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# Carbonic Anhydrase 9 as an Endogenous Marker for Hypoxic Cells in Cervical Cancer[1]

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## ABSTRACT

**The presence of radiation-resistant hypoxic cells in some solid tumors is known to predict for relapse after radiotherapy. Use of an endogenous marker of hypoxia would be a convenient alternative to current methods that measure tumor oxygenation, provided the marker could be shown to reliably identify viable, radiation-resistant, hypoxic cells. Carbonic anhydrase 9 (CA9) is a transmembrane protein overexpressed in a wide variety of tumor types and induced by hypoxia. Using a monoclonal antibody and cell sorting, CA9-positive cells in SiHa cervical carcinoma xenografts growing in immunodeficient mice were found to be clonogenic, resistant to killing by ionizing radiation, and preferentially able to bind the hypoxia marker pimonidazole. CA9 and pimonidazole immunostaining were compared in formalin-fixed sections from tumors of 18 patients undergoing treatment for cancer of the cervix. Excellent colocalization was observed, although the area of the tumor section that bound anti-CA9 antibodies represented double the number of cells that bound anti-pimonidazole antibodies. Occasional regions staining with pimonidazole but not CA9 could be indicative of transient changes in tumor perfusion. Results support the hypothesis that CA9 is a useful endogenous marker of tumor hypoxia.**

## INTRODUCTION

Tumors lacking or low in oxygen are often less curable not only by radiotherapy but also by surgery (reviewed in Ref. 1). Because the presence of hypoxic tumor cells is likely to indicate a poor outcome after therapy, it would be useful to identify hypoxic tumors at the start of treatment and then modify treatment accordingly. However, current methods used to detect hypoxic cells are often technically complex, invasive, or require administration of chemicals to mark hypoxic cells. Although the procedure is invasive and subject to the same sampling errors, an endogenous marker of hypoxia that could be identified in conventional formalin-fixed tumor sections would be an important step forward. It might also shed some light on the factors that create the more aggressive phenotype of hypoxic tumors and could possibly serve as a target for therapy.

CA9[3] (also called MN/CA9 or G250) is a member of the CA family that catalyzes the reversible hydration of carbon dioxide to carbonic acid. Transcription of this gene is known to be regulated by the Von Hippel-Lindau tumor suppressor gene, the protein product of which is part of a ubiquitin ligase complex (2). This complex is responsible for targeting HIF-1$\alpha$ for oxygen-dependent proteolysis (3, 4). The interaction of Von Hippel-Lindau protein with HIF-1$\alpha$ appears to be governed by iron-dependent hydroxylation of a specific proline in the oxygen-dependent degradation domain of HIF-1$\alpha$ (5, 6). Therefore, at low levels of oxygen, HIF-1$\alpha$ is stabilized causing an increase in expression of CA9. Areas of high expression of CA9 have been shown to colocalize with regions of tumor hypoxia in bladder and skin cancer, and incubation of tumor cells under hypoxia has been shown to induce expression of CA9 (7–9).

Focal expression of CA9 occurs in >90% of carcinomas of the cervix, digestive tract, head and neck, as well as glioblastomas and basal cell carcinomas (9). CA9 expression has been used to distinguish between malignant and preneoplastic lesions of the lung (10) and cervix (11). Recently, CA9 expression was found to be a significant predictor of disease-specific and metastasis-free survival in patients with locally advanced squamous cell carcinoma of the uterine cervix, after allowing for stage, age, and tumor grade (12).

Because CA9 antibody staining is often described as perinecrotic, a critical question is whether cells that express CA9 in solid tumors represent viable, hypoxic cells. This question can be addressed in tumor xenograft models because the antibody to CA9 recognizes an external epitope on this transmembrane protein. Therefore, unlike HIF-1$\alpha$ antibody staining, immunostaining for CA9 can be performed on viable cells. Two cancer cell types, a human cervical carcinoma cell line and a human glioma, were chosen for evaluation based on the high expression of CA9 in these tumor types (9) and on our previous experience characterizing hypoxia in these two xenograft models (13, 14). SiHa cervical carcinoma cells were grown as multicellular spheroids or xenograft tumors in immunodeficient mice, and fluorescence-activated cell sorting was used to determine whether CA9-positive cells were viable, resistant to radiation damage, or could preferentially bind the hypoxia marker, pimonidazole. Binding of pimonidazole was then compared with CA9 expression in sequential sections of tumor biopsies from 18 patients with cervical carcinoma undergoing radical radiotherapy.

