7 (VEGF-A165b); Potent anti-angiogenic factor	GAGTTAAACGAACGTACTTGCAGA	TCAGGTTTCTGGATTAAGGACTG	CATCACCA
Angiogenin/RNase5 (ANG); Potent stimulator of angiogenesis; the formation of new blood vessels.	CATTGTCCTGCCCGTTTC	CAGCACGAAGACCAACAACA	CATCACCA

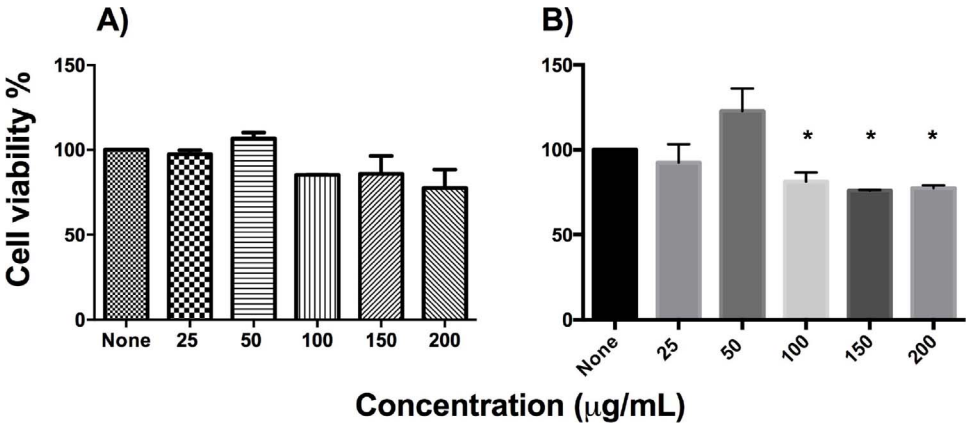

Fig. 1. Cell viability in keratinocytes and endothelial cells after treatment with IDR-1018. Using a WST-1 colorimetric assay, the viability of cells was determined after 24 h of stimulus with IDR-1018 for keratinocytes (A) and for endothelial cells. Results are represented as mean ± SD of 5 independent experiments performed in duplicate. *$p < 0.05$.

2. Material and methods

2.1. Cell cultures and stimulation

Human keratinocyte cell line (HaCaT, Cell Lines Service, Eppelheim, Germany) and the human endothelial cell line, (EA.hy926, ATCC, CRL-2922) were seeded into cell culture flasks (Corning, NY, USA) and cultured using high glucose Dulbecco's Modified Eagle's Medium, containing 24.97 mM glucose (DMEM HG 1X Gibco, Manassas, VA), supplemented with 10% fetal bovine serum (FBS) (Corning, Manassas, VA), Endothelial Cell Growth Supplement (ECGS, Sigma-Aldrich, Germany); 100 IU/L of penicillin and 100 mg/L of streptomycin (Gibco, Carlsbad, CA) was added for EA.hy926 cells. Incubations were performed in humidified 5% CO_2 at 37 °C and once cells reached 80–85% of confluence they were disaggregated with 0.05% trypsin-EDTA (Corning Manassas, VA). Subsequently 1×10^5 cells/well were seeded in 24 well plates and treated with different concentrations of IDR-1018 (25 or 50 μg/mL); Viability was assessed using a colorimetric cell proliferation and viability kit WST-1 (Roche, Mannheim, Germany) after treatment with 25, 50, 100, 150 or 200 μg/mL of IDR-1018.

2.2. Hypoxic conditions

For the assay under hypoxic conditions (1% O_2), cells were treated as described above. To create a hypoxic atmosphere, cells were placed in a hypoxia chamber (Panasonic MCO-5M-PE, New Brunswick, NJ), in humidified 5% CO2 at 37 °C for 24 h.

2.3. Western blot analysis

For Western blot analyses, cells were lysed in RIPA buffer (10 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 10 mM NaF, 0.05 mM PMSF) with added protease inhibitor cocktail (Promega, Madison WI). Equivalent amounts of protein were electrophoresed on SDS 10% polyacrylamide gels (Biorad, Hercules CA). The gels were then electroblotted onto nitrocellulose membranes (0.45 μm Biorad, Hercules, CA). After blocking with 5% milk for 30 min at room temperature, membranes were incubated with specific primary anti-human hypoxia induced transcription (HIF) −1α antibody (1:500; Abcam, Kendal Square, Cambridge, UK). The protein was then visualized by incubating with a secondary horseradish-peroxidase-labeled antibody (Goat anti-mouse 1:5000, Thermo Scientific, Rockford, IL) for 1 h at room temperature. The reactions were detected using an enhanced chemiluminescence assay (Super Signal West Femto, Thermo Scientific Rockford, IL). The densitometry analysis was carried using the Image-Lab software version 5.2.1 2014 from Bio-Rad Laboratories with automatic quantification tool, signal value of each band was normalized using β-actin as normalization protein.

2.4. RNA isolation, reverse transcription and gene expression analysis by real time PCR

Total RNA from each culture was extracted with Trizol (Invitrogen, Auckland, NZ) according to the manufacturer's instructions. Reverse mRNA transcription was performed using 1 μg total RNA, 2 μM Oligo (dT) 15 primer (Promega, Ontario, Canada), 10 mM deoxynucleotide triphosphates, 10 units ribonuclease inhibitor (Invitrogen, Carslbard, CA) and 4 units Omniscript Reverse Transcriptase (Qiagen, Mexico). Real-time qPCR was performed with a LightCycler 480 thermocycler (Roche Applied Science Inc, USA), using specific hydrolysis probes and primers (Table 1). Both primers and probes were newly designed using universal probe library software (Roche Applied Science Inc., USA). All data were analyzed using the expression of *hypoxanthine phosphoribosyl transferase (HPRT)* as a reference gene and an internal control. Relative quantification of gene expression was performed by the comparative quantification cycle (Cq) method, using the formula, $2^{-\Delta\Delta CT}$ as previously described [24]. This method is based on the expression levels of a target gene vs. a reference gene (*HPRT*) comparing between treated and untreated cells. The comparative threshold cycle method was used to assess relative changes in mRNA levels between untreated cells

