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Hypoxia, pseudohypoxia and cellular differentiation

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ABSTRACT

Tumor hypoxia correlates to aggressive disease, and while this is explained by a variety of factors, one clue to understand this phenomena was the finding that hypoxia induces a de-differentiated, stem cell-like phenotype in neuroblastoma and breast tumor cells. The hypoxia inducible transcription factors (HIFs) are regulated at the translational level by fluctuating oxygen concentrations, but emerging data reveal that both HIF-1α and HIF-2α expression can be induced by aberrantly activated growth factor signaling independently of oxygen levels. Furthermore, HIF-2α is regulated by hypoxia also at the transcriptional level in neuroblastoma and glioma cells. In cultured tumor cells, HIF-2α is stabilized at physiological oxygen concentrations followed by induced expression of classical hypoxia-driven genes, resulting in a pseudohypoxic phenotype. In addition, in neuroblastoma and glioma specimens, a small subset of HIF-2α positive, HIF-1α negative, tumor cells is found adjacent to blood vessels, i.e. in areas with presumably adequate oxygenation. These tumor niches are thus pseudohypoxic, and the HIF-2α expressing cells present immature features. We have postulated that this niche in neuroblastomas encompass the tumor stem cells. Oncogenes or tumor suppressor genes associated with pseudohypoxia are frequently mutated or deleted in the germline, implicating that the pseudohypoxic phenotype indeed is tumorigenic. In summary, the hypoxic and pseudohypoxic phenotypes of solid tumors are attractive therapeutic targets.

1. Introduction

Our interest in hypoxia and its effects on cell differentiation was initiated by the observation that a subset of the sympathetic nervous system (SNS)-derived childhood tumor neuroblastoma contained areas with spontaneous ganglionic-to-neuroendocrine differentiation in cells surrounding zones of overt necrosis [1,2]. A link between hypoxia and cellular differentiation was not obvious at that time, but the first *EPAS1/HIF2A* knockout study, demonstrating impaired development of mouse SNS ganglia [3], suggested a connection between SNS development, HIF-2 and hypoxia. We hypothesized that hypoxia, and stabilization of HIF-2α, would initiate neuroendocrine differentiation in cultured neuroblastoma cells, and contacted Dr. Lorenz Poellinger who together with members of his lab introduced us to the basics of hypoxia research. We found, however, that established neuroblastoma cell lines grown at hypoxia did not present with an enhanced neuroendocrine phenotype. Instead, data suggested the complete opposite, i.e. that hypoxia induced a de-differentiated, stem cell-like phenotype [4]. During the time of establishing our own research project studying neuroblastoma and hypoxia, Lorenz's knowledge and open mind were of great importance. Our continued collaboration generated findings important for the hypoxia as well as tumor biology research fields, and these findings are reviewed below.

2. Hypoxia blocks cellular differentiation – neuroblastoma as model system

Neuroblastomas are derived from immature cells of the ganglionic SNS lineage and the stage of neuronal differentiation at which the tumor cells are arrested, correlates to clinical outcome [5]. Neuroblastomas are phenotypically heterogeneous and the overt cellular differentiation stage within a given tumor specimen varies. In a small subset of neuroblastomas, we observed that cells in discrete areas surrounding necrotic zones presented with a neuroendocrine marker gene expression pattern ($IGF2^+$, $SCG2^+$, $CHGA/B^+$, TH^+, NPY^-, $BCL2^-$, $HNK1^-$), suggesting a spontaneous conversion from neuronal to neuroendocrine lineage [1,2]. Presumably, this spontaneous shift in lineages was driven by an altered milieu (hypoxia, low pH, nutrient deficiency etc.) in these peri-necrotic areas, and we decided to investigate the effect of hypoxia on neuroblastoma cell phenotypes. Although the expression of what was then looked upon as two neuroendocrine marker genes, *IGF2* and *TH*, was induced by hypoxia, the expression of neuroendocrine hallmark genes *CHGA* and *CHGB* decreased. As *IGF2* and *TH* were later confirmed as hypoxia driven