## MATERIALS AND METHODS

**Cells, Spheroids, and Xenografts.** SiHa human cervical carcinoma cells were obtained from American Type Culture Collection. SiHa spheroids, grown to ~600 $\mu$m in diameter as described previously (15), were incubated for 4 h in spinner culture with 100 $\mu$g/ml pimonidazole, followed by 20 min with 2 $\mu$M Hoechst 33342 to provide a fluorescence diffusion gradient for cell sorting (16). Spheroids were disaggregated in 0.25% trypsin, and single cells were sorted on a Becton Dickinson FACS 440 dual laser cell sorter.

M006 human glioma xenografts growing in NOD/SCID mice were originally obtained from Drs. Allan Franko and Joan Alallunis-Turner (13) and have not been established in culture in our laboratory. SiHa or MOO6 tumor cell aggregates were implanted s.c. in the backs of NOD/SCID immunodeficient mice, 8–10 weeks of age. Tumors were used when they reached a weight of 0.4–0.6 g. For analysis of the radiation response of the tumor cells, mice with tumors were exposed to 250 kV X-rays at a dose rate of 2.04 Gy/min. Then mice were injected i.v. with 0.1 ml of Hoechst 33342 (8 mg/ml) to provide a fluorescence diffusion gradient from the blood vessels into the tumor cords. Twenty min after injection, tumors were

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[3] The abbreviations used are: CA9, carbonic anhydrase 9; HIF-1$\alpha$, hypoxia-inducible factor 1$\alpha$; NOD/SCID, non-obese diabetic/severe combined immunodeficient; FBS, fetal bovine serum.

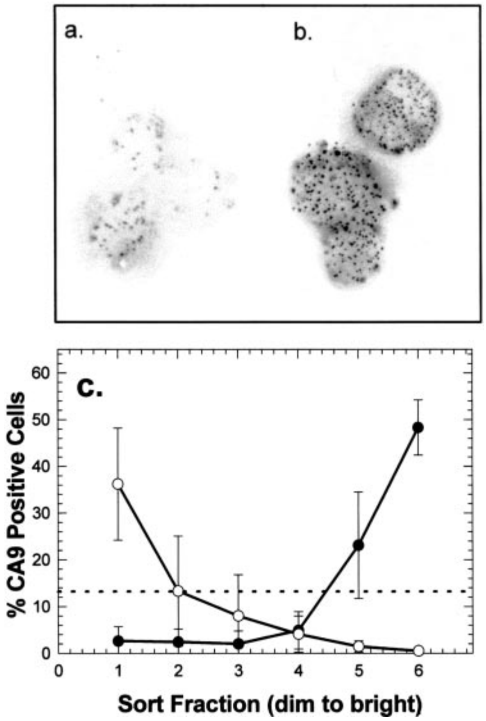


Fig. 1. Expression of CA9 on outer cell membranes of SiHa cervical carcinoma cells grown as spheroids. Two-week-old SiHa spheroids, ~600 $\mu$m in diameter, were incubated for 20 min with Hoechst 33342 to label the external cells of spheroids. After enzyme disaggregation, single cells were incubated with anti-CA9 antibodies and sorted on the basis of Hoechst 33342 or CA9 intensity. *a* and *b* show CA9 expression for external and internal cells of spheroids respectively. In *c,* SiHa cells were sorted on the basis of CA9 (●) or Hoechst 33342 (○) concentration and reanalyzed for the percentage of CA9-positive cells. The means for three independent experiments are shown; *bars,* SD. *Dotted line,* the response of unsorted cells.

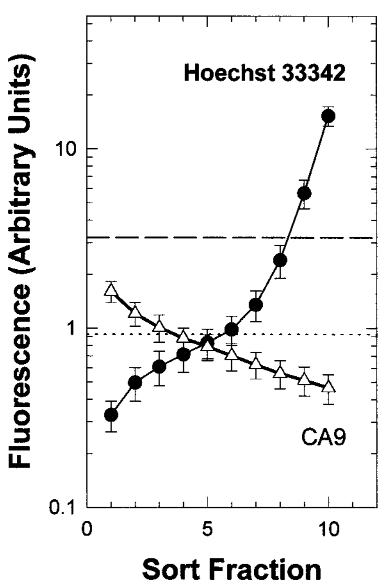


Fig. 2. Comparison between the Hoechst 33342 sorting gradient (●) and the CA9 fluorescence-tagged antibody sorting gradient (△) in SiHa xenografts. The means for 10 fractions from five tumors are shown; *bars,* SD. *Dotted lines,* the intensity of the unsorted populations.

excised, and a single cell suspension was prepared using mechanical dissociation and enzyme digestion (14).

