Activation of a prometastatic gene expression program in hypoxic neuroblastoma cells

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Abstract

The hypoxia inducible factor-1 α (HIF1 α) is a key regulator of oxygen homeostasis, modulating cell survival, and growth in cells exposed to hypoxia. In this study, neuroblastoma (NB) cells SH-SY5Y and SK-N-MC were employed to determine the mechanisms regulating adaptation to hypoxia. NB cells were cultured in a serum-free medium in the presence or absence of CoCl₂ (100 μ M, hypoxia mimic) for up to 48 h. SH-SY5Y and SK-N-MC cell numbers were not affected by CoCl₂ treatment, while mitochondrial activity was reduced by $\sim$ 50% in SH-SY5Y cells and by $\sim$ 70% in SK-N-MC cells. Intracellular accumulation of HIF1 α protein was detected as early as 30 min of post-hypoxia, followed by the increase of mRNA for vascular endothelial growth factor (VEGF) and nuclear accumulation of the ID1–2 transcription factors by 4 h. In hypoxic SH-SY5Y NB cells, real-time PCR analysis showed that the genes involved in maintenance of cell–cell and cell–matrix interactions (i.e. adenomatous polyposis coli, E-cadherin, catenin, EphB2, fibronectin-1, HTATIP2, tissue inhibitor of metalloprotease-4) were down-regulated by up to 90%, while genes involved in enhancement of metastatic behavior (integrin α 7b1, hepatocyte growth factor receptor, transforming growth factor- β 1, VEGF, kisspeptin, interleukin-1 β) were dramatically up-regulated above 200%. These changes were all consistent with the induction of epithelial–mesenchymal transition. We have thus demonstrated that NB cell adaptation to hypoxia, in addition to the modulation of HIF1 α and VEGF expression and nuclear translocation of ID1 and ID2 transcription factors, involve in the activation of a gene expression program consistent with the pro-metastatic events. These processes are probably responsible for the NB cell transition from an adherent phenotype to a highly migratory, invasive and aggressive NB cell type.

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Introduction

Hypoxia can be described as a reduction of oxygen supply below the normal level of tissue oxygen tension (Hockel & Vaupel 2001). This event may occur in acute or chronic vascular disease, pulmonary disease, and notably in cancer (Carmeliet & Jain 2000). In hypoxia, cellular mechanisms (i.e. mitochondrial activity) are compromised, and if severe or prolonged may lead to cell death (Semenza 1999, Wenger 2002). Conversely, exposure to hypoxic conditions may activate a number of adaptive/survival cellular responses (Bunn & Poyton 1996, Semenza 1999,

Wenger 2002, Lee *et al.* 2004, Wenger *et al.* 2005). This is the case in solid tumors that become hypoxic because of aberrant newly developed blood vessels and tumor overgrowth, causing inefficient local circulation (Harris 2002, Semenza 2003, Brown & Wilson 2004).

Although hypoxia is toxic to both cancer cells and normal cells, cancer cells often undergo genetic and adaptive changes that allow them to survive and even proliferate in an hypoxic environment (Harris 2002, Semenza 2003, Brown & Wilson 2004, Ikeda 2005). These processes contribute to the malignant and aggressive phenotype (Jogi *et al.* 2003, 2004, Axelson

et al. 2005, Belozerov & Van Meir 2005, Kaur *et al.* 2005, Chen *et al.* 2006, Holmquist *et al.* 2006). However, the mechanisms involved are not fully understood.

Mammalian cells, including cancer cells, adapt to hypoxia primarily through a transcriptional response pathway mediated by the hypoxia inducible factors (i.e. HIF1 α ; Semenza 1998, Wenger 2002, Safran & Kaelin 2003, Zarembek & Malech 2005). In normoxia HIF1 α protein levels are regulated at transcriptional, via factor inhibiting HIF1, and post-translational levels are regulated via ubiquitination and proteasome degradation of HIF1 α protein (Bunn & Poyton 1996, Semenza 1999, Wenger 2002, Lee *et al.* 2004, Mazure *et al.* 2004, Wenger *et al.* 2005). In hypoxia, HIF1 α inhibition and degradation are abrogated and this leads to a stable and functional HIF1 α and formation of HIF complexes. These complexes can thus bind to regulatory hypoxia responsive elements (HRE) of genes such as the survival cytokine erythropoietin (EPO) and the angiogenesis factor, vascular endothelial growth factor (VEGF; Semenza 1998, Wenger 2002, Lee *et al.* 2004).

