Carbonic Anhydrase IX, an Endogenous Hypoxia Marker, Expression in Head and Neck Squamous Cell Carcinoma and its Relationship to Hypoxia, Necrosis, and Microvessel Density

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ABSTRACT

Carbonic anhydrase IX (CA IX) is a transmembrane glycoprotein with an active extracellular enzyme site. We have shown previously that it was hypoxia inducible and may therefore be an endogenous marker of hypoxia. It is overexpressed in some tumors, particularly renal cell carcinoma. The aim of this study was to examine the expression and localization of CA IX in head and neck squamous cell carcinoma (HNSCC) and relate this to the location of tumor microvessels, angiogenesis, necrosis, and stage. Expression of CA IX was determined by immunoblotting in three HNSCC cell lines grown in normoxia and hypoxia (pO2 0.1%) and three paired tumor and normal tissue samples of HNSCC. Archived paraffin sections (79) of HNSCC were immunostained with antibodies to CA IX and CD34 to determine microvessel density (MVD). By double staining sections with CA IX and CD34, the distance between blood vessels and the start of CA IX expression and necrosis was calculated. CA IX was induced by hypoxia in all three HNSCC cell lines and overexpressed in HNSCC tumor tissue. Overexpression was localized to the perinecrotic area of the tumor on immunostaining, and the percentage area of the tumor expressing CA IX was significantly higher with more tumor necrosis ($P = 0.001$), a high MVD ($P = 0.02$), and advanced stage ($P = 0.033$) on univariate analysis and necrosis ($P = 0.0003$) and MVD ($P = 0.0019$) on multivariate analysis. The median distance between a blood vessel and the start of CA IX expression was 80 μm (range, 40–140 μm). CA IX is overexpressed in HNSCC because of hypoxia and is a potential biomarker for hypoxia in this tumor. Overexpression may help to maintain the intracellular pH, giving tumor cells a survival advantage and enhancing resistance to radiotherapy and chemotherapy. CA IX is a potential target for future therapy in HNSCC.

INTRODUCTION

Carbonic anhydrases are encoded by three independent gene families: α-CA, β-CA, and γ-CA. CA9 is one of the α-CA isoenzymes. Carbonic anhydrases catalyze the reversible conversion of carbon dioxide to carbonic acid and are involved in respiration, calcification, acid-base balance, and the formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid. The different carbonic anhydrases have different tissue distribution, subcellular localization, biological function, kinetic properties, and susceptibility to inhibitors (1).

CA9 is a novel member of the carbonic anhydrase family which codes for a transmembrane glycoprotein that possesses an extracellular catalytic domain with weak enzymatic activity. It has homology also to basic helix-loop-helix domain proteins. Transfection into murine fibroblast NIH mouse fibroblast cells promotes proliferation, and it may be involved in control of cell proliferation and oncogenesis (1, 2).

There is abundant expression of CA IX protein in normal human upper GI mucosa and GI-associated structures, such as pancreas, gallbladder, and liver (3–5). Expression is most prominent on the basolateral surfaces of the crypt enterocytes in the duodenum and jejunum. This suggests that it might serve as a ligand or a receptor for another protein that regulates intercellular communication or cell proliferation (6). Interestingly, expression is lost in gastric adenocarcinoma (5). Normal human heart, lung, kidney, prostate, peripheral blood, brain, placenta, and muscle do not express CA IX (3).

CA IX overexpression has been identified in a number of solid tumors, including renal carcinoma and particularly clear cell adenocarcinoma (7–10), cervical squamous carcinoma (11, 12), ovarian carcinoma (13), colorectal carcinoma (14), esophageal carcinoma (15), bladder carcinoma (16), and non-small cell lung carcinoma (17). In some epithelial tissues, expression has been observed in areas of severe dysplasia, e.g., cervix (12), but in most, no expression is present until malignant invasion occurs, where it is often an early indicator of malignancy, e.g., lung carcinoma (17). There appears to be an inverse correlation between CA IX expression and stage and grade in some tumors (7, 11, 14, 15), and low expression of CA IX has been correlated with poor prognostic factors, such as lymph node metastases and depth of invasion (11, 14, 15).

Clear cell renal carcinoma where CA IX expression is particularly high is almost always associated with mutation of the VHL tumor suppressor gene and loss of function of pVHL (18). CA IX is also overexpressed in mutant VHL renal cell carcinoma cell lines. This overexpression is reversed by transfection of the wt VHL gene back into the cell (19).

