Expression of a Truncated Active Form of VDAC1 in Lung Cancer Associates with Hypoxic Cell Survival and Correlates with Progression to Chemotherapy Resistance

M. Christiane Brahimi-Horn1, Danya Ben-Hail7, Marius Ilie2,3, Pierre Gounon4, Matthieu Rouleau5, Véronique Hofman2,3, Jérôme Doyen1, Bernard Man6, Varda Shoshan-Barmatz7, Paul Hofman2,3, Jacques Pouyssegur5, and Nathalie M. Mazure1

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 metalloproteases. Its formation was also reversible upon cell reoxygenation and associated with cell survival through binding to the antiapoptotic 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 show 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. Cancer Res; 72(8); 1–11. ©2012 AACR.

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.

Because mitochondria regulate both metabolism and apoptosis (4–6) and that fusion/fission participates in apoptosis (7), we investigated whether 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 Ca2+ and metabolites including ATP and NADH and interacts with antiapoptotic proteins such as Bcl-2 and hexokinase in controlling the release of cytochrome 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 3 highly homologous isoforms: VDAC1, VDAC2, and VDAC3, and VDAC1 is composed of 19 amphipathic α-helices that form a β barrel and of a mobile N-terminal α-helix, located inside the pore (13). Through binding to VDAC1 hexokinase, the enzyme that catalyzes the first step of glycolysis is optimally positioned for ATP capture (14) and hexokinase expression is increased by HIF (15). Thus, these interactions influence the function of
both hexokinase 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 Dulbecco’s Modified Eagle’s Medium (Gibco-BRL) supplemented with 5% or 10% inactivated FBS as appropriate in penicillin G (50 U/mL) and streptomycin sulfate (50 μg/mL). Dr. van de Wetering provided LS174 cells expressing the tetracycline repressor. A Bug-Box anaerobic workstation (Rus-kinn Technology Biotrace International Plc.) set at 1% oxygen, 94% nitrogen, and 5% carbon dioxide was used for hypoxia.

Transfection of short interfering RNA

The 21-nucleotide RNAs were synthesized (Eurogentec). siRNA sequences targeting SIMA (siCtl), and HIF-1α were described previously (16). The short interfering RNA (siRNA) sequences targeting human VDAC1, VDAC2, VDAC3, and hexokinase II are given in the Supplementary Materials and Methods. HeLa cells were transfected with 40 nmol/L of siRNA 24 hours before normoxia or hypoxia, as described (3).

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

VDAC1 and VDAC1-ΔC were purified from hypoxic HeLa cells after solubilizing in lauryldimethylamine-oxide (LDAO) and chromatography on hydroxyapatite, 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 (50 mg/mL) and purified VDAC1 or VDAC1-ΔC was added to the cis chamber containing 1 mol/L NaCl and 10 m mol/L HEPES, pH 7.4, unless otherwise indicated. After one or a few channels were inserted into the PLB, currents were recorded by voltage clamping with a Bilayer Clamp BC-525B Amplifier (Warner Instruments), the current trace duration was 4 or 10 seconds. Current was measured with respect to the trans side of the membrane (ground). The current was digitized on-line with a Digidata 1200 interface board and PCLAMP 6 software (Axon Instruments, Inc.).

