Carbonic Anhydrase IX Expression and Tumor Oxygenation Status Do Not Correlate at the Microregional Level in Locally Advanced Cancers of the Uterine Cervix
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Abstract

Purpose: Carbonic anhydride IX (CA IX) can be induced by hypoxia in vitro and shows an immunohistochemical expression pattern that is predominantly found in perinecrotic tumor areas and correlates with exogenous markers of hypoxia, such as pimonidazole. CA IX might therefore serve as an endogenous marker of tumor hypoxia, although comparisons of CA IX immunostaining with direct oxygenation measurements using pO2 microsensors have thus far yielded contradictory results.

Experimental Design: Because tumor heterogeneity may be among the factors responsible for the discrepancy between the two methods, CA IX expression in tissue samples originating from oxygen microelectrode tracks of locally advanced cervical cancers was assessed in this study. Seventy-seven biopsy specimens were analyzed immunohistochemically using an anti–CA IX rabbit polyclonal antibody and semiquantitative scoring.

Results: CA IX expression showed no correlation with the oxygenation variables median pO2 and hypoxic fraction 2.5, 5, or 10. Cases with higher International Federation of Gynecology and Obstetrics stages (IIb–IVa) exhibited stronger expression of CA IX (P = 0.035) and CA IX expression tended to be more prevalent in node-positive patients (P = 0.051).

Conclusions: These data indicate that CA IX cannot be recommended as a substitute for oxygen microelectrode measurements. That the expression of CA IX does not correlate with the oxygenation status may be due to the degree to which other factors, such as nutrient (e.g., glucose) deficiency or the action of oncogenic mutations, can modulate the in vivo expression of this protein, rendering a strict association with tumor hypoxia too unreliable for clinical use.

Microenvironmental hypoxia is a characteristic of solid malignant tumors that is associated with disease progression and resistance to therapy (1, 2). Of the various methods available for the detection of hypoxia in human tumors, direct oxygenation measurement using microelectrodes is still considered to be the "gold standard" in the clinical setting. Despite the proven validity (3), reproducibility (4), and prognostic relevance (5–7) of this method, major drawbacks are its invasiveness and applicability only to accessible tumors. Endogenous markers of hypoxia would therefore be of great value and could additionally provide an opportunity for the study of hypoxia in archived material. Carbonic anhydrase IX (CA IX), a member of the α-carboanhydrase family that possesses carbonic anhydrase and proteoglycan domains (8), is a candidate for such an endogenous marker. It is strongly hypoxia inducible in transformed cells in vitro, a process that is mediated through transactivation by hypoxia-inducible factor-1α (HIF-1α; ref. 9). Colocalization of CA IX protein expression with pimonidazole, which is commonly called an exogenous hypoxia marker, has been reported repeatedly (10–12). Whether in vivo expression of CA IX in human tumors is also primarily determined by the cells’ oxygenation status is, however, still a matter of debate. One aspect that is often neglected in this discussion is the fact that CA IX expression is predominantly found in cancer cells. Expression in normal tissue has been documented (13), but its distribution is not quite compatible with a primarily hypoxia-induced expression. Physiologically hypoxic renal medulla [mean pO2 ≤10 mm Hg (14) and a high glycolytic rate] has been found to be CA IX negative (13), whereas the strongest expression of CA IX was detectable in gastric mucosa [in human, rat, and mouse (15)], with mice homozygous for a null mutation of the CA IX gene showing gastric hyperplasia (16). In uterine cervix cancers, Loncaster et al. (17) found a moderate correlation of CA IX with direct oxygenation measurements, but variation between individual cases was substantial. These results are in contrast to the data presented by Hedley et al., who found no correlation (18) in an investigation of the same tumor entity. These discrepancies may at least partially be attributable to tumor heterogeneity. The aim of the present study was to clarify the status of CA IX as an endogenous hypoxia marker by assessing...
the expression of CA IX in biopsies taken directly from oxygenation measurement tracks, thereby reducing the influence of tumor tissue heterogeneity.

Materials and Methods

Patients. All patients in this study were enrolled in a prospective clinical trial for the evaluation of the significance of tumor oxygenation in primary, locally advanced carcinomas of the uterine cervix that commenced at the Department of Obstetrics and Gynecology, University of Mainz Medical School in June 1989. The study design was approved by the local medical ethics committee, with patients giving informed written consent before being enrolled. Forty-seven patients, from whom one or two tumor biopsy specimens of the oxygen measurement tracks were available, were included in the present study. This specific cohort of patients had been recruited between August 1991 and April 1997. Table 1 shows relevant patient and tumor characteristics at the time of the pretreatment pO2 measurements.