After the recent description of VHL regulation of HIF-1α (20), we investigated the hypoxic regulation of CA IX and have shown it is inducible by hypoxia. A HIF binding site in the 5′ promoter region of CA9 was found, and we showed that a hypoxia response element and HIF-1α were essential for CA9 transcription under hypoxia.

HNSCC is known to be a particularly hypoxic tumor with the degree of hypoxia having a significant impact on its response to radiotherapy chemotherapy and prognosis (22–25). The aim of this study was to examine the induction of CA IX by hypoxia in HNSCC cells lines and to analyze its expression and localization in HNSCC. Expression has been examined in relation to MVD as a measure of angiogenesis and necrosis as an indicator of the effects of hypoxia. Additionally, the distance of CA IX expression from blood vessels was analyzed as a surrogate assessment of the relation to hypoxia.

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Received 1/16/01; accepted 4/24/01.

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2 The abbreviations used are: CA9, carbonic anhydrase 9 (gene); CA IX, carbonic anhydrase IX (protein); HIF-1α, hypoxia inducible factor 1 α; HNSCC, head and neck squamous cell carcinoma; MVD, microvessel density; TBS, Tris-buffered saline; VHL, von Hippel Lindau; MoAb, monoclonal antibody; wt, wild type; pH[E], intracellular pH; pH[E], extracellular pH; GL, gastrointestinal.
**MATERIALS AND METHODS**

**Cell Lines.** Human HNSCC lines UM-SCC22A, UM-SCC22B (courtesy of Dr. T. Carey, University of Michigan; Ref. 26), and SCC-25 (American Type Culture Collection) were maintained in DMEM with 10% heat-inactivated FCS and 2 mM fresh glutamine. Cells were exposed to normoxia or hypoxia (94.9% N2, 5% CO2, and 0.1% O2) for 16 h. Cells were harvested on ice and homogenized in lysis buffer (8 mM urea, 10% glycerol, 1 mg/ml G418 (G418 was removed from the medium 24 h before collection of samples). Cells were harvested on ice and prepared in 8 M urea lysis buffer as above. Whole cell preparations for immunostaining were harvested and fixed in 10% neutral buffered formalin overnight. Cell pellets were embedded in paraffin and sectioned onto silanized glass slides.

**Fresh Tissue Samples.** Three paired tumor and normal tissue samples from primary HNSCC were snap frozen in liquid nitrogen. Tissue samples were frozen sectioned and stained with H&E to ensure that material contained normal or tumor tissue as appropriate. Samples were sectioned on ice and homogenized in 8 mM urea lysis buffer as above.

**CA IX Immunoblotting.** Cell and tissue extracts were protein quantified using the Bio-Rad detergent-compatible protein assay (Bio-Rad, UK) to ensure even protein loading between lanes. Samples were diluted in phosphate buffered saline to give 50 μg of protein/μl. RCC4 and RCC4/WHL extracts were used as positive and negative controls, respectively. Proteins were resolved in 8 M urea lysis buffer as above.

**CA IX Immunohistochemistry.** Human HNSCC lines UM-SCC22A, UM-SCC22B (courtesy of Dr. T. Carey, University of Michigan; Ref. 26), and SCC-25 (American Type Culture Collection) were maintained in DMEM, 10% heat-inactivated FCS, 2 mM fresh glutamine. Cells were maintained in DMEM, 10% heat-inactivated FCS, 2 mM fresh glutamine, and 1 mg/ml G418 (G418 was removed from the medium 24 h before collection of samples). Cells were harvested on ice and prepared in 8 M urea lysis buffer as above. Whole cell preparations for immunostaining were harvested and fixed in 10% neutral buffered formalin overnight. Cell pellets were embedded in paraffin and sectioned onto silanized glass slides.

** Archived Tumor Specimens.** Previously untreated patients (79) with HNSCC presented to the Oxford Center for Head and Neck Oncology between 1995 and 1999 were studied (Table 1). All had surgery as their first line of management, with some receiving postoperative radiotherapy. Specimens of complete resections rather than biopsies were selected so that both normal and tumor tissue were present on each slide. Seven samples of lymph node metastases from patients in this series were also selected.