Neuroblastoma (NB) is the most common neural crest derived extracranial solid tumor of childhood and arises from sympathetic nervous tissue origins (Bown 2001, Brodeur 2003). NB cells vary in relation to their differentiation stage, with aggressive NB tumors deriving from more immature NB cells (Brodeur *et al.* 1988, Bown 2001, Brodeur 2003, Holmquist *et al.* 2005, 2006). These NB tumors may be present as phenotypically distinct groups of NB cell types (i.e. neuroblastic, neuroendocrine) organized in lobular structures with a central necrotic/hypoxic zone (Jogi *et al.* 2002, 2003). Recent evidence has demonstrated that NB cells growing in these necrotic/hypoxic zones undergo a gene expression switch involving decreased expression of neuronal/neuroendocrine marker genes, but induced gene expression of neural crest sympathetic progenitor markers (Jogi *et al.* 2002, 2003, 2004, Axelson *et al.* 2005). These data (Jogi *et al.* 2002, 2003, Axelson *et al.* 2005), indicating that hypoxia causes dedifferentiation of NB cells to proliferative immature neuroblast-like cells, suggest potential activation of epithelial–mesenchyme transition mechanisms (EMT). Although EMTs occur during critical phases of embryonic development in many animal species, it is now well recognized that EMT is a potential mechanism for cancer progression (Huber *et al.* 2005, Lee *et al.* 2006, Thiery & Sleeman 2006). However, the activation of these events in hypoxic NB cells remains largely undetermined.

In these studies, we have exposed the NB cell lines SH-SY5Y (N type) and the SK-N-MC (S type) to the hypoxia mimicking agent CoCl₂ and analyzed cellular and molecular responses to these conditions. We have investigated for the first time whether exposure to hypoxia might produce changes in expression of genes regulating cell–cell and cell–matrix interactions, thus affecting cell adhesion, migration, and metastatic behavior or EMT.

Materials and methods

Reagents

Cobalt chloride, 0.01% sterile poly-L-lysine solution and paraformaldehyde were purchased from Sigma Chemical Co. Total cellular RNA was extracted from cells using RNeasy Mini Kit, Qiagen Pty Ltd. Tissue culture flasks, plates, and chamber slides were purchased from Nunc (Roskilde, Denmark). Chemical reagents (Analar grade) were purchased from BDH-Merck Pty Ltd. (Kilsyth, Victoria, Australia). Nitrocellulose membranes (0.45 μ M) were obtained from Schleicher and Schuell (Dassel, Germany). BioMax films were from Eastman Kodak Company. Intracellular pathway inhibitors AG490 (JAK2 inhibitor), SB203580 (p38 MAPK inhibitor), and U0126 (MEK1/2 inhibitor) were obtained from Calbiochem (Calbiochem-Novabiochem Corporation, San Diego, CA, USA), and the MAPK inhibitor (PD98059) and PI3K inhibitor (LY294002) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture

The two unrelated human neuroblastoma cell lines, SK-N-SH-SY5Y (Russo *et al.* 2004a,b) and SK-N-MC (Russo *et al.* 2004c) were cultured in DMEM (Trace Biosciences, Castle Hill, New South Wales, Australia) supplemented with 10% FCS (CSL Ltd, Parkville, Victoria, Australia), penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C.

Treatment

When cells reached the required confluence (70%), they were incubated, for up to 48 h or as indicated, in the presence or absence of CoCl₂ (100 μ M). Cobalt Chloride concentrations were initially based on those previously published for neuroblastoma cells (Kitamuro *et al.* 2001) with optimal concentration determine in preliminary experiments in our laboratory (not shown). Media was replaced every 24 h. Cells were then assayed at 24 or 48 h as indicated.

Signaling pathway inhibitors were used in some experiments, AG490 at 10 μ M, SB203580 at 10 μ M, U0126 at 25 μ M, PD98059 at 100 μ M, and LY294002 at 100 μ M for up to 24 h. Concentrations of the above pathway inhibitors were consistent with those previously shown to be effective in SH-SY5Y cells (Russo *et al.* 2004a,b).

Cell number assay

Cells were cultured in 24-well plates and upon reaching 70% confluence were treated as above, for up to 48 h. Cell number was determined by a colorimetric cell number (naphthol blue-black, NBB) assay protocol previously described by Janet *et al.* (1995). Assays were performed at least thrice and samples were run in triplicates.

Mitochondrial activity assays ($\pm$ inhibitors)

Cells were cultured in 24-well plates and were then treated as above, for 48 h. Alternatively cells were pre-treated for 3 h with SF media containing the various signaling pathway inhibitors (see above) in the absence or presence of CoCl_2 . After 3 h, CoCl_2 was added to the cells for 24 h before being harvested. Cell number was determined by a colorimetric mitochondrial activity (3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide, MTT) assay protocol previously described by Janet *et al.* (1995). Assays were performed thrice as indicated.

Immunocytochemistry

Cells were cultured in poly-L-lysine coated 4-well chamber slides and upon reaching 70% confluence were treated as above, for up to 24 h. Cells were fixed with 4% paraformaldehyde (20 min) followed by glycine 100 mM in PBS pH 7.4 (10 min) and washed with PBS. Wells/cells were then blocked with 1% denatured BSA in PBS pH 7.4 (DBSA, denatured by incubation at 60 °C for 30 min) prior to permeabilizing cells with 0.1% Triton-X 100, 1% DBSA in PBS pH 7.4 (10 min). The primary antibodies, described below, were applied in 1% DBSA in PBS and incubated overnight at 4 °C as indicated.