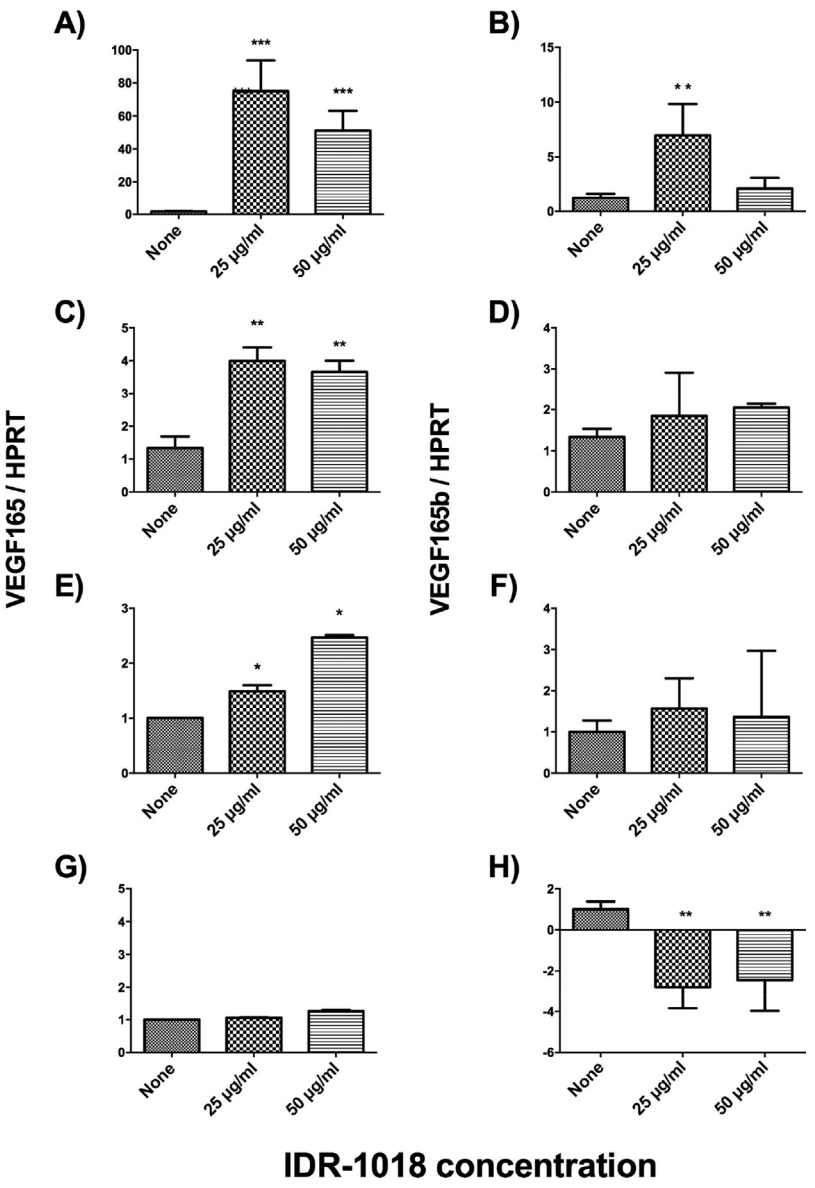

Fig. 2. Induction of the VEGF isoforms in keratinocytes. Keratinocytes (HaCat) were grown in high glucose media and stimulated with IDR-1018 for 1 (A and B), 6 (C and D), 12 (E and F) and 24 (G and H) hours and the expression of the proangiogenic isoform VEGF165 and the anti-angiogenic isoform VEGF165b was analyzed by RT-qPCR. Results are represented as mean ± SD of 5 independent experiments performed in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001.

(control) reflected in fold changes. Thus, untreated cells were uniformly normalized to a value of 1. The PCR products from the VEGF isoforms were isolated and submitted for sequencing to confirm sequence (Data not shown), this is especially important when the analytes are RNA splice variants with subtle distinctions.

2.5. In vitro wound closure assay

The in vitro wound closure assay was performed as described before [15]. Briefly, cells were seeded onto fibronectin-coated 24-well tissue culture plates in DMEM supplemented with 10% FBS until they reached 100% confluence. To avoid cell proliferation, the cells were pre-treated with mitomycin C (3.5 µg/mL) (Sigma-Aldrich, St. Louis MO, USA) for 2 h in humidified 5% CO2 at 37 °C. Then, the confluent monolayer of cells was scratched with a sterile 200 µL-pipette tip to create a uniform cell-free zone in each well. The cultures were washed twice with Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis MO, USA) and re-coated with fibronectin (5 µg/mL) for 1 h at 37 in humidified 5% CO2 at 37 °C. At this time point (t = 0 h) wound margins were photo-documented. Then, cells were treated for up to 72 h at 37 °C and 5% CO_2 with IDR-1018 (25 and 50 µg/mL, diluted in cell culture media) or, as positive controls, 40 ng/mL VEGF (Peprotech, Mexico) for EA.hy926 cells or 20 ng/µL EGF (Peprotech Mexico) HaCaT for cells. The re-population (cell migration) of wounded areas was observed under an inverted microscope (Leica, Germany) and the same fields of the wound margin were photo-documented at 24, 48 and 72 h. Each image was measured using Leica Application Suite Imaging Software version 4.4.0.

2.6. Statistical analysis

Normality of distribution for all data sets was analyzed using a non-parametric Kolmogorov-Smirnov test. This was followed by non-parametric multiple comparison test Kruskal-Wallis to identify differences between groups. In the case of finding statistical significance ($p < 0.05$), a Dunn's post hoc test was performed. Two-sided p values of < 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism Software (version 5.02, San Diego, CA).