**H&E-stained sections of all of the specimens were examined at low (×40) and medium (×100) power by two observers (N. B. and P. W.). Tumors were graded as well, moderate or poorly differentiated. The margin of tumor invasion into surrounding normal tissue was identified as either pushing or infiltrating. A pushing margin is recognized when there is a defined border between tumor and stroma; an infiltrating margin when there is no clearly defined border to the tumor and invasion occurs in thin filaments (28). The whole tumor area was examined, and the degree of necrosis was divided into three categories: 0 < 5%, 5% < 25%, and >25%.**

**Immunostaining.** Sections (4 μm) of formalin-fixed, paraffin-embedded tissue were cut onto silanized glass slides. They were cleared of paraffin in Citroclear (HD Supplies, UK) and rehydrated through graded alcohol baths. After a rinse in tap water, they were placed in 3% hydrogen peroxide for 15 min.

**CA IX.** Slides were blocked in 10% normal human serum for 15 min, then incubated with 1:50 MoAb M75, as described (27), at a dilution of 1:50 in assay diluent. Two representative slides demonstrating overexpression of CA IX and areas of necrosis were selected. CA IX immunostaining was carried out as above using the Horse Radish Peroxidase Envision System (Dako) without counterstaining. Slides were then washed for 15 min in TBS, and immunostaining for CD34 was carried out as above. Slides were counterstained and mounted as above.

The distance from blood vessels marked with CD34 to the start of CA IX expression and necrosis was assessed using an eyepiece graticule calibrated against a gradient slide. Measurements were taken from both tumors in three different areas carefully selected to represent cross sections of a tumor cord, avoiding oval or longitudinal sections. Measurements were done in three different directions from a single vessel.

**Statistics.** Correlation between the level of CA IX expression in sections of primary tumor and nodal metastases was examined using Spearman’s rank correlation. The difference in CA IX expression with age (two categories at the median age), MVD (two categories at the highest third), percentage of tumor necrosis (three categories: 0–5%, 5–25%, and >25%), sex, T stage (T 1–4), N stage (N0 and N+), grade of tumor, and margin of invasion was compared using a Mann-Whitney U test or Kruskall Wallis test as appropriate. The difference in necrosis scores compared. When CA IX expression was divided at the median into two categories of high expression (>20%) and low expression (<20%), necrosis into two categories present (>5%) or absent (<5%), and T stage into early (T 1) and advanced (T 3) All statistics were done using SPSS software v9.0.
RESULTS

Expression of CA IX on Western Blotting in HNSCC Cell Lines and Tissue Samples. Because VHL mutation constitutively up-regulates HIF-1α, controls for CA IX expression were extracts from the renal cell line RCC4 with VHL mutation. They showed marked up-regulation of CA IX in normoxia, in contrast to the control RCC4/VHL cell line transfected with wt VHL, where CA IX was minimal in normoxia. It appeared as two bands related to glycosylation. CA IX was up-regulated in all three head and neck cell lines exposed to hypoxia (0.1% O₂ for 16 h) with little or no expression in normoxia (Fig. 1a). CA IX expression was clearly up-regulated in tumor samples compared with paired normal tissue taken at operation from patients with HNSCC (Fig. 1b).

Localization of CA IX Expression in HNSCC. CA IX was over-expressed in 71 of 79 HNSCC tumor sections examined; expression was confined to the perinecrotic region of these tumors (Fig. 2a). It was absent or very low in the normal epithelium overlying tumor tissue (Fig. 2b). Expression was confined to the cell membrane (Fig. 2c). In the eight tumor sections where no CA IX expression was seen, there was little or no tumor necrosis observed (0% necrosis in four cases, 5% necrosis in two cases, 20% necrosis in two cases). The level of expression was similar in both the primary and lymph node metastases from the same patient in all seven cases, although no significant correlation could be demonstrated because of the small numbers (primary median CA IX 50% (range, 5–60%), secondary median CA IX 35% (range, 20–80%), n = 7, P = 0.093, Spearman’s correlation).

Distance between Blood Vessel and Necrosis and CA IX Expression. The median distance from a blood vessel to the start of necrosis was 130 μm (range, 80–200 μm; n = 18), and the median distance between a blood vessel and the start of CA IX expression was 80 μm (range, 40–140 μm; n = 18), as shown in Fig. 2f. Using a formula published by Tomlinson and Gray (31), which makes assumptions about oxygen diffusion and consumption on the basis of experiments done on squamous cell carcinoma of the lung, the partial pressure of oxygen at a given distance from the nearest vessel can be

Fig. 1. RCC4/VHL (wt VHL), negative control; RCC4 (empty vector), positive control. a, expression of CA IX in HNSCC cell lines (UM-SCC22A, UM-SCC22B, and SCC-25) on Western blotting in normoxia and hypoxia. N, normoxia; H, hypoxia (pO₂ 0.1%). b, expression of CA IX in paired tumor and normal tissue from 3 patients with HNSCC. T, tumor tissue; N, paired normal tissue.

