Hypoxia and Differentiation in Squamous Cell Carcinomas of the Uterine Cervix: Pimonidazole and Involucrin

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ABSTRACT

Purpose: Pimonidazole binding (hypoxia) and involucrin expression (differentiation) overlap extensively in squamous cell carcinomas. This study asks whether involucrin might serve as an endogenous marker for tumor hypoxia. A second question is whether differentiation affects hypoxia-inducible metallothionein (MT) expression in normal human epithelia and squamous cell carcinomas as it does in rodent epithelia.

Experimental Design: Thirty-four patients with squamous cell carcinoma of the uterine cervix were infused with pimonidazole hydrochloride solution. The next day, multiple biopsies were formalin-fixed, paraffin-embedded and sectioned at 4 μm. Qualitative and quantitative analyses for involucrin expression, pimonidazole binding, and human MT-IIa mRNA expression were performed.

Results: No overall correlation between the extent of involucrin expression and pimonidazole binding was observed. The lack of correlation was because of heterogeneous patterns of immunostaining for involucrin generally related to tumor grade. Colocalized immunostaining for involucrin and pimonidazole binding was observed in intermediate grade tumors but not in well-differentiated or poorly differentiated tumors. Human MT-IIa mRNA and MT protein were expressed in basal lamina of normal human epithelia and in the proliferative rims of tumor nests.

Conclusions: Colocalization of immunostaining for involucrin and pimonidazole binding is consistent with oxygen regulation, but the lack of involucrin expression in hypoxic regions of poorly differentiated tumors indicates that its transcriptional status with respect to hypoxia induction is altered by cell differentiation. The localization of MT message and protein in the outer rims of most tumor nests indicates that the transcriptional status of metallothionein is also altered by differentiation.

INTRODUCTION

The discovery that MT is not expressed in the majority of hypoxic cells in human squamous cell carcinomas (1) was unexpected because MT was known to be induced by hypoxia in experimental systems (2, 3). A possible explanation for this anomaly was found in studies that showed that differentiation alters the transcription status of MT. In particular, MT is expressed in the proliferating basal layers of rodent stratified epithelia and not in the more differentiated (4) and hypoxic (5) suprabasal layers. In an analogous way, MT is expressed in the proliferating, outer edges of tumor nests and not in the hypoxic, central regions (1, 6). These results supported the idea that squamous cell differentiation alters the transcription status of MT in a way that makes it unavailable for hypoxia induction. However, this notion was based on data from rodent tissues, and one of the aims of this study was to find out whether MT expression is related to differentiation in human stratified epithelia.

The extensive colocalization for pimonidazole binding and involucrin expression (1) was reminiscent of that reported for pimonidazole binding and CA9 expression in squamous cell carcinomas (7, 8). CA9 is known to be oxygen regulated, and it has been suggested that it might serve as an endogenous marker for human tumor hypoxia. Unlike CA9, involucrin is not generally recognized as an oxygen-regulated protein. However, its transcription is controlled by AP-1 (9) and, in principle, could be oxygen regulated given that c-Jun/AP-1 is responsive to hypoxia梭. This study asks whether involucrin expression might serve as an endogenous marker for tumor hypoxia.

MATERIALS AND METHODS

Chemicals. Pimonidazole hydrochloride (Hypoxyprobe-1) was obtained from NPI, Inc. (Belmont, MA) in the form of sealed vials containing 1.0 g of pimonidazole hydrochloride dissolved in 10 ml of sterile 0.9% saline. Liquid DAB peroxidase substrate was obtained from Dako Corp. (Carpinteria, CA). Alkaline phosphatase NBT/5-bromo-4-chloro-3-indoly1 phosphate substrate was obtained from Ambion, Inc. (Austin, Texas).
Neutral-buffered 10% formalin, Biomeda Pronase, enzyme grade polyoxyethylene ether (Brij 35), Biomeda CrystalMount, ProbeOn Plus glass slides, and miscellaneous reagent-grade chemicals were obtained from Fisher Scientific Company (Norcross, GA). Clear-rite 3, a nontoxic deparaffinizing agent, was obtained from Richard-Allan Scientific (Kalamazoo, MI) and Aqua Hema-toxylin from Innovex Biosciences (Richmond, CA).

