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Involvement of HIF-1 &HIF-2 in the hypoxic induction of VEGF and MMP-7 genes

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Abstract

Aim: To describe the use of siRNA technology to inhibit HIF-1α and HIF-2α expression in primary macrophages cultured *in vitro*, to determine whether pro- MMP-7 expression regulated by these two HIFs. In these experiments, the well described HIF-target, VEGF, is used as a positive control whenever possible.

Methods: Monocytes were isolated from buffy coats & grown for seven days to be differentiated into MDM, then HIF-1 α, HIF-2α expressions were inhibited by transfection with siRNA oligonucleotide, finally the expressions of VEGF & MMP-7 were examined.

Results: Complete inhibition of pro-MMP-7 & VEGF release by MDM showing that their hypoxic regulation is entirely dependent of HIF-1 and HIF-2.

Conclusion: These findings indicate that both genes identified could be important for the survival and functioning of macrophages in hypoxic diseased tissues. Moreover, these data emphasize that the role of HIF-2α in

addition to HIF-1α needs to be considered when developing agents that target the HIF pathway.

Introduction

The presence of multiple areas of hypoxia (low oxygen tension) is a hallmark feature of human and experimental tumors. Monocytes are continually recruited into tumors, where they differentiate into tumor-associated macrophages (TAM) and gather in hypoxic and/or necrotic areas. A number of recent studies have shown that macrophages respond to the levels of hypoxia found in tumors by up-regulating such transcription factors as hypoxia-inducible factors 1 and 2, which then activate the expression of a broad array of mitogenic, pro-invasive, pro-angiogenic and pro-metastatic genes. We showed the mRNA for the gene encoding the pro-tumour enzyme, matrix metalloproteinease-7 (MMP-7 or matrilysin), to be upregulated by human macrophages exposed to hypoxia *in vitro* (Burke *et al*., 2003). Here, we extend this finding to show that these cells also upregulate their release of Pro-MMP-7 protein when exposed to hypoxia and that this is a HIF-1 & HIF-2 dependent phenomenon.

Introduction

The presence of multiple areas of hypoxia is a characteristic feature of malignant tumours resulting from local imbalance between the supply of oxygen by blood vessels and its consumption by the surrounding tumour mass (Vaupel *et al*., 2001).

Many of the adaptations to hypoxia are mediated by the activation of specific genes through the action of hypoxia inducible transcription factors (HIF-1 and HIF-2) (Wiesener *et al*., 1998). These proteins are heterodimers consisting of two different α units and a common constitutive β subunit, both basic helix loop helix (BHLH)-PER ARNT SIM (PAS) domain protein (Semenza 2002). Under normoxic conditions the α subunit is hydroxylated

at specific proline residues resulting in ubiquitination through the interaction with von Hippel-Lindau protein (pVHL) and proteosomal degradation. This process inhibited under hypoxia, as a result HIFα accumulates in the nucleus and bind to short DNA sequences called hypoxia-response elements (HREs) ′5-RACGTG-3` near or in oxygen-sensitive genes, stimulating gene expressions which have important roles in tumour biology such as angiogenesis, glucose/energy metabolism, cellular growth, metastasis and apoptosis (Maxwell *et al*., 1997; Semenza *et al*., 2000; Zarember *et al*., 2005).

Tumour associated macrophages (TAMs) are present in large numbers in most types of human tumour. Indeed they can constitute up to 80% of the total tumour mass in breast carcinomas (Kelly *et al*., 1988). They originate mainly from the circulating monocytes rather than tissue macrophages (Yamashiro *et al*., 1994). In breast carcinomas, macrophages are found mainly in the poorly vascularized, hypoxic regions (Leek *et al*., 1996). Under hypoxic conditions, it is thought that they alter their gene expression and adapt their metabolic activity to anaerobic glycolysis, with increased production of lactate and pyruvate, and metabolic acidosis (Lewis *et al*., 1999). TAMs seem to have a complex role in tumour growth as they can exert both anti-tumour and pro-tumour effects through a variety of cytokines and enzymes (Bingle *et al*., 2002).

TAMs respond to hypoxia by expressing pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) (Lewis *et al*., 2000). Moreover, macrophages exposed to hypoxia in *vitro* up-regulate their release of VEGF (Harmey *et al*., 1998). Macrophages also known to release PR-39 amino acid peptide, that inhibits the degradation of HIF-1α by neighbouring cells and stimulate their expression of VEGF (Lie *et al*., 2000). Once macrophages reach hypoxic regions in the tumour they promote tumour progression by releasing factors which stimulate tumour angiogenesis and reduced patient survival in various forms of cancers such as breast cancer (Leek *et al*., 1996) and pulmonary adenocarcinoma

(Takanami *et al*., 1999). Macrophages express abundant HIF-1α in hypoxic areas of various tumour types (Burke *et al*., 2002).

MMP-7 (matrilysin) is one of the smallest MMPs, consisting of two domains, a pro-domain and a catalytic domain (wossner *et al*., 1998). MMP-7 is secreted as a 28 kDa proenzyme. Activation of the proenzyme involves a proteolytic removal of the N-terminal pro-region containing the cysteine switch motif conserved in MMPs. The resulting mature and active enzyme (19 kDa) consists of a catalytic domain with a zinc binding motif (Van Wart, *et al*., 1990).

