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Effects of hypoxia on transcription factor expression in human monocytes and macrophages

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Abstract

The presence of multiple areas of hypoxia (low oxygen tension) is a hallmark feature of human and experimental tumours. Monocytes are continually recruited into tumours where they differentiate into tumour-associated macrophages (TAM) and often accumulate in hypoxic and/or necrotic areas. A number of recent studies have shown that macrophages respond to hypoxia by up-regulating transcription factors such as HIF-1α and HIF-2α, which in turn up-regulate the expression of a broad array of mitogenic, pro-invasive, pro-angiogenic and pro-metastatic genes. Here we show that primary human macrophages but not monocytes rapidly up-regulate HIF-1α and HIF-2α proteins upon exposure to hypoxia, and that these proteins then translocate to the nucleus. We also demonstrate differences in the temporal expression and responses to re-oxygenation for HIF-1α and HIF-2α in macrophages. Here we found that, compared to HIF-1α, HIF-2α expression was prolonged and persisted with re-oxygenation. ATF-4 and Egr-1 were also found to be hypoxia-responsive transcription factors in macrophages but not monocytes, but only early after exposure to hypoxia. Taken together, these findings indicate that a number of transcription factors work together in a tightly regulated fashion to control macrophage activities in ischaemic areas of diseased tissues.

Keywords: Hypoxia; Macrophages; Monocytes; Transcription factors

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). Transcription factors provide cells with a means by which to sense and respond to environmental signals like hypoxia. Many of the adaptive responses of cells to hypoxia are mediated by the activation of specific genes via the well-characterized hypoxia-inducible transcription factors, HIF-1 and HIF-2. These proteins are heterodimers consisting of a distinct α subunit and a common constitutive β subunit (Semenza, 2000). Under normoxic conditions, the α subunit is hydroxylated at specific

Abbreviations: MDM, monocyte-derived macrophages; TAM, tumour-associated macrophages; HIF, hypoxia-inducible factor; ATF-4, activating transcription factor-4; Egr-1, early growth response-1; NFκB, nuclear factor κB; C/EBPβ, CCAAT/enhancer binding protein β; Ets-1, erythroblastosis virus E26 oncogene homolog 1.

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proline residues, resulting in ubiquitination through the interaction with von Hippel-Lindau protein (pVHL) and proteosomal degradation. This process is inhibited under hypoxia, and HIFs accumulate in the nucleus and bind to short DNA sequences near or in the promoters of oxygen-sensitive genes called hypoxia-response elements (HREs).

HIF-responsive genes aid in regulating such processes in tumour biology as angiogenesis, glucose/energy metabolism, proliferation, apoptosis and metastasis (Harris, 2002). Tumour-associated macrophages (TAMs) are present in large numbers in most types of human tumour and originate mainly from circulating monocytes rather than tissue macrophages (Yamashiro et al., 1994). In breast, ovarian and bladder carcinomas, TAMs have been shown to accumulate in poorly vascularized, hypoxic and/or necrotic regions (Leek et al., 1996; Negus et al., 1997; Onita et al., 2002). Under hypoxic conditions, macrophages show pronounced changes in gene expression and adapt their metabolic activity to anaerobic glycolysis, with increased production of lactate and pyruvate, and metabolic acidosis (Lewis and Murdoch, 2005; Lewis et al., 1999). They also up-regulate their release of the important pro-angiogenic growth factor, vascular endothelial growth factor (VEGF), when exposed to hypoxia *in vitro* and in hypoxic areas of human breast tumours (Lewis et al., 2000). A number of studies have shown that HIF-1 and HIF-2 play a major role in controlling the transcriptional response to hypoxia in macrophages. However, there is currently some degree of controversy about which form of HIF is the most important in regulating macrophage responses to hypoxia. Using PMA-differentiated U937 pro-monocyte cells, Talks et al. (2000) showed that HIF-2α is the predominant transcription factor up-regulated by hypoxia. Moreover, using adenoviral gene transfer to examine the effects on gene expression of the over-expression of HIF-1α versus HIF-2α in human macrophages, White et al. (2004) found that HIF-2α was the primary mediator of hypoxic induction of macrophage genes encoding angiogenic proteins. In contrast, Burke et al. (2002) found that human macrophages preferentially up-regulate HIF-1α after exposure to hypoxia *in vitro* and in avascular, hypoxic areas of various forms of human tumours *in vivo*.

Apart from HIF-1α and HIF-2α, macrophages may also up-regulate other transcription factors in response to hypoxia. A number of other transcription factors such as HIF-3α (Heidbreder et al., 2003), activating transcription factor-4 (ATF-4) (Ameri et al., 2004), early growth response-1 (Egr-1) (Yan et al., 1999), nuclear factor κB (NFκB) (Koong et al., 1994), CCAAT/enhancer binding protein β (C/EBPβ) (Hehlgans et al., 2001; Yan et al., 1995) and erythroblastosis virus E26 oncogene homolog 1 (Ets-1) (Oikawa et al., 2001) have been shown to be up-regulated by hypoxia in various cell types. However, the expression of these transcription factors by hypoxic macrophages has not been investigated to date.

