Expression of a truncated active form of VDAC1 in lung cancer associates with hypoxic cell survival and correlates with progression to chemotherapy resistance

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Running title: VDAC1-ΔC mediates hypoxic chemoresistance
Abstract

Resistance to chemotherapy-induced apoptosis of tumor cells represents a major hurdle to efficient cancer therapy. Although resistance is a characteristic of tumor cells that evolve in a low oxygen environment (hypoxia), the mechanisms involved remain elusive. We observed that mitochondria of certain hypoxic cells take on an enlarged appearance with reorganized cristae. In these cells, we found that a major mitochondrial protein regulating metabolism and apoptosis, the voltage-dependent anion channel 1 (VDAC1), was linked to chemoresistance when in a truncated (VDAC1-ΔC), but active form. The formation of truncated VDAC1, which had a similar channel activity and voltage-dependency as full-length, was hypoxia-inducible factor-1 (HIF-1)-dependent and could be inhibited in the presence of the tetracycline antibiotics doxycycline and minocycline, known inhibitors of metallo-proteases. Its formation was also reversible upon cell reoxygenation and associated with cell survival through binding to the anti-apoptotic protein hexokinase. Hypoxic cells containing VDAC1-ΔC were less sensitive to staurosporine- and etoposide-induced cell death, and silencing of VDAC1-ΔC or treatment with the tetracycline antibiotics restored sensitivity. Clinically, VDAC1-ΔC was detected in tumor tissues of patients with lung adenocarcinomas and was found more frequently in large and late-stage tumors. Together, our findings demonstrate that via induction of VDAC1-ΔC, HIF-1 confers selective protection from apoptosis that allows maintenance of ATP and cell survival in hypoxia. VDAC1-ΔC may also hold promise as a biomarker for tumor progression in chemotherapy-resistant patients.

Precis: Blockade of a mitochondria anion channel may improve lung cancer therapy by restoring apoptotic sensitivity and circumventing chemoresistance in hypoxic tumor cells.

Keywords: Apoptosis; Chemoresistance; Hypoxia-Inducible Factor-1; Mitochondria; Voltage-Dependent Anion Channel.
Introduction

It is well-established that cells exposed to the limiting oxygen microenvironment (hypoxia) of tumors acquire resistance to chemotherapy-induced apoptosis (1). However, the mechanisms involved and the implication of the key factor of the hypoxic response, the hypoxia-inducible factor (HIF), have not been extensively investigated (2). We recently reported that several types of cancer cells exposed to a hypoxic microenvironment showed enlarged mitochondria with reorganized cristae; a result of modifications to fusion/fission (3). In addition, we showed that these cells were resistant to chemotherapy-induced apoptosis.

Since mitochondria regulate both metabolism and apoptosis (4-6) and that fusion/fission participates in apoptosis (7), we investigated if certain mitochondrial proteins implicated in these processes play a role in resistance to apoptosis in a HIF-dependent manner.

The voltage-dependent anion channel (VDAC) regulates mitochondrial import and export of Ca\(^{2+}\) and metabolites including ATP and NADH, and interacts with anti-apoptotic proteins such as Bcl-2 and hexokinase (HK) in controlling the release of Cyto. c (8-11). Of note, screening by RNA interference identified VDAC1 as a protein implicated in resistance to cisplatin-induced cell death (12). In mammals VDAC is present in three highly homologous isoforms: VDAC1, VDAC2, and VDAC3 and VDAC1 is composed of 19 amphipathic beta strands that form a beta barrel and of a mobile N-terminal alpha helix, located inside the pore (13). Through binding to VDAC1 HK, the enzyme that catalyzes the first step of glycolysis is optimally positioned for ATP capture (14) and HK expression is increase by HIF (15). Thus, these interactions influence the function of both HK and VDAC in cell death and metabolism. However, the role of VDAC in metabolism and apoptosis in hypoxia is not known.
Materials and Methods

Cell culture

LS174, PC3, HeLa, 786-O, SKMel and A549 cells were grown in DMEM (Gibco-BRL) supplemented with 5 or 10% inactivated fetal bovine serum as appropriate in penicillin G (50U/ml) and streptomycin sulfate (50µg/ml). Dr. van de Wetering provided LS174 cells expressing the tetracycline repressor. A Bug-Box™ anaerobic workstation (Ruskinn Technology Biotrace International Plc) set at 1% oxygen, 94% nitrogen and 5% carbon dioxide was used for hypoxia.