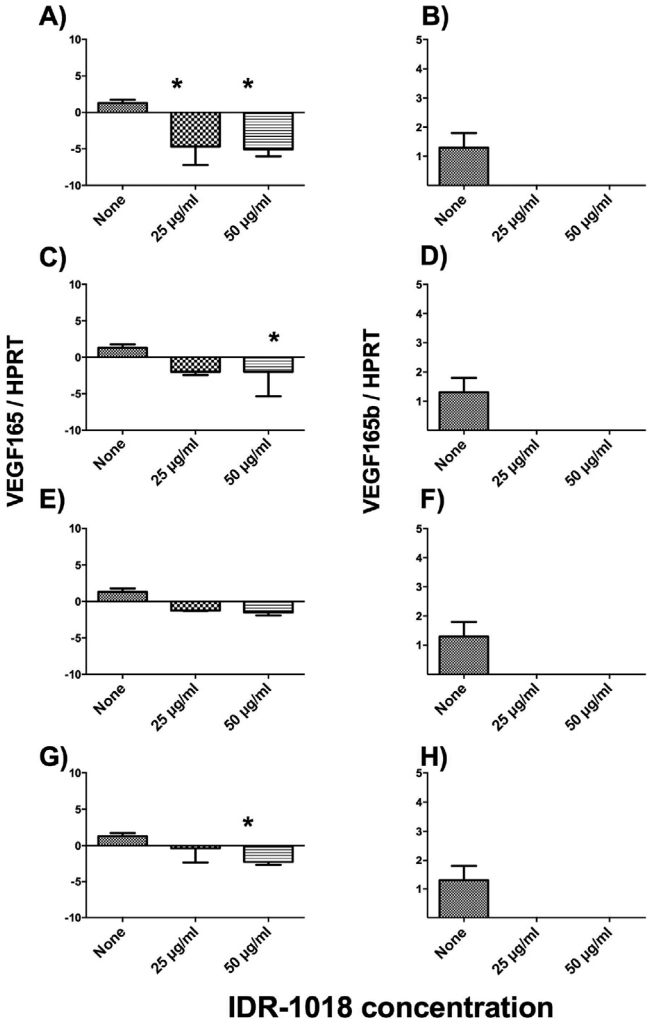

Fig. 3. Induction of the VEGF isoforms in endothelial cells. Endothelial cells were grown in high glucose media and stimulated with IDR-1018 for 1 (A and B), 6 (C and D), 12 (E and F) and 24 (G and H) hours and the expression of the proangiogenic isoform VEGF165 and the anti-angiogenic isoform VEGF165b was analyzed by RT-qPCR. Results are represented as mean ± SD of 5 independent experiments performed in duplicate. *$p < 0.05$.

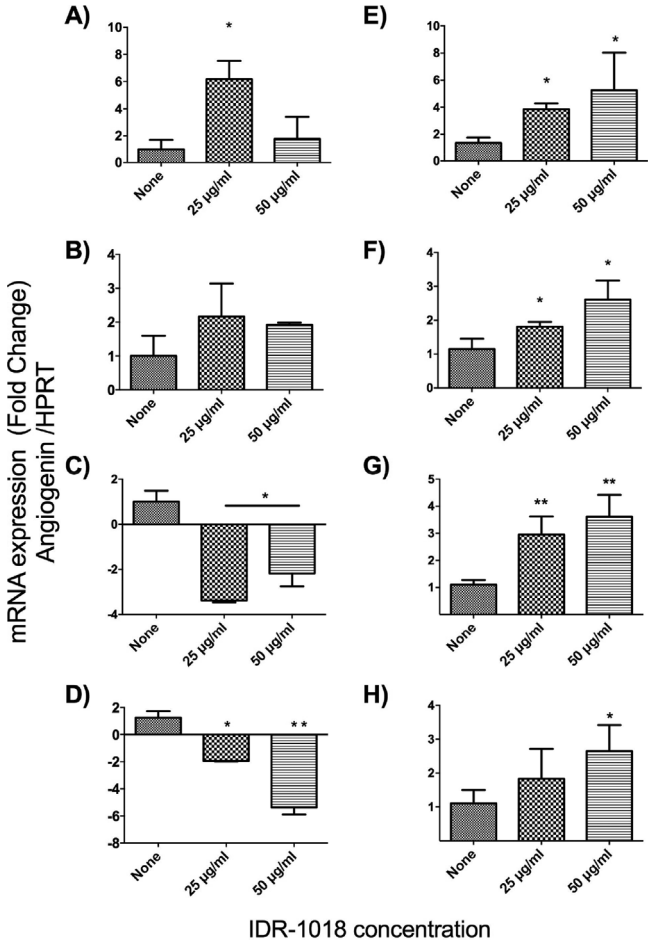

Fig. 4. Induction of angiogenin in keratinocytes and endothelial cells by IDR-1018. Keratinocytes and endothelial cells were grown in high glucose media then stimulated with different concentrations of IDR-1018. Keratinocytes and endothelial cells were incubated for 1 (A and E), 6 (B and F), 12 (C and G) and 24 h (D and H respectively). Results are represented as mean ± SD of 5 independent experiments performed in duplicate. *$p < 0.05$, **$p < 0.01$.

3. Results

3.1. Induction of VEGF isoforms by IDR-1018 in keratinocytes and endothelial cells

First, the toxicity of IDR-1018 against cells was evaluated by assessing residual respiratory activity with the WST-1 assay at different concentrations. Our results showed that in keratinocyte IDR-1018 did not significantly change viability at any concentration used (Fig. 1A), while in endothelial cells viability was decreased by ~20% at concentrations of 100–200 µg/mL (Fig. 1B); therefore, subsequent experiments were performed at 25 and 50 µg/mL peptide. Next, we evaluated 2 key growth factors involved in angiogenesis in both keratinocytes and endothelial cells. The results showed that IDR-1018 increased vascular endothelial growth factor (VEGF) 165 expression by 75-fold at 25 µg/mL after an hour of stimulation (Fig. 2A) while more modest increases of 4 and 1.5 fold were observed after 6 and 12 h stimulation respectively (Fig. 2C and E). No changes were seen after 24 h of stimulation (Fig. 2G). Regarding the anti-angiogenic isoform VEGF165b, after an hour of stimulation there was a 7-fold increase at 25 µg/mL of IDR1018 (Fig. 2B) but at 6 and 12 h (Fig. 2D and F) no changes were observed while after 24 h IDR-1018 decreased the expression of VEGF165b (Fig. 2H). The same general trends were observed at 50 µg/mL IDR-1018 but increases were smaller. In endothelial cells the induction of both isoforms, was suppressed by IDR-1018 at all timepoints (Fig. 3A–D), while anti-angiogenic isoform was abrogated by IDR-1018 treatment (Fig. 3B–F).

