Assessment of hypoxia and TNF-α response by a vector with HRE and NF-kappaB response elements

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1. ABSTRACT

Hypoxia and inflammatory cytokine activation (H&I) are common processes in many acute and chronic diseases. Thus, a single vector that responds to both hypoxia and inflammatory cytokines, such as TNF-α, is useful for assessing the severity of such diseases. Adaptation to hypoxia is regulated primarily by hypoxia inducible transcription factor (HIFα) nuclear proteins that engage genes containing a hypoxia response element (HRE). Inflammation activates a multitude of cytokines, including TNF-α, that invariably modulate activation of the nuclear factor kappa B (NF-kB) transcription factor. We constructed a vector that encompassed both a hypoxia response element (HRE), and a NF-kappaB responsive element. We show that this vector was functionally responsive to both hypoxia and TNF-α, in vitro and in vivo. Thus, this vector might be suitable for the detection and assessment of hypoxia or TNF-α.

2. INTRODUCTION

Hypoxia and inflammatory cytokine activation (H&I) are common processes in many acute and chronic diseases. Ischemic-reperfusion injury, due to blood vessel occlusion is a well-studied form of H&I. Following occlusion of the vessel, anterograde blood flow ceases and there is a shift from aerobic to anaerobic tissue metabolism. Upon reperfusion, a sudden influx of oxygen perturbs the adaptive metabolism, generating substantial toxic free radical stress, which may, in turn, lead to cell death. This is followed by an obligate inflammatory phase to clear necrotic tissues before adaptive remodelling and neovascularisation re-establishes normal tissue oxygen tension (1). H&I is also prevalent and clinically significant in chronic inflammatory diseases such as arthritis, atherosclerosis, heart failure, cancer, rhabdomyolysis, inflammatory bowel disease and autoimmune disorders. In such instances, inflammation alters the cellular micromilieu, and with the perpetual presence of
inflammatory cells, imposes greater metabolic demands to cause vasculopathy, further reducing tissue perfusion and increasing hypoxia (2).

Adaptation to hypoxia is regulated primarily by hypoxia inducible transcription factor (HIF) nuclear proteins that activate genes encompassing a hypoxia response element (HRE). Canonical oxygen sensing by HIFs occurs through perpetual synthesis and degradation by oxygen-dependent hydroxylation, rendering HIFs susceptible to ubiquitination and proteosomal degradation. As oxygen tension drops, the absence of hydroxylation stabilizes HIFs which translocate into the nucleus. In hypoxia, HIFα binds HRE-responsive gene promoter-enhancer elements and regulate homeostasis by induction of erythropoietin, vascular endothelial growth factor, glucose transporter or other molecules, essential to adaptation to hypoxia (3).

Inflammation is activated by leukocyte accumulation and release of a multitude of cytokines and other mediators of inflammatory responses. Inflammatory cytokines, such as TNF-α invariably modulate receptor-mediated inhibitor-kappa-kinase (IKK) phosphorylation of inhibitor-of-kappa-B (IkB), allowing dissociation and suppression by IkB of nuclear factor kappa B (NF-kB). Dissociation of IkB enables translocation of NF-kappaB into the nucleus, where it transcriptionally regulates the cellular response to inflammation. NF-kappaB has also been implicated as an oxygen-sensitive transcription factor (4). A growing body of evidence suggests that both the HIFα and NF-kappaB transcription factors establish mechanistic cross-talk (5), interdependent of specific gene expression (6-9). Further, HIFα can be stabilized by inflammatory actuators, such as lipopolysaccharides, tumor necrosis factors or interleukins (2). Given the concomitant prevalence of H&I in various acute and chronic diseases, it is logical to jointly utilize both HRE and NF-kb-mediated mechanisms within a single vector to detect and assess the severity of hypoxic and TNF-α mediated responses.

A strategy for reducing viral vector associated risks in vivo is to perform cell mediated vector transduction ex vivo prior to transplantantation in vivo. EPCs have been extensively studied as delivery vehicles for gene therapy due to their role in promoting therapeutic neovascularization in addition to their amenability to viral transduction. EPCs transduced with HRE and NF-kB-regulated viral vectors provide a means of identifying where these cells are incorporated and responsive to either hypoxia or TNF-α. These vectors can be used broadly to study hypoxia or TNF-α stimuli, physiologically or in clinically relevant vector design to regulate gene expression. Our results show that combined promoter regulation with HRE and NF-kb-responsive elements react proportionately to either hypoxia or TNF-α cytokine activation and provide a novel mechanism for the detection of these stimuli in vitro and in vivo.