In this study we compared the levels of expression of HIF-1α, HIF-2α and HIF-3α, ATF-4, Egr-1, NFκB, C/EBPβ and Ets-1 in primary human monocytes and monocyte-derived macrophages (MDM) exposed to hypoxic culture conditions for up to 24 h *in vitro*. We show that there are significant differences in the temporal expression between HIF-1α and HIF-2α in response to hypoxia in macrophages but not monocytes. Furthermore, these two transcription factors differ in their response to re-oxygenation, following exposure to hypoxia. Besides HIF-1α and HIF-2α, ATF-4 and Egr-1 were also transiently up-regulated in hypoxic conditions in macrophages but not monocytes. These data show that many more transcription factors are activated by hypoxia in macrophages than monocytes.

Materials and methods

Isolation and culture of primary human monocyte-derived macrophages (MDM)

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% heat inactivated, human AB serum, 2-mM L-glutamine (all from Sigma, Poole, UK). Totally 8×10^7 mononuclear cells were seeded into 10-cm tissue culture plates (Iwaki Inc., Iwaki, Japan) and cultured for 2 h after which non-adherent cells were removed by washing and the culture medium replenished. Monocytes were either used in experiments at this stage or cultured for 7 days to allow differentiation into MDM. The purity and full differentiation of the resultant MDM was checked using CD68 by immunocytochemistry and carboxypeptidase M by flow cytometry (Rehli et al., 1995). In all experiments the purity of differentiated MDM was greater than 90%.

Hypoxic culture and protein extraction

MDM 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 during and

immediately after 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 20 min, sheered by repeated passage through a 25-gauge needle and then centrifuged at 400*g* for 10 min at 4 °C to remove cell debris. Nuclear and cytoplasmic extracts were prepared using CelLytic Nuclear extraction kits (N-XTRACT, Sigma, Poole, UK). All extracts were stored at −20 °C 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 min in reducing loading buffer. Totally 60 μg sample was run on 10% SDS-PAGE gels and after separation the proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were incubated for 16 h at 4 °C in blocking buffer (5% skimmed milk powder in 10-mM Tris, 180-mM NaCl, 0.05% Tween-20, pH 8) then probed with anti-transcription factor monoclonal antibody or polyclonal antiserum (see Table 1) diluted in blocking buffer and incubated for 2 h at room temperature. After washing with TBST the membranes were then exposed to anti-mouse secondary antibody conjugated with horseradish peroxidase (Dako Ltd., Copenhagen, Denmark) at a 1:2500 dilution in blocking buffer for 1 h at room temperature. The secondary antibody was detected using enhanced chemiluminescence reagent (Amersham, Little Chalfont, UK). Expression of β-actin was used to determine equal loading of protein in all immunoblots. The expression of this constitutively expressed protein is not altered by hypoxia and can be used to examine protein levels in nuclear as well as cytoplasmic extracts (Krauss et al., 2003; Zhong and Simons, 1999). To determine equal loading of proteins in whole cell, cytoplasmic and nuclear extracts, immunoblots were stripped with stripping buffer (100-mM β-mercaptoethanol, 2% SDS, 62.5-mM Tris HCl, pH 6.7) at 50 °C for 30 min and re-probed with a mouse monoclonal antibody to β-actin (Sigma, Poole, UK) at 1:30,000 in blocking buffer.

Results

Temporal expression of HIF-1α, HIF-2α and HIF-3α by human monocytes and MDM in response to hypoxia

Isolated human peripheral blood monocytes or 7-day MDM were subjected to severe hypoxia (0.1% O_2) for 1, 6, 16 and 24 h and the accumulation of HIF-1α, HIF-2α and HIF-3α analysed by immunoblotting of total cell extracts. Expression of all three HIFs was compared to the expression of the constitutively expressed protein β-actin. Monocytes did not increase the expression of HIF-1α, HIF-2α or HIF-3α at any time point tested (Fig. 1). In contrast, HIF-1α protein was up-regulated in MDM after just 1 h of exposure to hypoxia; expression gradually increased by 6 h, reached maximum levels by 16 h, and by 24 h expression had declined. HIF-1α protein was not detected in total cell extracts from MDM exposed to normoxia for up to 24 h (Fig. 1). Similar to HIF-1α, HIF-2α protein was not detected in MDM exposed to normoxia (Fig. 1) and it was rapidly up-regulated in total cell extracts from MDM by 1 h exposure to hypoxia. These elevated levels were maintained at 6 h but were slightly reduced after 16- and 24-h exposure to hypoxia. HIF-3α was constitutively expressed by MDM for all time points analysed and its expression did not alter in response to hypoxia (Fig. 1).