3.2. IDR-1018 induced angiogenin in endothelial cells

Angiogenin, primarily expressed by endothelial cells, is one of the most potent angiogenic factors and has an important role in regulating vessel maturation, endothelial cell migration, adhesion and survival [35]. Therefore, we sought to determine whether IDR-1018 induced this molecule. It was observed that in keratinocytes, IDR-1018 induced angiogenin gene (ANG) expression by 4- to 5- fold relative to untreated cells after 1 h of stimulation with 25 µg/mL of the peptide (Fig. 4A). No significant changes were detected after 6 h of stimulation (Fig. 4B), and after 12 and 24 h of stimulation, ANG gene expression was actually down-regulated at both concentrations of IDR-1018 (Fig. 4C and D respectively). In contrast in endothelial cells, IDR-1018 increased the expression of ANG at all the investigated times at both concentrations (except 24 h treatment with 25 µg/mL) (Fig. 4E–H).

3.3. IDR-1018 increased TGF-β expression in endothelial cells

Chronic wounds tend to become chronically inflammed, producing

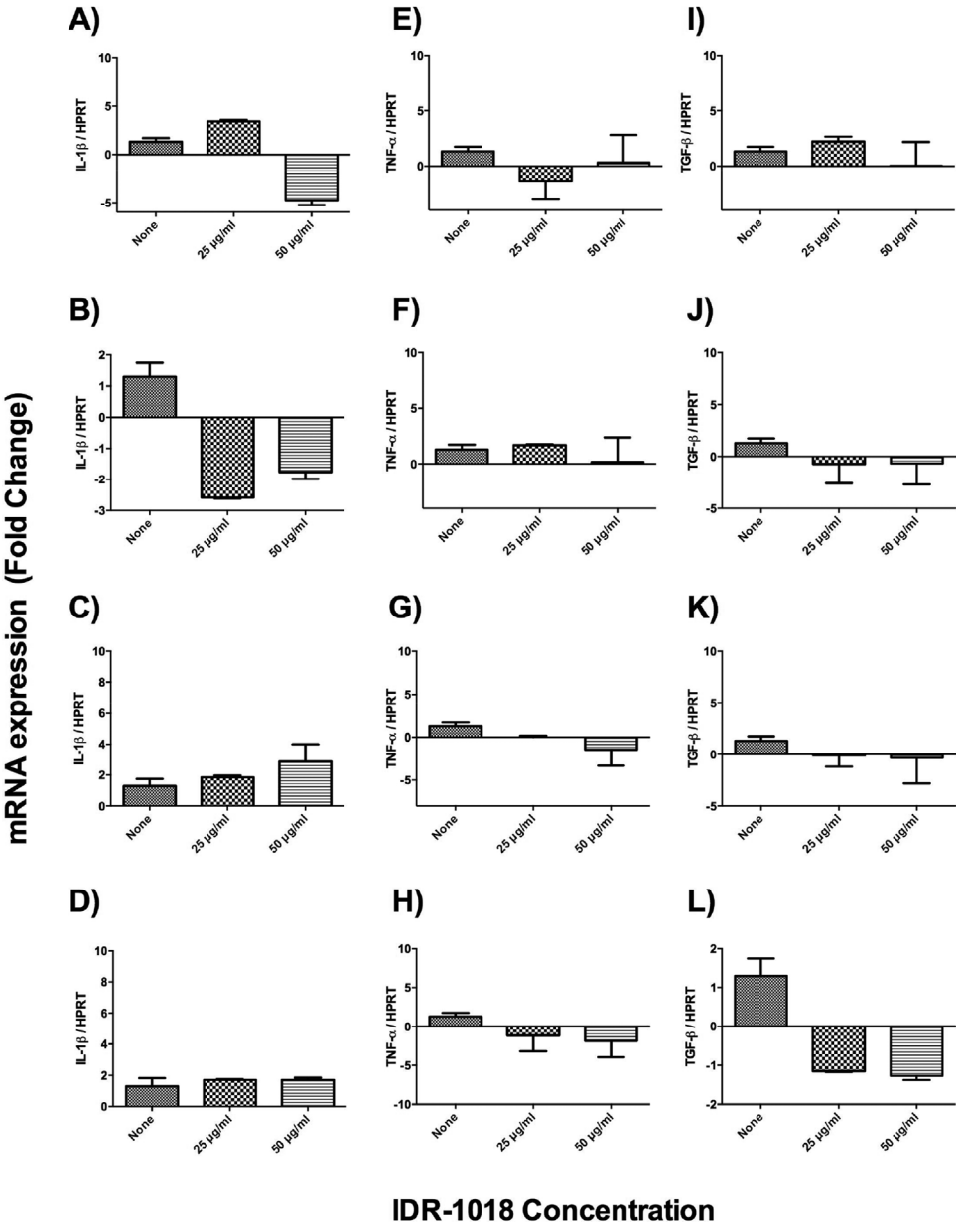

Fig. 5. Expression of cytokines in keratinocytes stimulated with IDR. Keratinocytes were grown in high glucose media then stimulated with different concentrations of IDR-1018. IL-1β, TNF-α and TGF-β gene expression was evaluated after stimulation for 1 (Panel A, E, I), 6 (Panel B, F, J), 12 (Panel C, G, K) and 24 h (Panel D, H, L). Results are represented as mean ± SD of 5 independent experiments performed in duplicate.

an environment that favors proteolysis due to the influx of inflammatory cells and to the chronic up-regulation of pro-inflammatory cytokines. These events inhibit the wound healing process, due to the degradation of growth factors and cytokines, thus diminishing their functionality [4,18]. Therefore, we evaluated the effects of IDR-1018 on the expression by keratinocytes and endothelial cells of cytokines IL-1β, TNF-α and TGF-β. The results showed that IDR-1018 did not induce significant changes in the expression of these cytokines in keratinocytes (Fig. 5). Similar results were obtained when cytokines were measured in supernatants from stimulated cells by ELISA (S1). With regards to endothelial cells, IL-1β expression levels were down-regulated after 1 h of stimulation at both IDR-1018 concentrations (Fig. 6A). No significant changes were observed after 6, 12 or 24 h of stimulation (Fig. 6C, E and G, respectively). Gene expression of TGF-β was significantly ($p < 0.05$) increased by greater than 2-fold by treatment with 50 μg/mL IDR-1018 for 1, 6, and 12 h (Fig. 6B, D and F, respectively), when compared with non-treated cells. Similarly after 24 h of stimulation, TGF-β expression increased significantly using both IDR-1018 concentrations (Fig. 6H). Similar results were obtained when cytokines were measured in supernatants from stimulated cells by ELISA (S2). No IDR-1018-induced TNF-α gene expression was detected in these cells (data not shown).