Transfection of siRNA

The 21-nucleotide RNAs were synthesized (Eurogentec, Seraing, Belgium). siRNA sequences targeting SIMA (siCtl), and HIF-1α were described previously (16). The siRNA sequences targeting human VDAC1, VDAC2, VDAC3 and HKII are given in the supplemental Materials and Methods. Hela cells were transfected with 40nM of siRNA 24h prior to normoxia or hypoxia, as described (3).

Reconstitution of purified VDAC1 and VDAC1-ΔC into a planar lipid bilayer (PLB), single channel current recording and data analysis

VDAC1 and VDAC1-ΔC were purified from hypoxic HeLa cells after solubilizing in LDAO and chromatography on hydroxyapetite, as described (17). Elution with increasing Pi concentrations separated VDAC1 and VDAC1-ΔC. The fractions containing either VDAC1 or VDAC1-ΔC were used for channel reconstitution into a planar lipid bilayer (PLB). A PLB was prepared from soybean asolectin dissolved in n-decane (50mg/ml) and purified VDAC1 or VDAC1-ΔC was added to the cis chamber containing 1M NaCl and 10mM Hepes, pH, 7.4, unless otherwise indicated. After one or a few channels were inserted into the PLB, currents were recorded by voltage-clamping using a Bilayer Clamp BC-525B amplifier (Warner Instrument, Hamden, CT), the current trace duration was 4 or 10sec. Current was measured with respect to the trans side of the membrane (ground). The current was digitized on-line using a Digidata 1200 interface board and PCLAMP 6 software (Axon Instruments, Inc. Union City, CA).
Patients and tissue sample preparation

44 patients who underwent surgery for lung adenocarcinoma between May 2007 and May 2010 at the Pasteur Hospital (Department of Thoracic Surgery, CHU of Nice) were selected. The patients received the necessary information concerning the study and consent was obtained. The study obtained approval of the ethics committee (CHU of Nice). The main clinical and histopathological data are summarized in Supplemental Table 1. Morphological classification of the tumors was assigned according to the WHO criteria (18). The tumors were staged according to the international tumor-node-metastasis system (19). Follow-up data for all the patients were collected regularly. The median follow-up was 21 months (3.8–38.2 months). Among these patients, 13 relapsed (29.5%) and 6 (13.6%) died. Protein and miRNA were extracted from the same tissue sample using the protocol AllPrep® DNA/RNA/Protein from QIAGEN.

Statistics

All values are the means±S.D. of the indicated number of determinations (n) and significant differences are based on the Student’s t test and p values indicated. All categorical data used numbers and percentages. Quantitative data were presented using the median and range or mean. Differences between groups were evaluated using the chi square test for categorical variables and the Student’s t test for continuous variables. SPSS 16.0 statistical software (SPSS Inc., Chicago, Ill) was used. All statistical tests were two-sided, and p values<0.05 indicated statistical significance whereas p values between 0.05 and 0.10 indicated a statistical tendency.

Results

Hypoxic cells with enlarged mitochondria are resistant to chemotherapy and resistance implicates mitochondrial proteins

We reported that certain tumor-derived cell lines exposed to hypoxia showed a tubular mitochondrial network (PC3, SkMel) while others showed enlarged mitochondria (LS174, HeLa, A549) (3). All cells showed a mitochondrial transmembrane potential (ΔΨm) that was unchanged compared to normoxic
cells but the latter group was resistant to staurosporine (STS)-induced apoptosis. We now show that when hypoxic LS174 cells with enlarged mitochondria were treated with STS, the $\Delta \Psi_m$ decreased in normoxia but remained unaffected in hypoxia (Figure 1A). In normoxia LS174 cells released mitochondrial Cyto. c when incubated with STS while hypoxic LS174 cells with enlarged mitochondria did not (Figure 1B). To address the implication of anti-apoptotic proteins of the Bcl-2 family in hypoxic resistance to STS-induced apoptosis, we tested the effect of the BH3 domain mimetic ABT-737, an inhibitor of Bcl-2 and Bcl-X$_L$ (20) on the apoptosis resistance of hypoxic cells. ABT-737 restored apoptosis as induced by STS in hypoxic LS174 cells (Figure 1C), suggesting that association with a BH3 domain protein is implicated in resistance.

To better understand the molecular mechanisms behind resistance we compared the normoxic and hypoxic levels of anti- and pro-apoptotic proteins of the Bcl-2 family (Figure 1D). LS174 and PC3 cells incubated in hypoxia (72h) were resistant or sensitive to STS-induced apoptosis, respectively. Bax and tBID were not or only slightly detected in LS174 cells (Figure 1D) while the expression of Bak and Bcl-X$_L$ were slightly enhanced in LS174 cells. Since Bcl-X$_L$ has been described to interact with VDAC1 (21) we examined the level of VDAC. We observed hypoxic induction of a faster migrating SDS-PAGE form of VDAC in LS174 but not in PC3 cells (Figure 1D). Immunoblots of mitochondrial fractions confirmed mitochondrial origin (Figure S1).

