Letter to the Editor

Hypoxia and HIF-1 α protect A549 cells from drug-induced apoptosis

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Dear Editor,

The impact of hypoxia on initiation of cell death *versus* cell survival as well as chemoresistance is still a matter of debate. This is reflected by divergent expression patterns of pro- as well as antiapoptotic regulators.¹ HIF-1 is overexpressed in many human cancers² and studies correlating increased HIF-1 α expression with patient mortality and/or treatment failure exist.³ Chemoresistance mediated by hypoxia has been described and provokes an aggressive tumor phenotype.^{3–5} However, little is known whether HIF-1 operates as an exclusive signal to protect from apoptosis under hypoxia or whether it does so in concert with other signals.

A549 cells, preexposed for 24 h to hypoxia (0.5% O₂), showed a significant reduction of apoptosis after staurosporine-treatment (1 μ g/ml, 4 h) compared to normoxic controls (Figure 1A). Similar results were obtained after etoposide-treatment (data not shown). Time-kinetics revealed significant chemoresistance from 16 h onwards (Figure 1B), which implies transcriptional mechanisms to be involved. In further experiments, the 24 h time-point was chosen, giving robust protection from apoptosis.

We asked whether, and to what extent, protection might be HIF-1-dependent and used HIF-1 α -specific siRNA (Dharmacon, USA). Complete knockdown of HIF-1 α protein by HIF-1 α -siRNA was proven by Western-analysis, while the use of mock-siRNA (siCONTROL™ nontargeting Duplex #1, Dharmacon, USA) excluded unspecific effects (data not shown). Mock-siRNA did not interfere with caspase-3 inhibition, but silencing HIF-1 α under hypoxia, reduced chemoresistance by roughly 30% (Figure 1C; lane 6 *versus* 2). In addition, the hypoxia mimetic CoCl₂, that stabilizes HIF-1 α and activates HIF-1, promoted drug resistance, although protection was less prominent compared to hypoxia. CoCl₂-evoked protection was eliminated following HIF-1 α knockdown, indicating a HIF-1 specific effect (Figure 1C). Similar results were obtained when desferrioxamine was used (data not shown).

These observations are in line with reports showing that knockdown of HIF-1 α reduced but not abolished chemoresistance.^{6,7} Considering the fact that, chemoresistance persists, although HIF-1 α is eliminated, points to alternative hypoxia-induced survival pathways. To explore these pathways, we used a number of target specific inhibitors. Blocking cyclooxygenase-2 (COX-2) with NS398 reduced protection compared to resistance evoked by hypoxia (Figure 1C). Importantly, knocking down HIF-1 α and blocking COX-2 resulted in additive sensitization towards staurosporine, which implies that parallel operating pathways contribute to protection independently of each other (Figure 1C).

Gathering information on regulation of COX-2 under hypoxia, we checked protein expression of HIF-1 α and COX-2. As expected, maximum HIF-1 α protein was observed after 4 h of hypoxia and declined afterwards, whereas COX-2 accumulated time dependently (Figure 1D). Many tumors show high COX-2 expression which is correlated with chemoresistance, and various human cells such as macrophages, hepatocytes, neutrophils, thymocytes and/or neurons acquire resistance towards apoptosis upon COX-2 induction.⁸ COX-2 expression and formation of prostaglandin E₂ (PGE₂), a COX-2-derived metabolite, has been reported under hypoxia although COX-2 upregulation is unrelated to HIF-1-signaling.⁹ Para- and/or autocrine signaling of PGE₂ with activation of adenylyl-cyclase and cAMP formation is known to regulate antiapoptotic proteins such as inhibitors of apoptosis. As a COX-2-dependent protective principle under hypoxia has not been described, we evaluated characteristics of HIF-1 α *versus* COX-2-elicited protection signals with respect to compartmentalization that is discriminating intra- *versus* extracellular effects. Conditioned media (CM) from hypoxic cells protected cells cultured under normoxia from drug-induced apoptosis based on caspase-3-activity determinations (Figure 1E). Considering, that NS398 reduced protection under hypoxia, we tested whether NS398 also blocked the antiapoptotic principle of CM. However, protection evoked by CM remained unaltered when NS398 was added to normoxic cells. Similar observations were made with LY294002 (30 μ M) to block PI3K-signaling (Figure 1E). We then changed the experimental setup and added NS398 or HIF-1 α -siRNA during the conditioning phase (Figure 1E). NS398 completely reversed protection by CM when applied during conditioning, while HIF-1 α -siRNA did not (Figure 1E). These results imply that COX-2, but not HIF-1 α , is essential for the production/formation of one or more secreted survival factors. Secreted survival factors produced under hypoxia are known, and typically comprise HIF-1 target gene products such as VEGF, EPO, or IGF.¹⁰ In our system these molecules can be excluded, as their production should have been sensitive to knock-down of HIF-1 α during generation, and their production should not have been antagonized by NS398. Furthermore, transducing mechanisms of these factors often are PI3K-mediated, a pathway that was excluded to convey protection of CM since LY294002 failed to antagonize inhibition of caspase-3-activity. Taking into consideration, that downstream of COX-2 PGE₂ may promote cell survival, we noticed that this prostanoid dose dependently attenuated staurosporine-induced cell death. PGE₂, 502 nM, protected