3.4. IDR-1018 down-regulated HIF-1α in keratinocytes and endothelial cells

One of the major components hindering healing of chronic wounds, such as DFUs, is the accompanying tissue hypoxia [9]. Prolonged hypoxia is often the result of decreased tissue perfusion and inadequate angiogenesis. The reason is that hypoxia can amplify and prolong the acute inflammatory response and thereafter inhibit the progression of healing [1]. To evaluate the effect of IDR-1018 on the expression of the key HIF-1α, cells grown under hypoxia conditions (1% 0_2) for 24 h in the presence of IDR-1018, cells were lysed and intracellular extracts were submitted for Western blot analysis. Densitometric analysis showed that both concentrations of IDR-1018 led to significantly decreased HIF-1α levels ($p < 0.01$ and $p < 0.001$, respectively) by around 3- to 4-fold in keratinocytes under hypoxic conditions (Fig. 7A). In endothelial cells, both concentrations of IDR-1018 significantly decreased HIF-1α in hypoxic conditions ($p < 0.01$), however in normoxia only 50 μg/mL of IDR decreased ($p < 0.05$) HIF-1α levels

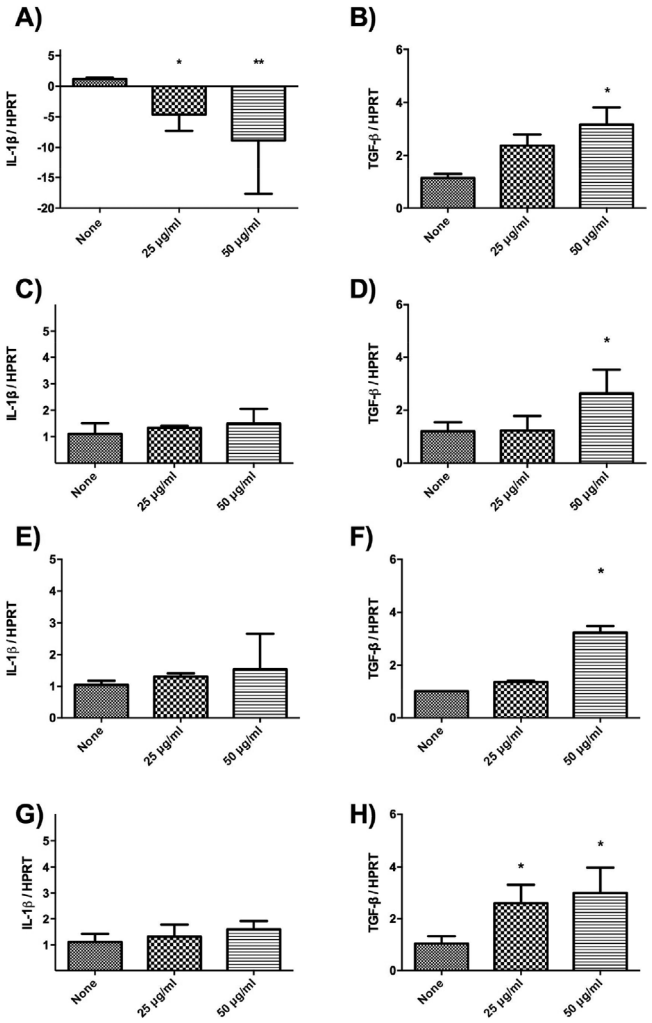

Fig. 6. Expression of IL-1β and TGF-β in endothelial cells. Endothelial cells were grown in high glucose media then stimulated with different concentrations of IDR-1018. IL-1β and TGF-β gene expression was evaluated after stimulation for 1 (Panels A and B), 6 (Panels C and D), 12 (Panels E and F) and 24 h (Panels G and H). Results are represented as mean ± SD of 5 independent experiments performed in duplicate. *p < 0.05, **p < 0.01.

(Fig. 7B).

3.5. IDR-1018 promoted cell migration in wounded endothelial cells

As shown above, IDR-1018 led to the induction of pro-angiogenic factors. Therefore we asked whether this peptide induced cell migration in wounded endothelial cells and keratinocytes, under both hypoxia and normoxia conditions. For all the experiments confluent layers of cells were scratched to create a denuded area and then the migration of cells into this denuded area was followed over time, using recombinant VEGF or EGF as positive controls with unstimulated (PBS treated) cells as the negative control. In keratinocytes there were statistically significant differences in cell migration of IDR-1018 treated keratinocytes when compared to PBS-stimulated cells, under conditions of normoxia (Fig. 8A) and hypoxia (Fig. 8B). In endothelial cells, 25 μg/mL of the peptide, induced significant migration under conditions of normoxia, showing a statistically significant increase in migration/wound healing after 24, 48 and 72 H of stimulation ($p < 0.01$, Fig. 8C). Similar results were obtained for endothelial cells under conditions of hypoxia, showing statistically significant differences after 48 and 72 h of infection (Fig. 8D). Representative microphotographs of the migration assay are shown in Fig. 9A–L.