To examine hypoxia marker binding, mice were sacrificed 90 min after i.p. injection of 100 mg/kg pimonidazole hydrochloride (Hypoxyprobe-1; NPI, Belmont, MA), and a single cell suspension was prepared from the tumors as described previously (14). Tumor cells (~150,000) were sorted on the basis of CA9 or Hoechst 33342 concentration, fixed in 70% ethanol, and kept at −20°C before analysis.

**CA9 Staining and Cell Clonogenicity Assay.** Single cell suspensions were incubated for 15 min at room temperature with a 1:5000 dilution of CA9 monoclonal antibody, prepared as described previously (17). Cells were then washed twice in PBS containing 4% FBS and resuspended in a 1:200 dilution of Alexa-488 conjugated anti-IgG antibody (Molecular Probes, Eugene, OR). Ten min later, cells were rinsed and analyzed within 10 min using a Becton Dickinson FACS 440 dual laser cell sorter. Cells were sorted on the basis of Hoechst 33342 concentration as described previously and on the basis of CA9 concentration. Sorted cells were plated in MEM + 10% FBS and antibiotics, and 2 weeks later, colonies were stained with malachite green and counted to obtain the surviving fraction.

**Anti-Pimonidazole Antibody Staining.** Ethanol-fixed cells were centrifuged, rinsed in PBS plus 4% FBS, and resuspended in primary anti-pimonidazole antibody as described previously (14). For spheroid experiments, cells were centrifuged, rinsed in PBS plus 4% FBS, and resuspended in Alexa-594 or phycoerythrin-conjugated secondary antibody before staining DNA with 0.1 $\mu$g/ml 4′,6-diamidino-2-phenylindole. Samples were analyzed using a Coulter Elite cytometer. The hypoxic fraction was determined from pimonidazole intensity profiles by assuming that hypoxic cells were 10 times more fluorescent than well-oxygenated cells (14).

**Patients.** Currently at the Vancouver Cancer Center, British Columbia Cancer Agency, there is an on-going clinical study assessing the importance of pretreatment tumor oxygenation status for patients with invasive epithelial cervical cancers. This protocol has been approved by the British Columbia Cancer Agency Ethics Board as well as the University of British Columbia Ethics Committee. Patients were deemed eligible if they had a histologically confirmed clinically visible invasive carcinoma of the cervix, either squamous cell, adenocarcinoma, or a variant of these. Patients were suitable candidates for radical curative therapy and could undergo tissue biopsy without anesthesia. Patients were considered ineligible if they were unable to give informed consent, had liver enzyme tests greater than twice the normal laboratory values, serum creatinine ≥150 $\mu$mol/l, or a history of a peripheral neuropathy.

After giving consent, patients received a 20-min i.v. infusion of 0.5 g/m$^2$ Hypoxyprobe-1 (pimonidazole hydrochloride; NPI) dissolved in 0.9% sterile saline. Approximately 24 h later (about 4 plasma half-lives), incisional biopsies of visible tumor were obtained from unanesthetized patients.

**CA9 and Pimonidazole Staining of Tissue Sections.** Biopsies (~150 mg) were fixed in formalin and embedded in paraffin. Sequential sections (5 $\mu$m thick) from a single biopsy were de-waxed in xylene and rehydrated in graded alcohols before staining for CA9 using the immunoperoxidase method with diaminobenzidine tetrahydrochloride (11, 17). Pimonidazole analysis was performed in a similar manner using sequential sections (18, 19). Sections were then stained with hematoxylin. Digitized images of entire tumor sections (stroma and tumor) were prepared by electronically tiling up to 200 individual frames (×63). Areas of obvious necrosis were not included. The percentages