3. MATERIALS AND METHODS

3.1. Cell culture

Human cord blood mononuclear cells were purchased from Lonza (Cambrex, Massachusetts) and seeded onto 100 mm² fibronectin-coated culture dishes (BD Biosciences). Cells were supplemented with endothelial growth medium (EGM-2) (Lonza), 10% FBS and 100 units/ml penicillin, 100 µg/ml streptomycin and cultured at 37°C, 5% CO₂. Endothelial progenitor colonies were isolated and characterized as previously described (10,11). Following 72 hours of mononuclear cell culture in fibronectin coated dishes with endothelial growth supplemented media (EGM-2 media, Lonza), cultures were then washed extensively to remove any remaining non-adherent mononuclear cells from the adherent endothelial progenitor colonies. The progenitor colonies were subsequently cultured in EGM-2 thereafter. After ~15 days, endothelial colony-forming cells produce a visible outgrowth of endothelial progenitor cells (EPC) with classical endothelial cobblestone morphology. The outgrowths were detached using Hy2tase solution (Thermo-Fisher Scientific) and expanded in EGM-2 media for no more than 5 passages on fibronectin-coated dishes prior to experimentation, so as to maintain the progenitor phenotype.

HEK293FT (Invitrogen) were maintained in DMEM supplemented with 10% FBS for viral packaging. When necessary, before validation in a primary human EPC, we performed vector optimization in the highly stable and transduction permissive HT1080 cell line in accordance with the provider’s directions, HT1080 cells (MEM 10% FBS media; American Type Culture Collection). This ensured cost effectiveness, availability and reduced risk of influencing the cell responses (such as by genetic drift due to multiple cell passages associated with a primary cell), while performing vector editing and served as a check to ensure vector design and responsiveness are not cell dependent.

3.2. Construction of lentiviral vectors

A 3’ polylinker (5’CACCGTTAACAACGAA TTCGCTAGCCTGAGTGACTCCGGAGTTACCGTACGATATCCGCAC-ACCGTACGGATATC) was cloned into a pLenti6/V5-D-TOPO vector to generate pLenti-linker according to the manufacturer’s protocol (Invitrogen). A 620-bp EcoRI-Ncol fragment containing the internal ribosome entry site (IRES) from plasmid pRES2-AcGFP1 (Clontech) was ligated into a 4.3-kb EcoRI-Xbal fragment of pRES (Clontech) along with a 1.7-kb Ncol-Xbal fragment containing the firefly luciferase (Luc) gene from plasmid pGL3 (Promega); this yielded plasmid pRES-Luc. The gene coding for the Aequorea coelebs green fluorescent protein (AcGFP1) was amplified from pRES2-AcGFP1 (Clontech) by PCR using EcoRI-containing (underlined) and SalI-containing (underlined) primers (Table 1). The PCR product was digested with EcoRI-SalI.
and ligated into EcoRI-SalI-digested pIRES-Luc to generate pGFP-IRES-Luc (GIL). The 2.9-kb EcoRI-SalI-fragment containing the GFP-IRES-Luc cassette was ligated with EcoRI-XhoI-digested vector pLenti-linker to generate pLenti-GFP-IRES-Luc (pLenti-GIL).

To excise the CMV sequence as pLenti-ΔCMV-GIL or minimally present the otherwise constitutively active CMV construct as pLenti-mCMV-GIL, the area upstream of CMV and the area behind CMV, or the area of the minimal CMV, were amplified by PCR using ClaI-containing (underlined) primers or a SpeI-containing (underlined) primer (Table 1). The two PCR products were digested with ClaI-SpeI and respectively ligated into ClaI-SpeI-digested pLenti-GFP-IRES-Luc to generate pLenti-ΔCMV-GIL and pLenti-mini-CMV-GIL. Eight copies of the hypoxia response element (HRE) consensus sequence (GCCCTACGTGCTCTACACAG-CCTGTCTGACC TCTCGACCTACCGGCGCTGAGGCCACAAGCTC) of the human erythropoietin (EPO) gene enhancer were respectively inserted into pLenti-ΔCMV-GIL, upstream of GFP and Luc gene to generate pLenti-HRE-GIL. Four copies of the NF-kappaB binding sequences (NF-kB) of the human vascular cell adhesion molecule (VCAM) promoter (CTGGGTTCCTCTGAAGGATTCTCCT) were respectively inserted into: 1) pLenti-ΔCMV-GIL 5’ of the GFP gene, 2) pLenti-HRE-GIL 3’ region of the HRE sequences and 5’ region of the GFP gene, or 3) pLenti-HRE-ACMV-GIL 3’ region of the HRE sequences and 5’ region of the minimal CMV promoter to generate: 1) pLenti-NF-kB-GIL, 2) pLenti-HRE-NF-kB-GIL and 3) pLenti-HRE-NF-kB-ΔCMV-GIL.