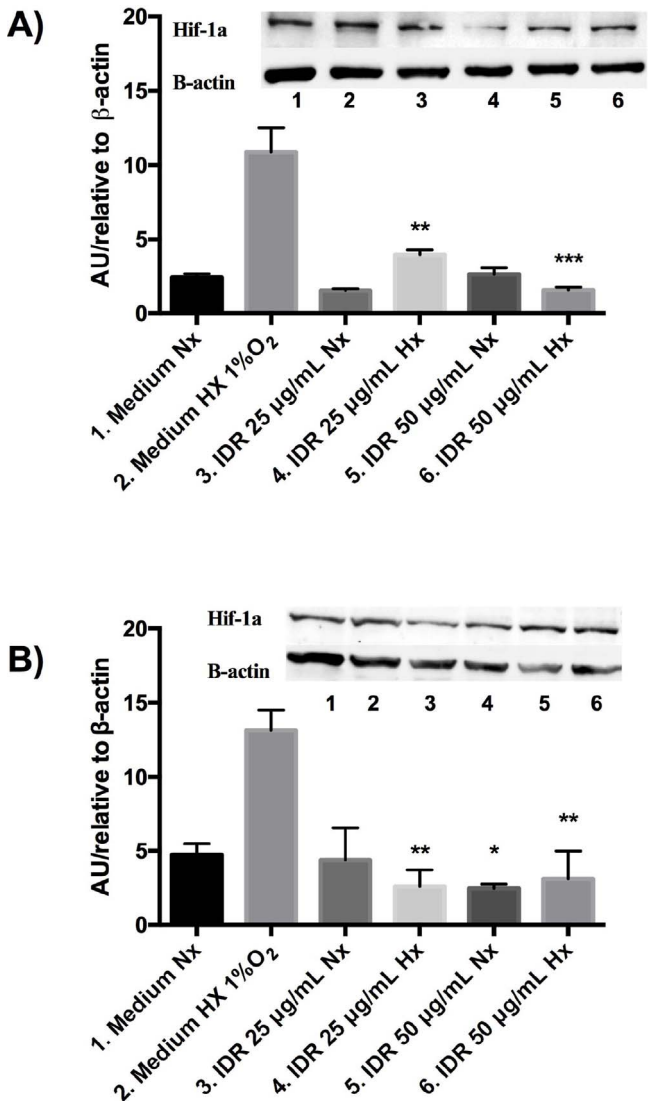

Fig. 7. Down-regulated of Hif-1α by IDR-1018 in keratinocytes and endothelial cells. Keratinocytes and endothelial cells were grown under hypoxic (HX) and normoxic (NX) conditions and stimulated for 24 h with 25 and 50 μg/mL of IDR-1018. The presence of HIF-1α, was evaluated with a Western blot assay and quantified by densitometry. Results are represented as mean ± SD of 3 independent experiments. IDR-1018 stimulated cells were compared with their respective non-treated condition. *p < 0.5, **p < 0.01, ***p < 0.001.

4. Discussion

In the past few years, there has been an increased effort to design and develop synthetic peptides, termed innate defence regulatory peptides (IDR), with enhanced immunomodulatory activities and modest or no antimicrobial activity, using natural antimicrobial peptides as templates [42]. However, several research groups have investigated and attributed other functions to IDRs, for instance it has been demonstrated that in vivo, IDR-1018 demonstrated significantly accelerated wound healing in mice and *S. aureus* infected porcine [36]. This study suggests that IDR-1018 promotes wound healing independent of direct antibacterial activity, interestingly, these effects were not observed in experimental wounds in diabetic mice [36]. The mechanism by which IDR-1018 induces wound healing in these models needs to be further elucidated.

Many factors have been shown to be involved in the poor wound healing ability of diabetic patients and chronic wounds, including a hyperglycemic environment, chronic inflammation, wound infection,

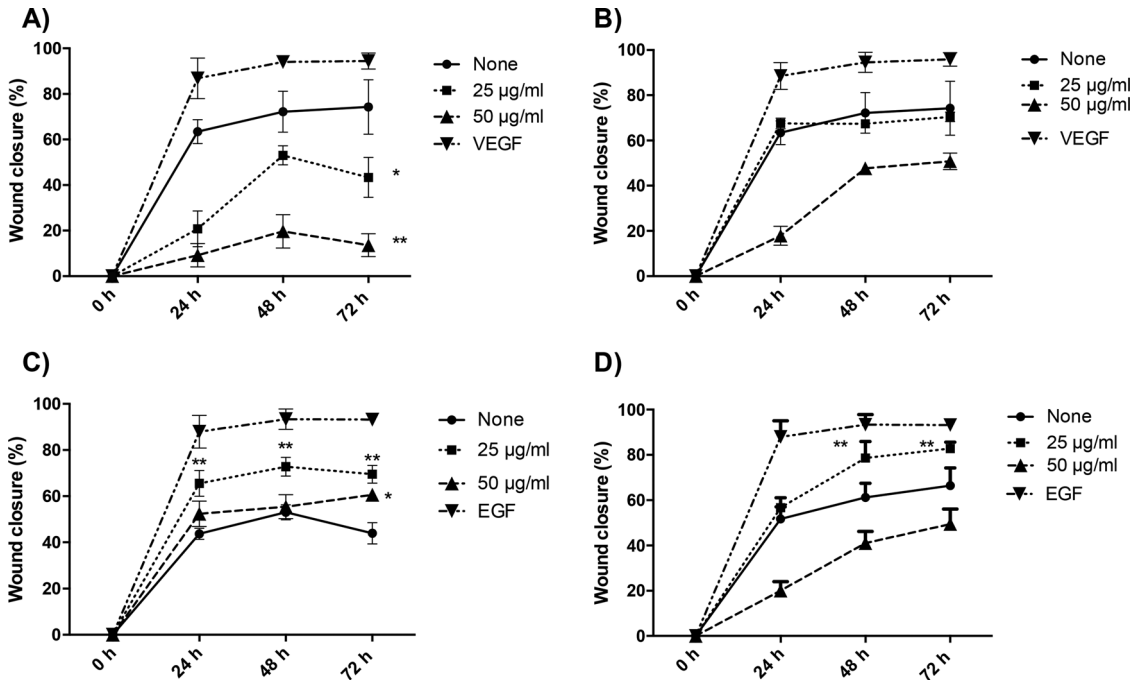

Fig. 8. IDR-1018 promoted endothelial cell, but not keratinocyte, migration in the scratch model under both hypoxic and normoxic conditions. Keratinocytes in normoxia (A) or in hypoxia (B) and endothelial cells in normoxia (C) and in hypoxia (D) were cultured as described in Methods and the in vitro closure assay was carried out measuring the percentage of closure under each experimental condition: IDR-1018 at 25 µg/mL and 50 µg/mL, none = untreated cells and rVEGF or rEGF used as positive controls. Results are represented as mean ± SD of 3 independent experiments performed in duplicate. *$p < 0.05$ and ** $p < 0.01$.