The monoclonal anti-human HIF1 α antibody (H1 α 67 antibody, Novus Biologicals, Inc., Littleton, CO, USA) was used at 1:200. The rabbit anti-human ID1-3 (C-20) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were also used at 1:200. Immunoreactivity for HIF1 α or ID1-3 was detected by the Alexa 488 labeled goat anti-mouse or goat anti-rabbit IgG both at 1:400 (Molecular Probes, Leiden, The Netherlands). Nuclear staining was

performed with DAPI (Millipore-Chemicon, Temecula, CA, USA) prior to mounting. Reactive cells were visualized by an inverted 1 $\times$ 70 u.v. microscope (Olympus). Images were taken at a 10 $\times$ -magnification objective. Immunocytochemistry was performed thrice and samples were run in duplicate. Omission of primary antibody or mouse or rabbit IgG was used as negative controls (data not shown).

HIF1 α immunoblotting

SH-SY5Y and SK-N-MC cells were grown as above (T80 cm² flasks, $\sim$ 80% confluency) in the presence or absence of CoCl_2 for 15 min up to 24 h as indicated. Following the treatment, cells were washed with ice-cold PBS, scraped, centrifuged, and cell pellet extracted with RIPA buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate) containing Complete protease inhibitor cocktails (Roche). Protein concentration, in cellular extract, was measured with BCA protein assay reagent (Pierce, Rockford, IL, USA). A hundred micrograms of total protein were then fractionated onto 7% SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. HIF1 α was detected by the anti-human HIF1 α antibody. Anti- α -tubulin (B-7) antibody (Santa Cruz Biotechnology, Inc.) was utilized to verify equal protein loading across samples. Experiments were performed thrice.

Preparation of RNA for RT-PCR and PCR-array

Cells were plated out in T80 cm² flasks and upon reaching 70% confluence were then cultured as above for up to 24 h. Cells were harvested by trypsin and total RNA was isolated using RNeasy mini kit (Qiagen), including the DNase treatment.

RT-PCR and DNA sequencing

cDNA was synthesized from 1 to 2 μ g of the total RNA using oligo(dT) primer and MuLV-RT (Perkin Elmer, Waltham, MA, USA, Roche) and first stand cDNA synthesis kit (Perkin Elmer), followed by PCR using the Taq Polymerase. Omission of total RNA (water control) or MuLV-RT (genomic DNA control) in the RT reaction was used to assess specific amplification for each gene (not shown). Primers were used as follows: GAPDH (forward 5'-CCATGGCACCCTCAAGGCTGA-3', reverse 5'-GGGCCATCCACAGTCTTCTGG-3'); HIF1 α (forward 5'-GAAAGCGCAAGTCCTCAAAG-3', reverse 5'-TGGGTAGGAGATGGAGATGC-3');

Figure 1 (A–D) Cell mitochondrial activity, but not cell number, is decreased by hypoxia. SH-SY5Y (1AB) and SK-N-MC (1CD) cells were treated with CoCl₂ (100 μ M) and NBB or MTT performed as described in Materials and methods. SH-SY5Y and SK-N-MC cell number was not affected by hypoxia (A and C) while mitochondrial activity (B and D) was reduced in SH-SY5Y cells by up to 50% (1B; 24–48 h) and by $\sim$ 70% in SK-N-MC cells (1D; 24–48 h).

VEGF (forward 5'-CCCACTGAGGAGTCCAACAT-3', reverse 5'-TTTCTTGCGCTTTTCGTTTTT-3'); ID1 (forward 5'-CTACGACATGAACGGCTGTTACTC-3', reverse 5'-CTTGCTCACCTTGCGGTTCT-3'); ID2 (forward 5'-TCAGCCTGCATCACCAGAGA-3', reverse 5'-CTGCAAGGACAGGATGCTGAT-3'); ID3 (forward 5'-CTGCAAGGACAGGATGCTGAT-3', reverse 5'-TGGCTCGGCCAGGACTAC-3'); SNAIL (forward 5'-CTGCGGGAAGGCCTTCTCT-3', reverse 5'-CGCCTGGCACTGGTACTTCTT-3'); SLUG (forward 5'-CGGACCCACACATTACCTTGTGTTT-3', reverse 5'-CACAGCAGCCAGATTCCCTCATGTTT-3'); TWIST (forward 5'-GGACAAGCTGAGCAAGATTGAGA-3', reverse 5'-TCTGGAGGACCTGGTAGAGGAA-3'). In order to confirm the identity of the amplified genes, PCR products were then sequenced (DNA-ABI Big Dye Terminator kit, Applied Biosystems, Foster city, CA, USA) and BLAST analysis was performed (data not shown).

Real-time PCR array (human tumor metastasis)

This method was used to determine if the tumor metastasis-related genes were regulated in response to the experimental conditions described above.

