Extracellular acidosis elevates carbonic anhydrase IX in human glioblastoma cells via transcriptional modulation that does not depend on hypoxia

ROBERT IHNATKO1, MIROSLAV KUBES1, MARTINA TAKACOVA1, OLGA SEDLAKOVA1, JAN SEDLAK2, JAROMIR PASTOREK1, JURAJ KOPACEK1 and SILVIA PASTOREKOVA1

1Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dubravská cesta 9, 845 05 Bratislava; 2Cancer Research Institute, Slovak Academy of Sciences, Vlarska 7, 833 91 Bratislava, Slovak Republic

Received April 19, 2006; Accepted June 16, 2006

Abstract. Most solid tumors display extracellular acidosis, which only partially overlaps with hypoxia and induces distinct adaptive changes leading to aggressive phenotype. Although acidosis is mainly attributable to excessive production of lactic acid, it also involves carbonic anhydrase (CA) IX-mediated conversion of CO2 to an extracellular proton and a bicarbonate ion transported to cytoplasm. CA IX is predominantly expressed in tumors with poor prognosis and its transcription and activity are induced by hypoxia. Here we investigated whether low extracellular pH in absence of hypoxia can influence CA IX expression in cell lines derived from glioblastoma, a tumor type particularly linked with acidosis. Our data show that extracellular acidosis increased the level of CA IX protein, mRNA and the activity of minimal CA9 promoter that contains binding sites for HIF-1 and SP-1 transcription factors. Mutation within each of these binding sites reduced the promoter activity, but did not eliminate the increase by acidosis. Transfection of HIF-1α cDNA produced additive inducing effect with acidosis. Normoxic acidosis was accompanied by HIF-1α protein accumulation and transiently increased phosphorylation of ERK1/2. Expression of a dominant-negative mutant of ERK2 reduced the CA9 promoter activity in both standard and acidic conditions. Similar result was obtained by inhibitors of MAPK and PI3K pathways, whose combination completely suppressed CA IX expression and abolished induction by acidosis. Altogether, our results suggest that acidosis increases the CA IX expression via a hypoxia-independent mechanism that operates through modulation of the basic CA9 transcriptional machinery.

Introduction

Extracellular acidosis and hypoxia frequently occur in microenvironment of solid tumors due to poor blood perfusion and/or oncogenic metabolism (1). These physiological stresses induce ample changes in genome, transcriptome and proteome of tumor cells, leading to acquisition of malignant tumor phenotype linked with invasion, metastasis and treatment resistance (2-4). Although there is a strong correlation between mean profiles of pH and partial oxygen pressure (pO2), they show discordant local relationships (5). Thus, acidosis and hypoxia may either act separately or cooperate to alter behavior of tumor cells.

Acidosis stimulates production of growth factors, angiogenic factors and proteases, impairs immune functions, stimulates invasion and motility, modulates responses to drugs and provides selective growth advantage to tumor cells (2). From the physiological point of view, acidosis may result either from inherently oncogenic metabolism with high rate of glycolysis in the presence of oxygen or from anaerobic metabolism induced by hypoxia, in which oxidative phosphorylation is replaced by glycolysis to satisfy energy demands of tumor cells (3,6). Both situations lead to excessive production of lactate and CO2, which cannot be efficiently eliminated from extracellular fluid because of defective vasculature and elevated interstitial pressure in solid tumors (7). Acidic microenvironment then causes transient intracellular acidification, which is incompatible with the cell growth and survival. However, tumor cells actively regulate their cytoplasmic pH to maintain neutral or slightly alkaline values favorable for various intracellular processes, via increased levels and/or activities of pumps, ion transporters and exchangers that extrude protons (1).
In addition to anaerobic glycolysis, hypoxia induces multiple responses including stimulation of angiogenesis and erythropoiesis, reduction of cell proliferation and adhesion, and modulation of cell survival to select for more aggressive tumor cells (4). These effects are mediated by a concerted action of numerous proteins including glucose and lactate transporters (GLUT-1, MCT-4), glycolytic enzymes (PGK, LDH), vascular endothelial growth factor (VEGF) and its receptors (VEGFR), erythropoietin (EPO-1) etc. All of these molecules are transcriptional targets of hypoxia inducible factor (HIF), which binds to hypoxia responsive elements (HRE consensus 5'-RCGTG-3') present in the regulatory regions of their genes. HIF is a heterodimer consisting of a constitutive β subunit and an oxygen-regulated α subunit, whose stability is determined by hydroxylation of two prolyl residues in oxygen-dependent degradation domain and transcriptional activity depends on hydroxylation of asparaginyl residue in C-terminal transactivation domain. In the presence of oxygen, HIF-α is modified by hydroxylases and targeted by pVHL tumor suppressor protein for proteolytic degradation. Under hypoxia, HIF-α remains non-hydroxylated, escapes recognition by pVHL, accumulates, translocates to nucleus and binds to HIF-β to form active HIF, which induces transcription of target genes (8).