Tumor oxygen tension measurements. Tumor pO2 was measured pretherapeutically with the computerized Eppendorf histography system (Eppendorf, Hamburg, Germany) using a protocol that has been described in detail previously (19). Briefly, pO2 measurements were done in the conscious patient along linear tracks, first in the s.c. fat of the mons pubis and subsequently in the cervix at the 12 and 6 o’clock sites in macroscopically vital tumor tissue. Within the tumor tissue, up to 36 pO2 measurements were made along each electrode track (72 readings in total), starting at a tissue depth of 5 mm. The individual pO2 measurement points were situated 0.7 mm apart, resulting in an overall measurement track length of ≈25 mm. Immediately following pO2 measurements, needle core biopsies (obtained using Biopry, Radioplast, Uppsala, Sweden) of ≈2 mm in diameter and 20 mm long were taken from those tumor areas where pO2 readings had been obtained. Both the pO2 readings and the needle core biopsies were done in all patients without general anesthesia. Intravaginal temperature, arterial blood pressure, heart rate, hemoglobin concentration, hematocrit, and arterial oxyhemoglobin saturation were monitored at the time when pO2 readings were taken. The pretherapeutic pO2 measurements were usually done 1 to 5 days before commencement of oncological treatment. After histologic examination of the biopsy specimens, pO2 measurements that were found not to have been done in vital tumor tissue were excluded from further analysis.

Immunohistochemistry. Expression of CA IX was assessed in 77 biopsy specimens taken from tumor pO2 measurement tracks obtained immediately after pO2 measurement in 47 patients. Two biopsies, corresponding to the 6 and 12 o’clock positions of the tumor center, were available for each of 32 patients and one biopsy for each of the remaining 13 cases. All material was fixed in formalin before being embedded in paraffin. Histologic slides were prepared from the paraffin blocks and dried overnight at 37°C. Subsequently, specimens were dewaxed in two changes of fresh xylene and rehydrated in a descending alcohol series. Retrieval of antigenic binding sites was done by heating specimens in 10:1 mmol/L Tris/EDTA buffer (pH 9.0) in a Braun FS10 steamer (Braun, Kronberg, Germany) for 40 minutes. Polyclonal rabbit anti-human CA IX (DakoCytomation, Hamburg, Germany) was used as the primary antibody at a concentration of ≈1 μg/mL in PBS. Incubation took place overnight at 4°C. A biotinylated goat anti-mouse/anti-rabbit secondary antibody was applied for 30 minutes at room temperature and further detection was carried out using a streptavidin-biotin-horseradish peroxidase system (Duet-Kit, DakoCytomation) in accordance with the manufacturer’s instructions. Color was developed with diaminobenzidine. Negative control specimens were incubated in PBS without the primary antibody under the same conditions. A tumor specimen with positive antibody under the same conditions. A tumor specimen with positive staining batch. Slides were counterstained with Mayer's hematoxylin, dehydrated in an ascending alcohol series, and covered with a coverslip using Eukitt mounting medium (Riedel-de Haen, Seelze, Germany).

Assessment of carbonic anhydrase IX expression. Although faint cytoplasmic or nuclear staining was also seen in some specimens, only distinct membranous staining was regarded as being specific/biologically relevant and evaluable as CA IX expression. A semiquantitative scoring system was used to assess the degree of CA IX expression in entire biopsy sections: score 0, no staining or very few positive cells (“absent”); score 1, <10% positive ("weak"); score 2, 11% to 50% positive ("moderate"); and score 3, >50% positive ("strong"). All specimens were reevaluated 2 weeks after the initial scoring. The reproducibility of the scoring was found to be at a satisfactory level (r = 0.87; P = 0.0001).

Statistical analysis. All statistical tests were done using the SPSS software package (version 12.0, SPSS, Inc., Chicago, IL). The significance level was set at α = 5% for all comparisons. Linear correlations between two variables were described by Spearman’s rank correlation coefficient (r). Two-sided Mann-Whitney U tests and Kruskal-Wallis tests were used for comparison of categorized variables.
Results

Carbonic anhydrase IX expression. A characteristic pattern of CA IX expression was observed, which showed increasing staining intensity as a function of distance from the vascularized tumor stroma (Fig. 1). Staining was particularly strong in the viable cell layers immediately adjacent to necrotic areas. Only one tumor (two biopsies) showed diffuse strong positivity in all cells (score 3). All other cases scored as “strong” in principle retained the gradient of the staining intensity described above. Rarely, expression of CA IX was found in the tumor stroma, but this was not included in the analysis. CA IX expression in tumor cells was present in 63 of 77 (≈82%) biopsies. Of these, expression was “weak” in 29 (≈38%) cases, “moderate” in 26 (≈34%) cases, and “strong” in 8 (≈10%) cases.