### 3.3. Lentivirus production

Lentiviral particles were generated using ViraPower™ Lentiviral Expression System (Invitrogen) according to the manufacturer’s directions. Lentiviral expression constructs were cotransfected with ViraPower™ packaging mix: pLP1, pLP2, and pLP/VSVG using Lipofectamine2000 (Invitrogen) into the HEK293FT producer cell line. Supernatants were collected 48 hours after transfection, centrifuged and filtered through a 0.45-µm filter. Viral titer was determined by blasticidin selection (10 µg/ml) on HT1080 cells 48 hours after transduction with 10-fold serial dilutions of the lentiviral supernatant. After 10 days of selection, the cells were stained with crystal violet and colonies were counted.

### 3.4. Lentiviral transduction

The titrated lentivirus was diluted into fresh medium to obtain a suitable Multiplicity of Infection (MOI). EPCs cultured in EGM-2 media (or HT1080 cells during vector optimization studies) were cultured in MEM and supplemented with 10% FBS were transduced with lentiviruses at MOI of 1 or 5 for 16 hours at 37°C, 5% CO₂ in a humidified atmosphere. Following transduction, the viral media was removed and replaced with fresh, complete culture medium. The transduced cells were further cultured for 4 days for in vitro or in vivo analysis.

### 3.5. In vitro hypoxia and TNF-α treatment

Lentiviral transduced EPCs were passaged into multi-well plates and distributed into four experimental groups: 1) normoxic conditions (5% CO₂), 2) inflammatory cytokine (hTNF-α, (10 µg/ml), 3) hypoxia (5% CO₂ and 1% O₂), and 4) H&I (T, B, and NK cell deplete; aged 6-10 weeks) received human EPCs transduced with lentiviral vectors. The experimental protocol was approved by the Queen’s University Animal Care Committee in accordance with national and international guidelines for animal care and the use of laboratory animals. Mice were only handled within laminar-flow hoods. Lentiviral-transduced EPCs were collected and suspended in 300 µl of sterile saline (total cell number ~1 × 10⁶) and slowly administered via tail vein injection. Mice were allowed to incorporate human EPCs for two-weeks post-injection and no adverse events were observed before separating animals into four groups: 1) normoxic, 2) inflammatory cytokine (10 µg/kg, hTNF-α), 3) hypoxic (10% O₂), and 4) H&I (10 µg/kg, hTNF-α, 10% O₂) for 16 h. Animals were immediately sacrificed in location and tissues from liver, spleen, heart, kidney, lung and brain were collected and analyzed for GFP and luciferase expression.

### 3.6. In vivo murine model of hypoxia and inflammatory cytokine activation

Immunodeficient male Rag2⁻/⁻Il2rg⁻/⁻ mice (T, B, and NK cell deplete; aged 6-10 weeks) received human EPCs transduced with lentiviral vectors. The experimental protocol was approved by the Queen’s University Animal Care Committee in accordance with national and international guidelines for animal care and the use of laboratory animals. Mice were only handled within laminar-flow hoods. Lentiviral-transduced EPCs were collected and suspended in 300 µl of sterile saline (total cell number ~1 × 10⁶) and slowly administered via tail vein injection. Mice were allowed to incorporate human EPCs for two-weeks post-injection and no adverse events were observed before separating animals into four groups: 1) normoxic, 2) inflammatory cytokine (10 µg/kg, hTNF-α), 3) hypoxic (10% O₂), and 4) H&I (10 µg/kg, hTNF-α, 10% O₂) for 16 h. Animals were immediately sacrificed in location and tissues from liver, spleen, heart, kidney, lung and brain were collected and analyzed for GFP and luciferase expression.