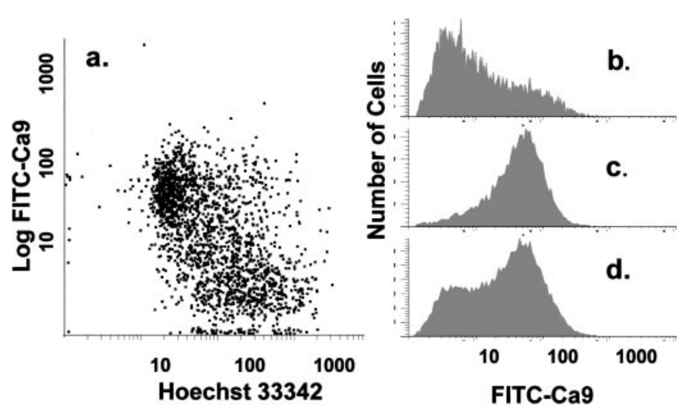


Fig. 3. CA9 expression in tumor xenografts in relation to Hoechst 33342 fluorescence. *a,* the gated bivariate plot of the relation between Hoechst 33342 fluorescence intensity and CA9 antibody staining in the M006 glioma xenograft. *b,* the CA9 distribution in the brightest Hoechst staining fraction (*i.e.,* the 10% of cells closest to the tumor blood vessels). *c,* CA9 in the dimmest Hoechst staining fraction (*i.e.,* the 10% of cells most distant from the tumor blood vessels). *d,* the distribution of CA9 in unsorted cells.

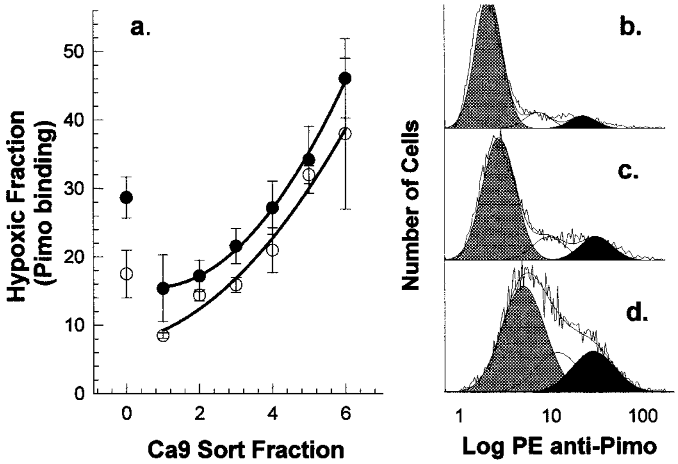


Fig. 4. Pimonidazole binding in SiHa and MOO6 xenografts. *a*, the percentage of hypoxic cells, determined by pimonidazole staining, as a function of CA9 antibody staining intensity. Results are the means for 4 SiHa tumors (●) and 3 M006 tumors (○); *bars*, SD. *b–d* are representative distributions showing pimonidazole binding in a M006 tumor for sort fractions 1, 3, and 5, respectively. The *hatched area* shows the well-oxygenated population, and the *filled area* represents the hypoxic cells within the tumor.

and flow cytometry. CA9 staining was punctate on the outer cell membrane and was significantly increased in the innermost cells of SiHa spheroids compared with the outer cell layer (Fig. 1, *a* and *b*). Only 0.5 ± 0.3% of the cells of these spheroids were sufficiently hypoxic to bind the hypoxia marker pimonidazole, yet 12% of cells bound CA9 antibodies (Fig. 1*c*). As expected, spheroid cells that bound the least Hoechst 33342 (*i.e.,* furthest from the spheroid surface) showed the most CA9 antibody binding.

Compared with SiHa spheroids, the overall intensity of CA9 antibody staining was reduced in SiHa cells grown as xenografts, perhaps because the suboptimal growth environment and the longer tumor disaggregation time affected the expression of this cell surface antigen. As might be expected, the average intensity and the gradient of fluorescence were considerably greater for Hoechst 33342 staining than for CA9 antibody staining in the same tumors (Fig. 2). Nonetheless, there was still a 4–5-fold difference in CA9 antibody staining in the brightest 10% of cells *versus* the dimmest 10% of cells (Fig. 2).

Bivariate analysis of Hoechst 33342 *versus* CA9 illustrated for the M006 xenograft confirmed that CA9-positive cells were inversely distributed relative to Hoechst 33342 (Fig. 3*a*). Histograms showing M006 glioma cell binding of anti-CA9 antibody indicated that a

