EPR Monitoring of Wound Oxygenation as a Biomarker of Response to Gene Therapy Encoding hCAP-18/LL37 Peptide

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INTRODUCTION

Pressure ulcers, vascular ulcers, and diabetic ulcers are common chronic wounds (1), and etiologies of impaired healing are multifactorial (2). In the complex process of wound healing, hypoxia plays a major role. Indeed, oxygen is a critical factor in the three phases of wound healing (i.e., inflammation, proliferative, and remodeling phases (3–6)). Although the measurement of tissue oxygenation could be of diagnostic and prognostic value, in the clinical practice, this parameter is predominantly evaluated by transcutaneous oxygen partial pressure (TcPO2), which only gives indirect measurements. Repeated absolute tissue partial pressure of oxygen (pO2) measurements should allow a more accurate prediction of wound healing, help for a better understanding of the physiopathology of impaired wound healing, and a better assessment of the value of treatments designed to modulate wound oxygenation. Such measurements can be obtained using electron paramagnetic resonance (EPR) oximetry. Determination of absolute pO2 by EPR oximetry is a well-described technique based on the interaction between unpaired electrons of oxygen and a paramagnetic probe. Most EPR oximetry studies rely on the shortening of the T2 transverse relaxation time of a probe, resulting in the broadening of the recorded EPR spectrum (7,8) (Fig. 1). In a previous work (9), we demonstrated that EPR oximetry with lithium phthalocyanine (LiPc) crystals (10) as the oxygen sensor can be applied to monitor oxygenation in wounds. Using EPR oximetry in a pedicled skin flap wound model, we observed a prolonged hypoxia in diabetic db/db mice presenting impaired wound healing, whereas a rapid wound healing and a faster reoxygenation was observed in nondiabetic mice (9).

The aim of the present study was to investigate the value of EPR oximetry to monitor the evolution of pO2 in wounds after administration of a treatment described to improve wound healing and to increase vascularization in acute wounds. The treatment we selected relied on the LL37 peptide. LL37 is primarily an antimicrobial peptide of 37 amino acids that belongs to the cathelicidin family (11,12). In addition to its role in innate and adaptive immunity (12), a proangiogenic activity was described for this peptide (13–17). Local delivery of LL37 by gene therapy (15) or nanoparticulate systems (17) has been previously described to accelerate wound closure in a full-thickness excisional skin wound and improve reepithelialization and angiogenesis in young diabetic mice. Nevertheless, the role of LL37 on tissue pO2 levels remained unknown. In addition, it was unknown whether this treatment was still efficient in chronic wounds observed in older mice, typically 12-week-old mice, in which microvascular complications caused by long-term hyperglycemia are observed (9).

NOTE

PurPOSE: To investigate the value of electron paramagnetic resonance oximetry to follow oxygenation in wounds treated by a plasmid-encoding host defense peptide hCAP-18/LL37.

METHODS: Flaps were created on diabetic mice (7- or 12-week-old db/db mice) presenting different levels of microangiopathy. The hCAP-18/LL37-encoding plasmids were administered in wounds by electroporation. Low-frequency electron paramagnetic resonance oximetry using lithium phthalocyanine as the oxygen sensor was used to monitor wound oxygenation in flaps during the healing process. Flaps were analyzed by immunohistochemistry to assess hypoxia and cell proliferation. Kinetics of closure was also assessed in excisional skin wounds.

RESULTS: A reoxygenation of the flap was observed during the healing process in the 7-week-old db/db treated mice, but not in the untreated mice and the 12-week-old mice. Histological studies demonstrated less hypoxic regions and higher proportion of proliferating cells in hCAP-18/LL37-treated flaps in the 7-week-old db/db treated mice compared with untreated mice. Consistently, the kinetics of excisional wound closure was improved by hCAP-18/LL37 treatment in the 7-week-old db/db but not in the 12-week-old mice.

CONCLUSIONS: Oxygenation measured by electron paramagnetic resonance oximetry is a promising biomarker of response to treatments designed to modulate wound oxygenation.


Key words: EPR; oximetry; wound healing; LL37; diabetes
Here, a plasmid coding for hCAP-18/LL37 was administered by electroporation in two wound models, a pedicled skin flap and a full-thickness excisional wound, either in young (7-week-old mice) or aged animals (12-week-old mice). The evolution of pO2 was monitored in pedicled skin flaps using EPR oximetry and correlated with the rate of excisional wound healing.