Patients and tissue sample preparation

Forty-four patients who underwent surgery for lung adenocarcinoma between May 2007 and May 2010 at the Pasteur Hospital (Department of Thoracic Surgery, CHU de Nice, Nice, France) were selected. The patients received the necessary information concerning the study and consent was obtained. The study obtained approval of the ethics committee (CHU de Nice). The main clinical and histopathologic data are summarized in Supplementary Table S1. Morphologic classification of the tumors was assigned according to the World Health Organization (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 (29.3%) 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 ± SD of the indicated number of determinations (n), and significant differences are based on the Student 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 χ² test for categorical variables and the Student t test for continuous variables. SPSS 16.0 statistical software (SPSS Inc.) was used. All statistical tests were 2-sided, and P values less than 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) whereas others showed enlarged mitochondria (LS174, HeLa, A549; ref. 3). All cells showed a mitochondrial transmembrane potential (ΔΨm) that was unchanged compared with 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 ΔΨm decreased in normoxia but remained unaffected in hypoxia (Fig. 1A). In normoxia, LS174 cells released mitochondrial cytochrome c when incubated with STS whereas hypoxic LS174 cells with enlarged mitochondria did not (Fig. 1B). To address the implication of antiapoptotic 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-XL (20) on the apoptosis resistance of hypoxic cells. ABT-737 restored apoptosis as induced by STS in hypoxic LS174 cells (Fig. 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 proapoptotic proteins of the Bcl-2 family (Fig. 1D). LS174 and PC3 cells incubated in hypoxia (72 hours) were resistant or sensitive to STS-induced apoptosis, respectively. Bax and BID were not or only slightly detected in LS174 cells (Fig. 1D) while the expression of Bak and Bcl-Xl was slightly enhanced in LS174 cells. Because Bcl-Xl 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 (Fig. 1D). Immunoblots of mitochondrial fractions confirmed mitochondrial origin (Supplementary Fig. S1).
The hypoxic induction of the formation of a smaller relative molecular mass form of VDAC is dependent on HIF-1 activation

Because 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-1α was silenced hypoxic cells did not contain the faster migrating form (Fig. 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 (Fig. 2B). siRNA directed to the mRNA of the 3 isoforms gave knockdown of the corresponding VDAC isoform (Fig. 2B). Knockdown was confirmed at the protein level and identified the different isoforms (Fig. 2C). The top band corresponded to VDAC1, the intermediate band to VDAC3, and the bottom 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 (Fig. 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 (Supplementary Fig. S2).

Finally, the faster migrating VDAC1 was not detected with a VDAC1 antibody directed to the C-terminus (Fig. 2D), suggesting that the C-terminus of the protein was truncated (VDAC1-ΔC). Doxycycline, a second-generation tetracycline that has cytoprotective and metal chelator effects, was found to diminish the formation of VDAC1-ΔC (Fig. 2E). Because 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 μg/mL doxycycline; Fig. 2E). Minocycline, another tetracycline antibiotic, which exerts uncoupling and inhibiting effects on mitochondrial respiration (22), also inhibited formation (Fig. 2F), and partially restored normal mitochondrial morphology (Fig. 2G). Because 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 posttranslational 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

---

**Figure 2.** Hypoxia induced a HIF-1α-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 2 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 hours of hypoxia. F, immunoblot of HIF-1α and VDAC1 (ab15895) of HeLa cells in hypoxia for 48 hours. Minocycline was added for the 48 hours of hypoxia. G, immunofluorescence to cytochrome c of HeLa cells in hypoxia without (+DMSO) or with minocycline. DMSO, dimethyl sulfoxide.
and restored the tubular mitochondrial morphology (Fig. 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 mitochondrial-dependent apoptosis, and to etoposide, a topoisomerase II inhibitor used in cancer therapy (Fig. 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 (Fig. 3B).

Two additional cell lines were examined for enlarged mitochondria, VDAC1-D and resistance to STS-induced apoptosis. SKMel cells did not show any of these features whereas A549 cells showed all of them (Supplementary Fig. S3), as for HeLa cells (Figs. 2 and 3 and Supplementary Fig. 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% (Fig. 3C) whereas VDAC1-D increased by around 50%, which supported posttranslational cleavage of VDAC1. The silencing of vdac1 in normoxic...
cells was associated with a change in mitochondrial morphology, as visualized with anticytochrome c (Fig. 3D) and a decrease in VDAC1 in hypoxia (Fig. 3E). However, these cells showed an increase in apoptosis with STS (Fig. 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 (Fig. 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 4 hours after reoxygenation, then progressively decreased after 8 hours and disappeared at 48 hours (Fig. 3F). As expected, the level of VDAC1 was inversely proportionate to that of VDAC1−ΔC. We showed previously that during the first 24 hours 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, pVDAC1-5kDaC (Fig. 3G), in cells exposed for 4 hours to STS showed a slight resistance to apoptosis (Fig. 3H). Finally, in the presence of doxycycline (Fig. 2E) or minocyclin (Fig. 2F), hypoxic cells were no longer protected from STS (Fig. 3I). Taken together, these results show 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 etoposide.