Eighty-four genes (RT² Profiler PCR Array, # APH-028A, Super Array, Inc., Bethesda, MD, USA), including gene involved in cell adhesion, extracellular matrix, cell cycle, cell growth proliferation, apoptosis,

Figure 2 The PI3K sustains mitochondrial functions in hypoxic conditions. Mitochondrial activity (MTT assay) of SH-SY5Y cells treated with CoCl₂ for 24 h is decreased by up to 80% ($P < 0.05$) the presence of the PI3K inhibitor (CoCl₂ LY versus CoCl₂ untreated '-'), or CoCl₂ LY versus SF-LY). Blockade of the JAK/STAT (AG) or MAPK (U) pathway did not significantly affect mitochondrial activity of SH-SY5Y cells treated with CoCl₂.

transcription factors, and controls (18SrRNA, RPL13A, HPRT1, β -actin and GAPDH) were thus simultaneously analyzed. Real-time PCR array was performed twice and experimental samples were generated as per conventional RT-PCR described above. Protocol was as per the manufacturer’s specifications.

Statistical analysis

GraphPad PRISM was utilized to perform one-way ANOVA. Experiments were performed at least thrice or as indicated, with samples run in duplicate–quadruplicate for each indicated time and data plotted as mean $\pm$ S.E.M.

Results

Neuroblastoma cell mitochondrial activity, but not cell number, is decreased by hypoxia

The two unrelated SH-SY5Y and SK-N-MC neuroblastoma cell lines were treated with hypoxia mimicking agent CoCl₂ (100 μ M) for up to 48 h (Fig. 1A–D). Cell proliferation or mitochondrial activity was determined by the colorimetric assays NBB or MTT respectively at each of the indicated time points as described in Materials and methods. As shown in Fig. 1A, SH-SY5Y cell number, in either SF or CoCl₂, was not significantly decreased (<5% Fig. 1A) over 48 h while mitochondrial activity was reduced by up to 50% (24–48 h; P <0.05) by the hypoxic agent, when

Figure 3 (A and B) Abundance of HIF1 α is increased by hypoxia in SH-SY5Y cells. (A) Cell extract from CoCl₂ treated (+), or untreated cells (–) as control, was fractionated onto a 10% SDS-PAGE transferred to a nitrocellulose filter and probed with anti-HIF1 α antibody as described in Materials and methods. The α -tubulin (black arrowhead) was used to verify loading equivalence between samples. HIF1 α was detectable by 30 min post-exposure to hypoxia (T30). (B) HIF1 α accumulation in the nuclear compartments is demonstrated by immunofluorescence with Alexa-488 combined with nuclear DAPI stain as described in Materials and methods.

compared with that of the untreated (SF) cells. A similar response to CoCl_2 was observed in SKNMC cells (Fig. 1C and D). However, SK-N-MC cell number (NBB assay) in SF at 48 h was significantly increased ($P < 0.01$) to more than that seen in SF conditions at 24 h, while there was no significant difference between NBB values for CoCl_2 treatment at 24 or 48 h versus SF at 24 h. Mitochondrial activity in SK-N-MC cells was reduced by up to 70% (48 h; $P < 0.01$) by the hypoxic agent CoCl_2 (Fig. 1C and D) when compared with MTT values is SF at 24–48 h. Mitochondrial activity (Fig. 1D) in SK-N-MC cells cultured in SF media for 48 h was increased to more than that seen at 24 h ($P = 0.05$), consistent with the increase in cell numbers, as determined by the NBB assay (Fig. 1C).

Mitochondrial activity of CoCl_2 -treated cells was dramatically decreased to 80% ($P < 0.05$) in the presence of the PI3K inhibitor (Fig. 2; CoCl_2 LY versus CoCl_2 untreated ‘–’, or CoCl_2 LY versus SF-LY), suggesting that this pathway is involved in the maintenance of mitochondrial functions in hypoxic conditions. A similar response to PI3K blockade was observed in SK-N-MC cells (data not shown).

Abundance of HIF1 α is increased by hypoxia in SH-SY5Y cells

The transcriptional complex HIF1, as described earlier, plays an essential role in oxygen homeostasis

(Semenza 1998, Wenger 2002, Safran & Kaelin 2003, Zarembek & Malech 2005). We therefore investigated whether the response to the hypoxia mimic CoCl_2 in neuroblastoma cells would involve modulation of HIF1 α abundance. Cell extract from CoCl_2 treated, or untreated cells as control, was fractionated onto a 10% SDS-PAGE, transferred to a nitrocellulose filter and probed with anti-HIF1 α antibody as described in Materials and methods. In SH-SY5Y cell in serum-free conditions and in the absence of CoCl_2 (normoxia), as shown in Fig. 2, levels of HIF1 α were extremely low/undetectable at each of the time points analyzed. HIF1 α was detectable by 30 min post-exposure to CoCl_2 (hypoxia; Fig. 3A, T30). The level of HIF1 α progressively accumulated intracellularly by 2 h and these levels were maintained for the duration of the experiment (24 h). HIF1 α protein was similarly induced in SK-N-MC cells. Hypoxia-stabilized HIF1 α appeared to accumulate in the cytoplasm at 4 h (Fig. 3B, CoCl_2) and translocated to the nuclear compartments as shown at 24 h (Fig. 3B, CoCl_2). HIF1 α was not detected in cells cultured in normoxia (Fig. 3B, UT). Cellular localization of HIF1 α in SK-N-MC was not determined. Consistent with IB and IF data, the abundance of HIF1 α mRNA (Fig. 4) in SH-SY5Y cells was significantly increased by 4 h, as per real-time PCR data (Table 1) and maintained up to 24 h. Similar regulation for HIF1 α mRNA was seen in SK-N-MC cells. These findings

