Toxicology and Applied Pharmacology 288 (2015) 330–338

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Dextran-shelled oxygen-loaded nanodroplets reestablish a normoxia-like pro-angiogenic phenotype and behavior in hypoxic human dermal microvascular endothelium

Nicoletta Basilico [a], Chiara Magnetto [b], Sarah D'Alessandro [c], Alice Panariti [d], Ilaria Rivolta [d], Tullio Genova [e], Amina Khadjavi [f], Giulia Rossana Gulino [g], Monica Argenziano [h], Marco Soster [h], Roberta Cavalli [h], Giuliana Giribaldi [g], Caterina Guiot [f], Mauro Prato [f,*]

[a] Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università di Milano, via Pascal 36, 20133 Milano, Italy
[b] Istituto Nazionale di Ricerca Metrologica (INRIM), Strada delle Cacce, 91, 10135 Torino, Italy
[c] Dipartimento di Scienze Farmacologiche e Biomolecolari, Università di Milano, via Pascal 36, 20133 Milano, Italy
[d] Dipartimento di Scienze della Salute, Università di Milano Bicocca, Via Cadore 48, 20900 Monza, Italy
[e] Dipartimento di Scienze della Vita e Biologia dei Sistemi, Via Accademia Albertina 13, 10123 Torino, Italy
[f] Dipartimento di Neuroscienze, Università di Torino, Corso Raffaello 30, 10125 Torino, Italy
[g] Dipartimento di Oncologia, Università di Torino, Via Santena 5 bis, 10126 Torino, Italy
[h] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via Giuria, 9, 10125 Torino, Italy

ARTICLE INFO

Article history:
Received 1 June 2015
Revised 6 August 2015
Accepted 10 August 2015
Available online 12 August 2015

Keywords:
Oxygen
Nanodroplet
Matrix metalloproteinase (MMP)
Tissue inhibitor of metalloproteinase (TIMP)
Human microvascular endothelial cell (HMEC)
Skin

ABSTRACT

In chronic wounds, hypoxia seriously undermines tissue repair processes by altering the balances between pro-angiogenic proteolytic enzymes (matrix metalloproteinases, MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) released from surrounding cells. Recently, we have shown that in human monocytes hypoxia reduces MMP-9 and increases TIMP-1 without affecting TIMP-2 secretion, whereas in human keratinocytes it reduces MMP-2, MMP-9, and TIMP-2, without affecting TIMP-1 release. Provided that the phenotype of the cellular environment is better understood, chronic wounds might be targeted by new oxygenating compounds such as chitosan- or dextran-shelled and 2H,3H-decafluoropentane-cored oxygen-loaded nanodroplets (OLNs). Here, we investigated the effects of hypoxia and dextran-shelled OLNs on the pro-angiogenic phenotype and behavior of human dermal microvascular endothelium (HMEC-1 cell line), another cell population playing key roles during wound healing. Normoxic HMEC-1 constitutively released MMP-2, TIMP-1 and TIMP-2 proteins, but not MMP-9. Hypoxia enhanced MMP-2 and reduced TIMP-1 secretion, without affecting TIMP-2 levels, and compromised cell ability to migrate and invade the extracellular matrix. When taken up by HMEC-1, nontoxic OLNs abrogated the effects of hypoxia, restoring normoxic MMP/TIMP levels and promoting cell migration, matrix invasion, and formation of microvessels. These effects were specifically dependent on time-sustained oxygen diffusion from OLN core, since they were not achieved by oxygen-free nanodroplets or oxygen-saturated solution. Collectively, these data provide new information on the effects of hypoxia on dermal endothelium and support the hypothesis that OLNs might be used as effective adjuvant tools to promote chronic wound healing processes.

Abbreviations: ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; DFP, 2H,3H-decafluoropentane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; OFN, oxygen-free nanodroplet; OLN, oxygen-loaded nanodroplet; OSS, oxygen-saturated solution; PBS, phosphate-buffered saline; PFP, perfluoropentane; TIMP, tissue inhibitor of metalloproteinase; US, ultrasound; UV, ultraviolet.