*The hypoxic induction of the formation of a smaller relative molecular mass form of VDAC is dependent on HIF-1 activation*

Since an additional VDAC form was observed in hypoxic resistant cells with enlarged mitochondria, and not in sensitive cells, we focused on the hypoxic induction of this form. As HIF-1 is essential in adaptation to hypoxia, we checked whether HIF-1 was involved in the formation of this form. When HIF-1$\alpha$ was silenced hypoxic cells did not contain the faster migrating form (Figure 2A), but a normal mitochondrial morphology was restored (data not shown). Similar results were obtained for LS174 and A549 cells (data not shown). Thus HIF-1 initiates hypoxia-induced VDAC.
Expression of VDAC isoforms is not induced at the mRNA level by hypoxia and the hypoxia-mediated form of VDAC is a C-terminal truncated VDAC1

We quantified the mRNA expression of VDAC1, VDAC2 and VDAC3 in normoxia and hypoxia but did not observe hypoxic induction of these isoforms (Figure 2B). SiRNA directed to the mRNA of the three isoforms gave knockdown of the corresponding VDAC isoform (Figure 2B). Knockdown was confirmed at the protein level and identified the different isoforms (Figure 2C). The upper band corresponded to VDAC1, the intermediate band to VDAC3 and the lower band to a faster migrating form of VDAC1. The identity of VDAC1 was confirmed with another VDAC1 specific antibody, but directed to the N-terminus, and both forms were silenced with siRNA (Figure 2D).

We considered the possibility that the fast migrating VDAC1 resulted from alternative splicing or hypoxia-mediated translation by internal ribosome entry, but did not find any evidence to support either possibility (Figure S2).

Finally, the faster migrating VDAC1 was not detected with a VDAC1 antibody directed to the C-terminus (Figure 2D), suggesting that the C-terminus of the protein was truncated (VDAC1-ΔC). Doxycycline (Dox.), a second-generation tetracycline that has cytoprotective and metal chelator effects, was found to diminish the formation of VDAC1-ΔC (Figure 2E). Since metal chelators increase the stability of HIF-1α we examined the level of HIF-1α but found only a slight increase when the fast migrating form of VDAC1 was significantly decreased (60%, 60 g/ml doxycycline) (Figure 2E). Minocycline, another tetracycline antibiotic, which exerts uncoupling and inhibiting effects on mitochondrial respiration (22), also inhibited formation (Figure 2F), and partially restored normal mitochondrial morphology (Figure 2G). Since tetracycline is an inhibitor of matrix metalloproteases we tested a number of protease inhibitors, but they did not inhibit formation of VDAC1-ΔC (data not shown). The possibility of post-translational cleavage of VDAC1, as described previously (23), is the most likely explanation for the appearance of VDAC1-ΔC.

VDAC1-ΔC is associated with cell survival in hypoxia
We then hypothesized that VDAC1-ΔC could be involved in hypoxic resistance to apoptosis. Silencing of VDAC1 and VDAC1-ΔC decreased the number of enlarged mitochondria and restored the tubular mitochondrial morphology (Figure 3A). To evaluate the sensitivity to an apoptotic stimulus of normoxic and hypoxic LS174 cells, we determined the caspase 3 and 7 activity in cells exposed to STS, an inducer of mitochondrion-dependent apoptosis, and to etoposide (Etop), a topoisomerase II inhibitor used in cancer therapy (Figure 3B). The caspase activity was the same in cells in normoxia or hypoxia, indicating that there was no induction of cell death in hypoxia. Silencing of VDAC1 in hypoxia partially reestablished the sensitivity of hypoxic LS174 cells to apoptosis (Figure 3B).