### 3.7. Analysis of GFP and luciferase expression

Cells and tissues were analyzed for GFP by FACS (FC500 Beckman Coulter) or microscopy (Leica DM-IRB), whereas luciferase was quantified
following cell/tissue lytic analysis using the Bright-Glo™ Luciferase Assay System (Promega) in a Lumat LB 9507 Luminometer (Berthold). The protein concentrations of the lysates were measured for normalization via BCA Protein Assay Kit (Thermo Fisher Scientific).

3.8. Statistical analysis
Data are expressed as mean ± SD. Statistical analyses were performed using Prism 4 software (GraphPad Software Inc.). Statistical significance between data sets was assessed using one-way ANOVA and Tukey's multiple comparison test (P<0.05 was considered significant).

4. RESULTS AND DISCUSSION

4.1. Combined promoter vector generation
An emerging requirement in vector development is to overcome simple monogenic delivery (the delivery of a single gene by a single vector) by using bicistronic vectors (delivery of two genes by a single vector) in order to better provide additional gene products and greater efficacy (i.e. gene adjuvants-genes that augment or synergize the efficacy of each other). This efficacy was recently demonstrated using dual protein kinase-B (Akt) and heme-oxygenase-1 (HO-1) expression in human EPCs for cell-mediated gene therapy (11), where combined Akt/HO-1 gene delivery into EPCs was more effective than either gene alone in promoting EPC-mediated recovery following infarction. However, this required adding two viral vectors, doubling the viral particle exposure to EPCs prior to transplantation. Further, the exogenous genes expressed in that model were constitutive and not regulated by stress. Additionally, constitutive viral transgene expression increases the risk of transactivation of neighbouring genome sequences (12). To jointly detect inflammatory and hypoxic responses, we created a single functional vector for high fidelity visualization and quantification of GFP and Luc activity using the promoters of NF-kappaB (Akt) and heme-oxygenase-1 (HO-1) expression in human fibrosarcoma cells (13). To construct this promoter, the pLenti6/v5-D-TOPO lentiviral vector was first modified by inserting nine restriction enzyme sites into the multiple cloning site (MCS) of the vector and generated the pLenti-linker vector (Figure 1A). A GFP-IRE-Luc cassette was then subcloned into pLenti-linker vector, under the ubiquitous and constitutive regulation of the cytomegalovirus (CMV) promoter to generate bicistronic combinatorial lentiviral vector, pLenti-GIL (GFP-IRE-Luc; Figure 1A). To reduce the risk of side effects to viral exposure, cells are first transduced ex vivo. When the human EPCs were stably transduced with pLenti-GIL, GFP was expressed in over 90% of cells at 5 multiplicities of infection (MOI) (Figure 1B). When compared to control cells that were either non-transduced or transduced with a Lac-operon at equal titer, luciferase activity was only observed in the pLenti-GIL EPCs (Figure 1C). When the bicistronic orientation was reversed by placing Luc upstream and the GFP downstream of the internal ribosomal entry site (IRES), the vector showed a similar transduction efficiency, however, intensity of GFP was lower (data not shown). This is consistent with prior reports that showed that the expression diminishes when Luc or GFP are placed downstream of the IRES promoter (13). When the CMV was excised from the expression vector, there was no measurable Luc activity (Figure 1D). When a minimal CMV enhancer was inserted into the vector, there was minimal promoter leakage or IRES drive, even when human fibrosarcoma cells were transduced and subjected to permissible and optimal condition (Figure 1E-F).

4.2. Construction of expression vectors responsive to hypoxia and inflammatory cytokine stimulation
To develop HRE and NF-kB-responsive vector, we first developed pLenti-∆CMV-GIL (deleted CMV) and pLenti-miniCMV-GIL (minimal CMV) by removing or partially deleting the internal CMV promoter. The cells transduced with pLenti-∆CMV-GIL and pLenti-miniCMV-GIL did not express GFP and Luc (Figure 1D-F). We subsequently generated three vectors, pLenti-NFkB-GIL, pLenti-HRE-GIL and pLenti-HRE-NFkB-GIL by respectively inserting NF-kB-responsive elements (four copies), HRE (eight copies) or both into pLenti-∆CMV-GIL (Figure 2A). The number of repeat sequences and orientation of each sequence was empirically changed for optimizing the responses to hypoxia or TNF-α.