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genes, and the expression of a range of neuronal/neuroendocrine markers decreased while early markers of the ganglionic lineage increased in hypoxic neuroblastoma cells, we concluded that these cells acquired an immature neural crest-like phenotype [4]. Interestingly, we recently discovered that *IGF2*, which we for years believed to be expressed exclusively in chromaffin cells during SNS development, is indeed expressed also in human embryonic pre-SNS ganglia (w6.5) [6]. Thus, the increase in *IGF2* expression in hypoxic neuroblastoma cells might both reflect a hypoxia-driven mechanism as well as a shift in expression coupled to an early stage of ganglionic differentiation. Although the finding that hypoxic neuroblastoma cells de-differentiate was unexpected, it was in line with the notion that poorly oxygenated tumors, as demonstrated in cervix carcinoma, are more aggressive than tumors with higher intra-tumoral oxygen [7].

3. Hypoxia blocks cellular differentiation – mammary gland and breast cancer as model systems

The observation that low oxygen in breast cancers correlated to aggressive disease, and that patients with highly differentiated tumors had more favorable outcome, led us to investigate the effects of hypoxia in breast cancer cells. We showed that hypoxic tumor cells adjacent to necrotic cores in human breast ductal carcinoma in situ (DCIS) lesions were less differentiated compared to the oxygenated cells in outer cell layers [8]. The link between hypoxia and an immature phenotype was further strengthened by our finding that cultured breast cancer cells de-differentiated when grown at hypoxic conditions. High levels of HIF-2α protein in breast tumors correlate to worse patient outcome and to distant metastasis [9]. These findings prompted us to investigate if hypoxia and expression of HIFs affected normal mammary epithelium.

The mammary epithelium goes through cycles of hormone driven proliferation, differentiation and regression during pregnancy, breast feeding and post lactational involution, but also monthly, with a lower amplitude, throughout female reproductive life. We found that 3D cultures of luminal mammary epithelial cells (MUC1 positive), derived from breast reduction surgery in healthy women, lost the ability to form acinar structures at hypoxia. Furthermore, the cells remained immature and proliferative during 21 days of culture at hypoxic conditions [10]. The hypoxic breast cells showed a cancer-like phenotype with a combination of sustained proliferation, decreased expression of differentiation markers, and loss of cellular polarization [10]. In an effort to understand a putative role of hypoxia and HIFs during breast development, we stained sections of mouse mammary glands from early puberty, virgin, lactating, and postlactational involution for HIF-1α and HIF-2α. In puberty, HIF-1α was expressed in ductal luminal epithelial cells, but not in basal/myoepithelial cells, the latter cell population supposedly encompassing the breast epithelial stem cells [11]. HIF-2α had a distinct expression in a subpopulation of cuboidal luminal cells during lactation. These milk-producing cells have a high metabolic rate, and HIF-2 may contribute to this increased metabolism and uptake of high-energy substrates, such as glucose. Interestingly, HIF-2α was expressed in epithelial cells of basal or stem cell phenotype in involution samples, one and two weeks after removal of the pups [11].

Hypoxia has profound effects on breast epithelial and precursor cells by inducing phenotypic changes similar to those seen during breast cancer development, and one might speculate if hypoxic niches in the mammary gland can promote carcinogenesis. Conditions like proliferation and growth of mammary epithelium into the less vascularized mammary fat pad, inflammation with highly metabolic, oxygen consuming infiltrating cells and fibrosis, might all lead to hypoxia with increased risk of developing cancer [12]. During mammary gland involution, the loss of hormonal stimulation leads to mammary epithelial cell death and expression of HIF-2α in a small subset of surviving cells. In breast cancer endocrine therapy, cancer cells also stop to proliferate and/or die in response to lowered estrogen levels (aromatase inhibitors) or inhibition of estrogen receptor binding (tamoxifen, fulvestrant). Interestingly, hypoxic conditions protected estrogen receptor positive cultured breast cancer cells against tamoxifen and fulvestrant treatment, and cells with acquired tamoxifen or fulvestrant resistance had increased protein levels of HIF-2α [13]. Silencing of HIF-2α or chemical inhibition of HIFs restored the anti-estrogen response in these cells suggesting that HIF-2α inhibition may overcome endocrine therapy resistance.