MMP-7 is expressed in epithelial cells of normal and diseased tissues (Wilson *et al*., 1996). The protein localizes in normal tissues to secretory and ductal epithelium in the endometrium, in various exocrine glands and macrophages (Busiek *et al*., 1992; Rodgers *et al*., 1994). MMP-7 up regulated by cytokines in inflammation result in increased level of TNF from macrophages to remodel the regenerated tissue (Gearing *et al*., 1994) It is also expressed in a variety of tumours ranging from breast, colon, prostate, stomach, skin and soft tissue tumours (Hashimoto *et al*., 1998; Shapiro *et al*., 1999; Deny *et al*., 2004; Yamamoto *et al*., 2004) and contributes to proliferation, angiogenesis and metastasis. Knockout mice lacking the gene have suppressed intestinal tumourigenesis (Wilson *et al*., 1997; Jiang *et al*., 2005). On the other hand, Patterson *et al*., 1997 has demonstrated that two member of human matrix metalloproteinase MMP-7 and MMP-9 hydrolyse human plasminogen to generate angiostatin fragment. Therefore, MMP-7 and MMP-9 may regulate new blood vessel formation through angiostatin which is a specific inhibitor of angiogenesis.

MMP-7 mRNA has been shown to be up regulated in hypoxic macrophages and that the promoter of MMP-7 is hypoxia- inducible in macrophages, and element from this promoter may prove useful in the

future development of hypoxia inducible therapeutic constructs optimized for use in macrophages.(Burke *et al*., 2003).

The aim of this study is to detect the MMP-7 and VEGF protein in hypoxic macrophages and use the siRNA technology to knockout HIF-1α & HIF-2α and see if these two genes are HIF-1 & HIF-2 dependent.

Materials and Methods

Isolation and culture of primary human monocyte-derived macrophages

Human monocytes were isolated from leukocyte enriched buffy coats obtained from healthy blood donors (National Blood Service, Sheffield, UK). Blood was diluted 1:1 with HBSS (without calcium or magnesium), layered on Ficol-Paque Plus (Amersham Biosciences, UK) and centrifuged for 40 min at 400 g. The mononuclear cell-rich band was removed, washed twice with HBSS and resuspended in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 2% human ABC serum, 2mM L-Glutamine, penicillin (100 IU/ml), and streptomycin (100µg/ml)(All from Sigma, Poole, UK). 8×10^7 mononuclear cells were seeded into 10 cm tissue culture plates (Iwaki, town,Japan) and cultured for 1 h after which non-adherent cells were removed by washing and the culture medium replenished. Adherent monocytes were cultured for 7 days, to allow differentiation into monocyte-derived macrophages. (Zuckerman *et al*., 1979). Purity and differentiation status of cells was checked using CD68 immunocytochemistry and carboxypeptidase M in flow cytometry. (Rehli, *et al*., 1995)

Hypoxic culture and protein extraction

Monocyte-derived macrophages were subjected to 0.1% (hypoxia) or 20.9% (normoxia) O_2 in 5% CO_2 humidified multi-gas incubators (Heto, Camberly, UK) for 1,6,16, and 24 h. Incubator oxygen levels were confirmed at the end of all experiments using mobile oxygen analyzers

(Analox Sensor Technology, Cleveland, UK). Culture medium depths of less than 2 mm were used throughout this study to ensure rapid removal of oxygen from the culture media during hypoxic experiments.

Preparation of MDM cell extracts and immunoblotting

Following hypoxic or normoxic incubation MDM were washed in PBS and total cell extracts obtained by lysing cells in lysis buffer (50 mM tris HCL pH 8.0, 150 mM NaCl, 1% triton X-100 and 1 protease inhibitor tablet (Roche, Mannheim, Germany). Cell lysates were incubated on ice for 40 minutes, sheered by repeated passage through a 25-gauge needle and then centrifuged at 400 g for 10 minutes at 4ºC to remove cell debris. Nuclear and cytoplasmic MDM extracts was prepared using CelLytic Nuclear extraction (Sigma, Poole, UK). All extracts were stored at -20ºC until immunoblot analysis. Protein concentration of cell extracts was estimated using QuantiPro BCA reagent (Sigma, Poole, UK). Samples for immunoblotting were prepared by heating to 100°C for 5 minutes in reducing loading buffer. 60µgsample was run on a 10% SDS-PAGE gels and after separation the proteins transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK). The membrane were incubated for 16 hours at 4ºC in blocking buffer (5% skimmed milk powder in TBST – 10mM Tris, 180mM NaCl, 0.05% Tween-20, pH 8) then probed with anti-HIF-1 (BD.Biosciences, San Jose, CA)or anti-MMP7 (R&D Systems,Abingdon, UK) antibody diluted to 1:250;1:100 concentration respectively in blocking buffer and incubated for 2 hatroom temperature. After washing with TBST the membranes were then exposed to anti-mouse secondary antibody conjugated with horseradish peroxidase (Dako Ltd, Copehnagen, Denmark) at a 1:2500 dilution in blocking buffer for 1 hour at room temperature. The secondary antibody was detected using enhanced chemiluminescence reagent plus (Amersham, Little Chalfont, UK). To confirm equal loading, membranes were stripped with stripping buffer (100 mM beta mercaptoethanol, 2% SDS, 62.5mM Tris HCl pH 6.7)

at 50°C for 30 minutes and re-blotted with a mouse monoclonal antibody to □-actin (Sigma, Poole, UK) at 1:15,000in blocking buffer. Protein expression was quantified by densitometry using Quantity One software (BioRad, Hercules, CA).