Figure 4 (A–D) The MAPK pathway mediates the induction of HIF1 α and VEGF mRNAs. Following exposure (0–24 h) to the hypoxia mimic agent CoCl_2 , total RNA was extracted and analyzed as described in Materials and methods. (A) HIF1 α mRNA was increased at 4–24 h, an event modulated by the MAPK pathway (C). (B) VEGF was up-regulated at 4 h. (C and D) The MAPK pathway inhibitor U0126, but not LY294002, modulated VEGF mRNA levels.

Table 1 Prometastatic gene expression in hypoxic NB cells

GeneBank	Symbol	Description	Fold-change CoCl ₂ 4 h/SF 4 h (c.o. > ±0.5; P<0.05)
<i>EMT and cell adhesion</i>			
NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.48
NM_000038	APC	Adenomatosis polyposis coli	0.29
NM_001903	CTNNA1	Catenin (cadherin-associated protein)- α 1, 102 kDa	0.44
NM_002011	FGFR4	Fibroblast growth factor receptor 4	0.57
NM_000245	MET/HGF	Met proto-oncogene (hepatocyte growth factor receptor)	1.43
NM_002206	ITGA7	Integrin- α 7	3.07
NM_000660	TGFB1	Transforming growth factor- β 1	1.50
NM_003376	VEGF	Vascular endothelial growth factor	5.38
NM_017902	HIF1 α	Hypoxia inducible factor-1 α -subunit	7.12
NM_005985	SNAIL	Human homolog of <i>Drosophila</i> Snail	NS
NM_003068	SLUG	Human homolog of <i>Drosophila</i> Slug	NS
NM_000474	TWIST	Human homolog of <i>Drosophila</i> Twist	NS
<i>Tumor growth and metastasis</i>			
NM_000576	IL1B	Interleukin-1 β	10.55
NM_002427	MMP13	Matrix metalloproteinase 13 (collagenase 3)	2.34
NM_003256	TIMP4	TIMP metalloproteinase inhibitor 4	0.34
NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4	1.89
NM_003650	CST7	Cystatin F (leukocystatin)	0.52
<i>Tumor suppressor</i>			
NM_002256	KISS1	KISS1 metastasis-suppressor	3.50
NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A	4.14
NM_001846	COL4A2	Collagen type IV, α 2	0.38
NM_004442	EPHB2	EPH receptor B2	0.12
NM_006410	HTATIP2	HIV-1 Tat interactive protein 2, 30 kDa	0.22

suggest that post-transcriptional rather than transcriptional events are responsible for accumulation of HIF1 α protein at the earlier time points (30 min–2 h), shown in Fig. 3A. Furthermore, use of the MAPK pathway inhibitor U0126 (Fig. 4C) indicated that this pathway plays a critical role in the up-regulation of HIF1 α mRNA seen at 4 h (Fig. 4A 4 h compared with Fig. 4C 4 h). PI3K blockade, using LY294002, had a negligible effect on HIF1 α mRNA levels at 30 min and 1 h, but had a reproducible inhibition at 4 h (Fig. 4A 4 h compared with Fig. 4D 4 h). Pathway blockade and its effect on HIF1 α mRNA level were not performed in SK-N-MC cells.

Abundance of HIF1 α correlates with induction of VEGF

Newly formed HIF1 complexes (HIF1 α / β) interact with the HRE of a large number of target genes including VEGF. We therefore used RT-PCR to analyze the expression of VEGF in response to hypoxia. The time course of expression in hypoxia versus normoxia control samples shows that at 4 h VEGF mRNA is up-regulated in both SH-SY5Y (Fig. 4A–D) and SK-N-MC cells (Fig. 5). Up-regulation of VEGF mRNA in CoCl₂-treated SH-SY5Y cells

was also seen at 24 h. The MAPK pathway inhibitor U0126 also affected VEGF mRNA levels (Fig. 4C, 30 min–1 h), while the blockade of the PI3K only minimally affected its expression (Fig. 4D). In similar experiments, CoCl₂ treatment also induced the mRNA levels of EPO and its receptor, both targets of HIF1 complex (data not shown). Pathway blockade and its effect on HIF1 α or VEGF mRNA level were not performed in SK-N-MC cells.

Figure 5 Up-regulation of HIF1 α and VEGF mRNAs in hypoxic SK-N-MC cells. SK-N-MC cells total RNA was extracted and analyzed as described in Materials and methods. HIF1 α mRNA was increased at 1 h, while VEGF mRNA was up-regulated at 4 h following exposure to the hypoxia mimic agent CoCl₂.

