Role of Carbonic Anhydrase IX in Human Tumor Cell Growth, Survival, and Invasion

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ABSTRACT

Carbonic anhydrase IX (CAIX) is a membrane-associated carbonic anhydrase (CA), strongly induced by hypoxia. CAIX is overexpressed in a variety of tumor types and associated with increased metastasis and poor prognosis. An inhibitor of CAs, acetazolamide has been reported to inhibit invasion. We used RNA interference (RNAi) to examine the function of CAIX in MDA468 and MDA231 breast carcinoma cells, which express high levels of CAIX under hypoxia. Hypoxia-induced CA activity was completely blocked by specific RNAi (P < 0.01). RNAi-treated cells showed growth delay in dense monolayer culture and a 50% reduction in clonogenic survival under hypoxia. In the MDA468 cells, there was no effect of RNAi treatment on invasion. In a cell line that did not induce CAIX under hypoxia, RT112, we found no effect on the ability of cells transfected with CAIX to invade or migrate. Thus, CAIX plays an important role in the growth and survival of tumor cells under normoxia and hypoxia, making it a potential target for cancer therapy, but is not involved in invasion.

INTRODUCTION

Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide (H₂O + CO₂ → H⁺ + HCO₃⁻) and as such are vital to many biological and physical functions. Carbonic anhydrase IX (CAIX) is one of four transmembrane isozymes and has been implicated in the control of cell proliferation and cellular transformation (1). Increased expression of CAIX has been shown in a wide spectrum of tumor types compared with normal tissues. CAIX expression is cell density dependent, and we have shown that it is strongly induced by hypoxia (2). There is significant overlap between CAIX expression and regions of hypoxia in solid tumors, suggesting a role for this enzyme in the adaptation of tumor cells to hypoxic conditions and in tumor cell progression (2–4).

Several clinical studies show a clear relationship between high CAIX levels in tumors and a poor prognosis (5, 6). CAIX contains an NH₂-terminal proteoglycan region that distinguishes it from other CA isoenzymes. Cells have been shown to adhere to purified CAIX protein via the proteoglycan domain, and this can be blocked by the monoclonal M75 antibody; therefore, a role in cell adhesion also is possible (7). However, whether CAIX expression is simply a marker of hypoxia or a mechanism of adaptation has not been determined, and the cellular function of CAIX remains unclear.

We have examined the cellular function of CAIX in human breast carcinoma cell lines using the technique of RNA interference (RNAi; ref. 8) to specifically block hypoxia-inducible CAIX. We also have transfected a plasmid containing the cDNA sequence for human CAIX into a bladder carcinoma cell line expressing negligible constitutive CAIX protein to further examine its role in migration and invasion. Therefore, the overall aims of this study were to specifically inhibit CAIX protein expression induced by hypoxia to examine its role in the adaptation of tumor cells to hypoxia and to assess its contribution to tumor cell migration and invasion.

MATERIALS AND METHODS

Cell Culture. Breast adenocarcinoma cell lines MDA468 (ATCC HTB-131) and MDA231 and RT112 bladder carcinoma cells were obtained from the Cancer Research United Kingdom Cell Services (London, UK) and maintained in exponential growth phase in DMEM supplemented with 10% FCS and glutamine. Normoxic incubations (CO₂ water-jacketed incubator; Thermo Electron, Waltham, MA) were carried out at 37°C in 5% CO₂ and 21% oxygen. Hypoxic incubations were carried out at 37°C in 95% humidity, 5% CO₂, and 0.1% oxygen in nitrogen-flushed hypoxic chambers (Heto Cellhouse 170 HI; RS Biotech, Irvine, United Kingdom).

Gene Silencing by RNA Interference. Our target sequence was selected from the ORF region of the human CAIX cDNA sequence according to manufacturer’s recommendations (Cruachem Limited, Glasgow, United Kingdom) and submitted to a Basic Local Alignment Search Tool search (National Center for Biotechnology Information database) to ensure targeting of a single gene. Two oligonucleotides consisting of ribonucleosides with 2′-deoxyribonucleosides (dTdT) at the 3′ ends, 5′-GAGGAUUCACCAAGAGG dTdT-3′ and 5′-CCUCUCGGGGUGAACUCdCdTdT-3′, were synthesized and annealed to form duplexes. The resulting duplexes were transfected into MDA468 and MDA231 breast carcinoma cells using oligofectamine reagent (Invitrogen, Carlsbad, CA) in serum-free OptiMEM (Invitrogen) for 4 hours. The cells were allowed to recover overnight in DMEM with serum and glutamine before further treatments. Reduction in CAIX protein expression, measured by Western blot analysis, was used to indicate gene silencing. Inverted control RNAi oligonucleotides (target sequence in reverse) were synthesized and annealed to examine the specificity of the RNAi effect.