Table 1. Antibodies and the concentrations used in immunoblot analysis employed in this study (Mo – mouse, Rb – rabbit)

Antibody	Concentration (mg/ml)	Antibody type	Manufacturer
HIF-1α	1	Mo monoclonal	BD Biosciences, San Jose, CA, USA
HIF-2α	4	Mo monoclonal	Gift of Prof. Adrian Harris, Oxford University, UK
HIF-3α	1.4	Rb polyclonal	Abcam, Cambridge, Cambridgeshire, UK
ATF-4	2	Rb polyclonal	Santa Cruz Biotechnology, Clane, Wiltshire, UK
Egr-1	2	Rb polyclonal	Santa Cruz Biotechnology, Clane, Wiltshire, UK
NFκB(p65)	1	Mo monoclonal	Santa Cruz Biotechnology, Clane, Wiltshire, UK
C/EBPβ	0.6	Mo monoclonal	Santa Cruz Biotechnology, Clane, Wiltshire, UK
Ets-1	2	Mo monoclonal	Lab Vision Corporation, Newmarket, Suffolk, UK

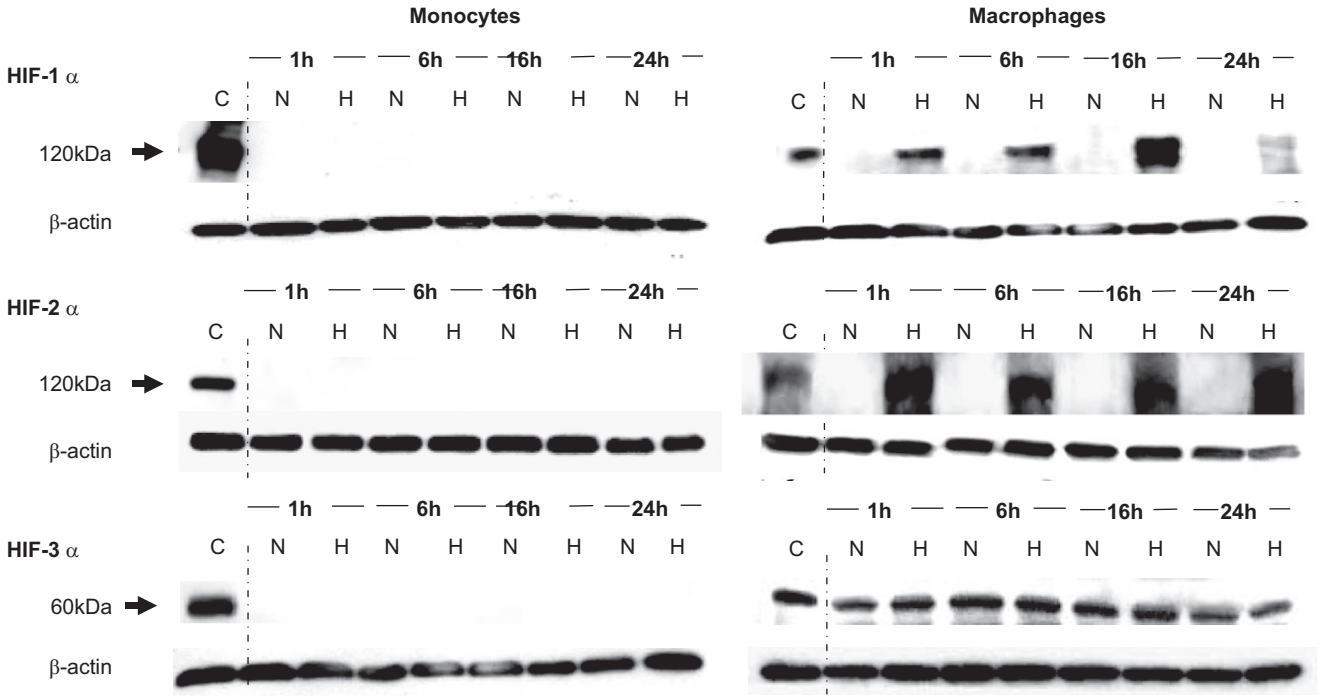

Fig. 1. Temporal expression of HIF-1α, HIF-2α and HIF-3α by human monocytes and MDM in response to hypoxia. Expressions of HIF-1α, HIF-2α and HIF-3α proteins were examined in total cell extracts from human monocytes and MDM following exposure to hypoxia ('H'; 0.1% O_2) or normoxia ('N'; 20.9% O_2) for 1, 6, 16 or 24 h. Monocytes (left panel) did not express these three HIFs under normoxic or hypoxic conditions. In MDM (right panel), HIF-1α and HIF-2α protein increased in a time-dependent manner in response to hypoxia. HIF-1α reached a maximum by 16 h, whereas HIF-2α rapidly increased by 1 h and remained elevated over 24 h. HIF-3α was constitutively expressed by MDM and was not responsive to hypoxic conditions. All immunoblots were stripped and re-probed for *β*-actin to ensure equal loading. 'C' is positive control; for HIF-1α – total cellular extract from HeLa cells stimulated with cobalt chloride for 4 h (Signal Transduction Laboratories); for HIF-2α – human breast carcinoma cells (T47D) exposed to hypoxia for 16 h, and for HIF-3α – human embryonic kidney cells (HEK 293) cells exposed to hypoxia for 16 h. Representative immunoblots of four separate experiments are shown.