The hypoxia mimic CoCl₂ affects abundance and/or cellular localization of Id1–2

As mentioned earlier, hypoxia promotes dedifferentiation of NB cells back to immature neuroblast-like cells, such that some of genes expressed in the neural crest cells might regain expression in hypoxic NB. The inhibitor of differentiation and DNA binding Id genes (Id1–3) appear to play a key role in these processes. Therefore, the expression of Id1–3 was investigated in SH-SY5Y cells exposed to hypoxic agent CoCl₂. The level of gene expression for each of the three genes was not significantly affected by hypoxia over the duration of the experiment (10 min–24 h time course, not shown). However, cellular localization and abundance for Id1 and Id2, and to a lesser extent Id3, protein were increased at 4–24 h of hypoxia (Fig. 6A–C, CoCl₂).

Cellular levels of Id1 were increased at 4 h with protein mostly localized to the peri-nuclear/nuclear compartment of hypoxic cells (Fig. 6A, 4 h CoCl₂). Id1 staining became localized mostly to the nuclear compartment after 24 h (Fig. 6B, 4 h CoCl₂). In untreated cells (normoxia, UT), Id1 immunoreactivity, at 4 and 24 h, was exclusively located to the cytoplasm (Fig. 6A, 4 h–24 h UT). Cellular levels of Id2 were also increased at 4 h of hypoxia, with protein localized to the nuclei of most cells (Fig. 6B, 4 h CoCl₂). Id2 protein abundance was further increased at 24 h, and localized to both nuclear and cytoplasm compartments (Fig. 6B, 24 h CoCl₂). In untreated cells, Id2 immunoreactivity, at 4 and 24 h, was exclusively located to the cytoplasm (Fig. 6B, 4 h–24 h UT). The levels of nuclear Id3 were increased more than the time in both untreated (Fig. 6C, 4–24 h UT) and CoCl₂ treated cells (Fig. 6C, 4–24 h CoCl₂), suggesting nuclear accumulation of Id3 under regulation of other factors, rather than by hypoxia.

Hypoxia promotes the activation of an EMT-like gene expression program in NB cells

In order to determine if the hypoxia triggers prometastatic processes in NB cells via induction of genes enhancing survival migration and invasion we utilized real-time PCR array.

As shown in Fig. 7 and Table 1, in hypoxic SH-SY5Y NB cells, genes involved in maintenance of cell–cell and cell–matrix interactions (i.e. adenomatous polyposis coli (APC), E-cadherin, catenin, EphB2, fibronectin-1, HTATIP2, tissue inhibitor of metalloprotease-4) were down-regulated by as much as 90%. However, Twist, Slug and Snail, other common markers of EMT, were not significantly affected by hypoxia.

Figure 6 (A–C) Hypoxia induces nuclear accumulation of Id1 and Id2. Immunofluorescence for ID13 was performed as described in Materials and methods. Nuclear localization and abundance for Id13 is shown by Alexa-488 with combined nuclear DAPI stain.

Figure 7 Prometastatic gene expression profile in hypoxic SH-SY5Y cells. Real time PCR-Array was performed as described in Materials and methods. Genes involved in maintenance of cell–cell and cell–matrix interactions (APC, E-cadherin, catenin, EphB2, fibronectin-1, HTATIP2, TIMP4) were down-regulated by as much as 90%. Under the same conditions, genes involved in enhancement of metastatic behavior (integrin $\alpha 7 \beta 1$, HGF, TGF $\beta 1$, VEGF, KISS1, IL1 β , MMP13) were dramatically up-regulated by over 200%. Real-time PCR data for HIF1 α , Snail, Slug and Twist are also included in this graph (see also Table 1).

Under the same conditions, genes involved in enhancement of metastatic behavior (integrin $\alpha 7 \beta 1$, hepatocyte growth factor receptor (HGF), transforming growth factor- $\beta 1$, VEGF, kisspeptin (KISS1), Interleukin-1 β (IL1 β), matrix metallo protease 13 (MMP13)) were dramatically up-regulated by over 200%.

Discussion

There is increasing evidence that hypoxia potently influences cellular phenotypes by altering the expression program of specific genes (Harris 2002, Semenza 2003, Brown & Wilson 2004). A major event in this regulation is the stabilization of the transcription factor HIF1 α leading to the induction of hypoxia responsive genes such as VEGF, one of the most extensively investigated. At normoxia, the HIF1 α subunit is degraded via the proteasomal pathway (Lee *et al.* 2004, Bardos & Ashcroft 2005).

In our study, and as previously reported by others (Rossler *et al.* 1999, Jogi *et al.* 2004, Das *et al.* 2005), hypoxia onset by CoCl₂ in SH-SY5Y and SK-N-MC cells produced intracellular accumulation of the HIF1 α and associated induction of its target gene VEGF, a process involving MAP kinase signaling. These events

are consistent with enhanced cell survival and tumor vascularization (Rossler *et al.* 1999, Jogi *et al.* 2004, Das *et al.* 2005).

