

Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species¹

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SPECIFIC AIMS

Small exponentially growing multicellular tumor spheroids robustly generate reactive oxygen species (ROS), whereas with the occurrence of hypoxic quiescent cell areas in large multicellular tumor spheroids ROS generation is down-regulated and an intrinsic multidrug resistance (MDR) is developed owing to the increased expression of the MDR transporter P-glycoprotein (P-gp). The aim of the present study was to demonstrate that P-gp expression under hypoxic conditions is correlated to expression of the hypoxia-related transcription factor hypoxia-inducible factor HIF-1 α and regulated by the intracellular redox state.

PRINCIPAL FINDINGS

1. Hypoxia in the depth of the tissue of multicellular tumor spheroids is correlated to down-regulation of ROS generation and increased expression of HIF-1

Multicellular tumor spheroids endogenously generate ROS that are used in signaling cascades involved in the regulation of tumor cell growth. It is demonstrated that with increasing size of tumor spheroids, the pericellular oxygen pressure in the spheroid center declined and endogenous generation of ROS was down-regulated. In parallel, an up-regulation of HIF-1 α and P-gp was observed, suggesting that P-gp is regulated by hypoxia and/or the intracellular redox state (Fig. 1).

2. P-gp expression is up-regulated on experimental physiological and chemical hypoxia

To investigate whether P-gp expression in multicellular tumor spheroids is regulated by the hypoxic microen-

vironment in the depth of the tumor tissue, small normoxic tumor spheroids were incubated for 72 h at 1% O₂. As expected, this treatment resulted in significant up-regulation of HIF-1 α . The expression of P-gp was significantly increased compared with the normoxic control. In a parallel set of experiments, tumor spheroids were incubated for 24 h with 100 μ M CoCl₂ or 130 μ M DFA, both compounds known to induce chemical hypoxia. Comparable to experimental physiological hypoxia, incubation with CoCl₂ and DFA significantly up-regulated Pgp in multicellular tumor spheroids, which indicates that P-gp expression in multicellular tumor spheroids is regulated by the pericellular O₂ pressure within the tumor tissue.

3. P-gp and HIF-1 α expression are regulated by the intracellular redox state

Hypoxic conditions are associated with a decreased intracellular redox state, i.e., reduced levels of intracellular ROS. It was therefore assumed that elevation of intracellular ROS levels should down-regulate P-gp and HIF-1 α . Large multicellular tumor spheroids expressing elevated levels of HIF-1 α and P-gp were treated for 24 h with 200 μ M H₂O₂ or 50 μ M BSO, known to raise intracellular ROS by depletion of the intracellular glutathione store. As expected, elevation of the intracellular redox state significantly down-regulated HIF-1 α . Tumor spheroids were incubated with the free radical scavengers APDC (0.2 μ M), DHA (2.5 mM), vitamin E (30 μ M), and NAC (20 mM). This treatment resulted in significant up-regulation of HIF-1 α and Pgp, indicating that the reduced intracellular redox state

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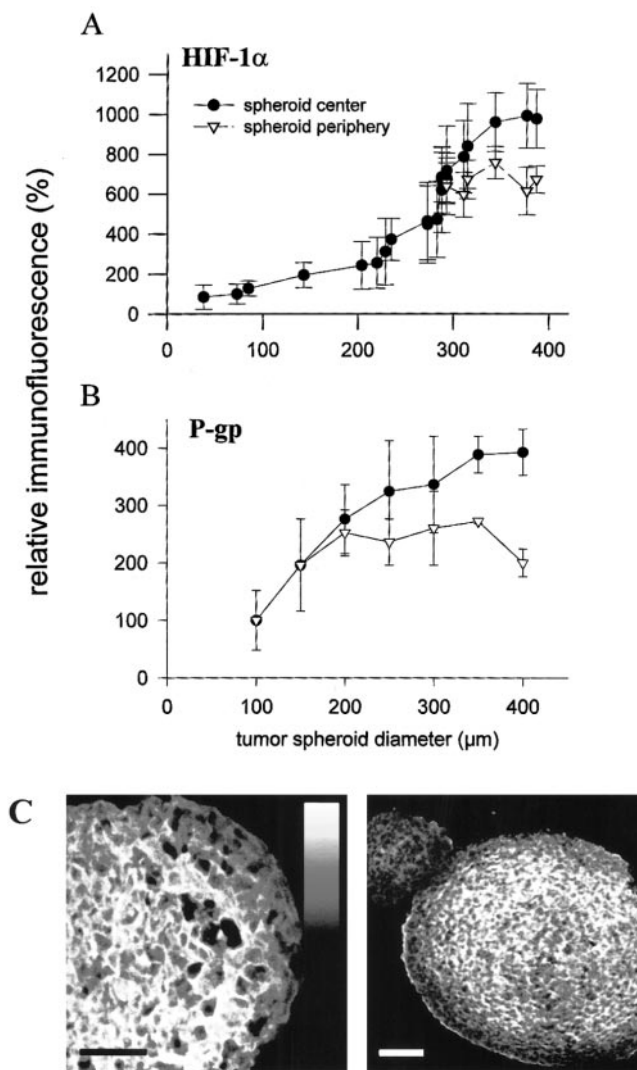