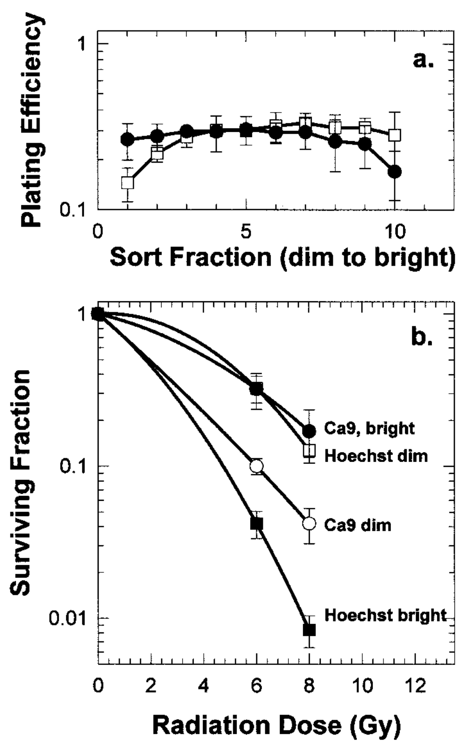


Fig. 5. Viability and radiation response of CA9-positive cells in SiHa xenograft tumors. *a*, the plating efficiency of unirradiated SiHa xenograft cells sorted on the basis of the fluorescence concentration gradients for Hoechst 33342 (□) or CA9 (●). The means for four tumors are shown; *bars*, SD. *b*, the radiation response of SiHa xenograft cells sorted based on the concentration of Hoechst 33342 (■, □) or CA9 (●, ○). □ and ○, the response of the 10% of cells that are least fluorescent within the tumor; ■ and ●, the response of the 10% of cells that are most intensely stained. The means for four tumors are shown; *bars*, SE.

Table 1 *Comparison between CA9 expression and pimonidazole binding in cervical cancers*

| Patient | Age | Smoker | FIGO[a] cervical cancer stage | Histology | % Pimopositive area | % CA9-positive area |
|---|---|---|---|---|---|---|
| 1 | 49 | No | IIb | Squamous | 13.6 | 27.7 |
| 2 | 71 | No | IVa | Squamous | 6.4 | 18.3 |
| 3 | 59 | No | IIb | Adeno | 3 | 14 |
| 4 | 34 | Yes | IIb | Squamous | 5.7 | 13 |
| 5 | 46 | Yes | IIIb | Squamous | 8.1 | 11.7 |
| 6 | 52 | No | $1b_2$ | Squamous | 5.7 | 11.4 |
| 7 | 59 | No | IIIb | Squamous | 5.1 | 10 |
| 8 | 33 | Yes | $1b_2$ | Squamous | 3.6 | 7.1 |
| 9 | 47 | No | $1b_1$ | Adeno | 4.3 | 7 |
| 10 | 42 | No | IIa | Adeno | 3.1 | 6.9 |
| 11 | 67 | No | $1b_2$ | Adeno | 4.6 | 6.2 |
| 12 | 33 | Yes | IIb | Squamous | 3.1 | 4.4 |
| 13 | 43 | No | IIb | Squamous | 2.3 | 4 |
| 14 | 38 | No | $1b_2$ | Squamous | 1.2 | 2 |
| 15 | 41 | No | $1b_1$ | Adeno | 1.1 | 2 |
| 16 | 41 | No | $1b_1$ | Squamous | 1.4 | 1.8 |
| 17 | 35 | Yes | $1b_2$ | Squamous | 5.1 | 1.6 |
| 18 | 41 | Yes | $1b_2$ | Squamous | 1.4 | 1.6 |

[a] FIGO, Fédération Internationale des Gynecologistes et Obstetristes; Pimo, pimonidazole; Adeno, adenocarcinoma.

of CA9- and pimonidazole-positive tissue were calculated from the ratio of diaminobenzidine tetrahydrochloride-positive area in pixels divided by the total area in pixels using NIH/Scion image software. Thresholds were optimized for individual sections.

## RESULTS

Initial experiments characterized CA9 binding in SiHa cells grown as multicellular spheroids and analyzed using immunohistochemistry

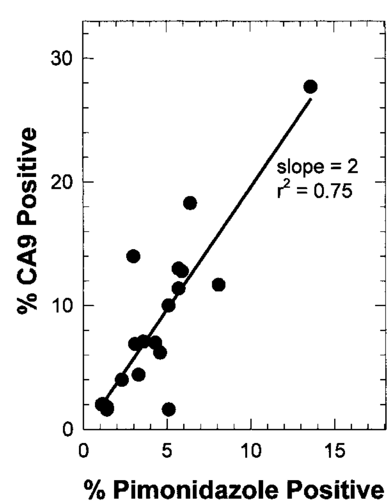


Fig. 6. Comparison between the percentage of the tumor area that binds CA9 antibodies with percentage of tumor area, measured in a sequential section, that binds pimonidazole (data from Table 1). The linear best-fit is shown.

subset of cells express higher levels of CA9 (Fig. 3*d*). Cells that expressed the most CA9 came from poorly perfused areas of the tumor that were relatively inaccessible to Hoechst 33342 (Fig. 3*c*). Conversely, tumor cells from well-perfused areas were less likely to express CA9 (Fig. 3*b*).