Transient transrection of cells with siRNA duplexes

The siRNA oligonucleotides used in this study were purchased from Eurogentec (Seraing, Belgium). The HIF-1α siRNA duplex targets nucleotides 1521-1541 of the HIF-1α mRNA sequence (NM001430) and is comprised of sense 5'–CUGAUGACCAGCA-ACUUGAdTdT-3 and antisense 5'–UCAAGUUGCUGGUCAUCAGdTdT-3. The inverted HIF-1α control duplex did not target any gene and comprised of sense 5'–AGUUCAACGACC-AGUAGUCdTdT-3 and antisense 5'–GACUACUGGUCGUU-GAdTdT-3. Duplexes were prepared by mixing 50µM concentration of antisense and sense oligonucleotides with annealing buffer (30mM HEPES pH 7.0, 100mM potassium acetate, and 2mM magnesium acetate) heat denaturing for 1 min at 85ºC and annealing at 37ºC for 1 hour. Duplex formation was confirmed by electrophoresis through 5% low melting temperature agarose (NuSieve GTC; FMC Bioproducts, Rockland, ME)

siRNA treatment of human monocyte-derived macrophages

Monocyte-derived Macrophages were cultured for 5days in 6 well plates (initial seeding density was 20 x 10^6 mononuclear cells/well). 5µg of 20 nM siRNA duplex was diluted in IMDM containing 2% AB serum, 2mM L-Glutamine, penicillin (100 IU/ml), and streptomycin (100µg/ml) to give a final volume of 100µl, then mixed by vortexing for 10 seconds. For complex formation 15µl of RNAiFect transfection reagent (Qiagen, Crawley, UK) was added to the diluted siRNA and mixed by vortexing for 10 seconds and then incubated for 15 minutes at room temperature. Adherent macrophages were washed with PBS and 1900µl of IMDM

medium added to the cells. 2000µl of the complexes were added drop wise onto the cells and the plate gently agitated before incubating 37ºC. Following 24 h incubation macrophages were washed and incubated for an additional 24 h before beginning the hypoxic experiments.

Measurement of VEGF and MMP-7

Tissue culture supernatants of monocyte-derived macrophages exposed to normoxia and hypoxia were collected, centrifuged for 10 minutes at 400 x g to remove cell debris and then concentrated 5 x using vivaspin Millipore columns.Secreted MMP-7 and VEGF were measured in the concentrated supernatant using the respective Quantikine ELISA kit (R&D Systems, Abingdon, UK).

Statistical analysis:

A parametric t-test was used to evaluate significance between experimental groups. *p*

values exceeding 0.05 were considered not significant.

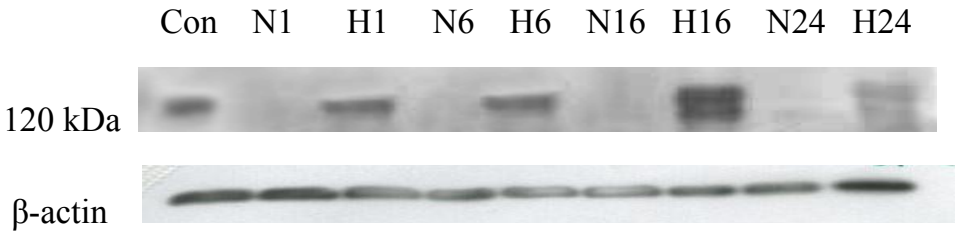
Results

Immunoblots for detection of HIF-1α in human MDM

A HIF-1α protein was detected in macrophages in total cell extract by using Western blotting. The protein was up-regulated at 1 and 6 hours reaching high level at 16 hours up to five folds and then start to decline at 24 hours of hypoxia (0.1% O_2) with no HIF-1α expression at 20.9% O_2 (Figure 1).

By looking at the cytoplasmic extract there is no HIF-1α expression detected unlike the nuclear extract where HIF-1α is up regulated after 16hours of hypoxia up to 7 folds and increase further when the cells treated

with LPS. There is small amount of HIF-1α protein detected in the hypoxic cytoplasmic fraction after LPS treatment (Figure2).