Figure 1. Expression of HIF-1 α (A) and P-gp (B) in the center or the periphery of cryosections obtained from multicellular prostate tumor spheroids. C) Representative cryosections of large tumor spheroids immunostained for HIF-1 α (left panel, bar=50 μm) and P-gp (right panel, bar=60 μm). The color bar represents HIF-1 α or P-gp immunofluorescence intensity. Note that P-gp and HIF-1 α expression is up-regulated with increasing size of multicellular tumor spheroids. Moreover, expression of P-gp and HIF-1 α is elevated in the center of the tumor spheroids compared with the spheroid periphery.

present in hypoxic regions of the tumor tissue may be responsible for the increased levels of HIF-1 α and P-gp.

4. Chemical hypoxia fails to up-regulate HIF-1 α or P-gp in multicellular hepatoma tumor spheroids mutant for HIF-1 β /ARNT

The data of the present study suggest that HIF-1 α and P-gp are regulated by the pericellular oxygen levels and/or the intracellular redox state in the depth of the tumor tissue of multicellular tumor spheroids. To validate our assumption that P-gp expression is under the control of HIF-1, multicellular hepatoma tumor spher-

oids wild-type (Hepa1) or mutant for HIF-1 β /ARNT (Hep1C4) were incubated for 24 h in the presence of 100 μM CoCl₂ or 130 μM DFA, and the expression of HIF-1 α and P-gp was investigated. A significant up-regulation of HIF-1 α and P-gp was observed in wild-type Hepa1 tumor spheroids under conditions of chemical hypoxia. In contrast, no significant increase in the expression of HIF-1 α and P-gp on chemical hypoxia was observed in HIF-1 β /ARNT-deficient Hep1C4 tumor spheroids, which clearly indicates that a functional HIF-1 heterodimer is a prerequisite for the observed up-regulation of P-gp expression under hypoxic conditions (Fig. 2).

CONCLUSIONS

It is well known that hypoxia in avascular micrometastases and avascular regions of solid tumors is associated with increased resistance toward anticancer agents. However, the molecular mechanisms underlying hypoxia-associated drug resistance are unknown. In the present study, it is demonstrated that with decreasing pericellular oxygen pressure in the depth of the avascular tissue of multicellular prostate tumor spheroids, elevated levels of HIF-1 α and P-gp occurred, suggesting that the expression and/or stability of both proteins is regulated by the same tissue microenvironment, i.e., pericellular oxygen pressure (see Fig. 3). It was demonstrated that the robust generation of intracellular ROS observed in small exponentially growing tumor spheroids was down-regulated with increasing size of the spheroids and decreasing pericellular O₂ pressures. ROS are known to play a role in signaling pathways that regulate cell growth and differentiation and have been demonstrated to mediate the proteolytic degradation of HIF-1 α and down-regulate the intrinsic P-gp expression in multicellular prostate tumor spheroids, indicating that HIF-1 levels in the tissue are regulated by the intracellular redox state of the tissue. To corroborate our working hypothesis of hypoxia/ROS-associated regulation of P-gp expression, small normoxic tumor spheroids that expressed low levels of P-gp were incubated under conditions of physiological or chemical hypoxia. This treatment significantly up-regulated P-gp expression, which implies that P-gp-mediated MDR in the depth of the tissue is regulated by the pericellular oxygen pressure. P-gp and HIF-1 α expression was up-regulated in the presence of free radical scavengers that lower the intracellular redox state whereas, as expected, an elevation of the intracellular redox state by the use of H₂O₂ or BSO resulted in down-regulation of both proteins. These observations are in line with the notion of reduced ROS levels occurring in hypoxic tissue and underscore previous results that hypoxia-induced changes in HIF-1 α and P-gp stability and subsequent gene activation are mediated by redox-induced changes (see Fig. 3).