**Immunological Reagents.** Supernatant from hybridoma clone 4.3.11.3 containing antipimonidazole IgG1 monoclonal antibody at a concentration of 70 μg/ml was used for the immunohistochemical detection of protein adducts of reductively activated pimonidazole (11). A biotin-conjugated F(ab')2 fragment of a rabbit antinouse IgG was obtained from Accurate Chemical Scientific Corp. (Westbury, NY). Protein blocker and peroxidase-conjugated streptavidin were obtained from Dako Corp. Streptavidin-conjugated alkaline phosphatase was obtained from Ambion, Inc. IgG mouse antihuman involucrin antibody clone SY5 used for the detection of involucrin was obtained from Sigma (St. Louis, MO).

**Pimonidazole Hydrochloride Infusion.** University of North Carolina at Chapel Hill Institutional Review Board approval was obtained for the clinical use of pimonidazole hydrochloride in studies of human tumor hypoxia. Informed consent was obtained from all patients before entry into the study. A volume of concentrated pimonidazole hydrochloride solution (1.0 g/10 ml; NPI, Inc.) equivalent to a calculated dose of 0.5 g/m² was added to 100 ml of sterile 0.9% saline and administered as an i.v. infusion lasting 20 min (6). The high water solubility of pimonidazole hydrochloride facilitates its administration as a routine i.v. infusion. As discussed previously, a dose of 0.5 g/m² is one-half of the well-tolerated single dose for pimonidazole hydrochloride (6, 12, 13). This dose produced neither general nor central nervous system toxicity in the present group of patients. In fact, patients did not experience the mildest of central nervous system effects such as a sensation of warmth that occurs with higher doses of pimonidazole hydrochloride. Our experience is similar to that of clinical investigators in six other centers (7, 8, 14–17).

**Biopsy.** Biopsies of squamous cell carcinomas of the uterine cervix were obtained in an outpatient setting in the Department of Radiation Oncology at University of North Carolina Chapel Hill. Multiple punch biopsies were taken from geographically distinct areas of the tumors 16–24 h after pimonidazole hydrochloride infusion. The biopsy procedure was well tolerated. Bleeding was generally <5 ml and did not exceed 10 ml in any case. No patient required vaginal packing or transfusion. Biopsy samples were placed in ice-cold 10% neutral-buffered formalin within seconds of harvesting and fixed at 4°C for 12–24 h. After fixation, the tissues were embedded in paraffin blocks and sectioned at 4 μm onto glass slides. In those cases where embedding was delayed, biopsies were transferred to and stored at 4°C in 70% aqueous ethanol to prevent excessive fixation. A total of 133 biopsies was obtained from 34 squamous cell carcinomas. The 34 tumors were taken from 34 patients of a total of 47 cervix carcinoma patients consented for the study. From the total of 47 patients, insufficient tumor material was available for study in 6 cases; in 1 case, no biopsy was taken after pimonidazole hydrochloride infusion because of a medical emergency unrelated to the study; and in 6 cases, patients were consented but for reasons unrelated to the study protocol (e.g., missed infusion session because of transportation difficulty) and did not receive infusions of pimonidazole hydrochloride.

Normal stratified epithelium of the cervix was obtained adventitiously during biopsy. Stratified epithelium of the human tongue was obtained from a wide excision of a squamous cell carcinoma of the head and neck. Normal epithelia were examined for patterns of involucrin protein expression, pimonidazole binding, and hMT-IIa mRNA expression. Dr. Michael Horstman of the Danish Cancer Center (Aarhus, Denmark) provided samples of normal mouse tongue for comparison with human epithelia.

**In Situ Hybridization Analysis of hMT-IIa and mMT-I mRNA.** Riboprobes for hMT-IIa mRNA were prepared from an American Type Culture Collection MT-IIa plasmid template (18). The hMT-IIa 3'-flanking region fragment was synthesized by PCR using the following primers: sense primer, 5'-GCGCCTCCCAAGATGTAAAGAAC-3'; and reverse primer, 5'-CCAGAGACAGAACATCGTCAAGGC-3'. The resulting 150-bp PCR product was cloned into the BamHIEcoRI sites of pBluescript II SK (+) (Stratagene, La Jolla, CA). Riboprobes for mouse mMT-I mRNA were prepared from a Bluescript (KS) vector containing the entire coding region of mMT-I cDNA. The vector was a generous gift from Dr. Richard D. Palmier (4). RNA transcripts were synthesized from the DNA templates by an *in vitro* MAXIscript *In vitro* Transcription Kit (Ambion, Inc.).