In contrast to hypoxia, no master regulator of responses to low extracellular pH has been identified to date. There are only few published studies investigating transcription factors mediating the effects of acidosis. They suggest some role for AP1 and SP1, whose DNA binding capacity is pH-dependent, and show that low pH may induce nuclear sequestration of pVHL and accumulation of HIF-α, thus supporting an involvement of HIF transcription factor (9-11).

Although extracellular acidosis in tumors is mainly attributable to increased accumulation of lactic acid, it also involves hydration of pericellular CO₂ by carbonic anhydrase IX (CA IX), a highly active, tumor-associated transmembrane carbonic anhydrase isoform, which works well even in the microenvironment rich in lactic acid (12-14). CA IX possesses an extracellular catalytic domain, whose performance is determined by a strong HIF-1α-mediated transcriptional activation of CA9 gene, which contains HRE element localized in 8-MG-BA and 42-MG-BA human glioblastoma cell lines derived from glioblastoma multiforme, which is a highly malignant tumor particularly linked with acidosis (23). In addition, it may be caused by microenvironmental factors other than hypoxia, particularly by extracellular acidosis.

In order to distinguish a contribution of low extracellular pH to expression of CA IX, we investigated the effect of normoxic acidosis on the level of CA IX protein and on the CA9 promoter activity in 8-MG-BA and 42-MG-BA human cell lines (24). On the other hand, CA IX is closely associated with a broad distribution of low pO₂ measured by microelectrodes and with distribution of other markers of hypoxia, such as pimonidazole, HIF-1α, GLUT-1 and VEGF. This difference can be explained by the position of HRE element near the transcription start site, which allows for especially tight transcriptional regulation of the CA9 gene, as well as by a high post-translational stability of CA IX protein, which reflects both actual and expired hypoxia (25). In addition, it may be caused by microenvironmental factors other than hypoxia, particularly by extracellular acidosis.

Materials and methods

Cell culture. 8-MG-BA and 42-MG-BA human glioblastoma cell lines (kindly provided by Dr Ivana Macikova and Dr Anna Perzelova, School of Medicine, Comenius University, Bratislava, Slovakia) were grown in MEM supplemented with 10% FCS under standard conditions as described (24). The cells were cultivated in sparse monolayer throughout the study to prevent the density-induced expression of CA IX. To simulate acidosis, MEM medium was supplemented with 5% FCS and adjusted to initial pH value of 5.8 with 1 M HCl immediately before the experiment. Then the medium was added to cells, where it spontaneously achieved pH 6.6 within one hour of cultivation and declined to pH 6.4 by the end of 24-h incubation period. No adjustment was done for control conditions, where the initial medium pH 8.2 equilibrated to pH 7.4 during the cell incubation. To mimic hypoxia, the cells were treated with an iron chelator deferoxamine mesylate (DFO, Sigma, St. Louis, MO) at 100 μM final concentration. Effect of acidosis on cell proliferation was determined by cell counting and [³H]-thymidine incorporation. Extent of cell death was assessed by FACS analysis performed with the Coulter Epics Altra flow cytometer using Annexin-V-FITC staining as described elsewhere (25).

Inhibitors, antibodies and plasmids. The MAPKK inhibitor PD98059 (Sigma) and the PI3K inhibitor LY294002 (Calbiochem, Cambridge, MA) were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored in aliquots at -20°C. Prior to use, the inhibitors were diluted in culture medium to working concentrations of 20 μM PD98059 and 5 μM LY294002. Cultures were pre-incubated with the inhibitors 1 h before the acidic treatment and/or addition of DFO.