**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 (Fig. 4A) and their channel activity was examined following reconstitution into a PLB. The current through lipid bilayer–reconstituted VDAC1 (fraction 12) or VDAC1−ΔC (fraction 22) in response to a voltage step from 0 to 40 or −40 mV (Fig. 4B and C) was the same for the 2 proteins. At −10 mV, the channel conductance of both proteins was the same (30 pA). At a higher voltage of 40 mV, the full-length channel showed 2 major conducting states with higher occupancy at the closed substate (S1), whereas VDAC1−ΔC showed higher open-state occupancy (O) in comparison to the occupancy of low-conducting substates (S1, S2; Fig. 4C). Both channels showed similar but not identical voltage-dependent conductance. At the high voltages, VDAC1−ΔC showed slightly higher conductance than VDAC1 (Fig. 4D), in agreement with the single-channel experiments [Fig. 4B(ii) and C(ii)]. The voltage sensitivity of VDAC1−ΔC suggests the presence of the N-terminus, conferring voltage gating of the channel (8). VDAC1−ΔC showed similar Ca2+-conductance to VDAC1, but at higher voltages spent a longer time in its open state, as reflected in the increased voltage sensitivity (Fig. 4E). For example, at +40 mV the Ca2+-conductance VDAC1−ΔC was about 1.4-fold higher than that of VDAC1 (Fig. 4E). VDAC1−ΔC, like VDAC1, interacted with purified Bcl-XL (ΔC) and decreased its channel conductance (Fig. 4F). Similar results were obtained with hexokinase I 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 hexokinase II is a major player in maintaining the highly malignant state of cancer cells (24), we focused on interaction between hexokinase II and VDACs in hypoxia. Immunoprecipitates with antihexokinase II contained VDAC1, VDAC3, and VDAC1−ΔC (Fig. 5A). In addition, the hexokinase II expression level was substantially increased in hypoxia (Fig. 5B). Silencing of more than 90% of the hypoxia-inducible expression of hexokinase II decreased considerably the level of VDAC1−ΔC (Fig. 5B). Silencing of hexokinase II in normoxia did not affect the level of VDAC1. Conversely, silencing of VDAC1 in normoxia and hypoxia decreased slightly the level of hexokinase II (Fig. 5B). These results were confirmed by immunofluorescence; no or little labeling was observed with anti-VDAC in cells silenced for either hexokinase II or VDACs (Fig. 5C). In addition, a more intense and punctate immunofluorescence was observed with anti-VDAC in VDAC1−ΔC–containing cells incubated in hypoxia than in normoxia (Supplementary Fig. S5). Clotrimazole (CTM) and bifonazole (BFN) induce apoptosis by detaching hexokinase from mitochondria (17, 25). Both agents increased mortality to a similar extent to that for VDAC silencing (Fig. 5D) and the mortality was enhanced in cells in hypoxia in their presence. This suggested that VDAC1−ΔC interacted with hexokinase II, as did purified hexokinase I, which decreased VDAC1−ΔC channel conductance. To better understand the role of VDAC1 and hexokinase II in cell survival in hypoxia, we silenced VDAC1 or hexokinase II 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 3-fold decrease in the area of colonies of cells after 10 days in hypoxia (Fig. 5E). Transient silencing of VDAC1 (siVDAC1) in hypoxia had no impact on cell survival but affected proliferation (P < 0.01), whereas silencing of hexokinase II (siHKII) strongly inhibited survival (Fig. 5E). As hexokinase II and VDAC1 form a complex and silencing of hexokinase II decreased the level of VDAC1 in cells (Fig. 5B), we hypothesized that hexokinase II interfered with ATP transport and thereby its production via its interaction with VDAC1−ΔC. Cells produced almost 2 times more ATP in hypoxia (Fig. 5F). Silencing of VDAC1 decreased hypoxic but not normoxic production of ATP, suggesting that VDAC1−ΔC influenced ATP production (Supplementary Fig. S6). This could reflect the impact of VDAC1 silencing on hexokinase II expression. As expected the silencing of hexokinase II in hypoxia decreased ATP. Lactate production, which reflects ATP synthesis via glycolysis, was increased in hypoxia and diminished with VDAC1 or hexokinase II silencing (Fig. 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 hexokinase.