Fig. 2. Expression of CA IX on immunostaining in HNSCC. a, perinecrotic expression of CA IX (×100). b, overlying normal squamous epithelium (×100). c, membrane expression of CA IX (×1000). Expression of CA IX is shown in paired primary (d) and lymph node metastases (e, ×100). f, range of distances between a blood vessel labeled with CD34 MoAb and the start of CA IX expression and necrosis (×250).
calculated (Fig. 3). In the HNSCC sections examined here, the median distance from a blood vessel to the start of CA IX expression of 80 μm (range, 40–140 μm) gives a tissue pO_2 of 1% (range, 0–2.8%). These results are only approximate because of the shrinkage of tissue on formalin fixation, the variation in sectioning of the tumor, and the assumptions made about oxygen diffusion and consumption.

**Difference in CA IX Expression with MVD, Necrosis Score, and T Stage.** CA IX expression was higher in tumors with a MVD in the top third of the range (P = 0.02, Mann-Whitney; Table 1), as shown in Fig. 4. CA IX expression was also higher as the percentage of tumor necrosis increased (P = 0.001, Kruskall Wallis; Table 1), as shown in Fig. 5. There was a significantly higher level of CA IX expression in more advanced tumors (T_2–4) compared with T_1 tumors (P = 0.033, Kruskall Wallis; Table 1). There was no significant difference in CA IX expression between categories of age, sex, N-stage, grade, or margin of invasion (Table 1). There was no significant difference in percentage of tumor necrosis with increasing T stage (P = 0.37, Kruskall Wallis, data not shown) or high and low MVD (P = 0.47, Mann-Whitney, data not shown).

When the difference in CA IX expression between high and low groups of MVD, categories of necrosis, and T stage was examined using multivariate analysis, percentage of tumor necrosis (P = 0.0003; odds ratio, 10.0) and MVD (P = 0.0019; odds ratio, 7.3) remained significant factors associated with CA IX expression. The effect of T stage on CA IX expression was no longer seen.

**DISCUSSION**

Hypoxic regions are common within solid tumors because of disorderly vasculature, shunting of blood, and oxygen consumption out of balance with oxygen supply as rapid growth outstrips the blood supply (32–35). Tumor hypoxia has been shown to be important in resistance to radiotherapy and chemotherapy (25, 36–38) and has a significant effect on disease free and overall survival in HNSCC (22, 23, 37).

This study demonstrates that CA IX is overexpressed in HNSCC. It was clearly induced by hypoxia in cell lines in vitro, and on immunostaining expression, it was clearly localized to the perinecrotic regions of the tumor, which are known to be hypoxic. CA IX expression was seen at a median distance from blood vessels of 80 μm correlating with a calculated pO_2 in the range of 1% at the edge nearest the blood vessels. At the edge nearest the area of necrosis, the pO_2 would be ~0.1%, which in vitro also gave strong induction of CA IX. There was a significant increase in CA IX expression as tumor necrosis increased. These observations suggest that CA IX is regulated by hypoxia in vivo. There was variability between tumors in distance from blood vessels to necrosis, which may reflect the susceptibility of the tumor cells to hypoxia-induced death, the heterogeneity of oxygen distribution within the tumor (39), or O_2 consumption by the tumor contributing to the final effect of necrosis. However, these variations also appear to affect CA IX expression concomitantly. CA IX expression was only seen in the cell membrane on immunostaining. It is a transmembrane glycoprotein that makes CA IX a potentially useful indicator of tissue hypoxia, as the protein cannot diffuse away from its point of origin. This is in contrast to vascular endothelial growth factor, which does not correlate with biomarkers of hypoxia, such as Pimonidazole (40). Although secreted proteins may be useful peripheral blood markers of hypoxia (41), CA IX is induced at the same oxygen tension at which HIF-1α and its downstream
target genes are induced and provides a measure of the percentage of the tumor population that is hypoxic (42).