The primary antibodies were as follows: M75 anti-human CA IX mouse monoclonal antibody was described earlier (26), anti-phospho ERK1/2 mouse monoclonal antibody and anti-phospho AKT mouse monoclonal antibody.
(Santa Cruz Biotechnology, Inc.), anti-ERK1/2 polyclonal rabbit serum (Sigma), anti-ß-actin mouse monoclonal antibody (Sigma) and anti-HIF-1α mouse monoclonal antibody (Chemicon Int.). Secondary anti-mouse antibodies conjugated with horseradish peroxidase were purchased from Sevapharma (Prague, Czech Republic) and anti-rabbit antibodies conjugated with peroxidase were obtained from Sigma.

Promoter constructs were generated by an insertion of PCR-amplified -50/+37 and -174/+37 CA9 genomic fragments upstream of the firefly luciferase gene in pGL3-Basic luciferase reporter vector (Promega). HRE and SP1 binding sites in -174/+37 construct were mutated by PCR-based in vitro mutagenesis. The resulting mutations were verified by sequencing, pRL-TK renilla vector (Promega) served for the control of transfection efficiency. HIF-1α cDNA in pcDNA1/Neo/ HIF-1α expression plasmid (27) was kindly provided by Professor Patrick Maxwell (Renal Section, Imperial College, London, UK). Dominant-negative mutants of ERK1 (pcDNA-ERK1) and ERK2 (pcDNA-ERK2) mutated in their ATP binding sites were kindly provided by Dr M.H. Cobb from Southwestern Medical Center, Dallas, TX (28).

**Transient transfection and luciferase assay.** The cells were plated into 30-mm Petri dishes to reach approximately 60% monolayer density on the next day. Transfection was performed with 1 μg of promoter-containing luciferase construct and 100 ng of pRL-TK plasmid DNA using a GenePorterII reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer’s recommendation. One day later, the transfected cells were trypsinized and plated in triplicates into 24-well plates. The cells were allowed to attach for 20 h. After pre-treatment with inhibitors for 1 h, the medium was removed and replaced with acidic or standard medium containing 5% FCS and appropriate concentration of inhibitor (where relevant). The cells were incubated for additional 24 h and then the reporter gene expression was assessed using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was normalized against the renilla expression. To analyze the effect of HIF-1α overexpression and ERK1/2 dominant-negative mutants, the promoter constructs (0.75 μg) were co-transfected with 0.75 μg of corresponding cDNAs in expression vectors as described elsewhere (29).

**RT PCR.** Total RNA was extracted from the cells using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Precipitated RNA was dissolved in DEPC-treated water and reverse transcribed with the M-MuLV reverse transcriptase (Finnzymes OY, Finland) using random hexanucleotides as primers. PCR reactions were performed using the primers specified below. Semiquantitative PCR was carried out with EX Taq DNA polymerase (Finnzymes). Following an initial denaturation at 94°C for 3 min, the amplification program was set as follows: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C during 40 sec for a total of 30 cycles, and finally 7 min at 72°C. The amplification products were analyzed on a 2% agarose gel. Quantitative PCR was performed with DyNamoe™ HS kit SYBR® Green qPCR kit (Finnzymes) on Techne Quantica (Duxford, Cambridge, UK) at similar amplification conditions. The primers were as follows (S, sense; A, antisense): CA9 S: 5′-CCGAGCGGACGCGCTTTGA-3′ and CA9 A: 5′-GTCAGTCTCGGCCTACCT-3′ (252 bp product), ß-actin S: 5′-CAACCGCGGAAGATAAGCC-3′ and ß-actin A: 5′-GATCTTCATGAGGTAGTCGT-3′ (236 bp product).

**Immunoblotting.** The cells were plated at different densities (10000-30000 cells/cm²) depending on the purpose of the experiment and incubated for 24 h. Then the cultures were pre-treated with inhibitors for 1 h and exposed to acidosis for 24 h. Parallel control dishes were pre-treated and maintained in the standard conditions for the same time period.