Two additional cell lines were examined for: enlarged mitochondria, VDAC1-ΔC and resistance to STS-induced apoptosis. SKMel cells did not show any of these features while A549 cells showed all of them (Figure S3), as for HeLa cells (Figures 2, 3 and S4). We then questioned which form of VDAC1 (full-length or truncated) was responsible for triggering resistance. In hypoxia, the level of VDAC1 decreased by around 50% (Figure 3C) while VDAC1-ΔC increased by around 50%, which supported post-translational cleavage of VDAC1. The silencing of vdac1 in normoxic cells was associated with a change in mitochondrial morphology, as visualized with anti-Cyto. c (Figure 3D) and a decrease in VDAC1 in hypoxia (Figure 3E). However, these cells showed an increase in apoptosis with STS (Figure 3B), suggesting that the decrease in VDAC1 in normoxia was not responsible for the protection against apoptosis. Moreover, silencing of HIF-1α (+Tet) in hypoxia with STS (Figure 2A) restored sensitivity to apoptosis (data not shown). We thus concluded that resistance to apoptosis was associated with VDAC1-ΔC. To evaluate this further, cells were placed in hypoxia and then reoxygenated. The level of VDAC1-ΔC was maintained for 4h after reoxygenation, then progressively decreased after 8h and disappeared at 48h (Figure 3F). As expected, the level of VDAC1 was inversely proportionate to that of VDAC1-ΔC. We showed previously that during the first 24h of reoxygenation, cells were protected from apoptosis (3). Transient exogenous overexpression of a small form of VDAC1 truncated by 5 kDa in the C-terminus, pVDAC1-5kDaΔC (Figure 3G), in cells exposed for 4h to STS showed a slight resistance to apoptosis (Figure 3H). Finally, in the presence of Dox (Figure 2E) or Minocyclin (Figure 2F) hypoxic cells were no longer protected from STS (Figure 3I). Taken together, these results demonstrate that both the enlarged morphology of mitochondria and
VDAC1-ΔC participate in protection against apoptosis in hypoxic LS174, A549 and HeLa cells exposed to STS or Etop.

**VDAC1-ΔC has the same channel activity and voltage-dependency as VDAC1 and binds Bcl-XL**

VDAC1 and VDAC1-ΔC proteins were purified from hypoxic HeLa cells (Figure 4A) and their channel activity was examined following reconstitution into a planar lipid bilayer. The current through lipid bilayer-reconstituted VDAC1 (fraction 12) or VDAC1-ΔC (fraction 22) in response to a voltage step from 0 to -10 or to -40mV (Figure 4B and C) was the same for the two proteins. At -10mV, the channel conductance of both proteins was the same (30pA). At a higher voltage of -40mV, the full-length channel showed two major conducting states with higher occupancy at the closed sub-state (S1), while VDAC1-ΔC showed higher open-state occupancy (O) in comparison to the occupancy of low-conducting sub-states (S1, S2) (Figure 4C). Both channels showed similar but not identical voltage-dependent conductance. At the high voltages, VDAC1-ΔC showed slightly higher conductance than VDAC1 (Figure 4D), in agreement with the single channel experiments (Figure 4BII and 4CII). The voltage sensitivity of VDAC1-ΔC suggests the presence of the N-terminus, conferring voltage gating of the channel (8). VDAC1-ΔC showed similar Ca\(^{2+}\) conductance to VDAC1, but at higher voltages spent a longer time in its open state, as reflected in the decreased voltage sensitivity (Figure 4E). For example, at +40mV the Ca\(^{2+}\) conductance VDAC1-ΔC was about 1.4-fold higher than that of VDAC1 (Figure 4E). VDAC1-ΔC, like VDAC1, interacted with purified Bcl-XL(ΔC) and decreased its channel conductance (Figure 4F). Similar results were obtained with HKII from rat brain (data not shown). These results suggest that the C-terminal domain is not required for the interaction of these anti-apoptotic proteins with VDAC1.