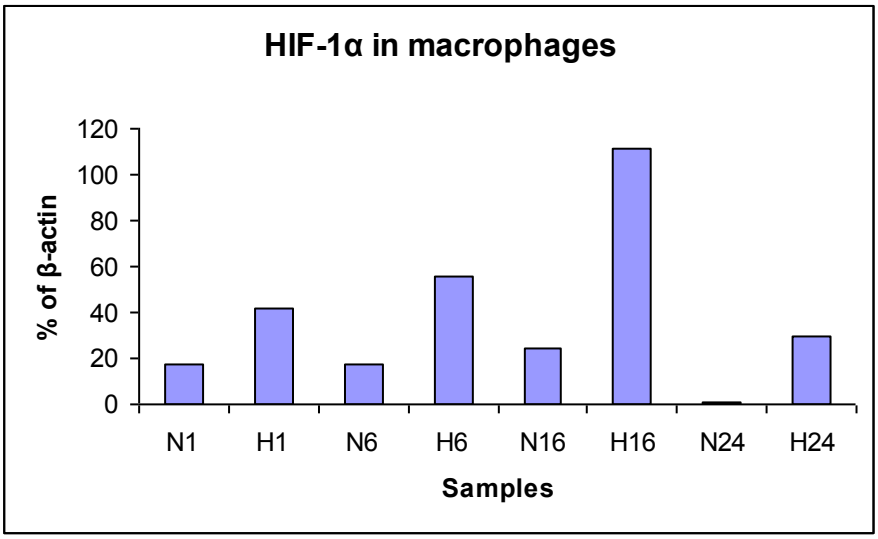


Figure1: HIF-1α expression in human MDM. Hypoxic induction of HIF-1α macrophage showing bands (at approximately120 kD) in total cell extracts following exposure to normoxia (N) (20.9% O_2) or hypoxia (H) (0.1% O_2) for 1, 6, 16 and 24 hours of hypoxia with the densitometry. The membrane was reblotted with the β-actin antibody to ensure equal loading.

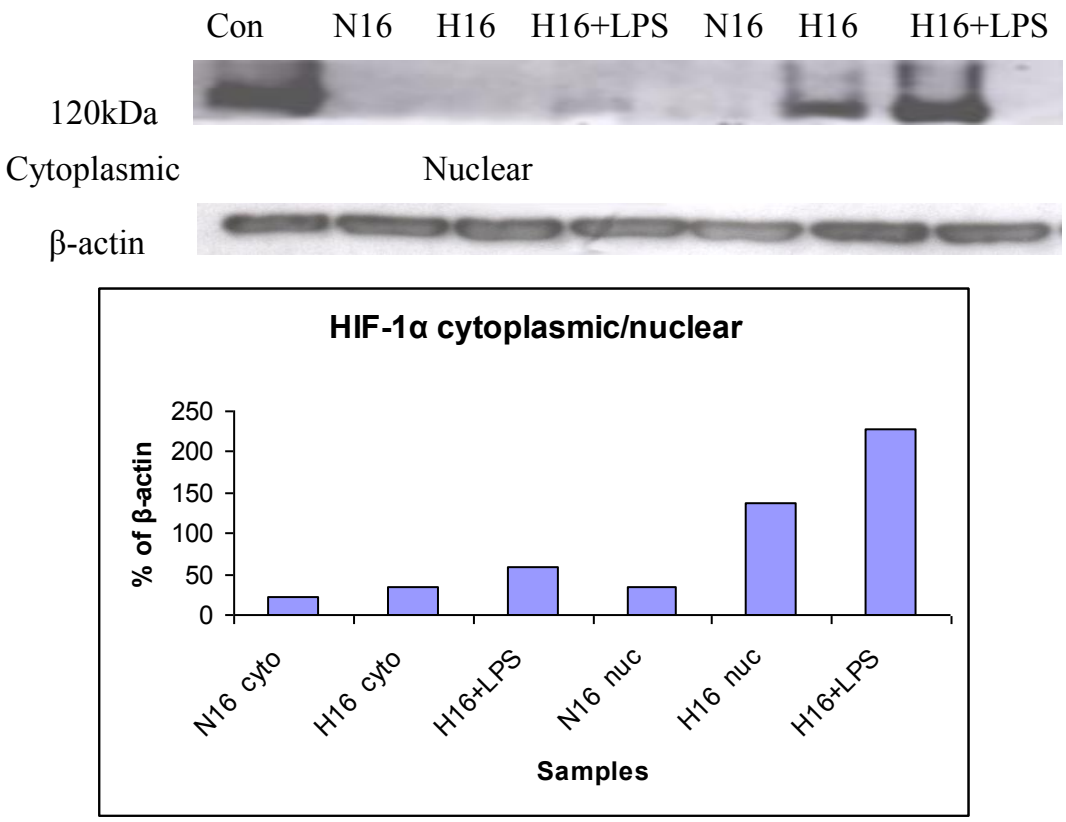
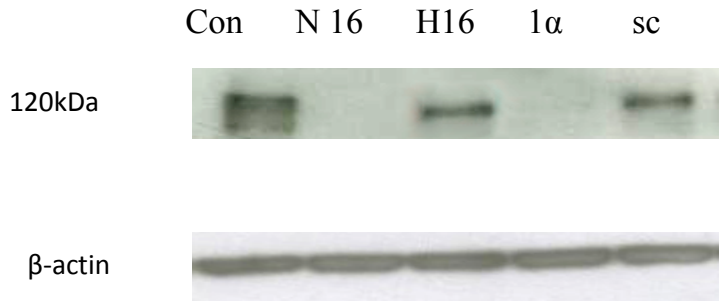


Figure 2: HIF-1α expression under the effect of LPS in the cytoplasmic and nuclear extracts in MDM. HIF-1α protein was detected mainly in the nuclear extract and increase further when the cell treated with LPS after 16 hour hypoxia (0.1% O_2). There was no HIF-1α expression in the cytoplasmic extract with the densitometry. The membrane was reblotted with the β-actin antibody to ensure equal loading.

Transient transfection of HIF-1α in human MDM

MDM were transfected with 5µg of 20 nM siRNA duplex and then the protein was checked through Western blotting after 48 hours transfection. HIF-1α protein was reduced in hypoxic macrophages treated with siRNA. There was no HIF-1α inhibition in the cells treated with the same concentration of the scrambled peptide (control) (Figure 3). The cells were viable after the siRNA transfection by using promidium iodide stain (PI) (data not shown).