* Corresponding author.
E-mail addresses: nicoletta.basilico@unimi.it (N. Basilico), c.magnetto@inrim.it (C. Magnetto), sarah.dalessandro@unimi.it (S. D'Alessandro), alice.panariti@mail.mcgill.ca (A. Panariti), ilaria.rivolta@unimib.it (I. Rivolta), tullio.genova@unito.it (T. Genova), amina.khadjavi@unito.it (A. Khadjavi), giuliarossana.gulino@unito.it (G.R. Gulino), monica.argenziano@unito.it (M. Argenziano), marco.soster@unito.it (M. Soster), roberta.cavalli@unito.it (R. Cavalli), giuliana.giribaldi@unito.it (G. Giribaldi), caterina.guiot@unito.it (C. Guiot), mauro.prato@unito.it (M. Prato).

http://dx.doi.org/10.1016/j.taap.2015.08.005
0041-008X/

Introduction

After injury, skin integrity must be restored promptly to reestablish the homeostatic mechanisms, minimize fluid loss, and prevent infection (Greaves et al., 2013). This is achieved through wound healing, a complex biological process where multiple pathways are simultaneously activated to induce tissue repair and regeneration. Traditionally, acute wound healing is defined as a complex multi-step and multi-cellular process distinguished in four phases involving different cell types: i) hemostasis, involving platelets; ii) inflammation, involving neutrophils, monocytes, and macrophages; iii) proliferation, involving keratinocytes, endothelial cells, and fibroblasts; and iv) matrix remodeling, involving keratinocytes, myofibroblasts, and endothelial cells (Diegelmann and Evans, 2004). In particular, during the third and fourth phases, the endothelium plays a pivotal role, since wound microvasculature is rebuilt through angiogenesis to restore the supply of oxygen, blood constituents and nutrients to the regenerating tissue, helping to promote fibroplasia and prevent sustained tissue hypoxia (Eming et al., 2014). Notably, oxygen represents a key regulator of normal wound healing since it is required for collagen deposition, epithelialization, fibroplasia, angiogenesis, and resistance to infection (Castilla et al., 2012; Sen, 2009). Once complete, these processes must be shut down in a precise order to prevent exaggerated or delayed responses.

In some cases, the combination of systemic (e.g. diabetes, vascular insufficiency, or aging) or localized (e.g. bacterial infections and dysregulated proteolysis) factors produce persistent pathological inflammation resulting in chronic wound formation (Diegelmann and Evans, 2004). A chronic wound is defined as a break in skin epithelial continuity lasting more than 42 days. Its prevalence varies with age, ranging approximately from 1% in the adult population to 3–5% in >65 year-old subjects (Greaves et al., 2013). Approximately 7 million patients are affected by chronic wounds in the United States, and an estimated $25 billion dollars is spent annually on the treatment of these wounds (Castilla et al., 2012).

A typical feature of chronic wounds is unbalanced proteolytic activity, which overwhelms tissue protective mechanisms (Diegelmann and Evans, 2004; Pepper, 2001). Within chronic wounds, activated cells such as endothelial, epithelial, and immune cells display increased production of proteases, including cathepsin G, urokinase and neutrophil elastase (Greaves et al., 2013). Furthermore, pro-inflammatory cytokines strongly induce the production of matrix metalloproteinases (MMPs) and down-regulate the levels of tissue inhibitors of metalloproteinases (TIMPs), thereby creating an environment with unbalanced MMP/TIMP ratios (Diegelmann and Evans, 2004; Pepper, 2001). Consequently, wound repair mediators become targets of proteases, and the resultant matrix degradation contributes to the delay in re-epithelialization, fibroplasia and angiogenesis (Pepper, 2001; Wells et al., 2015). However, the effects of hypoxia on the secretion of MMPs and TIMPs by the cellular environment of the wound are dramatically different depending on the considered cell type. Therefore, it is extremely important to assess carefully the effects of hypoxia on each single cell population participating to the wound healing process, from monocytes and keratinocytes to endothelial cells and fibroblasts. In a couple of recent works published by our group, hypoxia was shown to reduce MMP-9 and increase TIMP-1, not affect TIMP-2 secretion, in human monocytes (Gulino et al., 2015), whereas in human keratinocytes hypoxia was shown to reduce MMP-2, MMP-9, and TIMP-2 secretion without affecting TIMP-1 levels (Khadjavi et al., 2015). On the other hand, the effects of hypoxia on the secretion of gelatinases and their inhibitors by dermal microvascular endothelium or fibroblasts still need further investigation.