**VDAC1-ΔC forms a complex with hexokinase II and is associated with cell survival in hypoxia**

As HKII is a major player in maintaining the highly malignant state of cancer cells (24), we focused on interaction between HKII and VDACs in hypoxia. Immunoprecipitates with anti-HKII contained VDAC1, VDAC3 and VDAC1-ΔC (Figure 5A). In addition, the HKII expression level was substantially increased in hypoxia (Figure 5B). Silencing of more than 90% of the hypoxia-inducible
expression of HKII decreased considerably the level of VDAC1-ΔC (Figure 5B). Silencing of HKII in normoxia did not affect the level of VDAC1. Conversely, silencing of VDAC1 in normoxia and hypoxia decreased slightly the level of HKII (Figure 5B). These results were confirmed by immunofluorescence; no or little labeling was observed with anti-VDAC in cells silenced for either HKII or VDACs (Figure 5C). In addition, a more intense and punctate immunofluorescent was observed with anti-VDAC in VDAC1-ΔC-containing cells incubated in hypoxia compared to normoxia (Figure S5). Clotrimazole (CTM) and bifonazole (BFN) induce apoptosis by detaching HK from mitochondria (17, 25). Both agents increased mortality to a similar extent to that for VDAC silencing (Figure 5D) and the mortality was enhanced in cells in hypoxia in their presence. This suggested that VDAC1-ΔC interacted with HKII, as did purified HKI, which decreased VDAC1-ΔC channel conductance. To better understand the role of VDAC1 and HKII in cell survival in hypoxia, we silenced VDAC1 or HKII and tested cell proliferation/death and ATP and lactate production in normoxia and hypoxia. Hypoxia does not kill cells (26), but it slows proliferation, as shown by a three-fold decrease in the area of colonies of cells after 10 days in hypoxia (Figure 5E). Transient silencing of VDAC1 (siVDAC1) in hypoxia had no impact on cell survival but affected proliferation (p<0.01), whereas silencing of HKII (siHKII) strongly inhibited survival (Figure 5E). As HKII and VDAC1 form a complex and silencing of HKII decreased the level of VDAC1 in cells (Figure 5B), we hypothesized that HKII interfered with ATP transport and thereby its production via its interaction with VDAC1-ΔC. Cells produced almost two times more ATP in hypoxia (Figure 5F). Silencing of VDAC1 decreased hypoxic but not normoxic production of ATP, suggesting that VDAC1-ΔC influenced ATP production (Figure S6). This could reflect the impact of VDAC1 silencing on HKII expression. As expected the silencing of HKII in hypoxia decreased ATP. Lactate production, which reflects ATP synthesis via glycolysis, was increased in hypoxia and diminished with VDAC1 or HKII silencing (Figure 5G). Taken together, these results confirm that VDAC1 is involved in energy homeostasis and points to VDAC1-ΔC as an essential actor in both glycolysis and mitochondrial energy production in hypoxia probably through interaction with HK.
VDAC1-ΔC is present in tissues of patients with lung adenocarcinoma and is more frequently detected in late-stage rather than in early-stage tumors

Since we detected both VDAC1 and VDAC1-ΔC in hypoxic A549 lung carcinoma cells (Figure S3), we tested for VDAC1 and VDAC1-ΔC in lung adenocarcinomas tumor tissue from 44 patients. Tumor tissues were divided into two groups: stage IA and IB (n=25) and stage IIIA and IIIB (n=19). The clinical characteristics of the patients are listed in supplemental Table 1. The level of VDAC1-ΔC was determined in corresponding control matched healthy (C) and tumor (T) tissue of lung cancer patients (Figure 6A). The tumor tissue, but not healthy tissue contained VDAC1-ΔC and the level of VDAC1-ΔC in stage III was several fold higher than in stage I (see also below). In addition, electron micrographs of mitochondria of tumor and matched normal patients’ tissues showed enlarged mitochondria in only tumor samples (Figure S7).

The expression of CAIX (27, 28) and of miR-210 (29) (Figure 6B), two HIF-induced gene products, was analyzed to confirm the hypoxic status of the tissues (Figure 6A and B). Positivity for CAIX was about 76% and 71% for tumor tissues from stage I and stage III patients, respectively (Figure 6A). Only rare control tissues showed minimal CAIX expression. The quality control of the miRNA in the extracts was confirmed by the level of miR-21. Significant relative expression of miR-210 was observed in tumors (Figure 6B). The band intensity for VDAC1 and VDAC1-ΔC was determined using GeneTools software from Syngene and the ratio of the intensity of VDAC1-ΔC to VDAC1 was evaluated for early-stage I (A and B) and late-stage III (A and B) tumors (Figure 6A and C). The overall ratio for the early- and late-stages showed a significant increase when comparing healthy control and tumor tissue (Figure 6A, C and D). When patient tissues were sub-grouped into tissue showing either a low or high ratio (arbitrary threshold of 0.25) the number of patients showing a high ratio was slightly increased for stage III tumors (11 out of 19 versus 11 out of 25) i.e. 57.8% of stage III tumors were positive compared to 44% of stage I tumors (Figure 6C). The difference between the matched healthy and tumor tissue was substantially higher for both early- and late-stage positive tumors (sub-group high ratio). In particular, VDAC1-ΔC was detected more frequently in larger tumors (41.7% in T1 and T2 tumors versus 75% in T3 and T4 tumors p=0.08, statistical tendency) (Figure 6E–Table 1) and higher lung adenocarcinoma stage - patients not operated on (83.3% stage...
IIIB versus 42.1% in other stages, \( p=0.06 \), statistical tendency) (Figure 6F–Table 1). The level of necrosis of the patients’ tissues was neither high nor substantially different between stages I and III, 6 and 12%, respectively.
Discussion

Herein we demonstrated that hypoxia induces the appearance of a C-terminal truncated form of VDAC1. The mechanism regulating formation was HIF-dependent and the truncated form possessed channel activity, interacted with Bcl-xL and HKI, both of which protect against apoptosis.