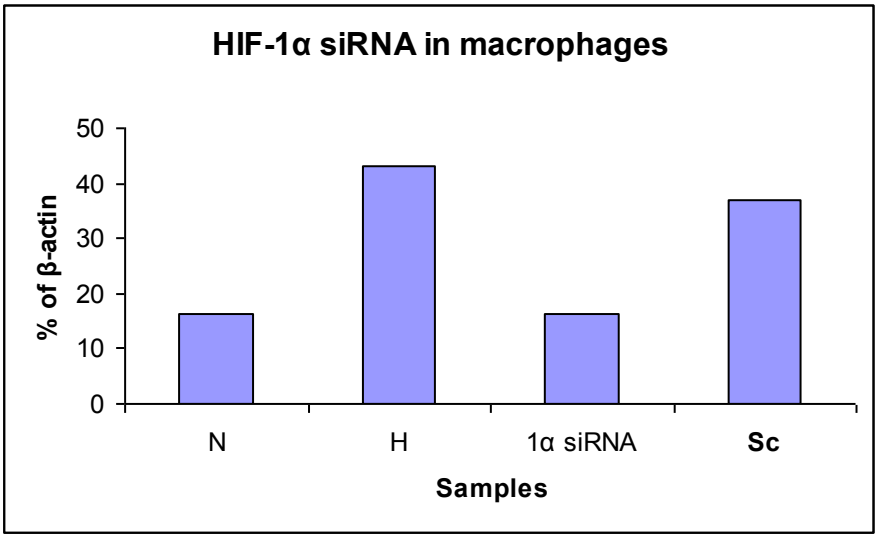
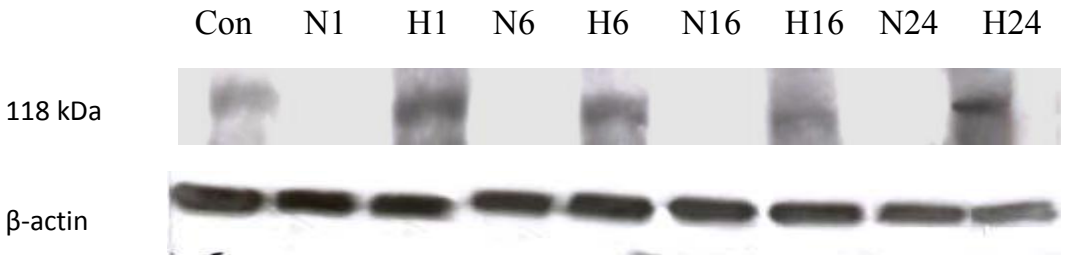


Figure 3: Transient transfection of HIF-1α in MDM. Cells treated with 5μg siRNA for 48 hours shows complete inhibition of the HIF-1α protein in the hypoxic macrophages as detected by Western blotting. There was no effect on HIF-1α expression in the cells treated with the scrambled peptide (control). The β-actin protein was used as a loading control.

Immunoblots for detection of HIF-2α in human MDM

A HIF-2α protein was tested in macrophages in total cell extract by using Western blotting. HIF-2α protein was not detected under normoxic conditions (20.9%) at 1, 6, 16 and 24 hours. However, macrophages were found to upregulate HIF-2α protein when subjected to hypoxia (0.1% O_2). In contrast to HIF-1α, maximal levels of HIF-2α were seen after 1 hour of hypoxia up to 6 folds. Thereafter, protein levels gradually declined. HIF-2α protein was stable at 24 hours, unlike HIF-1α, which had started to decline at this point (Figure 4).

As with HIF-1α, HIF-2α is translocated to the nucleus under hypoxic conditions HIF-2α was found not to accumulate in either the cytoplasmic or nuclear fractions of MDM in normoxia. However, after 16 hours exposure to hypoxia, HIF-2α protein was detected in the nuclear fraction up to 8 folds (Figure 5).



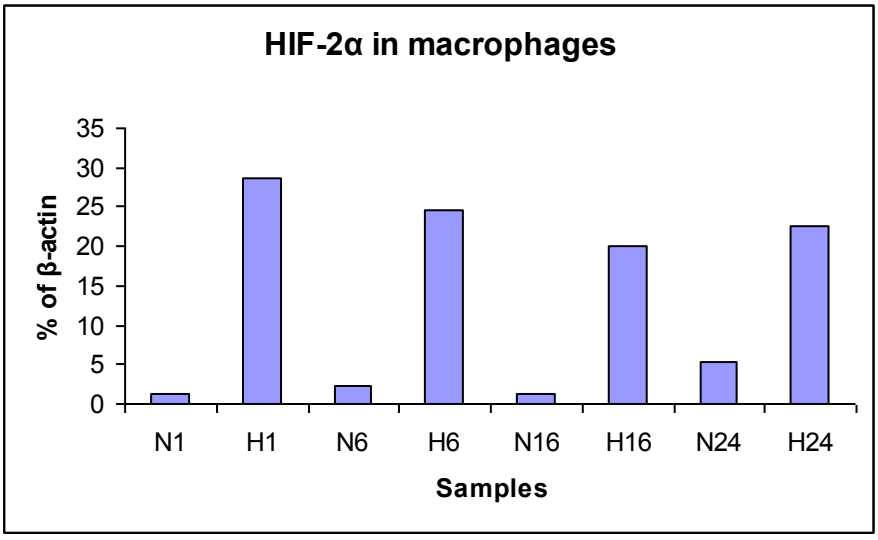
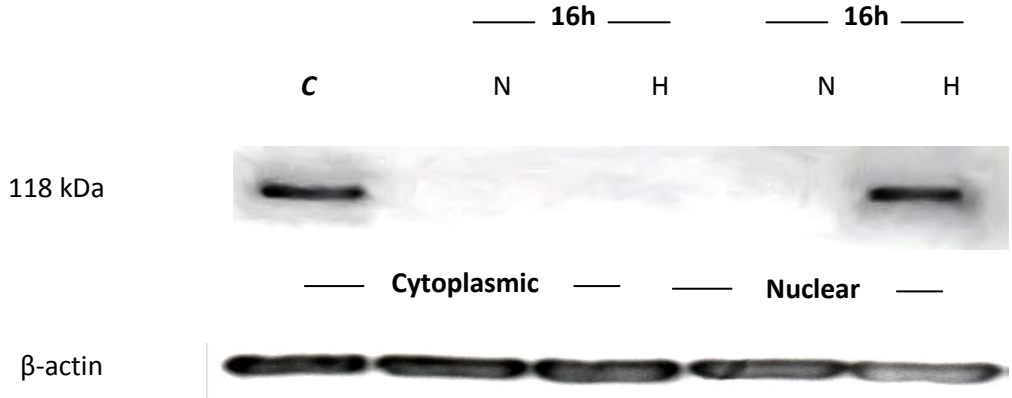