HIF-1α and HIF-2α accumulate in the nucleus of hypoxia-cultured MDM

To be functional HIF-1α and HIF-2α must accumulate in the nucleus where they can activate gene transcription by binding to HREs in the promoters of hypoxia-inducible genes. To determine if MDM accumulate HIF-1α or HIF-2α in the nucleus, we isolated nuclear and cytoplasmic extracts from MDM subjected to hypoxia (0.1% O_2) or normoxia for 16 h. Up-regulation of HIF-1α and HIF-2α transcription factors was detected in nuclear but not cytoplasmic extracts (Fig. 2). Expression of HIF-1α or HIF-2α was not detected in cells cultured under normoxic conditions.

MDM expression of HIF-1α and HIF-2α differs following re-oxygenation

Wiesener et al. (1998) reported that re-oxygenation rapidly degrades both HIF-1α and HIF-2α in HeLa cells. To examine the effect of re-oxygenation on both HIF-1α and HIF-2α in MDM, cells were cultured for 16 h in severe hypoxic conditions (0.1% O_2) and then re-oxygenated by returning the cells to normoxic culture conditions (20.9% O_2) for 1 or 2 h. Immunoblot analysis shows that HIF-1α expression is up-regulated by hypoxia after 16 h, but this is rapidly degraded by re-oxygenation, and HIF-1α expression is completely abrogated after 1 h exposure to normoxic culture conditions. Expression of HIF-2α was also elevated after 16 h in hypoxic conditions. However, in contrast to HIF-1α, HIF-2α expression remained elevated during re-oxygenation, even after 2 h exposure to normoxic conditions (Fig. 3).

Expression of other hypoxia-inducible transcription factors by human monocytes and MDM

To determine whether hypoxia-inducible transcription factors other than HIFs are up-regulated in human monocytes and MDM, these cells were subjected to 1-, 6-, 16- and 24-h normoxia (20.9% O_2) or hypoxia

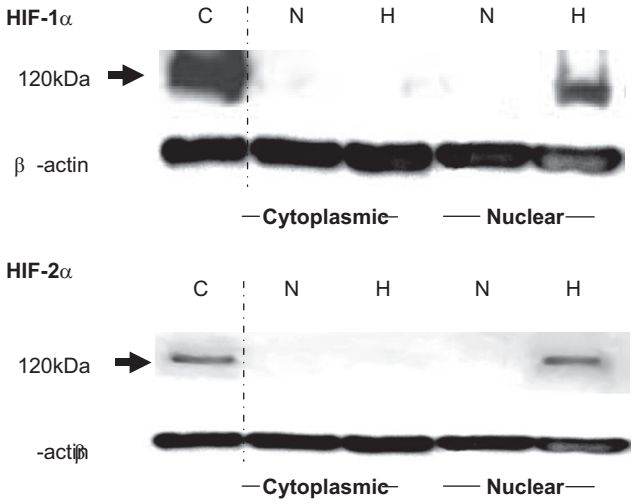

Fig. 2. HIF-1α and HIF-2α translocate to the nucleus of MDM, following exposure to hypoxia. Immunoreactive HIF-1α (upper panel) and HIF-2α (lower panel) protein bands were seen in nuclear but not cytoplasmic cell extracts following exposure of cells to normoxia (N) or hypoxia (H) for 16 h. All immunoblots were stripped and re-probed for β-actin to ensure equal loading. 'C' is positive control; for HIF-1α – total cellular extract from HeLa cells stimulated with cobalt chloride for 4 h (Signal Transduction Laboratories); for HIF-2α – human breast carcinoma cells (T47D) exposed to hypoxia for 16 h. Representative immunoblots of three separate experiments are shown.

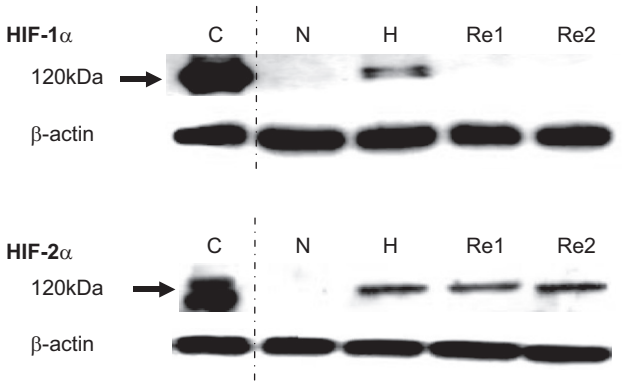

Fig. 3. MDM-derived HIF-1α and HIF-2α markedly differ in their response to re-oxygenation following 16 h hypoxia. Expression of HIF-1α and HIF-2α proteins were examined in total cell extracts from human MDM following exposure to hypoxia ('H'; 0.1% O_2) for 16 h followed by re-oxygenation (20.9% O_2) for 1 and 2 h (Re1 and Re2). HIF-1α expression was completely abrogated by re-oxygenation (upper panel), whereas HIF-2α expression was maintained even after 2 h re-oxygenation (lower panel). All immunoblots were stripped and re-probed for β-actin to ensure equal loading. 'C' is positive control; for HIF-1α – total cellular extract from HeLa cells stimulated with cobalt chloride for 4 h (Signal Transduction Laboratories); for HIF-2α – human breast carcinoma cells (T47D) exposed to hypoxia for 16 h. Representative immunoblots of three separate experiments are shown.