Stable Transfection of RT112 Cells. The cDNA sequence of human CAIX (a gift from Dr. J. Pastorek, Department of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovak Republic; ref. 1) was inserted between the NorI and KpnI sites of the pcDNA3.1 vector (Invitrogen). RT112 cells were seeded onto 10-cm dishes and transfected at ~70% confluence using FuGENE 6 Transfection Reagent (FuGENE; Roche Diagnostics, Basel, Switzerland) according to manufacturer’s instructions. Eighteen microliters of FuGENE reagent were diluted in serum-free medium and combined with 6 μg of DNA (either empty vector or CAIX-containing plasmid). The medium on the cells was replaced with 6 mL medium containing 10% FCS, and the FuGENE-DNA mix then was added dropwise. The cells were incubated under normal conditions for 24 hours, and the medium then was replaced with medium containing G418 (Life Technologies, Rockville, MD) at 2 mg/mL to select for transfected clones. After 48 hours, cells were trypsinized, counted, and plated onto 10-cm dishes at varying cell densities. These cells then were grown in the presence of G418 until individual colonies were apparent. Single clones were isolated using a pipette tip under sterile conditions and transferred to a 96-well plate and then maintained under selection until sufficient cell numbers allowed for extraction of protein and confirmation of CAIX protein expression by Western blot analysis.

Western Blot Analysis for CAIX Protein. Cells were lysed in 8 mol/L urea lysis buffer, and homogenized lysates were standardized for protein concentration using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Proteins were separated on 7.5% SDS polyacrylamide gels and transferred onto Immobilon membranes (Millipore, Bedford, MA) using a semidyblotter Imm-2 (WEP Company, Las Vegas, NV). Membranes were blotted with the primary M75 mouse monoclonal antibody to CAIX at 1:500 (a gift from Dr. J. Pastorek; ref. 1). Horseradish peroxidase-conjugated goat antimouse antibody (Dako, Glostrup, Denmark) was applied at 1:1,000, and chemiluminescence was detected with enhanced chemiluminescence Western blot kit (Ami-
CARBONIC ANHYDRASE IX AND HYPOXIA SURVIVAL

Reduction of CAIX Protein Expression in Breast Tumor Cells Using RNA Interference. Varying concentrations of RNAi duplex, from 0.1 nmol/L to 200 nmol/L, were transfected into MDA468 and MDA231 cells; the cells were exposed to 16 hours of hypoxia (0.1% O_2) on the following day (24 hours later); and protein then was extracted. Control cells were subjected to mock transfection conditions and exposed to 16 hours of normoxia or hypoxia in parallel. CAIX protein was constitutively expressed at a low level in control MDA468 cells under normoxia, but this expression was greatly increased under hypoxia (Fig. 1A). RNAi treatment significantly reduced protein expression in a dose-dependent manner, with CAIX protein reduced to normoxic levels at 200 nmol/L duplex concentration. Maximum reduction was achieved at duplex concentrations of ≥10 nmol/L; therefore, 10 nmol/L duplex was used for all of the subsequent experiments in this cell line. Double transfections (RNAi duplexes transfected into the cells on two consecutive days) also were used to prolong the RNAi effect because of the relatively long half-life of the CAIX protein (10). CAIX protein expression also was significantly reduced in MDA231 cells (Fig. 1B) in a dose-dependent manner. CAIX protein was not expressed under normoxia in this cell line, and all of the hypoxic expression was completely abolished after

**RESULTS**

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**Growth Curves.** RNAi-treated cells and controls were trypsinized and resuspended in medium and then counted using a hemocytometer. A total of 1 × 10^5 cells were seeded into each well of several 24-well plates in a total of 1 mL medium. A total of 5 × 10^5 cells were seeded for analysis of cell growth at high density. At various time points after seeding, cells in two or three individual wells were trypsinized and resuspended in medium and then counted using a hemocytometer to determine total cell numbers. Growth curves were repeated at least three times.