Inhibition of the MAPK pathway in CoCl₂ treated SHSY5Y cells produced the expected down-regulation of HIF1 α and VEGF expression, while mitochondrial activity (MTT) was not affected by MAPK inhibition. On the other hand, the PI3K inhibitor only minimally affected HIF1 α or VEGF expression. Therefore, activation of the PI3K pathway appears to be responsible for the survival of SH-SY5Y cells in hypoxia, a response likely to be sustained by VEGF signaling, as previously described in these neuroblastoma cell lines (Rossler *et al.* 1999, Jogi *et al.* 2004, Das *et al.* 2005).

A large number of transcription factors are known to be affected by hypoxia via HIF-dependent or -independent mechanism and these include members of the basic-helix–loop–helix family of transcription factors, namely the *Id* genes (Norton 2000, Jogi *et al.* 2002, 2004). The levels of mRNA for *Id1* and *Id2* are found up-regulated in some hypoxic neuroblastoma cell lines (Jogi *et al.* 2002, Lofstedt *et al.* 2004). Conversely, we found no significant change in *Id* mRNAs in hypoxic SH-SY5Y neuroblastoma cells, while the protein product of these genes (*Id1* and *Id2*, but not *Id3*) accumulated in the nuclei following hypoxia. The absence of effects of hypoxia treatment on the levels of *Id3* gene expression and protein, as we have shown, is however, consistent with that previously reported for *Id3* in SH-SY5Y cells by Jogi *et al.* (2002) and Lofstedt *et al.* (2004).

The modulation of *Id2*, as reported in our study, is particularly interesting since it is required for proper neural crest development (Martinsen & Bronner-Fraser 1998, Jogi *et al.* 2002). *Id2* is directly regulated by HIF1 α (Lofstedt *et al.* 2004), thus suggesting a link between HIF1 α activation and dedifferentiation of hypoxic neuroblastoma cells.

Chronic exposure to hypoxia mimic agent (24 h CoCl₂) appeared to alter cell morphology, suggesting alterations of cell–cell and cell–matrix interactions and indicating the acquisition of a ‘more motile’ phenotype, involving events similar to that described for EMT (Thiery & Sleeman 2006).

It is now well recognized that EMT is a potential mechanism for cancer progression (Huber *et al.* 2005, Lee *et al.* 2006, Thiery & Sleeman 2006) and several signaling pathways have been uncovered, which are common to EMTs in development and tumor progression (Huber *et al.* 2005, Lee *et al.* 2006, Thiery & Sleeman 2006).

Figure 8 Hypoxia adaptation and prometastatic gene activation in neuroblastoma cells. In solid tumors that become hypoxic because of aberrant newly developed blood vessels and tumor overgrowth, cells often undergo genetic and adaptive changes that allow them to survive and even proliferate in a hypoxic environment. These processes contribute to the malignant and aggressive phenotype. In normoxia (1) mitochondrial activity is optimal and levels of HIF1 α are constitutively regulated at transcriptional, via FIH1 (factor inhibiting HIF1, not shown), and post-translational levels via (2) hydroxylation, ubiquitination and (3) proteasome degradation of HIF1 α protein. In hypoxia (4), mitochondrial activity (5) is decreased and HIF1 α inhibition and degradation are abrogated (6). This leads to a stable and functional HIF1 α (7) and formation of HIF complexes (not shown). These complexes can thus bind to regulatory hypoxia responsive elements (HRE) (8) of genes such as the survival cytokine erythropoietin (EPO) and the angiogenesis factor, vascular endothelial growth factor (VEGF) and genes involved in enhancement of metastatic behavior and EMT-like events (9). The expression level of the Id13 genes was not significantly affected by hypoxia (10), however, cellular levels of Id1 protein were increased within the nuclear compartment of hypoxic cells. In normoxia Id1 and Id2 immunoreactivities (11) was exclusively located to the cytoplasm. The levels of nuclear Id3 were increased over the time in both normoxia (12) and CoCl₂ treated cells (10), suggesting nuclear accumulation of Id-3 under regulation of other factors, rather than by hypoxia. The PI3K or the MAPK pathway inhibitors LY294002 (13) and U0126 (14) indicated that these pathways play a critical role in the modulation of HIF1 α mRNA. U0126 (14) also affected VEGF mRNA levels, while the effects of LY294002 (13) were negligible.

In our study, we were able to demonstrate, for the first time in NB cells, that hypoxia strongly down-regulated E-cadherin (~60%), APC (~70%), α -catenin (~60%), FGFR4 (50%) but up-regulated HGF (~50%). These regulations are consistent with activation of EMT events (Thiery & Sleeman 2006) such as the previously reported dissociation of the cell adhesion complex (Bremnes *et al.* 2002, Ezzat *et al.* 2004, Blanc *et al.* 2005), and malignant progression of neuroblastoma (Hecht *et al.* 2004). Furthermore, the dramatically increased expression of the α 7 β 1 integrin by up to threefold in hypoxic SH-SY5Y cells suggests the transition of SH-SY5Y cells to a migratory cell phenotype (Gardiner *et al.* 2005).