To determine whether the CA9-positive cells of xenograft tumors preferentially bound the hypoxia marker pimonidazole, mice were injected with 100 mg/kg pimonidazole 90 min before sacrifice. Single cells obtained from SiHa or M006 tumors were then stained with CA9 antibody, and sorted cells were fixed in ethanol before incubation with anti-pimonidazole antibodies. Flow histograms were analyzed using three normal distributions representing aerobic, intermediate, and hypoxic populations (Fig. 4, *b–d*). Cells containing the least CA9 showed only 8–15% hypoxic cells, whereas up to 50% of the cells expressing high levels of CA9 were hypoxic (Fig. 4*a*). These results indicate that CA9-positive tumor cells preferentially bind the hypoxia marker pimonidazole.

To determine whether the CA9-expressing cells were viable, cells from SiHa tumors were incubated with CA9 antibody and sorted on the basis of the fluorescence concentration. Sorted cells were grown in culture to measure colony formation ability. Although there was a small decrease in plating efficiency for cells expressing the most CA9,

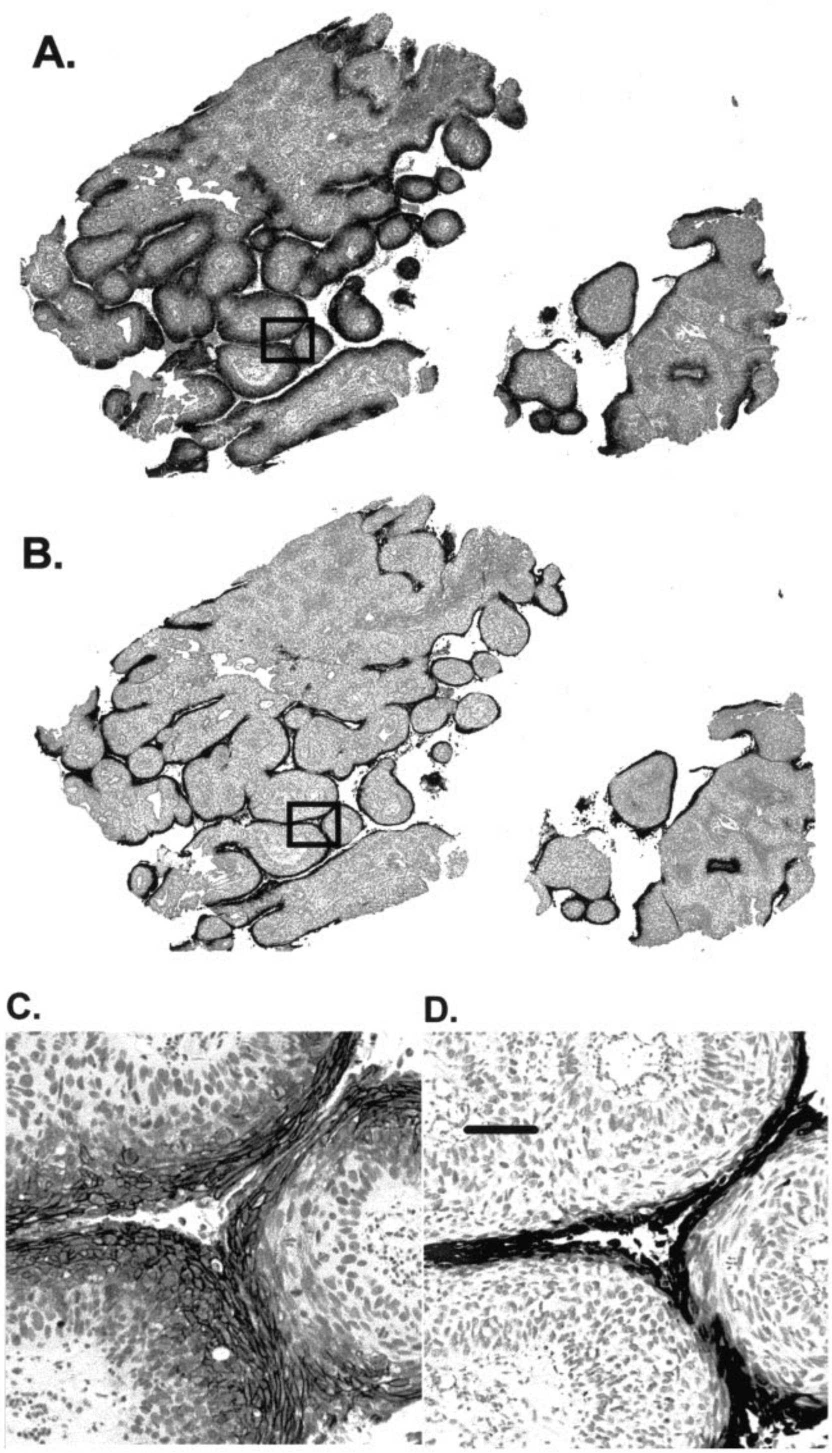


Fig. 7. Immunohistochemical analysis of pimonidazole adduct binding and CA9 expression on sequential sections of a cervical carcinoma biopsy for tumor 1. The area highlighted by the *squares* in *A* and *B* is enlarged in *C* and *D*. *A* and *C*, CA9 antibody binding. Note the localization to the membrane. *B* and *D*, anti-pimonidazole antibody binding. The *bar* is *D* is 85 μm.

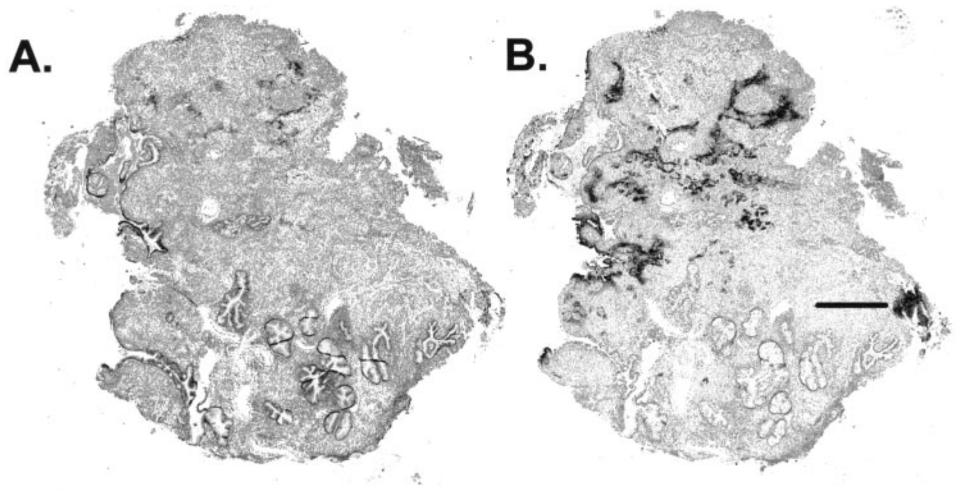


Fig. 8. A section of tumor 17 showing several regions staining for pimonidazole (*B*) with little or no CA9 staining (*A*), possibly indicating areas of transient hypoxia. The *bar* is ~850 $\mu$m.