Carbonic anhydrase IX expression and clinical and pathohistologic data. CA IX expression showed no correlation with clinical tumor size, pT stage, histology, histologic grading, patient age, menopausal status, parity, or pretherapeutic hemoglobin level. There was also no correlation with International Federation of Gynecology and Obstetrics (FIGO) stages when individual FIGO categories were tested. When however FIGO stage was dichotomized into two groups (stage Ib and IIa versus IIb–IVa), a significant correlation was observed (P = 0.035), with higher stages showing more pronounced expression of CA IX. There was also a borderline significant correlation with nodal status (P = 0.051). Biopsies from patients with positive lymph nodes tended to show a higher expression of CA IX. Additionally, a significantly greater expression of CA IX was found in smokers (P = 0.042). This correlation is strongly dependent on the fact that no instances of an entire absence of CA IX expression were found in tumor biopsies from smokers. Table 2 gives a summary of the associations with clinical data.

Carbonic anhydrase IX expression and oxygenation data. The Kruskal-Wallis test showed no differences in median pO2 and hypoxic fractions (HP) ≤2.5 mm Hg (HP 2.5), ≤5 mm Hg (HP 5), and ≤10 mm Hg (HP 10) among the four CA IX expression score groups (see Fig. 2). The same results were obtained when CA IX expression was tested as a dichotomized variable using different cutoff values (absent versus present expression; absent and weak versus moderate and strong expression, Mann-Whitney U test). There were also no statistically significant differences in CA IX expression between individual categories (e.g., absent expression versus strong expression).

Discussion

Comparison of the oxygenation status, as indicated by the variables median pO2 and HP 2.5, HP 5, and HP 10, and expression of CA IX in 77 biopsy specimens of advanced cancers of the uterine cervix as done in the present study showed no correlation. Strong expression of CA IX was evident in severely hypoxic tumors but also in their better oxygenated counterparts. Conversely, low expression of CA IX was found not only in less hypoxic tumors but also in severely hypoxic ones. Analyses of this problem have been done previously (17, 18), but with conflicting results. A special feature of the methodology employed in this study is that both oxygenation measurements and immunohistochemical analysis were carried out in the same tumor areas, thereby reducing the potentially interfering influence of tumor heterogeneity.

To assess the intensity of CA IX expression in this study, a simple “manual” scoring system was chosen, which has been widely applied in previous studies investigating CA IX expression (17, 20–22). Based on our own experience, image analysis methods are not yet reliable enough for the assessment of membranous staining. For example, calculation of the percentage of positive pixels per total tumor area (as carried out in refs. 11, 18) results in a bias caused by the size of individual tumor cells. Despite identical sizes of positive tumor areas, “small cell tumors” are likely to have an apparently higher expression than “large cell tumors,” because the former contributes a larger proportion of membrane substance. When using image analysis-based expression assessment, an exclusion of the tumor stroma is a prerequisite to avoid a pronounced interference due to differences in individual tumors’ proportions of stroma. This is a labor-intensive process that additionally requires specially qualified personnel. The clinical applicability of a potential method for hypoxia estimation would of course generally be greater if it did not depend on special methods and technical equipment.

Of the two available investigations comparing CA IX expression with oxygen microsensor measurements, only the study by Loncaster et al. (17) described a correlation. However, there are remarkable differences in the percentage of CA IX–positive cases between two parts of this particular study. Whereas a prospectively recruited patient cohort, which was employed for correlation of CA IX expression intensity with the oxygenation status (n = 68) showed 94% of cases to be positive, the retrospective branch of the study, used for prognostic correlations (n = 130), had a lower proportion of positive cases (71%). The study of Hedley et al. (18) found 70% CA IX–positive cases but, as in our own investigation, failed to find a correlation between CA IX expression and pO2 electrode measurements. The data presented here are in accordance with both cited studies (17, 18) in that (a) the absence of hypoxia cannot be predicted from a lack of CA IX expression and (b) strong CA IX expression can be identified in tumors that exhibit hypoxic fractions comparable with those found in tumors lacking CA IX expression.

A meaningful role for CA IX as a surrogate hypoxia marker has also been suggested based on comparisons with the expression patterns of “extrinsic hypoxia markers,” such as pimonidazole. Most of these studies found weak but significant correlations (10, 11), although, to our knowledge, only one study (12) described a strong correlation between the two markers, with CA IX expression constantly amounting to ≈2 times that of pimonidazole in the 18 cases undergoing assessment. In contrast, the initial report by Wykoff et al. (9), examining basal cell carcinomas and bladder cancer, could not find a correlation between CA IX and pimonidazole. In addition to these diverging results, the importance of 2-nitroimidazoles as clinically useful markers of hypoxia is still a matter of debate, because neither pimonidazole (23) nor EF5 binding (24) correlate with direct oxygenation measurements. Differences in the assessment of necrotic areas (25) and other factors [e.g., unspecific binding of pimonidazole to keratinizing areas (26)], may be responsible for the lack of agreement between these two methods. Nevertheless, prognostic relevance has mainly been shown for direct oxygenation measurements (see ref. 27 for a review).