**METHODS**

**Plasmids**

The gene-encoding human hCAP-18/LL37 peptide originates from the Ad5-hCAP-18 virus, which was kindly provided by the Vector Core (Gene Therapy Program, Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA). DNA cloning was performed using pQE-Trisystem plasmid vector (Qiagen, Hilden, Germany) (15). The pEGFP-N1 (Clontech, Palo Alto, CA) used as control encodes green fluorescent protein (GFP). Plasmids produced by E. coli XL-1 blue were purified by endotoxin-free plasmid Giga-prep (Qiagen).

**Animals**

All animal experiments were approved by the local ethical committee for animal care of the Université Catholique de Louvain (2014/UCL/MD/026). Male BKS(D)-Leprdb/JOrIrj (db/db) mice (Elevage Janvier, Le Genest St. Isle, France), ages 7 and 12 weeks old at the surgery, were used as the model of type 2 diabetes. All experiments were performed under gaseous anesthesia (isoflurane 3% for induction and 2% for maintenance, Abbott, Wavre, Belgium).

**Glycemia Measurements**

Just before surgery, blood was sampled from the tail vein and analyzed with a GlucoMen LX Plus glucometer (Menarini Diagnostics, Florence, Italy). Animals were considered diabetic when nonfasting blood glucose was greater than 250 mg/dL.

**Wound Models**

Two wound models were selected: a pedicled U-shape skin flap model (30 × 8 mm) and a full-thickness excisional skin wound (4 mm diameter) as previously reported (9,15).

**Wound Treatments**

The pQE-hCAP-18/LL37 plasmid was administered in 7- and 12-week-old db/db mice, both in pedicled skin flaps and excisional skin wounds. The pEGFP-N1 plasmid was administered in control animals.

In the flap model, 150 μg of plasmid was diluted in phosphate-buffered saline to a total volume of 240 μL and distributed in 12 intradermal injections of 20 μL at 10 sites around and 2 in the flap. Four electroporations were realized to cover the entire surface around (left, right, distal region) and in the flap. In excisional skin wounds, plasmids were administered as previously described (15).

The skin was placed between electrodes separated by 2 mm (BTX Caliper Electrodes, BTX Harvard Apparatus, Holliston, MA), and electroporations were performed with a Gemini System electroporator (BTX Harvard Apparatus) with the following settings: 500 V/cm, 10 pulses, 20 ms, 1-s intervals.

**Oxygenation Measurements**

**Implantation of LiPc Crystals**

The LiPc crystals, kindly provided by H.M. Swartz (Dartmouth Medical School, Hanover, NH), were implanted subcutaneously using a 26-G needle 10 days before surgery (9). The LiPc crystals were placed on the dorsal midline at 25 mm from the pedicle of the flap.

**EPR Oximetry Measurements**

An EPR spectrometer equipped with a low-frequency microwave bridge operating at 1.15 GHz and an extended loop resonator (ClinEPR, Lyme, NH) was used for pO2 measurements. Animals were positioned in the EPR spectrometer, the region containing LiPc crystals placed under the surface coil positioned in the center of the magnet. The acquisition parameters were as follows: modulation amplitude: 0.01 mT; modulation frequency: 21.3 kHz; power: 0.794 mW; sweep width: 0.25 mT; sweep time: 3 s; time constant: 5 ms; number of scans: 3 to 20; number of points/scan: 1024.

Line width was calculated using a one-component fitting (LiPc), developed at the Dartmouth EPR Center, that accounts for instrumental...
modulation parameters. The model uses a fixed modulation amplitude value as a fixed fitting parameter and gives the same intrinsic line width regardless of the modulation amplitude used for the acquisitions. The EPR linewidth of the recorded signal was then converted to pO₂ value using an ex vivo calibration determined previously for the same batch of LiPc crystals used in the present study (Fig. 1).

Measurements were realized one day before the surgery and at days 1, 2, 3, 4, 5, 7, 9, and 11 after surgery.

Histology and Immunohistochemistry

At day 9 after surgery, 7-week-old db/db mice treated with LL37 peptide and controls received an intraperitoneal injection of 60 mg/kg pimonidazole hydrochloride (Hypoxyprobe, Burlington, MA) 2 h before sacrifice. Pimonidazole binds to peptides thiol groups in cells when pO₂ is less than 10 mmHg, forming adducts detectable by immunohistochemistry.

Samples of skin flap (at the distal site) were harvested, fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. Five-μm sections were realized and stained with hematoxylin/eosin. Other sections were incubated with rabbit anti-mouse Pab2627 antibody (1:200 dilution, Hypoxyprobe) or rabbit anti-mouse anti-Ki67 antibody (1:400 dilution, Cell Signaling Europe, Leiden, the Netherlands) overnight at 4°C, followed by 30-min incubation at room temperature with Envision anti-rabbit secondary antibody (Dako, Heverlee, Belgium) and stained with diaminobenzidine for 5 min (Dako).