this difference was not found to be significant (Fig. 5*a*). As is typically seen in the SiHa tumor and other xenograft tumors (20), cells distant from the blood supply (*i.e.,* that bind the least amount of Hoechst 33342) showed a small reduction in plating efficiency, probably because of inclusion of some debris in this sort fraction.

Resistance of xenograft cells to killing by ionizing radiation was used as an indicator of the presence of "radiobiologically hypoxic" tumor cells. Mice bearing SiHa tumors were irradiated and administered an i.v. injection of Hoechst 33342. Ten min later, tumors were removed, and single cells were labeled with anti-CA9 antibody. Cells were sorted on the basis of CA9 and Hoechst 33342 staining and were plated for survival using a colony formation assay. The 10% of cells expressing the highest amount of CA9 were more resistant to killing by ionizing radiation compared with the 10% of cells expressing the least amount of CA9 (Fig. 5*b*). As expected because of its steep diffusion gradient into the tumor cord, Hoechst 33342 staining was better able to identify the well oxygenated, radiation-sensitive cells of the tumor than CA9. However, both sorting strategies were equally efficient in identifying the most radioresistant tumor cells.

Having established that CA9 expression could be used as an indicator of hypoxia in tumor xenografts, the next step was to compare CA9 immunostaining with pimonidazole binding in formalin-fixed sections from tumors of 18 patients undergoing treatment for cancer of the cervix. Demographics of the patients, stage and histopathological diagnoses of the tumors are described in Table 1.