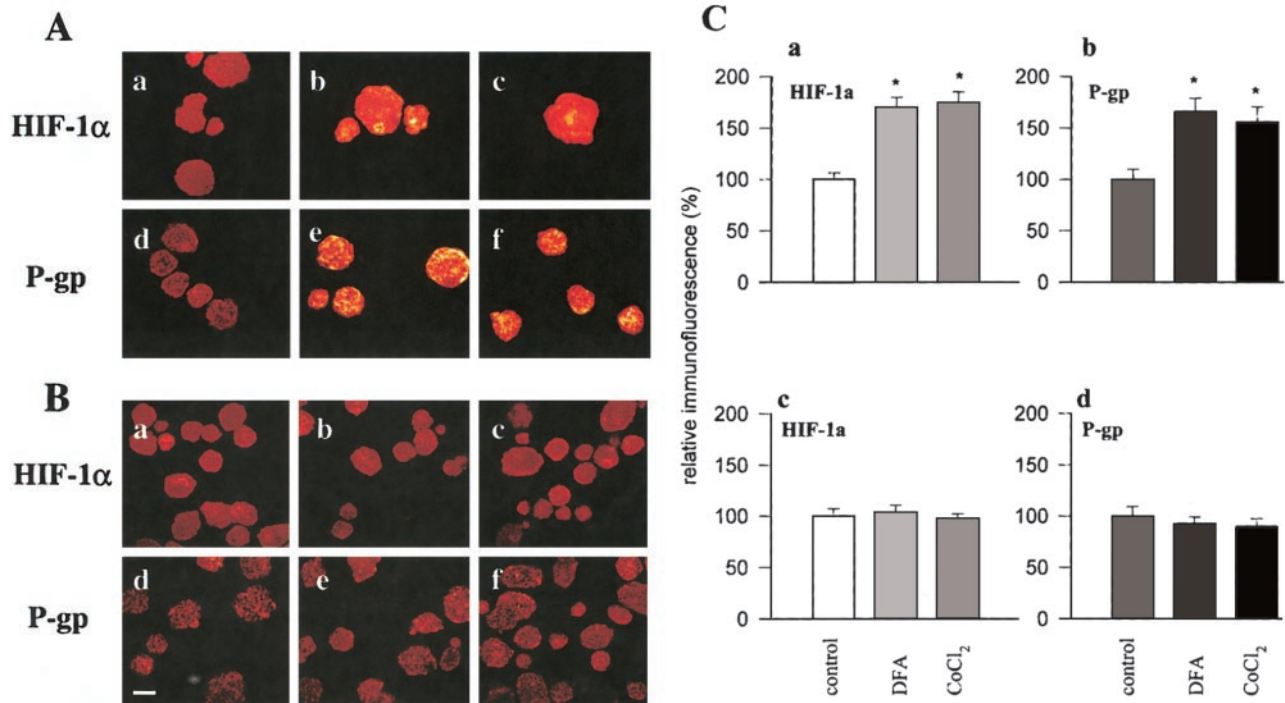


Figure 2. Effects of chemical hypoxia on the expression of HIF-1 α and P-gp in multicellular hepatoma tumor spheroids of the Hepa-1 cell line (wild-type) or the Hep1C4 cell line, which is deficient for ARNT. *A, B*) Representative images of tumor spheroids of the Hepa-1 cell line (wild-type) (*A*) or Hep1C4 cell line (*B*) under control conditions or conditions of chemical hypoxia. (*A, a*) HIF1- α , control; (*A, b*) HIF1- α , 130 μ M DFA; (*A, c*) HIF1- α , 100 μ M CoCl₂; (*A, d*) P-gp, control; (*A, e*) P-gp, 130 μ M DFA; (*A, f*) P-gp, 100 μ M CoCl₂; (*B, a*) HIF1- α , control; (*B, b*) HIF1- α , 130 μ M DFA; (*B, c*) HIF1- α , 100 μ M CoCl₂; (*B, d*) P-gp, control; (*B, e*) P-gp, 130 μ M DFA; (*B, f*) P-gp, 100 μ M CoCl₂. Bar = 50 μ m. *C*) quantitative evaluation of HIF-1 α (*a, c*) and P-gp (*b, d*) expression in Hepa-1 (*a, b*) and Hep1C4 (*c, d*) multicellular hepatoma tumor spheroids.

Our observation of a correlation of HIF-1 α and P-gp expression was validated by the use of multicellular tumor spheroids derived from a HIF-1 β /ARNT mutant hepatoma cell line that is unable to form a functional HIF-1 α /ARNT heterodimer (Hepa1C4). It was supposed that in HIF-1 β /ARNT mutant hepatoma tumor spheroids experimental hypoxia should fail to up-regulate P-gp. As expected, P-gp expression remained unchanged under conditions of chemical hypoxia in Hepa1C4 tumor spheroids, which clearly demonstrates that a functional HIF-1 α /ARNT heterodimer is required for the hypoxic up-regulation of P-gp.