Previous reports demonstrated cross talk between HREs and NF-kappaB promoter activity (14-15). However, we failed to see such a cross talk by the vectors that we developed (Figure 2B) since hypoxia did not increase Luc activity in the pLenti-NF-kB and neither did TNF-α increase Luc activity in the pLenti-HRE alone. The responsiveness of the initial vector to hypoxia was lower than that expected for use in assessment of hypoxia and TNF-α mediated responses. To overcome this problem and to enhance transactivation without loss of promoter specificity, we reinserted a minimal CMV promoter into the vector, a strategy previously shown to amplify transcriptional sensitive activity (16). This significantly improved the promoter response to hypoxia or TNF-α (Figure 2C). Importantly, as previously reported, the minimal CMV eliminated the significant differences between responses to hypoxia and TNF-α, synergistically amplifying the combined promoter effect of HRE and NF-kappaB response elements (Figure 2C, purple bar & D).

4.3. In vitro response of vectors to hypoxia and inflammatory cytokine stimulation
We tested the responsiveness of the pLenti-NFkB-GIL, pLenti-HRE-GIL and pLenti-HRE-NFkB-GIL in response to normoxia, hypoxia and TNF-α that drives the expression of NF-kB promoter.
Hypoxia-inflammatory cytokine detection

4.3.1. In vitro response to hypoxia
Hypoxia induced up to three-fold increase in luciferase activity in pLenti-HRE-GIL and pLenti-HRE-NFkB-GIL transduced EPCs as compared to transduced EPCs that were exposed to normoxia (Figure 2B, blue).

4.3.2. In vitro response to TNF-α
EPCs transduced with the same vectors showed twelve-fold increase in luciferase activity when exposed to normoxia and treated with TNF-α, as compared to transduced EPCs that were exposed to normoxia alone (Figure 2B, red).

4.3.3. In vitro response to hypoxia and TNF-α
EPCs that were transduced with pLenti-HRE-NFkB-GIL showed up to fifteen-fold higher luciferase activity when exposed to hypoxia and treated with TNF-α as compared with control EPCs exposed to normoxia alone (Figure 2B, purple).
These findings show that pLenti-HRE-NFkB-GIL is responsive to both hypoxia, and TNF-α alone or both. For this reason, the vector, can be used for the assessment of all conditions that lead to hypoxia and inflammatory cytokine activation. Hypoxia and inflammatory cytokine activation coincide in a multitude of disease processes. Recent studies have established that regulation of HRE and NF-kappaB elements may be cooperative. pLenti-HRE-NFkB-GIL vector can be employed in vitro for the assessment of hypoxia and TNF-α (Figure 2).

4.3.4. In vivo response to hypoxia and TNF-α

To examine the expression system in vivo, human EPCs were transduced with the pLenti-BHRE-4NFkB-mini CMV-GIL-regulated lentivirus (Figure 3A). To avoid rejection, these cells were injected via tail vein into Rag2−/−Il2rg−/− immune deficient mice. Two weeks later, luciferase activity was measured in various tissues of animals that were exposed to hypoxia for 16 hours and treated with and without TNF-α (16 hours). In heavily vascularized tissues such as lung, liver and spleen, luciferase activity was ~2.5-fold higher in response to hypoxia or TNF-α alone (17). However, when hypoxia was combined with treatment with TNF-α there was > thirty-fold increase in luciferase activity in these tissues (Figure 3B). This could be the result of differential EPC distribution after intravenous infusion as previously reported, which can vary according to cell size or mechanisms of EPC adhesion (18-20). Alternatively, these organs may be more or less susceptible to H&I due to their perfusion (21). A limitation to our study is that
these organs were homogenized for luciferase analysis; future studies should examine EPC distribution and the colocalization of nuclear translocation in NF-kB and HIF-transcription factors by immunohistochemistry. In constrast in the heart, brain and kidney, there was only a 2-3 fold increase in luciferase activity when these tissues were subjected to both hypoxia and TNF-α (Figure 3B). Based on such evidence, the vector may be used to assess hypoxic and TNF-α cytokine-associated effects in vitro and in vivo.

5. ACKNOWLEDGMENTS

This work was funded in part by the Heart and Stroke Foundation of Canada Grant to LGM and CAW; Natural Sciences and Engineering Council grant to KRB. During this work Dr. Luis G. Melo was deceased. The authors do not declare any conflicts of interest.

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Abbreviations: AAV9: adeno-associated-virus

Key Words: Hypoxia, Inflammation, HRE, NF-kappa B, Vector design

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