**Clonogenic Assays.** RNAi-treated cells and controls were trypsinized and resuspended in medium and then counted using a hemocytometer. Eight hundred cells were plated out onto 10-cm dishes, in triplicate, and then allowed to attach for 2 hours. The dishes were incubated for 48 hours under normoxia or hypoxia, and all of the dishes then were returned to normoxia for 14 to 21 days to allow for the formation of colonies. Colonies (>50 cells) then were stained with crystal violet and counted by eye. Clonogenic assays were repeated at least three times in triplicate.

**Carbonic Anhydrase Activity Assay.** RNAi-treated cells and controls were homogenized on ice in H&E buffer (20 mmol/L HEPES, 1.5 mmol/L EDTA, and protease inhibitors) and centrifuged at 3000 rpm at 2°C for 10 minutes.

The supernatant then was ultracentrifuged at 37,500 rpm (100,000 × g) for 44 minutes at 4°C, and the resulting pellet was resuspended in cold Tris-buffered saline buffer for the assay. Membrane-related CA activity was measured using the method described by Wilbur and Anderson (9). This is an electrometric reaction in which the time required (seconds) for a saturated CO_2 solution to lower the pH of 0.02 mol/L Tris HCl buffer from 8.3 to 6.3 at 0°C is determined. Time without enzyme = T_0 and time with enzyme = T. Activity units/mg protein = 2 × (T_0 − T)/T × mg protein in sample.

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RNAi treatment at duplex concentrations of ≥100 nmol/L. Control and active duplexes (10 nmol/L) were transfected into MDA468 cells on two consecutive days; the cells were exposed to 16 hours of normoxia or hypoxia; and protein was extracted (Fig. 1C). RNAi-treated cells showed ~50% reduction in CAIX protein expression compared with mock-transfected controls under normoxia, and hypoxic induction was blocked to that of basal normoxic levels. In contrast, control inverted RNAi duplex had no effect on CAIX protein expression compared with the active RNAi.

**RNAi Reduces Membrane CA Activity in MDA468 Cells.**
MDA468 cells were transfected with 10 nmol/L RNAi duplex on two consecutive days and then placed in normoxia or hypoxia for 16 hours on the following day. Membrane extracts were prepared from the cells and used to measure membrane-related CA activity (Fig. 2). In the control cells, CA activity increased ~50% under hypoxia, as would be expected from the large induction in protein expression shown under these conditions (see Fig. 1C). After RNAi treatment, basal CA activity was reduced by 50% under normoxia, and basal levels under hypoxia were not significantly different from normoxia. Specific RNAi inhibited all of the increase in activity under hypoxia (P < 0.01). Because RNAi-treated cells had the same basal level of CA activity remaining under normoxia and hypoxia, this shows that all of the increase in activity under hypoxia is caused by CAIX as opposed to any other membrane-bound CA.

**RNAi Results in Growth Delay of MDA468 and MDA231 Cells.**
RNAi-treated cells and inverted controls were seeded into 24-well plates in equal numbers following double RNAi transfection of 10 nmol/L duplex, and the total cell numbers in individual wells were determined at various time points afterward (Fig. 3A and B). Each point represents the mean of three individual wells counted. In MDA468 (Fig. 3A), the RNAi-treated cells showed a 3-day growth delay in monolayer culture compared with inverted controls. For MDA231 cells (Fig. 3B) the growth delay was less (1 or 2 days), but the RNAi-treated cells failed to reach the same degree of confluence as the inverted control cells. This result is not surprising because RNAi treatment using the same duplex concentration had a lesser effect on CAIX protein levels in MDA231 cells compared with MDA468 cells (Fig. 1B). The experiment was repeated in MDA468 cells using a higher initial cell density (5 × 10^4) to more closely examine the maximum cell densities achieved (Fig. 3C). Reduction in CAIX protein expression by RNAi had a significant effect on the total cell numbers present at confluence in this experiment (~50% reduction in the RNAi-treated cells compared with mock-transfected controls).

**CAIX Involved in Survival of MDA468 Cells under Normoxia and Hypoxia.** A clonogenic survival assay was used to assess the impact of CAIX protein expression on longer-term survival of MDA468 cells after exposure to normoxia or hypoxia for 48 hours.