Interaction of VDACs with Bcl-2 family members is implicated in translocation of metabolites across the mitochondrial outer membrane (21). Nonetheless, it has been reported that the three isoforms are dispensable for mitochondrial-dependent cell death, but this was demonstrated in a cellular and environmental context that was neither malignant nor hypoxic (30). In addition, VDAC2 but not VDAC1 has been shown to inhibit Bak-mediated mitochondrial apoptosis (31). It is possible to hypothesize that changes in the expression of Bcl-2 proteins are implicated directly in resistance.

Cytoprotection of lung cancer cells to cisplatin correlated with suppression of activation of Bax but not Bak by cisplatin (12). However, in our study the Bax protein was not detected in LS174 cells. In addition, there exists an intricate crosstalk between the machineries of mitochondrial dynamics (fusion and fission), thus morphology, and apoptosis (32, 33). Both anti-apoptotic (Bcl-2) and pro-apoptotic (Bak and Bax) proteins interact with proteins involved in mitochondrial fusion (mitofusins) and fission (dynamin-related GTPases). Thus, we may speculate that modifications in the expression of Bak and Bcl-xL correlate with the morphological alterations observed. In addition, the increases in Bak and Bcl-xL in hypoxic LS174 cells correlated with the morphological alteration in hypoxia. It may also be hypothesized that the increase in the expression of Bcl-XL and the modification of the open configuration of the VDAC1-ΔC channel by Bcl-XL inhibits mitochondrial ATP/ADP exchange, which favors ATP production through glycolysis. A shift toward glycolysis is a characteristic of hypoxic cancer cells and may explain survival and thus resistance when confronted with a potentially lethal agent. The observed change in organization of the cristae of the mitochondria may also rupture the interaction between VDAC and the adenine nucleotide translocase thereby leading to a change in VDAC-mediated ATP transport. Additionally, if VDAC oligomerization is responsible for Cyto c. release in apoptosis (11) a change in its conformation may block Cyto. c in the mitochondrial intermembrane space and thus diminish apoptosis.
The notion that resistance of hypoxic regions of tumors to chemotherapy (34) is associated with hypoxic VDAC1-ΔC and Bcl-XL is supported by reports showing that protection of HepG2 cells against Etop-induced apoptosis was HIF-1α-dependent (35) and that Bcl-XL is induced by HIF (36).

Since we detected VDAC1-ΔC in tumor tissue of patients (50%) and that the frequency of positivity for VDAC1-ΔC was higher in late-stage tumors than in early-stage tumors, we believe that VDAC1-ΔC represents a product of tumor progression. Gene expression of VDAC1 has been reported to predict poor outcome in early stage non-small cell lung cancer (37). In addition, VDAC1 was shown to be up-regulated in prednisolone sensitive acute lymphoblastic leukemia cells but not in resistant cells (38).

In conclusion, our results point to modifications in mitochondrial dynamics and production of VDAC1-ΔC as a survival response in hypoxic cancer cells that resist apoptosis. Since agents that promote apoptosis may hold therapeutic benefit, these results may have important repercussions for combating cancer cell resistance to chemotherapy. A synthetic lethality approach targeting RAS tumor cells identified a small molecule inhibitor of VDAC2 that induced changes in mitochondrial morphology and cell death (39). We propose that VDAC1-ΔC may be a potential biomarker to stratify patients with respect to tumor progression and that the VDAC1-ΔC/HK complex may be a cancer specific target for therapy.
Acknowledgements

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References


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<td>Male</td>
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Table 1. Comparison of the characteristics of the patients and their tumors with VDAC1-ΔC-detection.
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**Figure Legends**

**Figure 1.** Anti- and pro-apoptotic proteins in the blockade of cytochrome c release. A, Flow cytometric analysis of ΔΨ of LS174 cells in normoxia (N) or hypoxia (H) without (-) or with (+) staurosporine (STS); 3 experiments. STS was added for 18 h at 1 µM. B, Immunofluorescence to Cyto. c of LS174 cells in normoxia or hypoxia with STS, bar 7.3 m. The percentage of apoptotic cells is given. C, Caspase activity of cells incubated in normoxia or hypoxia with or without the Bcl-2 and Bcl-Xl inhibitor ABT-737 (10 M) for the last 4 h and without or with STS treatment for the last 4 h (n=8, two experiments). D, Immunoblot of anti- and pro-apoptotic proteins and VDACs in LS174 and PC3 cells in normoxia or hypoxia.