vascular insufficiency, a decreased levels of cathelicidin, hypoxia, sensory neuropathy, and abnormal neuropeptide signaling [3,33]. One of the most important factors to achieve wound healing is a proper blood vessel formation, i.e. angiogenesis. It has been reported that this process is impaired in diabetic wounds, while the administration of angiogenic factors to diabetic mice promotes angiogenesis and stimulates wound healing [14]. In the present study, we wanted to determine whether IDR-1018 induced pro-angiogenic molecules and endothelial cell migration at non-toxic concentrations. First, confirmed the cytotoxicity of IDR-1018 for the cells used in this study. As reported before [36] IDR-1018 had insignificant toxicity for keratinocytes at concentrations as high as 200 µg/mL. However in the endothelial cell line toxicity was higher, so we decided to use non-toxic concentrations of 50 µg/mL and below. Although we used several positive and negative controls during the development of our experiments, we could use IDR-1 as a negative peptide given that this peptide does not significantly alter immune responses similar to IDR-1018 [41], nevertheless the controls used are good and discern well between treated and not treated cells.

VEGF (or VEGF-A) is one of the most potent factors associated with angiogenesis and is considered a key regulatory molecule for both physiological and pathological angiogenesis. The *VEGF* gene gives rise to more than 12 isoforms of VEGF grouped into two major families due to differential RNA splicing. In general terms VEGFa isoforms promote angiogenesis whereas VEGFb isoforms are anti-angiogenic [12,16,31]. Angiogenesis is necessary for formation of granulation tissue as well as for providing oxygen and nutrition to wounds. Inadequate angiogenesis in diabetic patients inhibits wound healing [23]. Indeed, it has been demonstrated that the induction of VEGF165 using adenovirus vector (ADV)-mediated gene transfer, accelerates experimental wound healing in a diabetic mouse model [7], In the present study we showed the ability of IDR-1018 to induce VEGF165 in keratinocytes; we detected a downregulation of this growth factor in endothelial cells. Interestingly IDR-1018 abolished the production of the anti-angiogenic VEGFb isoform in endothelial cells and decreased the expression in keratinocytes at 24 h. This indicates that IDR-1018 inhibits the anti-angiogenic isoform whilst moderately promoting VEGF in keratinocytes, which should promote angiogenesis and wound healing. Nevertheless, we asked whether other pro-angiogenic molecules, such as the potent angiogenic molecule angiogenin [13], could be up-regulated by IDR-1018 since angiogenin can induce new vessel formation at femtomolar doses. Indeed IDR-1018 significantly induced the expression of angiogenin largely in endothelial cells. Since angiogenin is able to protect against neuron degeneration through the promotion of angiogenesis, neurogenesis and neuronal survival under conditions of stress [35], and given that these features are dampen in chronic wounds such as DFU, thus IDR-1018 has a promising therapeutic use for this condition, however noteworthy to have in mind that angiogenin influences nearly all steps of tumorogenesis including protecting tumor cells from poor survival conditions, promoting tumor cell proliferation, enhancing tumor cell migration and invasion [19,40], thus for future clinical research it is important to avoid the use of IDR-1018 by systemic delivery instead it is important to evaluate local administration.

As mentioned above, in chronic wounds the chronic up-regulation of pro-inflammatory cytokines inhibits the wound healing process degrading growth factors [18]. Taking this reasoning into account based on data showing that IDR-1018 is able to modulate immune response [42], we determined IL-1β, TNFα and TGF-β in our cell models. Results indicated that in keratinocytes there were no significant changes whereas in endothelial cells IDR-1018 promoted mRNA expression of anti-inflammatory TGF-β, which affects cell regulation, growth, differentiation, angiogenesis, extracellular matrix formation, and the immune response and it has shown to promote wound healing [10,39]. Therefore, IDR-1018 promoted several molecules involved in the optimal wound healing. We found no significant changes in IL1-β, which is a cytokine that is overexpressed during inflammation. However this is consistent with data in previous studies showing little effect on pro-inflammatory cytokines except to suppress production of these cytokines after their induction with an appropriate inflammatory stimulus [5,25] however unlike the situation for chronic wounds we did not stimulate inflammation in our studies.