TGF- β is a major regulator of EMT (Muraoka *et al.* 2002, Thiery & Sleeman 2006) and TGF- β 1 is induced in hypoxic regions of solid tumors (Toomey *et al.*

2001). It was recently demonstrated (Shah *et al.* 2006), that transcriptional activation of TGF- β 1 requires HIF1 α . This finding suggests that the up-regulation of TGF- β 1 expression (~150%) observed in our studies, might be a direct consequence of the increase of HIF1 α abundance. Although the up-regulation of TGF- β 1 and modulation of other genes (E-cadherin, APC, α -catenin, VEGF, MMPs, etc.) are consistent with EMT, we found that the expression of Snail, Slug and Twist, other common markers of EMT, was not significantly affected. Whether this indicates that our *in vitro* system fails to fully mimic the *in vivo* conditions relevant to the induction of Snail, Slug and Twist, or that the described prometastatic gene expression program leads instead to a EMT-like or an incomplete EMT event, as recently reported by Christiansen & Rajasekaran (2006), remains unclear.

The involvement of pro-inflammatory cytokines such as IL-1 β in hypoxia response, angiogenesis, tumor growth, and metastasis is largely documented (Apte *et al.* 2006, Elaraj *et al.* 2006, Zhang *et al.* 2006). In our study, IL1 β was the most potently up-regulated gene (10 fold) and this might lead to a more aggressive SH-SY5Y cell phenotype, including activation of EMT events (Chaudhuri *et al.* 2007) and invasion/metastasis (Song *et al.* 2003). IL-1 β promotes expression and release of MMPs, including collagenase MMP13 (Klatt *et al.* 2006), which is up-regulated in our study.

The induction of MMP13 (2.3 fold) in our studies is intriguing, since this collagenase has an important and specific role in skeletal biology, but its role in neuroblastoma is unknown. Of relevance is that neuroblastoma primary tumor metastases to bone and bone marrow (Cotterill *et al.* 2000, Russell *et al.* 2004, 2005), tissues which develop and mature under low ($\sim$ 1–2%) oxygen (Wang *et al.* 2007a,b). It is thus likely that in these circumstances metastatic neuroblastoma cells could develop the ability to degrade bone tissue by modulating MMPs (i.e. MMP13) and TIMPs (i.e. TIMP-4, down 2.2 fold), as we have demonstrated in our *in vitro* hypoxia mimicking model for neuroblastoma cells. A further supportive finding is that chemokine receptor-4 (CXCR4), whose expression in neuroblastoma primary tumors is associated with clinical presentation of bone and bone marrow metastasis (grade 3 and 4; Russell *et al.* 2004), was up-regulated by 180% in our study.

Another intriguing finding was the strong up-regulation of two tumor suppressor genes, namely *KISS1* and cyclin-dependent kinase inhibitor 2A (*CDKN2A*, p16/ARF/MTS1). Expression studies for *KISS1* in human breast cancer (Martin *et al.* 2005) and *CDKN2A* in human colorectal carcinomas (Ohhara *et al.* 1996) clearly demonstrated that the expression of these two genes is increased in aggressive tumors and also correlates with increased mortality.

Another group of tumor suppressor genes was in contrast strongly down-regulated (2–5 fold) in hypoxic SH-SY5Y cells. This includes the α 2 chain of collagen type IV (Kamphaus *et al.* 2000, Maeshima *et al.* 2000), the receptor tyrosine kinase Ephrin-B2 (Alazzouzi *et al.* 2005, Jubb *et al.* 2005, Davalos *et al.* 2007), the HIV-1 tat-interacting protein 30 (NicAmhlaibh & Shtivelman 2001). The down-regulation or inactivation of these genes has been shown to accelerate tumorigenesis (Kamphaus *et al.* 2000, Maeshima *et al.* 2000) disrupt cell–cell adhesion and cell positioning (Alazzouzi *et al.* 2005, Jubb *et al.* 2005, Davalos *et al.* 2007) and increase metastasis and angiogenesis (NicAmhlaibh & Shtivelman 2001). Of particular

interest is the dramatic down-regulation ($\sim$ 90%) of EPHB2 observed in our study. Suppression or inactivation of EPHB2 has been shown to accelerate tumorigenesis initiated by APC mutations in the colon and rectum of APC^{Min/+} mice, demonstrating that EPHB2 is an important tumor suppressor in the large intestine (Batlle *et al.* 2005). The dramatic down-regulation of APC ($\sim$ 80%) and EPHB2 ($\sim$ 90%) observed in our *in vitro* study is thus consistent with Batlle's *in vivo* findings (Batlle *et al.* 2005), and might point to a potential prometastatic synergism of these genes in hypoxic NB cells.

In conclusion (see also Fig. 8), we have demonstrated for the first time that hypoxia in neuroblastoma cells activates a complex prometastatic gene program resembling EMT-like events, contributing to cellular dedifferentiation and transition to an invasive and metastatic NB cell phenotype. This cellular and molecular adaptation is probably promoted by the 'master' hypoxia regulator HIF1 α . We therefore propose that similar mechanisms might exist and contribute *in vivo* to enhancing metastatic behavior of neuroblastoma. Our findings thus point to the identification of new potential candidates for the development of more targeted and or alternative therapies in neuroblastoma.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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