For the detection of CA IX, the cells were rinsed and extracted with the cold extraction buffer composed of 0.3% SDS, 20 mM Tris pH 8.8, Complete Mini protease inhibitors (Roche Applied Science) and 20 μl of the solution containing 0.5 M Tris pH 7.0, 50 mM MgCl₂, 0.032 mM DNase I and 0.036 mM RNase (MP Biomedicals, Inc.). The extracts were centrifuged (15 min at 13000 rpm) and total protein concentrations were determined by BCA assay (Piecz, Rockford, IL). Samples of 40 μg total proteins were separated by the electrophoresis using 10% SDS-PAGE and blotted onto the PVDF membrane (Hybond-P, Amersham). Before immunodetection, the membrane was treated by the blocking buffer containing 5% non-fat milk in PBS with 0.2% Nonidet P-40 for 1 h and incubated for 1 h with M75 MAb diluted 1:2 in the blocking buffer. Then the membrane was washed, incubated for 1 h with the anti-mouse secondary antibody, washed again and developed with the ECL detection system.

To analyze the accumulation of HIF-1α protein, the cells were scraped into extraction buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, supplemented with Complete Mini protease inhibitors), sonicated on ice for 30 sec and centrifuged. Samples containing 100 μg of total proteins were electrophoresed on 8% SDS-PAGE, transferred onto PVDF membrane and detected with anti-HIF-1α antibody diluted 1:500 in blocking buffer using the same procedure as described for CA IX.

Activation of ERK1/2 was detected in cell extracts obtained with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, containing the protease inhibitor cocktail). Samples of 40 μg total proteins were run on 8% SDS-PAGE gel and blotted. Two parallel membranes were treated either with antibodies to phospho-ERK1/2 at 1:500 dilution or with antibodies to total ERK1/2 at 1:40 000. After incubation with the secondary antibodies, the membranes were processed and the signals were visualized as above.

**Results**

**Effect of extracellular acidosis on cell growth and survival.** Glioblastoma multiforme is the most malignant type of brain tumor, which is characterized by hypoxia and acidosis. Recent immunohistochemical analyses of human tissue sections revealed that this tumor type readily expresses carbonic anhydrase IX and that high CA IX level is significantly and independently associated with poor prognosis of brain tumor patients (30). Due to these facts, we have chosen two human glioblastoma cell lines as a suitable model for our present investigation. Both 8-MG-BA and 42-MG-BA cell lines possess...
typical morphological features of glioblastoma multiforme and express corresponding protein markers (24,31).

In the initial experiments, we examined the effect of acidic culture medium adjusted to pH 6.4 on the growth properties and survival of cells in comparison with the cells maintained in medium with pH (pHe) of 7.4 (Fig. 1A). In accordance with the published data obtained with other glioblastoma cell lines (32), proliferation of both 8-MG-BA and 42-MG-BA cells was decreased following their exposure to low extracellular pH for 24 h as determined by cell counting (Fig. 1B). This was confirmed by [3H]-thymidine incorporation, which revealed that the proliferation of acidic cultures was reduced to about 20% of the controls (data not shown). Nevertheless, Annexin-V staining has detected only a negligible increase in the fraction of dead cells (Fig. 1C). As a consequence, the cell cultures subjected to acidosis remained viable, but reached lower density than the control cultures incubated in the standard cultivation medium (Fig. 1D).

Acidosis increases CA IX protein levels in the absence of hypoxia. In spite of the reduced cell proliferation, lowered extracellular pH led to significantly elevated expression of CA IX protein. The CA IX level was higher under more acidic conditions (pH 6.4) than in the moderate acidosis (pH 6.8) and was proportional to initial cell density at plating, with higher expression of CA IX in more dense cells (Fig. 2). Positive relationship to cell density indicated possible involvement of pericellular hypoxia, which is typical for crowded cell cultures and which could augment the effect of acidosis on CA IX expression (21). Therefore, both acidic and control cultures were subjected to continuous stirring in order to ensure thorough cell oxygenation throughout the experiment. Expectedly, stirring led to overall reduction of CA IX levels irrespective of extracellular pH (Fig. 2B). However, the cells maintained in acidic medium (pH 6.4) still revealed considerably higher expression of CA IX protein than the control cells (pH 7.4), suggesting that acidosis induces CA IX independently of pericellular hypoxia and that both factors have additive effects.