(0.1% O_2) and the levels of ATF-4, Egr-1, NFκB, C/EBPβ and Ets-1 were analysed by immunoblotting.

ATF-4 protein was found to be constitutively expressed in human monocytes and similar levels of expression were found in both normoxic and hypoxic cultured monocytes for all of the time points tested. ATF-4 therefore does not appear to be regulated by hypoxia in monocytes. In contrast, ATF-4 protein levels were found to be markedly up-regulated in MDM, following incubation in hypoxia for 1 h when compared to their normoxic counterparts. However, thereafter ATF-4 was found to be expressed in both normoxic and hypoxic cells at similar levels and remained constant for up to 24 h (Fig. 4). The localisation of ATF-4 expression was assessed by analysing nuclear and cytoplasmic cell extracts after culture in normoxia or hypoxia for 1 h. ATF-4 expression was not detected in the cytoplasmic fractions of either normoxic- or hypoxic-cultured cells. ATF-4 expression was detected in nuclear extracts generated from MDM cultured in normoxic and hypoxic conditions. However, the levels of ATF-4 were markedly up-regulated in hypoxic MDM compared to normoxic MDM (Fig. 5).

Egr-1 was not detected in normoxic or hypoxic monocytes for the first 6 h of incubation. However, after 16 and 24 h, levels of Egr-1 protein were up-regulated in both normoxic and hypoxic conditions. Furthermore, Egr-1 protein levels under hypoxic conditions were twice that seen in the normoxic monocytes at both 16 and 24 h. Similar to ATF-4, expression of Egr-1 protein in MDM was found to be markedly up-regulated by hypoxia after 1 h when compared to levels seen in normoxic cells. However, by 6-h incubation Egr-1 protein levels within normoxic MDM had increased to reach similar levels as the hypoxic cells and these levels remained elevated for up to 24 h (Fig. 4). The localisation of Egr-1 expression was also assessed by analysing nuclear and cytoplasmic cell extracts after culture in normoxia or hypoxia for 1 h. In contrast to ATF-4, HIF-1α and HIF-2α, Egr-1 accumulated in both the cytoplasmic and the nuclear extracts. Expression in the hypoxic cytoplasmic extract was twice that of extracts from normoxic-cultured MDM, whereas EGR-1 expression in the nuclear extract was similar for both normoxic and hypoxic conditions (Fig. 5).

Expression of C/EBPβ was not observed in monocytes after 1 h in either normoxic or hypoxic conditions. However, similar to Egr-1, expression of C/EBPβ gradually increased in both normoxic and hypoxic conditions from 6 to 24 h. Furthermore, expression of C/EBPβ in hypoxic culture conditions was greater than in normoxic conditions at these time points (Fig. 4). In contrast to monocytes, C/EBPβ protein was constitutively detected in human MDM. Protein levels were similar for both normoxic and hypoxic cultured macrophages and levels did not alter throughout the 24 h time-course of the experiment (Fig. 4).