Figure 4. Effect of hypoxia on HIF-2α accumulation in human monocyte-derived macrophages *in vitro*. Immunoreactive HIF-2α protein bands (at 118 kD) were seen in total cell extracts (A) following exposure of cells to hypoxia ('H'; 0.1% O_2) for 1, 6 or 16 & 24 h. No bands were visible in extracts after cells experienced normoxia for the same periods of time ('N'; 20.9% O_2). The membrane was stripped and re-blotted with a β-actin antibody to ensure equal loading of all lanes.



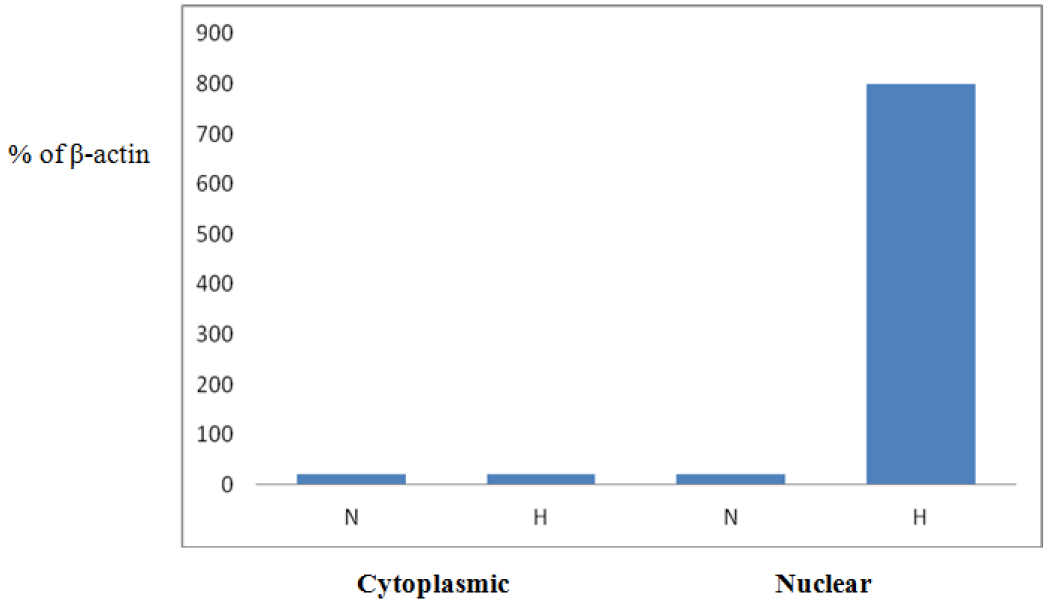


Figure 5. Immunoreactive HIF-2α protein bands were seen in nuclear but not cytoplasmic cell extracts following exposure of cells to hypoxia for 16h. The control is human breast carcinoma cells (T47D), exposed to hypoxia for 16h.

Transient transfection of HIF-2α in human MDM

Macrophages were transfected with HIF-2α siRNA at concentration of 250 nM. HIF-2α protein expression was inhibited in these cells after 16 hours hypoxia. There was no effect on HIF-2α expression after transfection with the scrambled oligonucleotide siRNA probe (Figure 6).