Preclinical models *in vitro* and *in vivo* have shown that tumor hypoxia alters the malignant cell phenotype, selecting for p53 mutations, stimulating angiogenesis and metastasis, and markedly reducing the efficacy of radiotherapy and chemotherapy. Clinical studies confirm that the presence of hypoxia confers a negative effect on local control, disease-free survival, and overall survival. Knowledge of the association of P-gp-mediated MDR and hypoxia will help to fight cancer by unconventional therapeutic approaches that alter the intratumor oxygenation status. Increased impetus should be directed toward the design of new therapeutics that target hypoxic cells and reverse the MDR phenotype. [F]

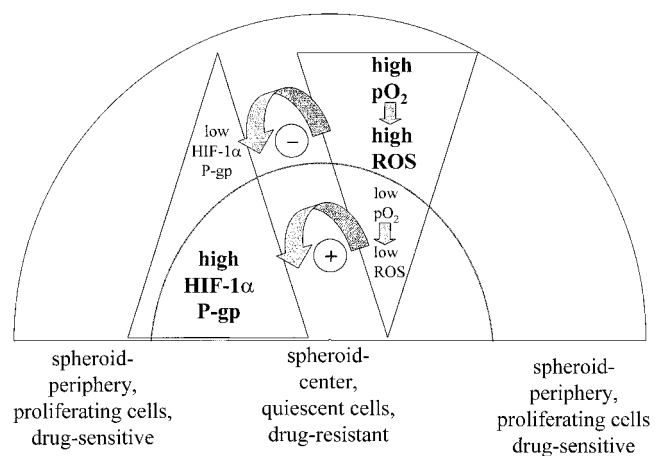


Figure 3. Effects of gradients in pO₂ and ROS in multicellular tumor spheroids on expression levels of HIF-1 α and P-gp. In the normoxic periphery of large multicellular tumor spheroids, proliferating cells prevail and ROS are robustly generated from their substrate O₂. These proliferating cell layers are drug sensitive and express only low levels of HIF-1 α and P-gp, presumably owing to increased degradation of HIF-1 α under normoxic conditions. In the center, pO₂ and ROS generation is reduced. In this hypoxic tissue microenvironment, up-regulation of HIF-1 α and P-gp occurs that results in drug-resistant phenotype of the quiescent central cell layers. It is concluded that MDR in hypoxic, avascular regions of solid tumors occurs via HIF-1-dependent up-regulation of P-gp.