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**Fig. 3. Effect of RNAi on growth and survival of breast tumor cells.** RNAi-treated cells and inverted control-treated cells were seeded into 24-well plates in equal numbers following double RNAi transfection, and the total cell numbers in individual wells were determined at various time points afterward for MDA468 (A) and MDA231 (B). The experiments were repeated at least three times, and the data shown are from representative experiments for each cell line. Each point represents the mean of three individual wells with SD. Comparison of means on individual days using Student’s t test (P < 0.05). C: The experiment was repeated in MDA468 cells using a higher initial cell density (5 × 10^4) to more closely examine the effect on maximum cell densities achieved. Each point represents the mean of three individual wells with SD. Comparison of means on individual days using Student’s t test (P < 0.05). D. A clonogenic survival assay was used to assess the impact of CAIX protein expression on longer-term survival of MDA468 cells after exposure to normoxia or hypoxia for 48 hours. The experiment was repeated at least three times, and the data shown are from a representative experiment. Each point represents the mean of triplicate samples with SD.
The survival of control cells appeared to be reduced after hypoxic exposure alone, although the difference was not significant. There was a significant reduction in clonogenic growth after hypoxia compared with controls. However, RNAi treatment significantly reduced survival further, by \(\sim 50\%\) \((P < 0.05)\) under hypoxia, suggesting that CAIX may be functionally involved in the survival of cells within the tumor microenvironment.

**Invasion and Migration of MDA468 Cells after RNAi.** To examine the possible involvement of CAIX in tumor progression, the capacity of the MDA468 cells for invasion or migration after RNAi treatment was assessed using a modified Bowden chamber assay with a Fluoroblok membrane. The Fluoroblok membrane acts to absorb \(>99\%\) of visible light within the 400 to 700 nmol/L range; therefore, only cells that have migrated or invaded across the membrane will be recorded fully, giving a quantitative estimate of cell numbers in real time. There was a significant increase in fluorescence during the first 12 hours for the control and RNAi-treated cells (Fig. 4A). From 12 to 48 hours, the fluorescence generated by the RNAi-treated cells increased slightly above that seen for the control cells; however, this difference only became significant after 48 hours. For the invasion assay, the Fluoroblok membrane was coated with Matrigel (Fig. 4B). As with the migration assay, the fluorescence increased sharply during the first 12 hours but then leveled off for the control and RNAi-treated cells. The invasion capacity of the RNAi-treated cells appeared to be slightly reduced compared with the control, but this difference was not significant.

**Expression of CAIX in RT112 Cells: Effect on Migration and Invasion.** Although blocking CAIX up-regulation had no effect on migration, we also examined the effect of up-regulating expression in a cell line with no detectable CAIX. RT112 cells were stably transfected with pcDNA3.1 plasmid containing cDNA sequence for human CAIX and with pcDNA3.1 plasmid alone (empty vector controls). Individual clones were expanded under selection, and CAIX protein expression was determined by Western blot analysis. Fig. 5A shows CAIX protein expression in two empty vector control clones (EV/2 and EV/3) and two CAIX-positive clones (CA9/2 and CA9/8). Exponentially growing cells were exposed to 16 hours of normoxia or hypoxia (0.1\% O\(_2\)), and protein then was extracted. EV/2 and EV/3 did not express CAIX, whereas CA9/2 and CA9/8 showed high expression under normoxia and hypoxia. The capacity of the four clonal cell lines for migration (Fig. 5B) and invasion (Fig. 5C) was determined. There was no overall difference in the ability of the CAIX-positive cells to migrate compared with the empty vector controls. The invasion rate of CA9/8 cells was significantly greater \((P < 0.05)\) than the two empty vector controls at 24, 36, and 48 hours after the start of the experiment. However, the invasion rate of CA9/2 cells was lower than that shown by the EV/2 cells despite expressing similar levels of CAIX protein to the CA9/8 cells (Fig. 5A). Therefore, no clear relationship between the expression of CAIX protein in these cells and their ability to migrate or invade was evident.

**Effect of Acetazolamide on Invasion or Migration.** Inhibition of total CA activity in one of the CAIX-expressing clones by acetazolamide \((100 \mu\text{mol/L})\) did not result in any significant alterations in the capacity of the cells for migration or invasion (Fig. 6).