The correlation between CA IX and MVD is likely to be attributable to the overexpression of CA IX at the same oxygen tension as hypoxia-induced proangiogenic cytokines, such as vascular endothelial growth factor. The lack of correlation between MVD and necrosis may be because necrosis indicates both severe hypoxia, metabolic O$_2$ consumption by the tumor, and the ability of a cell to withstand hypoxia rather than being a simple measure of hypoxia, although one study in breast carcinoma did find a correlation between MVD and necrosis (43).

Tumor cells can undergo apoptosis in hypoxia, and therefore, there is strong selection for pathways to escape this fate, e.g., p53 mutation (44). Selection of these cells in the hypoxic microenvironment is an important mechanism for malignant progression (45). Hypoxic apoptosis is substantially mediated by an acidic pHi, which occurs because of the switch from aerobic to anaerobic metabolism in hypoxia, in vitro studies (46). In that study, buffering the pH changes in hypoxia either by changing the medium or more concentrated buffers allowed cell survival under hypoxia. Thus, induction of genes able to regulate extracellular or pH may help hypoxic cells survive. Several mechanisms exist at a cellular level to generate an acidic pH and maintain the pHi. It has been proposed that increased activity of the mitogen-sensitive Na/ClH-exchanger, increased function or expression of H+ pumping ATPases, or an interaction between the tumor cell and its immediate environment may maintain the pH at a normal level while lowering the pH. Carbonic anhydrases have been suggested as one mechanism whereby the cell could maintain a difference in pH across its membrane (19, 47, 48).

Carbonic anhydrases catalyze the reversible conversion of H$_2$O and CO$_2$ to carbonic acid. During aerobic metabolism, CO$_2$ is generated. This moves out of the cell down a diffusion gradient as the extracellular CO$_2$ is maintained at a lower level by conversion to carbonic acid. During aerobic metabolism, CO$_2$ is generated. This moves out of the cell down a diffusion gradient as the extracellular CO$_2$ is maintained at a lower level by conversion to carbonic acid. The bicarbonate ions are pumped back into the cell in exchange for chloride ions while the H+ ions remain in the extracellular environment and lower the pH. The induction by hypoxia of CA IX with its active extracellular enzyme site could theoretically help to lower the extracellular CO$_2$ and maintain the pHi at a normal level, preventing apoptosis and giving the cell a major survival advantage. A reduction in the pH has advantages to tumor cells as it helps in the breakdown of the extracellular matrix, migration and invasion of tumor cells, induction of expression of growth factors, and reduction of the viability of nearby cells (49). This potential ability of hypoxia-induced CA IX to affect the pH and pHi in vitro and in vivo is currently under investigation. The pHi of a tumor is one of the most significant factors in mathematical models of tumor survival. (50).

Chronic lowering of the pH by inhibitors of the Na+/H$^+$ exchanger or Na$^+$-dependent HCO$_3^-$/Cl$^-$ exchanger is directly cytotoxic to tumor cells and inhibits tumor growth (48).

The pH of a tumor may significantly alter the uptake of chemotherapy drugs, particularly if they are weak electrolytes (51). Chlorambucil and 5FU, both weak acids, have increased toxicity and are retained in tumors when there is a low pH (52, 53). Doxorubicin and mitoxantrone, both weak bases, have reduced intracellular accumulation with low pH (54, 55). Doxorubicin toxicity has been enhanced in an animal model by raising the pH with bicarbonate in the drinking water (49).

Carbonic anhydrase inhibitors have been shown to inhibit tumor cell invasion in vitro (47), and in xenograft experiments, carbonic anhydrase inhibitors as part of a chemotheraphy regimen enhanced the effect of chemotherapy drugs and helped delay tumor growth (56).

Thus, our demonstration of up-regulation of CA IX in vivo in a perinecrotic pattern suggests this may be an important pathway in hypoxia, possibly regulating pHe to allow survival of a viable rim of cells under hypoxic conditions. This subpopulation of cells may be a suitable target for inhibitors of carbonic anhydrase. Use of CA IX as a target for radioimmunotherapy with MoAbs or use of CA IX to convert a pro-drug to an active drug may have potential problems because of its abundant expression normal human GI mucosa and GI-associated structures.

CA IX expression correlates with the oxygen diffusion distance and is expressed in a perinecrotic manner: this may be a marker for hypoxia in HNSCC. It is induced by hypoxia in HNSCC cells and is up-regulated in HNSCC. Up-regulation correlates with tumor necrosis and MVD. Overexpression may help to maintain the pHi, give tumor cells a survival advantage, and enhance resistance to radiotherapy and chemotherapy. CA IX provides a potential target for future therapy.

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