The results of the present study cannot be considered as surprising, bearing in mind our previous findings on the independence of HIF-1α (28) and GLUT-1 (29) expression from...
Fig. 1. Expression patterns and score of CA IX expression (histologic photographs; magnification, ×10) with respective oxygen tension (pO2) histograms for hypoxic (top) and normoxic tumors (bottom). Examples of high and low expression of CA IX are depicted. $n$, number of pO2 readings in the respective measurement track.
direct oxygenation measurement values. Although the period for induction and degradation of HIF-1α by hypoxia and reoxygenation is roughly comparable with the selective nature of microelectrode oxygen measurements, this is not the case for CA IX and GLUT-1, because these proteins have longer half-lives. Therefore, both of the latter markers may, in addition to chronic hypoxia, capture repeated episodes of intermittent hypoxia that are missed by the former methods.

CA IX expression is primarily regulated by HIF-1α, which in turn is modulated by a variety of growth factors, cytokines, and further mediators other than hypoxia (30). Basal levels of HIF-1α protein have been shown to be regulated by the activity of the phosphatidylinositol-3-kinase/AKT pathway (31), whereas the regulation of the transcriptional activity of HIF-1α seems to be a target of the RAS/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway (32). Both of these pathways are intracellular effectors for receptor tyrosine kinase activation (e.g., platelet-derived growth factor), which therefore seems to be “hardwired” to a certain degree of activation of the hypoxic response (33). On the other hand, these mechanisms may partially explain (a) why there is no direct correlation between CA IX expression and hypoxia and (b) the finding that CA IX expression is more prevalent in tumors with higher FIGO stages, because deregulation of signaling pathways is known to increase with tumor progression. On the other hand, the differential regulation of protein levels and transcriptional activity of HIF-1α may theoretically also allow for a tighter hypoxic regulation of downstream factors, such as CA IX, compared with HIF-1α. The transcriptional activity of HIF-1α has been identified as an hypoxia-regulated step that may independently remain inhibited when protein stabilization already takes place (34). A major role for this mechanism, however, is obviously not supported by our data.

Other mechanisms of hypoxia-independent or only partially hypoxia-dependent expression of CA IX are currently undergoing investigation. In a study by Rafajova et al. (35) in HeLa cells, low glucose and bicarbonate (HCO₃⁻/CO₂) levels, which were applied as accompanying microenvironmental stress factors, added to the effect of hypoxia on CA IX expression. Contrary to this, Vordermark et al. (36) found a reduction or abolition of hypoxia-induced CA IX expression in two cell lines exposed to low glucose levels. These authors convincingly argued that the glucose concentration of 5.5 mmol/L used in the study of Rafajova et al. was not genuinely “low” but rather within the

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**Table 2. Correlations of CA IX expression with clinical data**

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*Only applicable in surgically treated patients.
†Information on smoking habits was not documented in seven cases.

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**Fig. 2.** Fraction of pO₂ readings ≤5 mm Hg (HP 5) and CA IX staining (0, absent; 1, weak; 2, moderate; 3, strong) in identical microareas of locally advanced cancers of the uterine cervix. Note that some circles represent more than one measurement.
physiologic range. Previously, the expression of CA IX has even been described as being primarily cell density dependent (37), although it is now presumed that this mechanism partially depends on pericellular hypoxia, which occurs as a subsequence of high cell density (38). Even so, a cell density–dependent activation of the phosphatidylinositol-3-kinase/AKT pathway also seems to have an effect on CA IX expression via a specific target in the CA IX promoter. This activation seems to be predominantly a direct process, because it requires only minimal HIF-1α activation (39).

CA IX has also been associated with cell proliferation. The initial report on CA IX by Pastorek et al. (37) described an increased proliferation of NIH 3T3 cells when CA IX was expressed. Colocalization of CA IX staining and Ki-67 has been found in colorectal tumors (40), and there is evidence that the size of the fraction of cells that is both CA IX positive and undergoing proliferation (as measured by iododeoxyuridine incorporation) is predictive of prognosis in head and neck squamous cell carcinomas (41).

In conclusion, the comparison of CA IX expression and oxygenation measurements in the same areas of locally advanced cervical cancers could not identify a direct relationship. This study, together with results from a growing body of in vitro experiments, raises doubts concerning the suitability of CA IX as an endogenous hypoxia marker.

Acknowledgments

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References

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