Kinetics of Closure in Excisional Wounds

Kinetics of closure of excisional skin wounds treated with LL37 peptide or controls were determined by taking digital photographs repeatedly during the healing process. Photographs were taken with a Nikon Digital D90 camera equipped with an AF-S DX NIKKOR 18–55-mm f/3.5-5.6G VR lens (Nikon, Tokyo, Japan) placed at a fixed distance from the wounds. Pictures were analyzed with ImageJ 1.47v software (National Institutes of Health, Bethesda, MD). Wound areas were determined using the polygon selection tool and normalized with areas contained in the silicone ring. Results are expressed as the time to reach 50% of the wound closure.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Unpaired t-test was used to compare the mean pO₂ between control and LL37-treated group at days 9 and 11, and the mean time to reach 50% of wound closure. P values less than 0.05 (*) were considered to be statistically significant. Statistical analyses were performed using GraphPad Prism 7.01 (GraphPad Software, La Jolla, CA).

RESULTS

Excisional Wound Closure Rate

All mice presented hyperglycemia at the moment of the surgery with nonfasting blood glucose above 250 mg/dL. Nonfasting blood glucose was 396 ± 102 mg/dL and 449 ± 71 mg/dL for the 7-week-old and the 12-week-old db/db mice, respectively.

In the 7-week-old mice, 4-mm excisional skin wounds treated with hCAP-18/LL37 showed significantly faster closure compared with control GFP-treated wounds. Wounds reached 50% of closure in 12.3 ± 1.3 days for hCAP-18/LL37-treated mice compared with 18.1 ± 1.5 days for the control GFP-treated mice (P = 0.0199) (Fig. 2a). In the 12-week-old mice, no significant difference was observed in hCAP-18/LL37-treated wounds compared with GFP-treated wounds (Fig. 2b).

Wound Oxygenation

As described previously (9), it was not possible to measure wound oxygenation in full-thickness excisional skin wounds. For that reason, pO₂ was measured in pedicled skin flaps realized on the same animals. Measurements of the pO₂ in the distal part of pedicled flaps were determined using the EPR oximetry.
realized repeatedly during the 11 days after surgery in 7-week-old and 12-week-old db/db mice (Figs. 3a and 3b). Presurgical pO2 values were approximately 20 mmHg in all groups except in the 12-week-old control GFP-treated group, in which the pO2 was approximately 12 mmHg. These values are coherent with previous measurements of normal basal skin pO2 and demonstrate the absence of inflammatory reaction, which is known to decrease pO2. A similar drop of the pO2 was observed just after the surgery in all groups with pO2 near 0 mmHg. In the 7-week-old hCAP-18/LL37-treated group, pO2 increased from day 1 to day 7 to reach significantly higher pO2 values than those measured in the control group at day 9 at day 11. The distal part of the flap in this control group remained hypoxic during all of the monitoring. In the 12-week-old group, the pO2 remained low during all of the monitoring in the control and hCAP-18/LL37-treated groups.

Histology: Hypoxia and Proliferation

Hematoxylin/eosin staining and pimonidazole stains are presented in Figure 4. The sections illustrate regions in the flap (F) and in the normal skin (NS). Histological studies realized 9 days after the surgery on skin flaps of 7-week-old control mice showed highly hypoxic regions (dark brown in the section) near and inside the flap (Fig. 4c). In contrast, the hCAP-18/LL37-treated mice showed limited hypoxia in the flap, with only the superficial layer of the epidermis stained with anti-pimonidazole antibody (Fig. 4d).

Anti-Ki67 staining was used to delineate proliferation sites in the flaps (Fig. 5). In the control mice, only few proliferative cells were observed in the flap region (Figs. 5a and 5c). In the 7-week-old mice treated with hCAP-18/LL37, highly proliferative sites were evidenced in the subcutaneous tissue under and near the flap (Figs. 5d and 5e).
and 5f). This proliferation was high compared with normal skin (at the distance of the wound), in which proliferation was only observed in the deep layer of the epidermis and in the hair follicles (Fig. 5). Histological studies realized on 12-week-old mice showed that mice treated or not with hCAP-18/LL37 peptide presented highly hypoxic zones in the flaps, which were associated with a few proliferative cells and the absence of tissue regeneration (data not shown).