CA9 gene responds to acidosis via minimal promoter. As demonstrated on Fig. 3A by RT PCR analysis using mRNAs from glioblastoma cells incubated for 24 h in medium with pH adjusted to 7.4 and 6.4, extracellular acidosis stimulated the transcription of CA9 gene. Both 8-MG-BA and 42-MG-BA cell lines showed remarkably increased levels of the CA9 mRNA when maintained in acidic medium. This fact prompted us to look at the activation of the CA9 promoter. We first compared the effect of extracellular acidosis on activities of two constructs, in which CA9 promoter regions were cloned upstream of the reporter luciferase gene. The longer construct consisted of the CA9 genomic region corresponding to -174/+37 nucleotides with respect to transcription initiation site. This promoter fragment contained HRE element just upstream of the transcription start and five regions (PR1-PR5) protected in DNAse I footprinting (33), see the scheme on Fig. 3. PR1 and PR2 regions bind SP1/3 and AP1 transcription factors important for the basic activation of CA9 transcription (34). HRE plays a crucial role in response of CA9 to hypoxia and density via HIF-1 transcription complex cooperating with
The shorter construct contained minimal CA9 promoter encompassing nucleotides from -50 to +37 and corresponding to the PR1-HRE module. Each promoter-luciferase construct was co-transfected with the renilla coding plasmid to 8-MG-BA glioblastoma cells, which were then maintained for 24 h in acidic medium. Interestingly, both promoter regions exhibited approximately the same increase of the activity in the cells exposed to extracellular acidosis (Fig. 3B). This finding strongly suggested that the -50/+37 promoter region containing PR1-HRE module is both responsible and sufficient for the observed inducing effect of low extracellular pH.

Transcriptional activation of the CA9 gene is principally mediated via two cis-regulatory elements, namely the HIF-binding HRE site and the SP1-binding site within PR1. In order to reveal their possible participation in response to acidosis, we analyzed the CA9 promoter activity using the -174/+37 promoter constructs with mutations introduced separately into each of these two elements. Luciferase activities measured in 8-MG-BA cells transfected with the promoter construct containing the 5'-TCCTCCC-3' to 5'-TCCTAAAC-3' mutation in SP1 site were significantly lower in both acidic and control conditions, but the acidic induction remained preserved to similar extent as observed with the wild-type promoter (Fig. 3C). The other promoter construct with the 5'-TGCAGTA-3' to 5'-TGCTTTTA-3' mutation in HRE behaved analogously as it exhibited generally lowered activity, but the ratio between the luciferase values normalized against renilla at extracellular pH 6.4 versus 7.4 was approximately equal to the ratio obtained with the wild-type promoter (Fig. 3C). These data indicated that both HRE and SP1 sites primarily determine the basic level of the CA9 promoter activation, on top of which then acidosis can apply its modifying effect.
The above assumption was indirectly supported by the next experiment. The -174/+37 CA9 promoter construct was co-transfected to 8-MG-BA cells together with the cDNA coding for HIF-1α subunit of HIF-1 transcription factor and then its activity was analyzed in relationship to extracellular pH. As could be expected, overexpression of HIF-1α led to highly elevated activity of the CA9 promoter in the standard conditions (medium pH 7.4). This elevation was further improved by extracellular acidosis (pH 6.4) so that the ratio between the acidic and control luciferase values was roughly the same in the absence and presence of HIF-1α cDNA (Fig. 3D).

Acidosis induces accumulation of HIF-1α and transient phosphorylation of ERK1/2. HIF-1 plays a crucial role in activation of CA9 transcription by hypoxia and high cell density. However, it has been recently demonstrated that acidosis can cause accumulation of an α subunit of HIF and thereby influence the expression of the HIF-regulated genes (11). Because this phenomenon is dependent on the cell type and on the extent of extracellular acidosis, we wanted to know whether it occurs in the glioblastoma cells at an extracellular pH 6.4 and whether HIF-1α accumulation might be involved in acidic induction of CA IX observed in this study. Therefore, we performed immunoblot analysis to visualize the level of HIF-1α in lysates of 8-MG-BA cells maintained in acidic medium in comparison to control cells grown in the standard conditions. We also followed the levels of CA IX protein in the same samples. Moreover, we included a sample of the control cells treated by a hypoxia-mimicking chemical deferoxamine mesylate (DFO). As shown on Fig. 4A, acidosis induced slow but clearly visible accumulation of HIF-1α over the 24-h incubation period. The resulting level of HIF-1α was similar to the level of HIF-1α obtained by DFO treatment for 4 h. The acidosis-induced elevation of HIF-1α was accompanied by considerably increased level of CA IX protein. However, despite the similar levels of HIF-1α in 24-h acidosis and in 4-h hypoxia, the cells exposed to acidosis for 24 h contained higher amount of CA IX protein suggesting contribution of additional factor(s)/signal(s).