4. Different oxygen sensitivity between HIF-1α and HIF-2α

HIF-1α and HIF-2α are structurally similar and share 48% primary amino acid sequence homology [14], where the most conserved domains are DNA binding (basic helix-loop-helix, bHLH) and ARNT interacting (Per-Arnt-Sim, PAS). Much less is known about HIF-3α, but it has been shown to negatively regulate HIF-1 and HIF-2 by blocking Hypoxia Response Element (HRE) binding [15,16]. In the presence of oxygen, the alpha subunits are prolyl hydroxylated and bound by the von Hippel Lindau (VHL) protein, targeting HIF alpha to degradation. The alpha subunits are also targeted by factor inhibiting HIF (FIH1), leading to steric hindrance of HIF complex formation and inhibited transcriptional activity. These processes require oxygen, and hence, during hypoxic conditions HIF alpha subunits are free to form transcriptionally active complexes together with ARNT and co-factors CREB-binding protein (CBP) and p300.

Fluctuating oxygen levels have since long been known to regulate the activities of HIF-1 and HIF-2 via VHL and FIH1 binding. More recently, however, it has been shown that oxygen affects HIF expression also by other means. In neuroblastoma, glioma and breast cancer cells, hypoxia induces increased *HIF2A* transcription, in turn leading to accumulated HIF-2α protein [6,13,17–20]. Transcript levels of *HIF1A* on the other hand are virtually unaffected by oxygen, implicating a strong regulation of HIF-1α solely at the protein level [17]. Further, hypoxia affects the protein synthesis machinery through a newly discovered pathway involving HIF-2α, but not HIF-1α or ARNT. A complex consisting of HIF-2α, RNA-binding RBM4 and eIF4E2 is recruited to a large, but defined, set of mRNAs at low oxygen levels, favoring active translation at polysomes. This mechanism leads to avoidance of otherwise hypoxia-induced repression of protein synthesis [21]. Several genes targeted by the HIF-2α-RBM4-eIF4E2 complex are associated with hypoxia, including *HIF2A* itself, *ARNT*, *PHD2*/*EGLN1*, and *FIH1*, arguing for continued selective expression of HIF-2α, whereas HIF-1α seems to be repressed as hypoxia prevails [21]. This mechanism of action might very well explain the observed down-regulation of HIF-1α after acute phases of hypoxia in e.g. neuroblastoma and breast cancer cells [9,22].

While oxygen-governed regulation of the HIF alpha subunits is the main determiner of cellular levels of these proteins, HIF-1α and HIF-2α expression can also be influenced via oxygen-independent mechanisms. For example, presumably as part of a preventive program to precondition cells for augmented oxygen requirements, growth factor-induced signaling has been shown to elevate HIF-1α expression [23]. All cells, at least in acute phases, express HIF-1α protein in response to low oxygen levels, whereas growth factor signaling seems to induce expression in a more cell type and context specific pattern. Various growth factors have been demonstrated to play a role in determining HIF-1α expression, including insulin growth factor (IGF-I) [24,25], insulin [26], vascular endothelial growth factor (VEGF) [27] and epidermal growth factor (EGF) [28]. Effects are further transduced mainly via the PI3K- and/or Ras/MAPK pathways [28,29]. Several individual proteins that are part of these growth factor signaling pathways can be deregulated in human cancers. Thus, aberrant expression of the HIF proteins and their target genes in oxygenated milieus create a so-called pseudohypoxic state (see below). Although oxygen-independent regulation of HIF-2α has been much less studied, recent findings show that HIF-2α can be transcriptionally regulated by