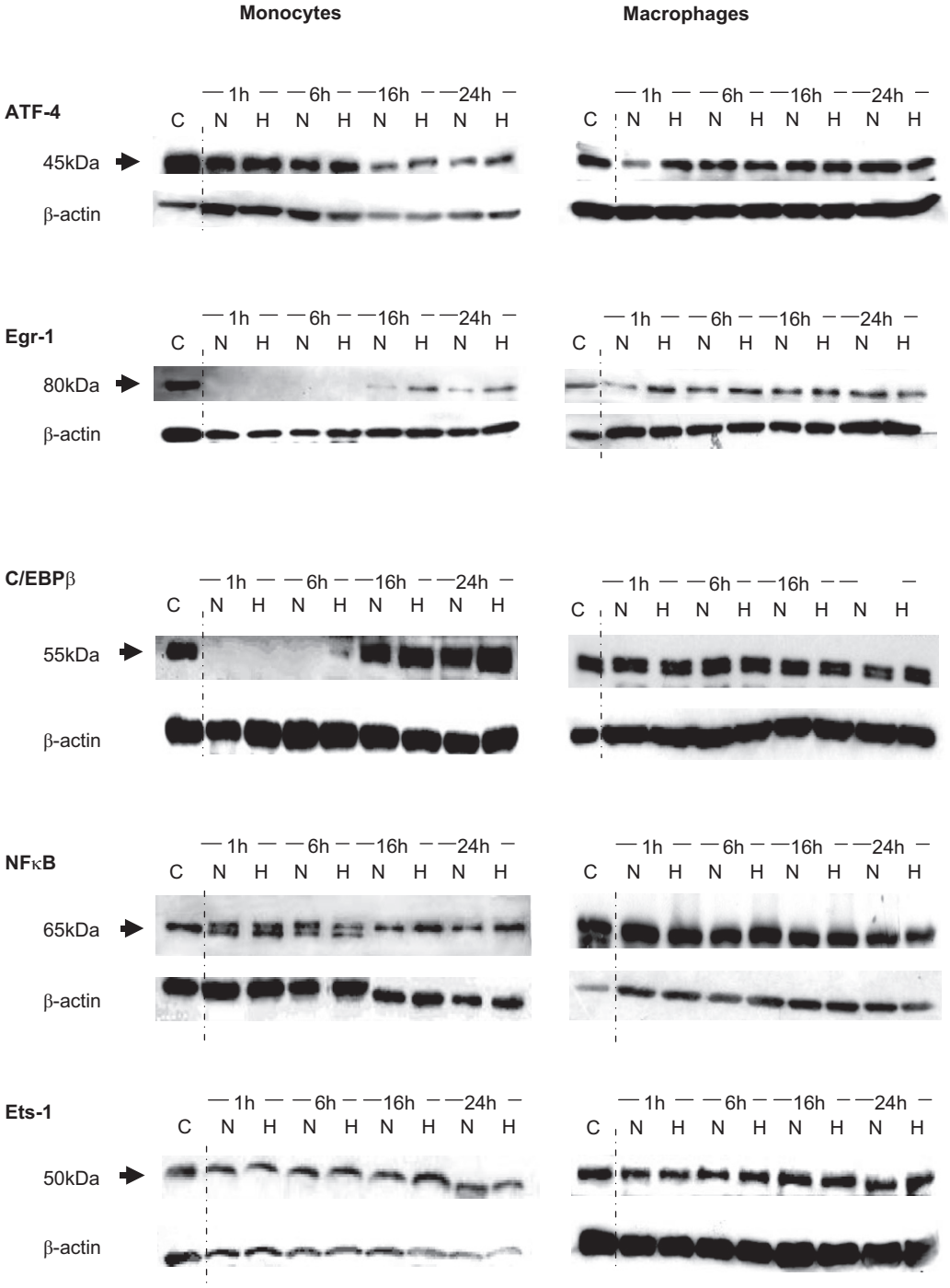

Fig. 4. Temporal expression of non-HIF transcription factors by hypoxia. Total cell extracts from human monocytes and MDM following exposure to hypoxia ('H'; 0.1% O_2) or normoxia ('N'; 20.9% O_2) for 1, 6, 16 or 24 h were examined for the expression of non-HIF hypoxia-inducible transcription factors. Levels of ATF-4 and Egr-1 in MDM were increased by exposure to 1-h hypoxia and remained elevated for hypoxia and normoxia thereafter. Expressions of Egr-1 and C/EBPβ increased in a time-dependent manner in monocytes as they differentiated towards a macrophage phenotype and expression was exacerbated by hypoxia. Ets-1, NFκB (p65/RelA) were constitutively expressed in both monocytes and macrophages under normoxic and hypoxic conditions. All immunoblots were stripped and re-probed for β-actin to ensure equal loading. 'C' is positive control; for ATF-4 total cellular extract from human fibrosarcoma cells (HT1080) exposed to hypoxia for 16 h; for Egr-1 and NFκB (p65/RelA) – total cellular extract from mouse fibroblast cells (NIH/3T3 – supplied by Santa Cruz Biotechnology); for C/EBPβ total cellular extract from HeLa cells (supplied by Santa Cruz Biotechnology) and for Ets-1 total cell extracts form acute lymphoblastic human T-cell leukemia cells (supplied by NeoMarkers). Representative immunoblots of three separate experiments are shown.

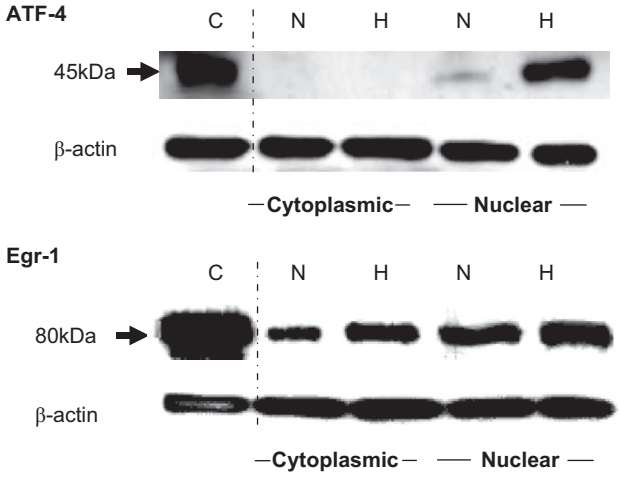

Fig. 5. ATF-4 and Egr-1 are up-regulated after 1-h exposure to hypoxia in MDM. ATF-4 (upper panel) and Egr-1 (lower panel) protein bands were seen in nuclear but not cytoplasmic cell extracts following exposure of cells to normoxia (N) or hypoxia (H) for 1 h. 'C' is positive control; for ATF-4 total cellular extract from human fibrosarcoma cells (HT1080) exposed to hypoxia for 16 h; for Egr-1 – total cellular extract from mouse fibroblast cells (NIH/3T3 – supplied by Santa Cruz Biotechnology). Representative immunoblots of two separate experiments are shown.