One of the possible players is the MAPK pathway that increases the transactivation capacity of HIF-1α without influencing its level (28,35). Indeed, in our experimental conditions acidosis induced transient activation of MAPK pathway via phosphorylation of ERK1/2, which temporally preceded the accumulation of CA IX (Fig. 4B). This result indicated that MAPK pathway could be involved in acidosis-induced signaling leading to augmented expression of CA IX, but its precise role requires further study.

Simultaneous inhibition of MAPK and PI3K pathways completely blocks CA IX expression and eliminates the effect of acidosis. MAPK pathway has been already implicated in molecular response of tumor cells to acidosis elsewhere (9). In order to better understand potential involvement of MAPK pathway in acidosis-induced expression of CA9 gene, we co-transfected the -174/+37 promoter construct with the cDNAs coding for the dominant-negative (DN) mutants of ERK1 and ERK2. These DN mutants lack the kinase activity and prevent the

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Involvement of signal transduction in the acidosis-induced CA IX expression. (A) Immunoblot analysis of HIF-1α accumulation in 8-MG-BA cells exposed for 24 h to normoxic acidosis. The cells were seeded in multiple parallels, exposed to acidosis and analyzed at indicated time intervals. Expression of HIF-1α and CA IX was detected by immunoblotting on the same membrane (divided to two parts based on the molecular weights of the analyzed proteins) using specific monoclonal antibodies. The cells incubated in the standard medium for 24 h served as a negative control for the effect of acidosis. The cells incubated in the standard medium and treated for 4 h with 100 μM DFO served as a positive control for the effect of hypoxia. (B) Immunoblot analysis of ERK1/2 activation during the exposure to extracellular acidosis. The cells were incubated as described above and parallel samples of extracts were analyzed using phospho-ERK1/2-specific and total-ERK-1/2-specific antibodies. (C) Transcriptional activity of the CA9 promoter following overexpression of dominant-negative mutants of ERK (DN-ERK) and treatment with MAPKK inhibitor PD98059. Transfections, cell incubations, measurements and calculation of results were as in Fig. 2. Treatment of transfected cells with PD98059 was initiated 1 h before and continued throughout the exposure to acidic and standard medium, respectively. (D) Expression of CA IX following inhibition of MAPK pathway (20 μM PD98059), PI3K pathway (5 μM LY294002), or both together. The cells were plated, pre-treated with inhibitors for 1 h, exposed to acidosis and inhibitors for additional 24 h and analyzed by immunoblotting with M75 MAb. The blot was reprobed with β-actin-specific antibody for the control of loading.
obtained with different cell lines in different experimental extracellular pH 6.7. Also Kaluz sarcoma cells and FaDu pharyngeal carcinoma cells at to achieve CA IX induction in the normoxic HT 1080 fibro-

signal transduction that activates basal transcription of These data indicate that the effect of acidosis requires ongoing leading to complete block of CA IX expression (Fig. 4D). However, the induction by acidosis was finally lost upon simultaneous inhibition of MAPK and PI3K pathways (Fig. 4D). Nevertheless, this reduction by acidosis was finally lost upon simultaneous inhibition of MAPK and PI3K pathways leading to complete block of CA IX expression (Fig. 4D). These data indicate that the effect of acidosis requires ongoing signal transduction that activates basal transcription of CA9.