**Figure 2.** Hypoxia induced a HIF-1-dependent novel form of VDAC1. A, Induction in hypoxia of a faster migrating SDS-PAGE form of VDAC is dependent on HIF-1α. Immunoblot of HIF-1α and VDACs in HIF-1α silenced HeLa cells in normoxia or hypoxia in the absence (-) or presence (+) of HIF-1α siRNA. B, Expression of the mRNA of VDAC1-3 in normoxia and hypoxia in HeLa cells. Expression of VDAC1, 2 and 3 after transfection with control (siCtl) or VDAC1 (siVDAC1), VDAC2 (siVDAC2) or VDAC3 (siVDAC3.1 or siVDAC3.2) siRNA, results are representative of two different siRNA for each isoform. C, Immunoblot to HIF-1α and VDAC1 (ab15895) in control (siCtl) or VDAC1 (siVDAC1), VDAC2 (siVDAC2) or VDAC3 (siVDAC3.1 or siVDAC3.2) silenced HeLa cells in hypoxia. Hypoxia-induced fast migrating VDAC1. D, Immunoblot using antibodies against the N- or C-terminus of VDAC1 in HeLa cells incubated in normoxia or hypoxia transfected or not with siRNA. E, Immunoblot of HIF-1α and VDAC1 (ab15895) of LS174 cells incubated in hypoxia. Doxycycline was added for the first 24 h of hypoxia. F, Immunoblot of HIF-1α and VDAC1 (ab15895) of HeLa cells in hypoxia for 48 h. Minocycline was added for the 48 h of hypoxia. G, Immunofluorescence to Cyto. c of HeLa cells in hypoxia without (+DMSO) or with minocycline.

**Figure 3.** Hypoxic induction of VDAC1-ΔC in cells with enlarged mitochondria show protection from stimuli-induced apoptosis. A, Immunofluorescence to Cyto. c in LS174 cells silenced for VDAC1 in...
hypoxia. Arrows indicate fragmented mitochondria; bar 7.3 m. The percentage of enlarged
mitochondria is given. B, Caspase activity of control and VDAC1 silenced LS174 cells (n=8, 2
experiments, * p<0.001) in normoxia or hypoxia without (-) or with (+) staurosporine (STS) or
eptopside (Etop.) for the last 4h. C, Immunoblot of VDAC1 in HeLa cells in normoxia or hypoxia.
Histograms show the quantification of the bands. D, Immunostaining with anti-Cyto. c of normoxic
HeLa cells transfected with siRNA to VDAC1. E, Immunoblots of HIF-1α or VDAC1 of HeLa cells
transfected with increasing concentrations of siVDAC1. F, Immunoblots to HIF-1α or VDAC1 of
HeLa cells incubated first in hypoxia and then reoxygenated. Histograms show quantification of the
signal to VDAC1 and VDAC1-ΔC. G, Expression of pVDAC1-5kDaΔC (0.5 and 5.0µg plasmid) in
normoxia in HeLa cells and endogenous VDAC1-ΔC expression in hypoxia. H, Caspase activity of
HeLa cells, in normoxia, transfected with a control plasmid or a plasmid expressing a 5kDa C-terminal
truncated VDAC1 with or without STS treatment. The percentage of cells with enlarged mitochondria
is given. I, Caspase activity (%) of HeLa cells incubated in normoxia or hypoxia with or without
Doxycycline (Dox) or Minocycline and without or with STS for the last 4h (n=8, two experiments).

**Figure 4.** VDAC1-ΔC channel activity and binding of Bcl-XL-ΔC are identical to that of VDAC1. A,
VDAC1 and VDAC1-ΔC purification from HeLa cells identified by immunoblotting (anti-VDAC1,
Calbiochem); fractions 21 or 22 were used. B-C, Channel activity of bilayer-reconstituted purified
VDAC1 or VDAC1-ΔC. currents through bilayer-reconstituted VDAC1 or VDAC1-ΔC in response to
a voltage step from 0 to -10mV (BI, and CI) or to -40mV (BII and CII,) were recorded. The dashed
lines indicate the zero current level. The total current amplitude histogram traces (in the same
recording), showing the relative occupancy of the open state (O) and closed substate (S) or, for
VDAC1-ΔC, of 2 or more substates (S1 and S2) during a 4sec recording are shown (BI,II and CI,CII).
D, Currents through the VDAC1 (●) or VDAC1-ΔC (○) channels were recorded in the presence of 1M
NaCl and in response to a voltage step from 0mV to voltages between -60 to +60mV. Relative
conductance was determined as the ratio of conductance at a given voltage (G) to the maximal
conductance (Go). The results are representative of 9 similar experiments in which the value of each
voltage represents the average of 3-6 swipes. E, Currents through VDAC1 (●) or VDAC1-ΔC (○) as
recorded in the presence of 0.2M CaCl_2 and in response to a voltage step from 0mV to voltages between –60 to +60mV. The results are the average of 2 similar experiments with 3 swipes for each voltage. F, VDAC channel conductance was recorded before and 10min after the addition of purified Bcl-X_l-ΔC to the cis chamber. A representative experiment of 3 similar experiments is shown.