Figure 1 (A) A549 cells were incubated for 24 h under normoxia or hypoxia (0.5% O₂) followed by staurosporine (1 μg/ml) treatment for 4 h without serum. Apoptosis was followed by DAPI-staining (a–c) or caspase-3-activity determinations (d) (*n* = 20). (B) A549 cells were incubated for 0–48 h under hypoxia followed by staurosporine treatment (1 μg/ml, 4 h). Apoptosis was followed by caspase-3-activity measurements (*significantly different from normoxia/staurosporine; lane 1; *n* = 3). (C) A549 cells were exposed to hypoxia, CoCl₂, the COX-2-inhibitor NS398, HIF-1α-siRNA or mock-siRNA. siRNA was preincubated for 48 h under normoxia. Apoptosis was induced by staurosporine (1 μg/ml, 4 h). Data show effects of CoCl₂, NS398 and/or siRNA on chemoresistance observed under hypoxia (# significantly different from hypoxia/staurosporine; lane 2; *n* = 5). (D) Time-dependent expression of HIF-1α and COX-2 (Anti-HIF-1α; BD Biosciences, USA; Anti-COX-2; Cell Signaling, USA) under hypoxia. Actin served as a loading control (Anti-Actin; Sigma, Germany). (E) Apoptosis in A549 cells under the influence of differentially conditioned media (CM) or PGE₂ (PGE). Conditioned media were generated for 24 h under normoxia (N) or hypoxia (H) in the presence or absence of 100 μM NS398 (NS) or 100 nM siRNA-HIF-1α (si). CM was then added for 24 h to cells cultured under normoxia with the further addition of either 100 μM NS398 or 30 μM LY294002 (LY) as indicated. Normoxic incubations were performed for 24 h in the presence or absence of 500 nM PGE₂. Subsequently, 1 μg/ml staurosporine (Sts) was added for 4 h (*n* = 5). Asterisks and rhombs mark *P*-values of *P* ≤ 0.05. All methods were previously described¹¹

cells significantly from drug-induced cell death under normoxia (Figure 1E).

We conclude that under hypoxia at least two pathways operate in parallel to protect A549 cells from apoptosis. First, induction of HIF-1α contributes to protection by promoting intracellular alterations, while a second signaling circuit uses HIF-1α-independent but COX-2-derived signals that

act para- and/or autocrine. As the production of these factor(s) is sensitive to NS398 but not to HIF-1α-siRNA, a COX-2-derived prostanoid such as PGE₂ seems likely. The expression of intracellular proteins may control receptor- and/or mitochondria-evoked proapoptotic pathways, whereas soluble antiapoptotic factors transmit protective responses to their neighborhoods. This may preserve the integrity of not

only single cells but also tissue organization and organ structures.

Exemplified by tumors, cells often acquire resistance to apoptosis under long-term hypoxia, although the role of HIF-1 α in contributing to resistance or enhancing cell death still is controversial. To understand the controversy on HIF-1-dependent *versus* -independent mechanisms of cell protection under hypoxia other factors than HIF-1, such as COX-2, deserve consideration. Cell specific expression of COX-2 and/or phenotype distinct responses towards COX-2 metabolites may serve as additional regulatory devices to understand the interplay between hypoxia and apoptosis.

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1. Bacon AL and Harris AL (2004) *Ann. Med.* 36: 530–539
2. Zhong H *et al.* (1999) *Cancer Res.* 59: 5830–5835
3. Semenza GL (2002) *Trends Mol. Med.* 8: S62–S67
4. Unruh A *et al.* (2003) *Oncogene* 22: 3213–3220
5. Erler JT *et al.* (2004) *Mol. Cell. Biol.* 24: 2875–2889
6. Yu EZ *et al.* (2004) *Lab. Invest.* 84: 553–561
7. Zhang Q *et al.* (2004) *Int. J. Cancer* 111: 849–857
8. von Knethen A and Brüne B (2000) *Mol. Cell. Biochem.* 212: 35–43
9. Schmedtje Jr JF *et al.* (1997) *J. Biol. Chem.* 272: 601–608
10. Semenza GL (2003) *Nat. Rev. Cancer* 3: 721–732
11. Callapina M *et al.* (2005) *Exp. Cell. Res.* 306: 274–284