Discussion

In this study, we investigated the effect of extracellular acidosis on expression of carbonic anhydrase IX. This was important from several reasons: a) acidosis is a hallmark of most solid tumors with direct impact on their phenotype and response to anticaner treatment; b) acidosis partly overlaps and cooperates with hypoxia in tumor progression; c) it induces adaptive responses of tumor cells to maintain neutral or slightly alkaline intracellular pH via activation of ion transport mechanisms that increase proton extrusion; c) carbonic anhydrase IX contributes to extracellular acidosis under hypoxia; d) CA IX is involved in ion transport machinery as a component of bicarbonate transport metabolon; e) CA IX is expressed in many solid tumors, but not in the corresponding normal tissues and its expression is strongly induced by hypoxia; f) intratumoral distribution of CA IX extends beyond the hypoxic regions detected by other markers of hypoxia. All these facts evoked a question, whether the expression of CA IX is induced by acidosis in absence of hypoxia. Positive answer to this question could nominate CA IX among the candidate mediators of adaptive responses of tumor cells to low extracellular pH and support its role in tumor progression.

Herein we showed that this is true for the glioblastoma multiforme-derived tumor cell lines 8-MG-BA and 42-MG-BA, which exhibited significantly increased CA IX levels with decreasing extracellular pH under normoxic conditions. This increase was clearly observed even though the acidic cultures achieved considerably lower density than the control cells and their basic, density-regulated expression was thus reduced. Similar effect was demonstrated by Willam et al (36) in HeLa cervical carcinoma cells and Hep3B hepatoma cells at pH 7.0 in both normocapnic-low bicarbonate and hypercapnic acidosis, whereas Vordermark et al (37) failed to achieve CA IX induction in the normoxic HT 1080 fibrosarcoma cells and FaDu pharyngeal carcinoma cells at extracellular pH 6.7. Also Kaluz et al (21) could not see any increase in CA IX level in highly dense HeLa cells in the normoxic acidosis at pH 6.6. These inconsistent data obtained with different cell lines in different experimental conditions indicate that extracellular acidosis may have cell-specific consequences. Nevertheless, the available evidence obviously supports the general idea that acidosis can induce the expression of CA IX in tumor cells independently of hypoxia.

The above conclusion is not surprising, since acidosis influences also some other hypoxia-regulated genes, including VEGF (38). However, acidosis and hypoxia work separately to stimulate expression of VEGF and have no additive effect when applied simultaneously (9). In the case of CA IX, both pericellular hypoxia in dense culture and overexpression of HIF-1α from the transfected DNA resulted in increased expression level, which was further augmented by acidosis. This finding was compatible with the earlier observations that CA9 mRNA levels induced by low oxygen or chemical hypoxia were further elevated by acidosis in HeLa and Hep3B cells (36). It is well possible that CA IX, which efficiently catalyzes conversion of CO2 to bicarbonate and proton, and which is believed to deliver bicarbonate to transmembrane transporters in order to buffer cytoplasmic pH, is increasingly needed for the improved protection of tumor cells exposed to combined hypoxic and acidic stress. Irrespective of the functional involvement, the additive inducing effect of hypoxia and acidosis provides further explanation for the intratumoral distribution of CA IX, which incompletely overlaps with distribution of VEGF and other hypoxic markers and which has been so far mainly attributed to high posttranslational stability of CA IX protein (23,39).

In an attempt to better understand a mechanism through which acidosis operates to induce CA IX expression, we analyzed the transcriptional activation of CA9 promoter. The CA9 promoter is characterized by HRE element localized between the downstream transcription start site and the upstream SP1 binding site. Due to this particular position, both HIF-1 and SP1 transcription factors are critically encompassed within the basal transcriptional machinery and elimination or mutation of their binding sites significantly decreases the density- and hypoxia-induced transcription of CA9 gene (20-22). Based on our data, the -50/+37 core promoter region containing intact HRE and SP1 sites appears sufficient to mediate the inducing effect of extracellular acidosis under normoxia. However, it remains unclear how this could be accomplished, since mutation of each of these sites leads to generally reduced CA9 expression, but does not abolish the effect of acidosis. One possible explanation is offered by Torigoe et al (10) who showed that low pH significantly enhances the DNA binding activity of SP1 in vitro and proposed that this could be a part of an adaptive response to transient decrease of intracellular pH due to hypoxia and acidosis in tumor microenvironment. Although the experimental evidence remains to be obtained, it seems quite plausible that the basal CA9 transcription might be induced by acidosis via tighter SP1 binding to CA9 promoter. Alternatively or concurrently, acidosis-induced stabilization of HIF-1α subunit of HIF transcription factor could be involved as recently described by Mekhall et al (11). They demonstrated that normoxic acidosis can neutralize the function of pVHL by triggering its nuclear sequestration, which enables HIFs to evade destruction in the presence of oxygen and activate its target genes. In accordance with this idea, we did find
accumulation of HIF-1α protein in 8-MG-BA glioblastoma cells exposed to acidosis and this increase was accompanied with an increase of the CA IX protein level.