NFκB (p65/RelA) and Ets-1 transcription factors were found to be constitutively expressed by both monocytes and MDM and there were no differences in the expression levels of these transcription factors when these cell types were cultured in normoxic or hypoxic conditions for all time points examined (Fig. 4).

Discussion

There is increasing evidence to show that TAM residing in hypoxic/necrotic areas within tumours secrete factors that promote tumour angiogenesis (Lewis et al., 2000; Lewis and Pollard, 2006). Many of these pro-angiogenic factors are regulated by such hypoxia-inducible transcription factors as HIF-1 and HIF-2, which also regulate the expression of metabolic factors that are required for cells to survive in such harsh conditions (Harris, 2002). Previous studies have shown that hypoxia causes accumulation of HIF-1α and HIF-2α in primary cultures of human macrophages or TAM *in vitro* and *in vivo* (Burke et al., 2002; Talks et al., 2000; White et al., 2004). However, little work has been done on the temporal pattern of expression of these transcription factors in these cells or their response to re-oxygenation.

Our data presented show that both HIF-1α and HIF-2α proteins are rapidly up-regulated in macrophages under severe hypoxia (i.e. within 1 h of exposure). A similar temporal pattern of hypoxic induction of HIF-1α and HIF-2α has been recorded in various tumour cell types. Wiesener et al. (1998) found that up-regulation of both HIF-1α and HIF-2α in HeLa cells occurred within 30-min exposure to hypoxia. HIF levels increased further from 2- to 4-h hypoxia but then declined from 8- to 48-h hypoxia. In the present study, the level of HIF-1α in total cell extracts from macrophages peaked after 16 h of hypoxia and then gradually declined by 24 h, whereas the total cellular level of HIF-2α protein was elevated at all of the time points examined (1–24 h). These data suggest that HIF-2α may be more stable than HIF-1α in hypoxia and remain biologically active for longer in macrophages experiencing hypoxia. Interestingly, this phenomenon has been observed in other cell types. Holmquist et al. (2005) showed that prolonged (72 h) hypoxia in neuroblastoma and breast carcinoma (MCF-7) cells diminished HIF-1α protein but further stabilized HIF-2α. In addition, increased HIF-2α and decreased HIF-1α protein levels during prolonged hypoxia have also been seen in A549 lung epithelial cells (Uchida et al., 2004).

To be able to transactivate expression of target genes under hypoxic conditions, HIF-1α and HIF-2α have to translocate to the nucleus (Jiang et al., 1996). Hypoxic stimulation of macrophages for 16 h caused the nuclear accumulation of both HIF-1α and HIF-2α, with no protein found in the cytoplasm, indicating that both were available to transactivate gene expression in these cells.

Due to the expression of angiogenic factors like VEGF by hypoxic tumour cells and macrophages (Harmey et al., 1998; Shweiki et al., 1992), hypoxic areas of tumours are likely to re-vascularize and re-oxygenate over time. Levels of both HIF-1α and HIF-2α decrease very rapidly (after 30 min) in HeLa cells subjected to re-oxygenation (Wiesener et al., 1998). This decrease is mediated by an increase in prolyl hydroxylase (PHD) activity, leading to hydroxylation of Pro-564 in the oxygen-dependent degradation domain of HIF-1α that controls its degradation during normoxic conditions (D'Angelo et al., 2003). The present study shows that macrophages up-regulate HIF-1α and HIF-2α in response to hypoxia. However, upon re-oxygenation for 1 or 2 h, macrophages rapidly reduced their levels of HIF-1α but levels of HIF-2α remained elevated, suggesting that these two highly related transcription factors are regulated differently by re-exposure to normoxic conditions in macrophages and indicate that HIF-responsive genes may be expressed in a HIF-2α-dependent manner even during re-oxygenation. Furthermore our study shows that HIF-3α protein can be detected in macrophages under both normoxia and hypoxia, with no apparent hypoxic induction, suggesting that HIF-3α transcription factor may not be involved in the hypoxic up-regulation of genes in macrophages. Similar observations have been found

with COS-7 cells transfected with the gene for human HIF-3α (Hara et al., 2001).

In contrast to macrophages, HIF-1α, HIF-2α and HIF-3α proteins were not detected in total cell lysates of human blood monocytes, cultured in either normoxic or hypoxic conditions for up to 24 h. One possible explanation for this is that monocytes frequent the bloodstream rather than tissues and are not equipped with such a response pattern since they do not usually experience hypoxia. Interestingly, HIF-1α has recently been shown to be essential for cell-mediated inflammation and phagocytosis by murine myeloid cells (Cramer et al., 2003; Peyssonnaux et al., 2005). Thus, it is likely that inflammatory stimuli regulate HIF-1α by a different mechanism compared to hypoxia-induced HIF-1α expression.