**Figure 5.** Hypoxic VDAC1-ΔC forms a complex with HKII and cell survival in hypoxia requires VDAC1-ΔC and HKII. A, Coimmunoprecipitation of endogenous VDAC1 with HKII. Lysates of hypoxic LS174 cells were immunoprecipitated with an anti-HKII antibody. VDAC proteins in the lysates and the immunoprecipitates are shown (arrow). Cytochrome c was used as a control. IB, immunoblot; IP, immunoprecipitate. B, Immunoblot of HKII and VDAC in control (siCtl), VDAC (siVDAC1) or HKII (siHKII) transfected HeLa cells in normoxia or hypoxia. Histograms show the intensities normalized to α-tubulin for VDAC1 and VDAC1-ΔC (left panel), and HKII (right panel). C, Immunofluorescence of VDAC1 in HeLa cells silenced for VDAC (siVDAC1) or HKII (siHKII) in hypoxia. Bar 7.3 m. D, Percentage mortality (trypan blue exclusion) of HeLa cells incubated in normoxia or hypoxia. Cell were transfected with or without a siRNA to VDAC1 and incubated or not with clotrimazole (CTM-50µM) or bifonazole (BFN-50µM) for the last hour of normoxia or hypoxia. E, Viability assay of HeLa cells in control (siCtl), VDAC (siVDAC1) or HKII (siHKII) silenced cells in normoxia or hypoxia. Cells were transiently transfected twice with the indicated siRNA (40nM), then seeded, and incubated in normoxia or hypoxia for 10 days before staining. The colony number and area was quantified using ImageJ software. Three experiments with two different sets of siRNA. Student’s t test (*p< 0.01). F, ATP production of HeLa cells silenced (siVDAC1) or not (siCtl) for VDAC1 or HKII (siHKII) (n=8, 3 experiments *p< 0.001) in normoxia or hypoxia. G, Lactate production of HeLa cells silenced (siVDAC1) or not (siCtl) for VDAC1 or HKII (siHKII) (n=2, 3 experiments, *p<0.01) in normoxia or hypoxia.

**Figure 6.** The ratio of VDAC1-ΔC to VDAC1 was higher in tumor tissue compared to control tissue and proportionately more stage III tumors had a high ratio. A, Representative immunoblots of VDAC in tissue extracts of healthy control (C) and tumor (T) tissue from 6 individual lung cancer patients
with either early-stage I or late-stage III tumors. CAIX was an indicator of hypoxia in tumors. B, The fold induction of miR-21 and miR-210 was determined for control and tumor tissues from patients with stage I and III tumors. The level of miR-21 indicated the quality of the miRNA in extracts while miR-210 is a hypoxia-induced miRNA. C, Ratio of the intensity of the immunoblot signal of VDAC1-ΔC to VDAC1 determined with GeneTools software (Syngene) for healthy (C) and tumor (T) tissue from lung cancer patients with either stage I or III tumors. D, Ratio of the immunoblot signal to VDAC1-ΔC to VDAC1 for each individual patient for healthy (C) and tumor (T) tissue. Lung cancer patients with stage I tumors (upper graph) and stage III tumors (lower graph). E, Evaluation of the number of lung cancer patients either negative or positive for VDAC1-ΔC, respectively (VDAC1-ΔC neg.) and (VDAC1-ΔC pos.), with small- (T1 and T2) or large- (T3 and T4) sized tumors. F, Evaluation of the number of lung cancer patients either negative or positive for VDAC1-ΔC, respectively (VDAC1-ΔC neg.) and (VDAC1-ΔC pos.), with stages IA–IB –IIIA or stage IIIB tumors.
Figure 1
Figure 2
Figure 4
Figure 5
Expression of a truncated active form of VDAC1 in lung cancer associates with hypoxic cell survival and correlates with progression to chemotherapy resistance

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