In contrast to CA9 gene, neither SP1 nor HIF, but rather AP1 transcription factor has been implicated in acidosis-stimulated expression of VEGF gene (9). The mechanism involved an improved binding of AP1 to cis-element located rather distally from the VEGF transcription start site at -937/-931 position downstream of the HRE element. However, any important role of AP1 transcription factor in acidosis-mediated effect on CA9 promoter in glioblastoma cells can be declined, since AP1 binding site is located out of the -50/-37 region, which showed approximately equal induction as the larger -174/+37 region that includes AP1 site.

Despite the differences in the composition of VEGF and CA9 promoters, particularly with respect to positions of the binding sites for HIF, SP1 and AP1 transcription factors and their utilization in transcriptional responses to microenvironmental stresses, regulation of both genes was shown to involve signaling from the MAP kinase pathway. Both Xu et al (9) and we showed transient ERK activation in glioblastoma cells exposed to extracellular acidosis and found reduced VEGF/CA9 promoter activities following MAPK pathway inhibition. Based on these findings, Xu et al (9) concluded that acidic extracellular pH induces VEGF transcription via ERK1/2 MAPK signaling pathway. This conclusion seemed tempting also for CA9. However, when we analyzed the effect of MAPK pathway inhibition either by the chemical compound PD98059 or by expression of the dominant-negative mutant of ERK2, we observed reduced CA9 expression also in the absence of acidosis, in the cells cultivated under standard conditions. This negative control, which was missing in the VEGF-study (9), revealed that the inducing effect of low extracellular pH was preserved also upon inhibition of MAPK signaling. The same deduction could be raised from the experiment, in which we inhibited PI3 kinase pathway by LY294002 inhibitor. These results are fully consistent with the previous investigations showing that MAPK and PI3 kinase pathways concomitantly mediate the signaling to HIF and SP1 and via these transcription factors influence the expression level of the CA9 gene under both basal and hypoxia/density-induced conditions (21,29). Similarly as shown before in HeLa cells, simultaneous inhibition of both pathways in glioblastoma cells resulted in complete block of basal CA9 expression under standard conditions. This block could not be overcome by acidosis, which failed to show an inducing effect. Altogether, the data presented here reinforce the proposal that the response of CA9 gene to acidosis is accomplished through an adverse mechanism, which does not operate on its own. It rather appears to modulate the transcriptional machinery assembled at the minimal CA9 promoter that involves HRE and SP1 sites and is activated by MAPK and/or PI3K signal transduction. Nonetheless, precise acidosis-induced modifications that occur at the CA9 promoter require further detailed study.

It also remains to be determined, how acidosis is sensed to modulate the activity of MAPK and/or PI3K pathways which ultimately lead to increased CA9 expression. Based on recent knowledge, it is quite plausible that ion transporters and other components of metabolons, which are functionally involved in adaptive responses of tumor cells to extracellular acidosis, can sense a transiently increased concentration of intracellular protons and transmit this signal via their cytoplasmic domains to signal transduction pathways, which then trigger their own expression and/or activation. This would represent a meaningful feedback mechanism facilitating the expression/activation of ion transport machinery in stressful conditions. Indeed, activity of several ion transporters is regulated by signal transduction-induced phosphorylation of their cytoplasmic domains in response to changes in pH and vice versa, activated ion transporters transmit this phosphorylation signal to the signal transduction pathways, including ERK (40). Interestingly, analogous signal transduction feedback has been recently described for CA IX whose intracellular tail can be phosphorylated following EGF stimulation and this phosphorylation leads to activation of PI3K-Akt pathway in renal carcinoma cells (41). Future studies will show whether this phenomenon is involved in CA IX regulation by microenvironmental stresses.

Acknowledgements

This work was supported by the grants from EU (EUROXY project LSHC-CT-2003-502932), from the Slovak Government (Cancer Genomics SP 51/0280800) and from the Slovak Scientific Grant Agency (VEGA-2/5107/05).

References