**DISCUSSION**

In this study, the expression of CAIX protein has been specifically reduced in human breast tumor cell lines using the technique of RNAi. Reduced membrane-related CA activity and significant effects on growth and survival under normoxia and hypoxia accompanied the decrease in protein expression, emphasizing the important role of CAIX in tumor cell survival. A plasmid containing the cDNA sequence for CAIX also has been transfected into bladder carcinoma cells, resulting in high expression of CAIX protein; however, this had no clear effect on the capability of these cells for migration or invasion.

CAIX previously has been suggested to play a role in tumor cell invasion \((11)\); however, in our study there was no clear evidence to support this. Previous evidence came simply from the effect of high concentrations of acetazolamide on invasion, whereas our study has concentrated on one particular CA that is highly up-regulated in malignancy and examined the effect of decreasing and increasing its expression. Hypoxia or hypoxia-inducible factor (HIF)-1α overexpression has been shown to increase Matrigel invasion in a human colon carcinoma cell line, and small interfering RNA directed toward HIF-1α inhibited this process \((12)\). In that study, HIF-1α was shown to regulate the expression of a number of proteins known to play established roles in the pathophysiology of invasion, including cathepsin D, matrix metalloproteinase-2, urokinase plasminogen activator receptor, fibronectin 1, keratins 14, 18, and 19, vimentin, transforming growth factor α, and autocrine motility factor.

However, it was not clear which HIF-1 targets were most important for the mechanism. CAIX is tightly controlled by HIF-1. Our results suggest that under normoxia CAIX alone did not act to influence
tumor cell invasion; however, in the tumor microenvironment it may be acting within a complex interplay of proteins, many of which will be influenced by tumor hypoxia. There also is evidence to suggest that CAIX further reduces cell-cell adhesion that is already diminished under hypoxic conditions (13).

CAIX reduces E-cadherin–mediated cell adhesion via interaction with B-catenin (14). E-cadherin is a key adhesion molecule whose loss or destabilization is linked to tumor invasion. Coexpression with cell adhesion molecules, as well as disruption of their function, may contribute to the aggressiveness of CAIX-expressing tumors. In a study of non–small-cell lung cancer, Giatromanolaki et al. (6) showed a significant association between CAIX and up-regulation of epidermal growth factor receptor, c-erbB-2, and MUC1, proteins that have been implicated in the invasion, migration, and metastasis of cancer. A significant association was found between CAIX positivity and c-erbB-2 overexpression in malignant breast tissue (14). Nevertheless, our experiments failed to show a convincing relationship between CAIX expression and migration or invasion potential.

In this study we found that CAIX is important for the growth and survival of tumor cells under normoxia and hypoxia. It is likely that hypoxia-inducible CAIX exerts biological effects via its influence on microenvironmental pH. It previously has been suggested that the extracellular CAs could convert CO₂ diffusing from oxygenated areas to carbonic acid, thereby promoting the generation of bicarbonate and hydrogen ions (15, 16). CAs have an important role in hydrogen ion transport (17) and therefore could help eliminate the acid load generated by hypoxia through glycolysis. Measurement of intracellular pH transients by Seminaphthorhodafluor (SNARF) fluorescence in the RNAi-treated cells and the RT112 transfectants revealed altered transmembrane CO₂ and proton fluxes, suggesting that CAIX does contribute to the maintenance of intracellular pH. ¹

Acidic pH is a major contributor to cell death under hypoxia (18), and hypoxia will occur in dense cultures and in colonies of cells. Extracellular acidosis and intracellular alkalosis also have been postulated to increase tumor growth (19); therefore, modification by

¹ R. Vaughan-Jones, unpublished data.
CARBONIC ANHYDRASE IX AND HYPOXIA SURVIVAL

CAIX could be a factor in tumor survival. CAIX previously has been reported to be up-regulated under confluence and the promoter shown to be regulated by microenvironmental factors under such conditions (20). Our results show the potential physiologic effects of this modulation under confluence and induced hypoxia.

The role we have shown here for CAIX makes it an attractive target for cancer therapy. CA inhibitors previously have been shown to elicit synergistic effects when used in combination with other chemotherapeutic agents in animal models (21). Small molecule inhibitors of CAIX (aromatic and heterocyclic sulfonamides), which do not enter therapeutic agents in animal models (21). Small molecule inhibitors of CAIX could be a factor in tumor survival. CAIX previously has been reported to be up-regulated under confluence and induced hypoxia.

REFERENCES

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