**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**

Because we detected both VDAC1 and VDAC1−ΔC in hypoxic A549 lung carcinoma cells (Supplementary Fig. S3),
we tested for VDAC1 and VDAC1-ΔC in lung adenocarcinomas tumor tissue from 44 patients. Tumor tissues were divided into 2 groups: stage IA and IB (n = 25) and stage IIIA and IIIB (n = 19). The clinical characteristics of the patients are listed in supplementary Table S1. The level of VDAC1-ΔC was determined in corresponding control matched healthy (C) and tumor (T) tissue of patients with lung cancer (Fig. 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 Figure 4). VDAC1-ΔC Mediates Hypoxic Chemoresistance

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 and 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 −10 mV (Bi(i) and Ci(i)) or to −40 mV (Bi(ii) and Ci(ii)) 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 4-second recording are shown (B and C). D, currents through the VDAC1 (●) or VDAC1-ΔC (○) channels were recorded in the presence of 1 mol/L NaCl and in response to a voltage step from 0 mV to voltages between −60 to +60 mV. Relative conductance was determined as the ratio of conductance at a given voltage (G) to the maximal conductance (G0). The results are representative of 9 similar experiments in which the value of each voltage represents the average of 3 to 6 swipes. E, currents through VDAC1 (●) or VDAC1-ΔC (○) as recorded in the presence of 0.2 mol/L CaCl2 and in response to a voltage step from 0 mV to voltages between −60 to +60 mV. The results are the average of 2 similar experiments with 3 swipes for each voltage. F, VDAC channel conductance was recorded before and 10 minutes after the addition of purified Bcl-XL-ΔC to the cis chamber. A representative experiment of 3 similar experiments is shown.
In addition, electron micrographs of mitochondria of tumor and matched normal patients' tissues showed enlarged mitochondria in only tumor samples (Supplementary Fig. S7).

The expression of carbonic anhydrase IX (CAIX; refs. 27, 28) and of miR-210 (ref. 29; Fig. 6B), 2 HIF-induced gene products, was analyzed to confirm the hypoxic status of the tissues (Fig. 6A and B). Positivity for CAIX was about 76% and 71% for tumor samples.

