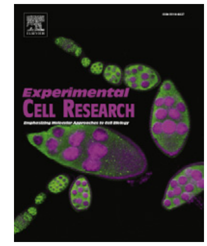
Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Changes in inflammatory gene expression induced by hyperbaric oxygen treatment in human endothelial cells under chronic wound conditions

Alexandra C. Kendall[a, b], Jacqueline L. Whatmore[a], Lorna.W. Harries[a], Paul G. Winyard[a], Gary R. Smerdon[b], Paul Eggleton[a,*]

[a]Peninsula Medical School, University of Exeter, Exeter, Devon, EX1 2LU, UK
[b]Diving Diseases Research Centre, Tamar Science Park, Research Way, Plymouth, PL6 8BU, UK

ARTICLE INFORMATION

Article Chronology:
Received 3 June 2011
Revised version received
7 October 2011
Accepted 23 October 2011
Available online 28 October 2011

Keywords:
Oxidative stress
Nitric oxide
Interleukin-8
Gene regulation
Chronic inflammation

ABSTRACT

Hyperbaric oxygen (HBO) therapy involves the inhalation of 100% oxygen, whilst inside a chamber at greater than atmospheric pressure. It is an effective treatment for chronic diabetic wounds, although the molecular mechanisms involved remain unclear. We hypothesised that HBO could alter inflammatory gene expression in human endothelial cells via a reactive oxygen/nitrogen species-mediated pathway. Endothelial cells were exposed to a chronic wound model comprising hypoxia (2% O_2 at 1 atmosphere absolute (ATA); PO_2 ~2 kPa) in the presence of lipopolysaccharide and TNF-α for 24 h, then treated with HBO for 90 min (97.5% O_2 at 2.4 ATA; PO_2 ~237 kPa). 5 h post-HBO, 19 genes involved in adhesion, angiogenesis, inflammation and oxidative stress were downregulated. Notably, only angiogenin gene expression, which promotes both angiogenesis and nitric oxide production (reflected by increased eNOS protein expression in this study), was upregulated. This led to a decrease in endothelial IL-8 mRNA and protein, which could help alleviate inflammatory processes during chronic wound healing. This was no longer evident 22.5 h post-HBO, demonstrating the importance of daily exposures in HBO treatment protocols. These studies indicate that elevated oxygen transiently regulates inflammatory gene expression in endothelial cells, which may enhance chronic wound healing.

Introduction

Chronic wounds are a huge burden on health services and the economy. In 2000, it was estimated that between three and six million people in the United States suffered from one of the three main types of chronic wound – a diabetic, venous or pressure ulcer, and that the total cost of wound care was around $3 billion per year [1]. As the population continues to age, this problem will grow, as people over 65 years old account for around 85% of chronic wound patients [1,2]. In normal wound healing, the wound progresses through three main overlapping phases – an inflammatory phase, a proliferative phase and a remodelling phase. These phases have characteristic cell involvement and involve intercellular signalling between platelets, macrophages, lymphocytes, polymorphonuclear leukocytes (PMNs), keratinocytes and endothelial cells, and the release of growth factors and cytokines from these cells orchestrates the healing process itself [3]. Wounds that fail to progress successfully through the normal

* *Corresponding author at:* Peninsula College of Medicine and Dentistry, University of Exeter, Exeter, EX1 2LU, UK. Fax: +44 1392 722926.
E-mail address: paul.eggleton@pms.ac.uk (P. Eggleton).
Abbreviations: CW, chronic wound; PC, pressure control.

0014-4827/$ – see front matter
doi:10.1016/j.yexcr.2011.10.014

healing process become chronic wounds, and healing is typically interrupted in the inflammatory phase [1].