On one hand, activation of HIF-1α, has been associated with a good

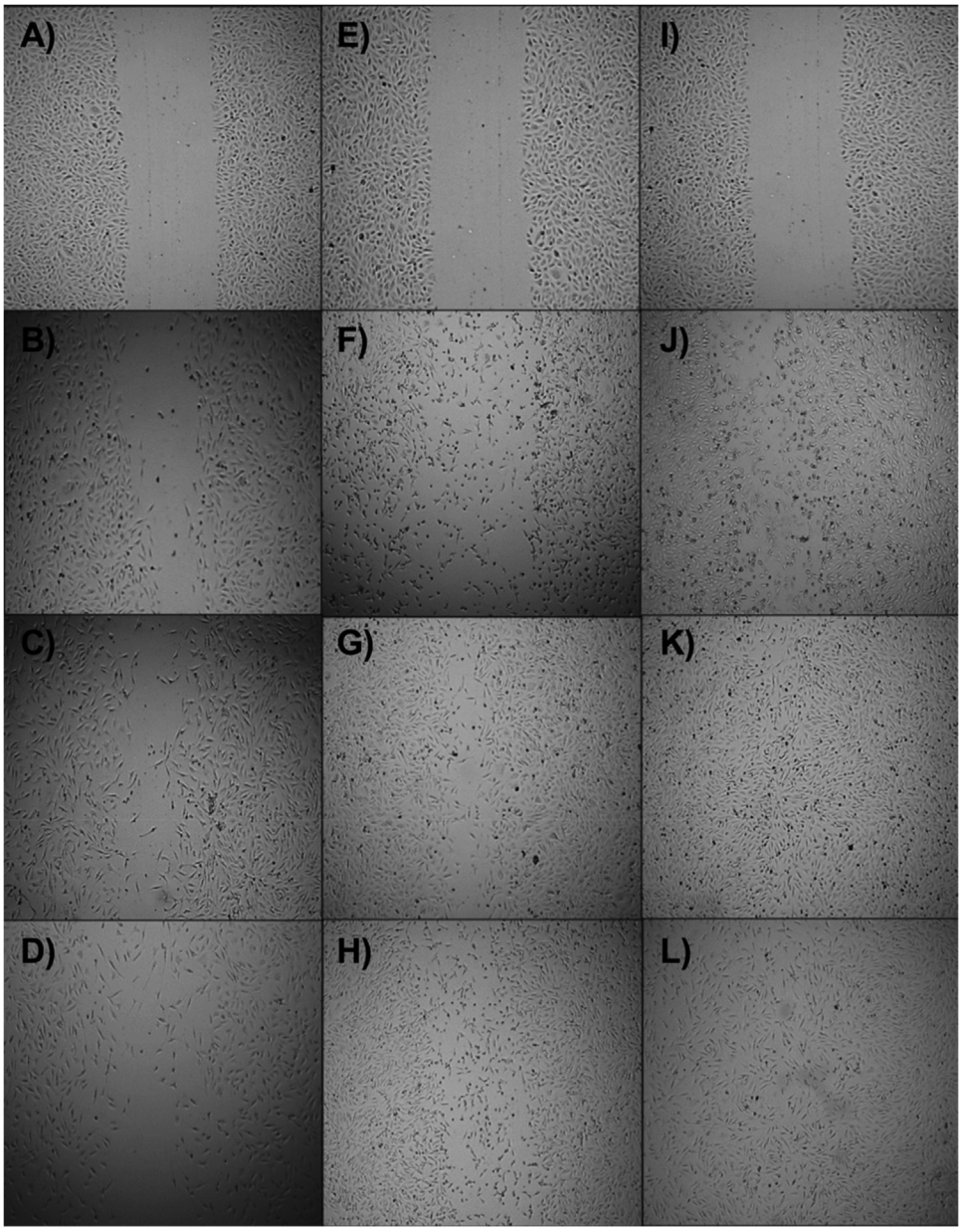

Fig. 9. Endothelial cell migration under hypoxic condition. Endothelial cells were seeded onto fibronectin-coated 24-well tissue culture plates until confluence. Then, the confluent monolayer of cells was denuded with a sterile 200- pipette tip to create a uniform cell-free zone in each well. To avoid proliferation mitomycin C was added. The cultures were washed twice with PBS and re-coated with fibronectin. At this time point wound margins were photo-documented. Cells were stimulated as follows. None stimulated cells after 0, 24, 48 and 72 h (panels A, B, C, D, respectively). Cells stimulated with 25 μg/mL of IDR-1018 for 0, 24, 48 and 72 h (panels E, F, G, H, respectively) and cells stimulated with EGF (20 μg/mL) for 0, 24, 48 and 72 h (panels I, J, K, L, respectively). Pictures are representative of 3 independent experiments. Magnification 40×.

wound healing in diabetic mice [6,38] while on the other hand, extended hypoxia can result in poor tissue perfusion and excessive inflammation that delays healing progression [1]. Thus to date the function of this factor during diabetic wound healing is not completely clear. Here we showed that IDR-1018 down-regulated the expression of HIF-1α under hypoxia. This seems counterintuitive since it has been described that HIF-1α is important to activate angiogenin and VEGF expression in cells exposed to hypoxia [34] while our experiments showed that IDR-1018 actually induced angiogenin in keratinocytes and endothelial cells and VEGF in keratinocytes. It is thus plausible to assume that IDR-1018 promoting their expression through an independent pathway such as the Akt/PI3K/Sp1 signaling pathway, which is an HIF-1α-independent pathway to activate angiogenesis [11,20,30]. Indeed this pathway is clearly used by cationic peptides since IDR-1018 suppresses IL-6 production in Flagellin-stimulated airway cells in an Akt-dependent manner[26] related peptide IDR-1002 induces CCL2 in human peripheral blood mononuclear cells in a PI3K-dependent manner [28] and cathelicidin LL-37 induces cell proliferation through Akt/PI3K [22].

Cell migration is fundamental not only for wound re-epithelialization but also for angiogenesis, within this process many growth factors are involved, including cytokines, adhesion molecules and angiogenin [21]. Given that our experiments showed that IDR-1018 induced most of these components, we sought to determine whether cell migration could also be promoted. We used a widely and well-characterized scratch model for in vitro wound closure [8,15]. In keratinocytes, IDR-1018 did not affect positively the cell migration under either hypoxia or normoxia conditions. Actually, under normoxia condition IDR delayed wound closure in keratinocytes, conversely other studies in animal models have shown that IDR-1018 induces an optimal wound healing [36], probably in the animal model, the wound closure is given because IDR-1018 increases angiogenesis thus promoting other molecules needed for wound closure or induces other molecules such as growth factors that can induce wound healing under normoxic condition. Worthwhile to mention that in DFU, there is a hypoxic condition, thus keratinocyte migration could be well induced, nevertheless clinical trials need to be performed to evaluate its effectiveness in humans. On the other hand, our results showed that IDR-1018 do not induce a significative cell proliferation in keratinocytes (S3), therefore wound closure is given for cell migration. In endothelial cells, IDR-1018

induced cell migration at 25 μg/mL under both conditions. There are differences in response to IDR-1018 between keratinocytes and endothelial cells, we do not know exactly which is the mechanism that confers such differences, we speculate that endothelial cells differentially express different receptors or the density of these receptors varies on the surface of each kind of cell, nonetheless, further molecular mechanisms to address this important question need to be further investigated.

In the present study, we have focused in the study of IDR-1018, however other peptides such as IDR-1002 have demonstrated similar activities [27,43] and can be studied in the same model as well.

5. Conclusions

IDR-1018 induced pro-angiogenic molecules in endothelial cells and in keratinocytes while reduced anti-angiogenic molecules. Besides promoted endothelial cell migration, these results suggest the potential use of this IDR to induce angiogenesis in chronic wounds such as DFUs. Nevertheless, molecular mechanism involved in this process need to be further elucidated.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.peptides.2018.01.010.

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