Figure 5. Hypoxic VDAC1-∆C forms a complex with hexokinase II and cell survival in hypoxia requires VDAC1-∆C and hexokinase II. A, coimmunoprecipitation of endogenous VDAC1 with hexokinase II. Lysates of hypoxic LS174 cells were immunoprecipitated with an antihexokinase II 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 hexokinase II and VDAC in control (siCtl), VDAC (siVDAC1), or hexokinase II (siHKII) transfected HeLa cells in normoxia or hypoxia. Histograms show the intensities normalized to α-tubulin for VDAC1 and VDAC1-∆C (left) and hexokinase II (right). C, immunofluorescence of VDAC1 in HeLa cells silenced for VDAC (siVDAC1) or hexokinase II (siHKII) in hypoxia. Bar, 7.3 μm. D, percentage of mortality (Trypan blue exclusion) of HeLa cells incubated in normoxia or hypoxia. Cells were transfected with or without a siRNA to VDAC1 and incubated or not with clotrimazole (CTM-50 μmol/L) or bifonazole (BFN-50 μmol/L) for the last hour of normoxia or hypoxia. E, viability assay of HeLa cells in control (siCtl), VDAC (siVDAC1), or hexokinase II (siHKII) silenced cells in normoxia or hypoxia. Cells were transiently transfected twice with the indicated siRNA (40 nmol/L), then seeded, and incubated in normoxia or hypoxia for 10 days before staining. The colony number and area was quantified with ImageJ software. Three experiments with 2 different sets of siRNA. The Student t test (*, P < 0.01). F, ATP production of HeLa cells silenced (siVDAC1) or not (siCtl) for VDAC1 or hexokinase II (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 hexokinase II (siHKII; n = 2, 3 experiments; *, P < 0.01) in normoxia or hypoxia. DAPI, 4′, 6-diamidino-2-phenylindole.
tissues from stage I and stage III patients, respectively (Fig. 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 (Fig. 6B). The band intensity for VDAC1 and VDAC1-ΔC was determined with 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 (Fig. 6A and C). The overall ratio for the early- and late-stages showed a significant increase when comparing healthy control and tumor tissue (Fig. 6A, C, and D). When patient tissues were subgrouped 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 of 19 vs. 11 of 25), that is, 57.8% of stage III tumors were positive compared with 44% of stage I tumors (Fig. 6C). The difference between the matched healthy and tumor tissue was substantially higher for both early- and late-stage positive tumors (subgroup high ratio). In particular, VDAC1-ΔC was detected more frequently in larger tumors (41.7% in T1 and T2 tumors vs. 75% in T3 and T4 tumors, $P = 0.08$, statistical tendency; Fig. 6E, Table 1) and higher lung adenocarcinoma stage—patients not operated on (83.3% stage IIB vs. 42.1% in other stages, $P = 0.06$, statistical tendency; Fig. 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 showed 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 Bel-X<sub>L</sub> and hexokinase I, 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 3 isoforms are dispensable for mitochondrial-dependent cell death, but this was shown 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 Bel-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 antiapoptotic (Bcl-2) and proapoptotic (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 Bel-X<sub>L</sub> correlate with the morphologic alterations observed. In addition, the increases in Bak and Bel-X<sub>L</sub> in hypoxic LS174 cells correlated with the morphologic alteration in hypoxia.

It may also be hypothesized that the increase in the expression of Bel-X<sub>L</sub> and the modification of the open configuration of the VDAC1-ΔC channel by Bel-X<sub>L</sub> 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. In addition, if VDAC oligomerization is responsible for cytochrome c release in apoptosis (11), a change in its conformation may block cytochrome 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 Bel-X<sub>L</sub> is supported by reports showing that protection of HepG2 cells against etoposide-induced apoptosis was HIF-1α dependent (35) and that Bel-X<sub>L</sub> is induced by HIF (36).

Because 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 upregulated 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. Because 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/hexokinase complex may be a cancer specific target for therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are thankful to N. Pons for helping to extract RNA/proteins from patients’ tissues and to J. Hickman for giving ABT737.

Grant Support

This research was supported by grants from the LNCC (Equipe labellisée), the ANR, the INCA, METOXIA (FP7-EU program), ARC, and Cancéropole PACA. The laboratory is funded by the Centre Antoine Lacassagne, CNRS, and INSERM. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 5, 2011; revised February 3, 2012; accepted February 15, 2012; published OnlineFirst March 2, 2012.
References

Expression of a Truncated Active Form of VDAC1 in Lung Cancer Associates with Hypoxic Cell Survival and Correlates with Progression to Chemotherapy Resistance

M. Christiane Brahimi-Horn, Danya Ben-Hail, Marius Ilie, et al.

Cancer Res Published OnlineFirst March 2, 2012.