Hypoxia is a key feature of chronic wounds, where destruction of the vasculature in the wound area, and high oxygen consumption by cells such as leukocytes and fibroblasts, result in low oxygen levels [4]. The PO_2 in chronic wounds has been measured using invasive oxygen electrodes, and has been found to lie in the range ~0.67–2.67 kPa (compared with typical values of ~4.0–6.67 kPa in healthy tissue) [5]. This prolonged hypoxia impairs several processes involved in wound healing, including collagen synthesis and deposition, epithelialisation, and phagocytic activity [6–8]. Additionally, chronic wounds are typically colonised by numerous species of bacteria, particularly opportunistic pathogens, as the hypoxic environment allows the growth of anaerobic bacteria, comprising 49% of bacterial species in chronic wounds compared with just 36% in acute wounds [9]. The prolonged hypoxia and bacterial contamination in the chronic wound leads to the release of large amounts of inflammatory cytokines by endothelial and other cells, exacerbating and propagating inflammation [10].

In order to provide nutrition and oxygen to wound sites to aid repair, the formation of new blood vessels via the process of angiogenesis is critical. The proliferation and migration of endothelial cells during the development of new capillary vessels help restore an oxygen supply to the ischaemic tissue. Understanding the molecular mechanisms that regulate wound healing in an inflammatory environment is essential if we are to provide new and more efficient treatment for chronic wounds.

HBO is an approved adjunctive therapy for the treatment of chronic diabetic wounds, used in conjunction with conventional treatments, including antibiotics, topical dressings, correction of vascular problems and debridement of tissue, when these latter treatments alone have proved ineffective. HBO involves the intermittent inhalation of 100% oxygen whilst inside a treatment chamber at a pressure greater than one ATA [11]. Breathing HBO forces high levels of oxygen to dissolve in the patient's plasma, which increases oxygen delivery to hypoxic wound tissue, where large intercapillary distances limit the diffusion of haemoglobin-bound oxygen. Oxygen dissolved in the plasma can diffuse further into the hypoxic tissue, raising wound oxygen levels from 0.67–2.67 kPa to 133.3–226.6 kPa [5,12]. A number of studies have provided evidence supporting the use of HBO in the treatment of chronic diabetic wounds, and an example of a wound treated successfully with HBO is shown in Fig. 1. HBO treatment has been shown to cause significant decreases in wound size area compared to control subjects [13] and more rapid healing rates [14], and a recent double-blinded placebo-controlled trial provided strong evidence to support the use of HBO in selected patients with chronic diabetic foot ulcers [15]. Despite these empirical observations, very few studies have investigated the effect of HBO on inflammatory gene expression over time in human endothelial or other cells at the site of wound damage. To address this lack of knowledge, we established a wound model employing human endothelial cells cultured under hypoxic conditions and exposed to tumour necrosis factor-alpha (TNF-α) and bacterial lipopolysaccharide (LPS), similar to the conditions observed in chronic wound sites. We monitored the real time expression of 92 pro- and anti-inflammatory genes under the wound model conditions pre- and post-HBO treatment. In addition, we focussed our attention on a number of gene products that appeared to be affected by HBO treatment in order to understand the wound healing processes that occur following HBO treatment.

Materials and methods

Reagents

Ham's F-12 medium and fetal bovine serum (FBS) were purchased from Lonza. Endothelial cell growth supplement (ECGS), collagenase, gentamicin, LPS and horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibody were purchased from Sigma-Aldrich UK. TNF-α was purchased from the NIBSC. Gas mixes were purchased from BOC. RNeasy® Mini Kit, RNA stabilisation reagent, TURBO DNA-*free*™ kit, TaqMan® Reverse Transcription Reagents kit, custom TLDA plates, RNase-free water and TaqMan® Universal PCR Master Mix were purchased from Applied Biosystems. Rabbit polyclonal anti-human eNOS antibody was purchased from Abcam. IL-8 sandwich ELISA kit was purchased from eBioscience, Inc.

Cell culture and viability

Human umbilical cords were obtained within 24 h of birth from normal pregnancies. The anonymous collection of umbilical cords for this project was approved by the North and East Devon

Fig. 1 – HBO is an effective treatment for chronic diabetic wounds. An example of a wound is shown before the initiation of HBO treatment, and five months post-treatment.