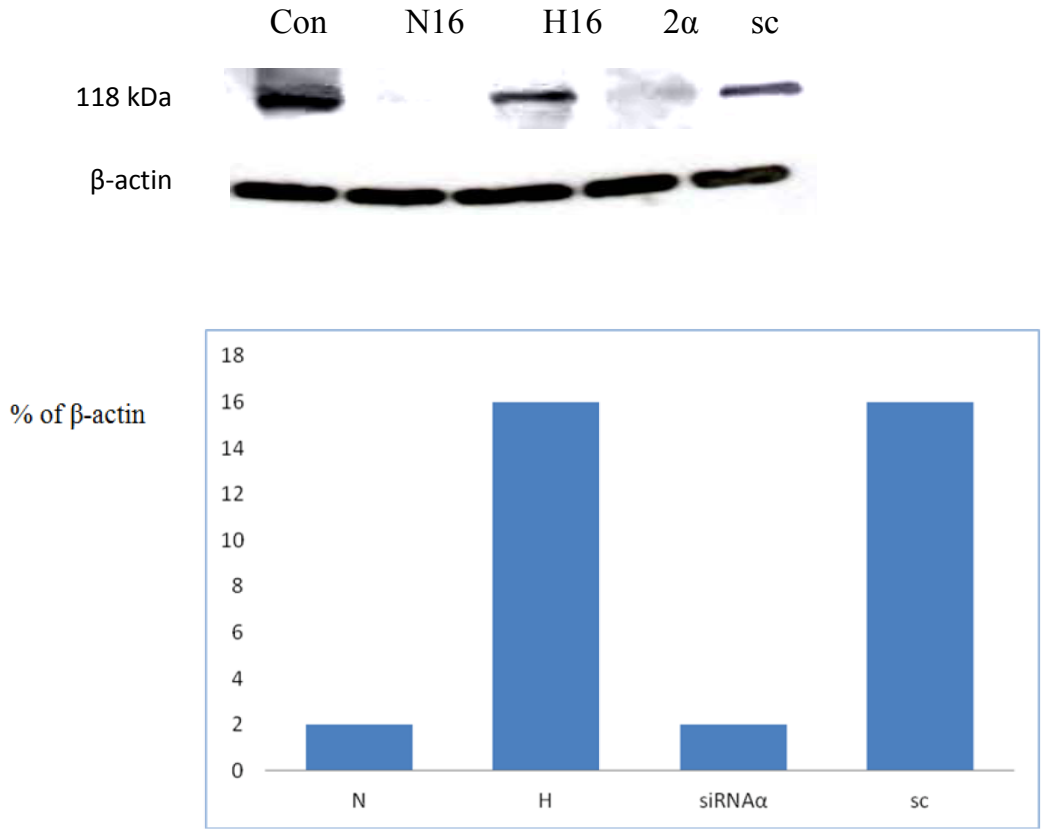


Figure 6: Transient transfection of HIF-2α in MDM. Cells treated with 5μg siRNA for 48 hours shows complete inhibition of the HIF-2α protein in the hypoxic macrophages as detected by Western blotting. There was no effect on HIF-1α expression in the cells treated with the scrambled peptide (control). The β-actin protein was used as a loading control.

VEGF expression in HIF-1α knockout macrophages

Media was collected after the siRNA transfection and concentrated before the ELISA experiment. VEGF protein was decreased to the normoxic level

in the HIF-1α knockout cells. This result suggests an important role for HIF-1α in the expression of VEGF protein under hypoxia (Figure 7).

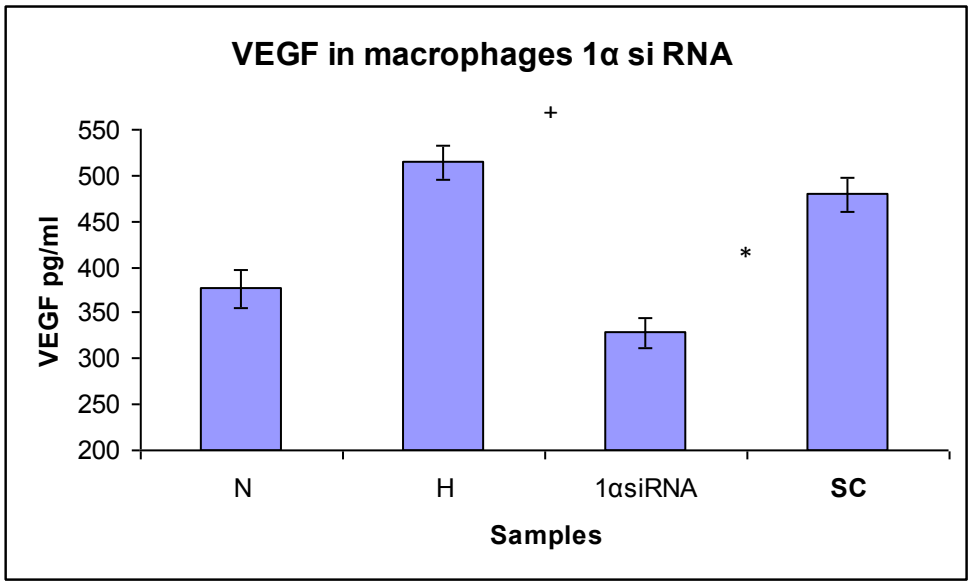


Figure 7: ELISA for VEGF expression in HIF-1α knockout in macrophages. VEGF protein was detected at the normoxic level in the absence of HIF-1α. + P<0.001 w.r.t. MDM cultured under normoxic conditions.* P<0.001w.r.t scrambled siRNA-treated MDM cultured under hypoxic conditions.

Hypoxic induction of pro-MMP-7 release by human macrophages: role of HIFs:

Pro-MMP-7 release was significantly ($P < 0.001$) upregulated by hypoxic macrophages compared to macrophages cultured in normoxic conditions. Treatment of macrophages with HIF-1α siRNA & HIF-2α siRNA significantly ($P < 0.001$) reduced the levels of hypoxia-induced pro-MMP-7

released when these macrophages were exposed to hypoxia for 16 hours. The levels of pro-MMP-7 found in HIF-1α siRNA & HIF-2α siRNA-treated macrophages were comparable to those found in the supernatants of macrophages cultured in normoxic conditions. Transfection of macrophages with the scrambled oligonucleotide siRNA probe had no effect on levels of pro-MMP-7 found in macrophage culture supernatants and these were similar to those found in macrophages subjected to hypoxia without siRNA treatment (Figure 8).