The percentage of the tissue area staining with anti-pimonidazole antibodies varied from 1.1 to 13.6% (Table 1). The percentage of total area associated with CA9 expression varied from 1.6 to 27.7%. A good correlation was observed between pimonidazole binding and CA9 expression measured in sequential sections (Fig. 6). Binding of both antibodies occurred in the same regions of the tumors, although it was apparent that CA9 staining extended beyond the region binding pimonidazole in almost all cases (Fig. 7). Only one tumor (no. 17) exhibited pimonidazole binding at much higher levels than CA9 expression, and this was confirmed by repeating the staining protocol for a second set of slides. Fig. 8 shows a section of this tumor with areas of pimonidazole binding in the absence of CA9. Areas of pimonidazole binding with minimal or no CA9 binding were also seen infrequently in other tumors.

## DISCUSSION

CA9 expression is up-regulated in many tumor types and can be a useful biomarker for malignancy (9, 17, 21). Recently, the appreciation of the fact that CA9 is regulated by HIF-1$\alpha$ has led to the suggestion that this might also be a useful endogenous marker for hypoxia (7, 9, 12). However, because focal expression of CA9 is often seen in association with necrosis, cells that express CA9 could be nonviable. Viable cell sorting allowed us to address this question directly. Results confirmed that cells within the SiHa xenograft tumors that express the most CA9 are clonogenic. Moreover, CA9-positive cells are more likely to be resistant to killing by ionizing radiation, and they bind significantly more pimonidazole than cells expressing little or no CA9, consistent with their reduced oxygenation.

Although the degree of CA9 staining is correlated with pimonidazole binding and radiation resistance, there is a continuum of increasing expression and no clear demarcation between CA9 expression in oxic and hypoxic cells (Fig. 3, *a* and *d*). This means that it may not be possible to use flow cytometry to identify a "hypoxic" population based on CA9 antibody binding in tumors with high hypoxic fractions. This is not surprising because half-maximal expression of HIF-1$\alpha$ occurs at an oxygen concentration of 1.5–2% (22), and even well-oxygenated tumor cells close to blood vessels can be equilibrated with oxygen concentrations as low as 2% (23). In comparison, pimonidazole binding is typically measured for cells at an oxygen concentration $<$10 mm Hg or as low as 0.1% oxygen (24, 25). Therefore, CA9 expression is indicative of cells that are maximally resistant to ionizing radiation as well as those of intermediate sensitivity.

A similar conclusion can be made for the clinical tumor samples. Images in Fig. 7 and the comparison shown in Fig. 6 indicate that twice as many cells, on average, express CA9 than bind pimonidazole. If CA9 expression is stimulated by oxygen concentrations that are too high to allow adequate nitroreduction and subsequent binding of pimonidazole, one would expect CA9 antibody staining to extend beyond the region able to bind pimonidazole. The pattern observed for cervical carcinomas seems to differ from the pattern reported for skin and bladder cancers. In the latter study, pimonidazole staining extended beyond the region stained with anti-CA9 antibodies (7). Whether this represents true intratumor differences in the pattern of CA9 expression or technical differences in antibody staining remains to be determined.

The tumor shown in Fig. 8 is the one example where large areas bound pimonidazole but did not demonstrate CA9 immunostaining. Because some regions within this tumor did show CA9 staining, it is unlikely that the protein was not expressed. In addition, other tumors showed occasional small regions of pimonidazole staining in the absence of CA9. A likely explanation is that the duration of hypoxia in some regions may be insufficient to up-regulate HIF-1$\alpha$ but adequate to allow pimonidazole metabolism and binding. In some murine tumors, localized transient changes in perfusion lasting on average 15–30 min have been observed (26, 27). Fluctuations in tumor blood flow are likely to create "healthy" hypoxic cells that can maintain repair capacity and are therefore better able to survive (28). Perfusion-limited hypoxia may require different treatment approaches than conventional diffusion-limited hypoxia (29), and analysis of pimonidazole and CA9 staining in individual cells could potentially offer a method for detecting both forms of hypoxia in human tumors.

In summary, cell sorting experiments using xenograft tumors in NOD/SCID mice confirmed that anti-CA9 antibody binds preferentially to viable, hypoxic cells in these xenografts. Good colocalization of CA9 and pimonidazole was observed in 17 of 18 invasive cervical cancers. It appears that CA9 may be a useful intrinsic marker of tumor hypoxia, and although it may overestimate the fraction of cells maximally resistant to ionizing radiation, it may also include the important category of cells that are intermediate in oxygenation. Outcome analysis of a larger patient cohort is necessary to determine the prognostic value of this marker.

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