Providing the phenotype of the cellular environment at the milieu of the wound is better understood, new therapeutic approaches addressing hypoxia might help to face chronic wounds. For this reason, the major role played by oxygen in essential wound healing processes has attracted considerable clinical interest and yielded compelling data (Sen, 2009). Additionally, scientific studies targeting the signaling pathways underlying oxygen response within the milieu of the wound tissue are helping to better understand the biochemical pathways involved in hypoxia sensing/response systems. This appears extremely crucial in order to exploit new oxygenating treatments targeting hypoxia-response mechanisms within the healing tissue, thus making them useful in the clinical management of chronic wound.

So far, hyperbaric oxygen therapy remains a well-established, adjunctive treatment for diabetic lower extremity wounds, when refractory to standard care practices (Sen, 2009). However, hyperbaric oxygen therapy is expensive and uncomfortable. Moreover, further rigorous randomized trials are needed to properly validate the outcomes of hyperbaric oxygen therapy on chronic wounds associated with other pathologies (arterial ulcers, pressure ulcers, and venous ulcers). Topical oxygen therapy, based on an O_2 gas emulsion applied to the superficial wound tissue, represents another promising approach to enhance the oxygenation of wounded tisues (Sen, 2009). Major advantages of topical oxygen therapy appear to be its independence of the wound microcirculation, its lower cost with respect to systemic oxygen therapy, lower risks of oxygen toxicity, and its relative simplicity of handling and application.

In this context, intensive research is being pursued to develop new carriers able to release therapeutically significant amounts of oxygen to tissues in an effective and time-sustained manner, such as hemoglobin- or perfluorocarbon-based systems (Cabrales and Intaglietta, 2013; Schroeter et al., 2010). Among the options currently under investigation, perfluoropentane (PFP)-based oxygen-loaded nanobubbles have been proposed as efficient and biocompatible ultrasound (US)-responsive tools for oxygen delivery (Cavalli et al., 2009a,b). Furthermore, oxygen-loaded nanodroplets (OLNs), constituted by 2H,3H-decafluoropentane (DFP) as core fluorocarbon and dextran or chitosan as shell polysaccharides, have been recently developed, characterized, and patented by our group as an innovative and nonconventional platform of oxygen nanocarriers, available in formulations suitable for topical treatment of dermal tissues (Magnetto et al., 2014; Prato et al., 2015). Intriguingly, while keeping all the advantages of nanobubbles, OLNs display higher stability and effectiveness in oxygen storage and release, lower manufacturing costs and ease of scale-up. Encouragingly, chitosan-shelled OLNs proved effective in counteracting the dysregulating effects of hypoxia on secretion of gelatinases and TIMPs by human keratinocytes (Khadjavi et al., 2015), whereas dextran-shelled OLNs abrogated hypoxia-dependent alteration of MMP-9/TIMP-1 balances in human monocytes (Gulino et al., 2015).

To go beyond the current knowledge on MMP/TIMP dysregulation in the different cell populations within the milieu of chronic wounds and expand the available evidence on OLN effectiveness, in the present work we explored the effects of hypoxia and OLNs on the pro-angiogenic phenotype and behavior of human dermal endothelium. To this purpose, a human dermal microvascular endothelial cell line (HMEC-1) was cultured in vitro both in normoxic and hypoxic conditions, in the presence or absence of dextran-shelled OLNs. Then, cells were challenged for their viability, proteolytic phenotype (secretion of gelatinases and their inhibitors), and wound healing abilities (migration, invasion of the extracellular matrix (ECM), and formation of microvessel-like structures).

Methods

Materials

All materials were from Sigma-Aldrich, St Louis, MO, aside from those listed below. Sterile plastics were from Costar, Cambridge, UK; MCDB 131 medium was from Invitrogen, Carlsbad, CA; fetal calf serum was from HyClone, South Logan, UT; epidermal growth factor was from PeproTech, Rocky Hill, NJ; Cultrex was from Trevigen, Gaithersburg, MD; LDH Cytotoxicity Assay kit was from Biovision, Milpitas, CA; enzyme-linked