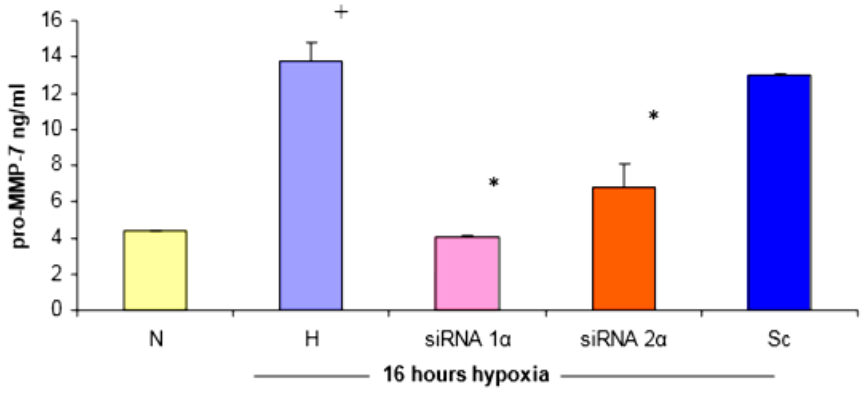


Figure 11: Inhibition of HIF-1α & HIF-2α ablates hypoxia-induced release of pro-MMP-7 by macrophages. The media from macrophages was collected and assayed for pro-MMP-7 expression. N = normoxia, H = hypoxia, siRNA 1α = hypoxic macrophages treated with siRNA for HIF-1α, siRNA 2α = hypoxic macrophages treated with siRNA for HIF-2α, Sc = hypoxic macrophages treated with a scrambled siRNA control. The level of pro-MMP-7 protein was reduced in both HIF-1α & HIF-2α-deficient macrophages compared with controls. A representative ELISA is shown of three separate experiments. + P<0.001 w.r.t. MDM cultured under normoxic conditions. * P<0.001 w.r.t scrambled siRNA-treated MDM cultured under hypoxic conditions.

Figure 8. The level of pro-MMP-7 protein was reduced in both HIF-1α & HIF-2α-deficient macrophages compared with controls. + P<0.001 w.r.t. MDM cultured under normoxic conditions. * P<0.001 w.r.t scrambled siRNA-treated MDM cultured under hypoxic conditions.

Discussion

In this study MMP-7 was shown to be up regulated in hypoxic macrophages at 16 hours of hypoxia but not at 1 and 6 hours using Western blotting for both whole cell extract and the media. This result was confirmed by zymography and ELISA. Induction of MMP-7 protein expression by hypoxia explain the high levels of this enzyme expressed by macrophages in human atherosclerotic lesions (Halpert *et al*., 1996)) as these sites are known to be ischemic (Bjornheden *et al*., 1999). The fact that MMP-7 levels are high at sites of plaque rupture suggests that it may be an important feature in the progression of certain cardiovascular diseases (Shah 1998). The hypoxic up regulated gene identified (MMP-7) could be important for the survival and functioning of macrophages in hypoxic diseased tissues and their promoters could prove useful in macrophage delivery gene therapy.

MMP-7 expression has been linked to resistance to doxorubicin chemotherapy. Owing to the ability of MMP-7 to cleave Fas ligand from the surface of the tumour cells, blocking the action of the drug (Mitsiades *et al*., 2001). TAMs express high level of MMP-7 in hypoxic areas of human breast carcinoma (Burke *et al*., 2003) suggesting that hypoxic induction of this protease in macrophage may contribute to the resistance of hypoxic tumour cells to treatment with doxorubicin.

In this study we used siRNA that specifically target degradation of mRNAs encoding HIF-1α. After treatment with siRNA, the expression of HIF-1α protein was greatly reduced under hypoxic conditions in the HIF-1α knockout cells. The siRNA oligonucleotides used was specific to HIF-1α protein tested. Inverted siRNA control had no effect on the expression of HIF-1α protein.

Two hypoxia inducible genes (VEGF and MMP-7) (Harmey *et al*., 1998; Burke *et al*., 2003)) were tested in macrophages. Both VEGF and MMP-7 protein were decreased to the normoxic level in macrophages HIF-1α knockout. This result indicates that both VEGF and MMP-7 expression is HIF-1α dependent in primary macrophages. Human MMP-7 promoter contain a sequence closely resembling a HRE at position -617 to -590, containing a consensus HIF binding site (HBS; 5ó –ACGTG ó 3) (Burke *et al*., 2003 133). Although the hypoxic regulation of MMP-7 and VEGF appears to involve HIF-1 in human MDM, we have yet to ascertain whether this is a direct or an indirect effect in the transcription of these two genes. In addition HIF-2α knockout worth to be tested in macrophages to check if it has any effect in expression of these genes in macrophages. As the activity of individual HREs varies markedly between different cell types. Therefore, the most appropriate HRE for use in macrophage based gene therapies would be derived from a gene highly up regulated in hypoxic macrophages.

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