As well as HIFs, other transcription factors like ATF-4 (Ameri et al., 2004), Egr-1 (Yan et al., 1999), NFκB (Koong et al., 1994), C/EBPβ (Hehlgans et al., 2001; Yan et al., 1995) and Ets-1 (Oikawa et al., 2001) have also been shown to be up-regulated by hypoxia in various cell types. We show that ATF-4 rapidly accumulates in the nucleus of macrophages after only 1 h of exposure to hypoxia, whereas in monocytes ATF-4 was equally present in normoxic or hypoxic cells at all time points examined. ATF-4 contains a nuclear targeting signal (Cibelli et al., 1999), which may account for its nuclear translocation during hypoxia. Blais et al. (2004) reported that ATF-4 is induced in HeLa cells after 1, 2 and 4 h of hypoxia. Whilst other investigators have reported the effect of anoxic, rather than hypoxic, induction of ATF-4 after 12 and 16 h in fibroblasts, human breast cancer cell lines and human melanoma cell lines (Ameri et al., 2004; Estes et al., 1995). Since ATF-4 is known to be up-regulated in response to amino acid or glucose deprivation in human hepatoma cells (Siu et al., 2002), the observation that ATF-4 was abundantly expressed in both hypoxic and normoxic macrophages by 6 h could be due to the rapid utilization of nutrients in the media by macrophages rather than represent a response to hypoxia *per se*. Based on these findings, acute hypoxia appears to result in immediate activation of ATF-4 in macrophages. However, the specific genes activated by ATF-4 in macrophages have yet to be identified.

Egr-1 was also seen to accumulate in macrophages within 1 h of exposure to hypoxia, with the protein present in both the cytoplasm and the nuclei of hypoxic cells. These data contrast with those for HIF-1α, HIF-2α and ATF-4, where the proteins were found to accumulate in the nuclei of macrophages under hypoxia. This difference may be due to the lack of hypoxia-targeted removal of Egr-1 from the cytoplasm or slow/inadequate translocation to the nucleus under hypoxia. In contrast to macrophages, Egr-1 is up-regulated in freshly isolated monocytes after 16 and 24 h of normoxia and hypoxia with no protein detected at 1 and 6 h. These data compare favourably with Yan et al. who showed a similar induction of Egr-1 in 2-day old monocytes and Kharbanda et al. (1991) who demonstrated rapid up-regulation of Egr-1 in monocytes stimulated to differentiate into macrophages upon stimulation with colony-stimulating factor-1 (Park et al., 2001). Thus, Egr-1 appears to be important in monocyte differentiation toward a macrophage phenotype. Moreover, since hypoxia increased Egr-1 levels above that seen under normoxia; hypoxia may accelerate the differentiation process.

Similar to Egr-1, C/EBPβ was also induced in monocytes after 6, 16 and 24 h of hypoxia with no protein detected at 1 h in normoxia or hypoxia, and is constitutively present in macrophages. It is tempting to speculate that, like Egr-1, C/EBPβ may also be involved in the process of monocyte differentiation into macrophages. In support of this Scott et al. (1992) and Pan et al. (1999) have demonstrated increased C/EBPβ mRNA levels in monocytes as they differentiate into macrophages. These results suggest that C/EBPβ may be an important transcription factor in the process of generating differentiated macrophages.

NFκB (p65/RelA) is one of the NFκB families of transcriptional activator proteins. NFκB enters the nucleus and binds to DNA in a dimerized state, typically with NFκB p50. The p65 subunit is known to be responsible for initiating transcription via DNA binding, whereas p50 is thought to provide a helper function in DNA binding (Schmitz and Baeuerle, 1991). Previous studies have shown NFκB to be activated by hypoxia in murine macrophages (Fong et al., 2007). However, we found that NFκB (p65/RelA) was constitutively detected in total cell extracts in both monocytes and macrophages subjected to either hypoxia or normoxia. This disparity could be due to murine macrophages responding differently to hypoxia than human macrophages. Another reason for this difference could be due to activation of other NFκB subunits by hypoxia. Recently, Battaglia et al. (2008) showed that in human macrophages hypoxia specifically up-regulates the expression of the chemokine CCL20 via activation of NFκB p50. Thus, it appears that p50 rather than p65 is the hypoxic-responsive NFκB factor that is responsible for driving the expression of NFκB regulated factors under hypoxic conditions.

Ets-1 has previously been shown to be induced by hypoxia in a human bladder cancer cell line (T24) (Oikawa et al., 2001). However, in both human monocytes and macrophages, Ets-1 is constitutively expressed under normoxia and hypoxia, suggesting that in these cells Ets-1 is not regulated by hypoxia.

The data presented here show that macrophages respond to pathologically low levels of oxygen by up-regulating a number of transcription factors in a

time-dependent manner. Although such immunoblot analyses show that the above transcription factors are up-regulated by hypoxia, further studies are now warranted to investigate their DNA-binding activity and role in regulating gene expression in macrophages in healthy and diseased tissues.

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