**DISCUSSION**

The main findings of the present study are as follows: (i) Electron paramagnetic resonance oximetry is able to monitor longitudinally the evolution of the pO₂ in wounds, and assess the effect of treatment on wound oxygenation; (ii) the efficacy of gene-encoding hCAP-18/LL37 peptide is strongly dependent on the age of the mice, underlying the relevance of microvascular complications caused by long-term hyperglycemia; and (iii) the evolution of the wound reoxygenation is correlated to the excisional wound healing kinetics.

Consistent with our previous study (9), the pO₂ dramatically dropped early after wounding and remained chronically low in both the db/db diabetic mice (7 and 12 weeks old) used in the present study (Fig. 3). We have previously shown that this hypoxia was consistent with microangiopathies observed by intravital microscopy (9). Here, we found that hCAP-18/LL37 encoding plasmid promoted the reoxygenation of a pedicled skin flap in 7-week-old diabetic mice, but not in 12-week-old db/db mice, for which no beneficial effect was observed on flap oxygenation that remained very low (Fig. 3). These results were consistent with the histological studies (Fig. 4) that showed less hypoxic regions in treated flaps in young mice treated with hCAP-18/LL37 compared with untreated mice (Fig. 4). The presence of hypoxia in untreated mice was also associated with a lack of cell proliferation in wounds (Fig. 5). Interestingly, the results were correlated with the kinetics of closure of full-thickness excisional skin wounds, which was faster in young diabetic mice treated with the hCAP-18/LL37 plasmid as compared with the untreated mice (Fig. 2). No difference was observed between treated and untreated groups of old mice. The dose of hCAP-18/LL37-encoding plasmid was chosen from a previous study (15), which showed that highest plasmid expression was obtained in an excisional wound after administration and electroporation of 50 μg of plasmids. In the flap, 150 μg was administered to reach the highest peptide expression possible. To compare the treatment efficacy on young and old diabetic mice, we kept the...
plasmid dose constant throughout the study. Differences in the healing process were observed depending on the age of the diabetic mice. The absence of treatment effect on older diabetic mice was previously observed in other wound healing studies (9,20,21). These results can be explained by a more pronounced development of diabetes complications in older mice, which is in agreement with our previous observations of microangiopathies, impaired healing, and a lack of reoxygenation of flaps in 12-week-old db/db mice (9).

These current results demonstrate that low-frequency EPR oximetry is a convenient technique used to monitor the evolution of wound oxygenation in vivo, noninvasively, and repeatedly during the healing process after treatment administration. The response of the oxygen sensor used in the study (LiPc) to $pO_2$ variations during the wound healing time frame was demonstrated previously, with the response to $pO_2$ variations observed for at least 30 days (9). The LiPc was demonstrated neither to influence the healing process, to induce fibrosis, nor to induce any inflammatory reaction (9,22–27). In addition, presurgical EPR oximetry measurements confirmed the absence of inflammation, as no hypoxia reflecting an inflammatory state was observed before wounding. However, LiPc crystals allow localized measurements at the site of implantation, which is a limitation because it cannot inform us about spatial variations of the $pO_2$ in the wound. Implantation of several LiPc crystals at distant sites for separate measurements is needed to obtain limited information about spatial variation. More experiments are needed to conclude that EPR oximetry can be a predictor of wound healing. It is likely that $pO_2$ measurement will be one of many clinical endpoints, as wound healing is multifactorial. These first results are encouraging, notably for the implementation of the technique for human wounds measurement with adapted EPR spectrometers. In patients, tissue oxygenation is currently monitored by the indirect TcPO$_2$ measurement, and is used in clinical practice to predict healing or to envisage amputation, such as in patients with a diabetic foot. In this context, development of clinical EPR spectrometers allowing direct, accurate, and repeatable measurements of tissue oxygenation (28,29) would be of particular interest in chronic wound care. A comparison between EPR and TcPO$_2$ would be interesting, as there are currently no published data that compare these two techniques. In this perspective, appropriate oxygen sensors for clinical use also need to be validated. Biocompatible sensors have recently been developed (30–33) and are currently being evaluated in clinical trials (28).

CONCLUSIONS

In conclusion, the measurement of wound oxygenation using EPR oximetry allows the assessment of the effect of treatments that are designed to modulate hypoxia in wounds. Further studies are needed to evaluate the effectiveness of EPR oximetry in comparison with other predictors of the wound healing process. Translational studies are ongoing that will determine whether the technique can be envisaged in humans in the future.

ACKNOWLEDGMENTS

The authors want to thank Michèle de Beukelaer and Chantal Fregimilicka for the histological studies, and Prof. Etienne Marbaix (Anatomopathology, Cliniques Universitaires Saint-Luc) for the helpful discussion about the results and the histological sections.

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