Riboprobes were labeled with biotin by a nonisotopic labeling kit (BrightStar Psooralen-Biotin; Ambion, Inc.). *In situ* hybridization was carried out on formalin-fixed, paraffin-embedded 4-μm tissue sections by a commercially available kit (GenPoint; Dako Corp.). Briefly, tissue sections were deparaffinized, rehydrated, equilibrated, and then digested with 1 μg/ml proteinase K for 30 min at 37°C. After washing with Tris buffer, sections were hybridized with biotin-labeled riboprobes overnight at 37°C. Positive signal was visualized as a brown color from the use of a DAB chromogen as the substrate for Streptavidin-HRP. In initial studies, *in situ* hybridization mRNAlocator-Hyb and mRNAlocator-Biotin detection kits (Ambion, Inc.) were used. The signal was visualized as a blue/purple color by the NBT/5-bromo-4-chloro-3-indolyl phosphate substrate for Streptavidin-conjugated alkaline phosphatase. A shift to the GenPoint kit was made when Ambion, Inc., discontinued their mRNAlocator-Hyb kit. Developed slides were mounted with aqueous/dry CrystalMount (Biomeda, Foster City, CA). Hybridizations with sense riboprobes or in the absence of any ribo-probe served as controls.

**Immunohistochemistry.** Sections from formalin-fixed, paraffin-embedded biopsy samples were immunostained for pimonidazole binding and involucrin by techniques described previously (1, 6). Briefly, sections were deparaffinized, rehydrated, and incubated with 3.0% aqueous hydrogen peroxide for 5 min at room temperature to inactivate endogenous peroxidase. After antigen retrieval by Pronase digestion for 25 min at 40°C, the primary antibody for pimonidazole adducts (1:50) was applied for 40 min at room temperature. The primary antibody for involucrin (1:100) was applied for 40 min at room temperature on a contiguous slide without antigen retrieval. Biotin-conjugated F(ab')2 secondary antibody (1:500) was then applied for
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10 min at room temperature followed by incubation with streptavidin conjugated peroxidase for 10 min at room temperature. Positive signals were detected by incubation of the tissue sections with DAB chromogen for 10 min at room temperature. The sections were counterstained in Aqua Hematoxylin for 35 s and mounted with Crystal/Mount. As a negative control, immunostaining was carried out without primary antibodies. From each biopsy a section proximal to sections stained for involucrin expression and pimonidazole binding was stained with H&E and assessed for the presence of tumor.

Image Analysis. Quantitative image analysis of the immunostaining of pimonidazole adducts and involucrin was performed on contiguous sections. Color detection threshold and default width settings were chosen for the DAB chromogen on the basis of an immunostained region at ×200 magnification in tissue sections. The settings for the chromogen staining were optimized for intensity, saturation, and hue so that all cells that were identified as labeled above background by visual inspection were also recognized as labeled by image analysis software. Cells immunostained above threshold intensity were scored as labeled with no distinction being made between light and heavy immunostaining. In the case of the hypoxia marker, pimonidazole, this approach provides the number of tumor cells that are labeled. This is of most interest and obviates concerns about variations in marker binding intensity arising from individual differences in pharmacokinetics, tumor cell redox properties, time to biopsy, and chronic versus acute hypoxia. It was assumed that all cells labeled with pimonidazole adducts, irrespective of immunostaining intensity, were at pO₂ < 10 mm Hg for a significant time during the initial 5–6 h half-life of pimonidazole hydrochloride (1, 12, 13, 19). Multiple fields from each section from each biopsy were captured at ×200 magnification by an Axioskop 50 microscope (×10) and Fluor objective (×20; Carl Zeiss, Inc., Thornwood, NY) linked through a high resolution three-chip video camera (model ZVS-3C750E; Carl Zeiss, Inc.) to a high resolution Sony Trinitron RGB color monitor (model PVM 1343 MD) and workstation running Metamorph software (Universal Imaging Corp., West Chester, NY). Each tissue section was exhaustively analyzed, field by field. The number of fields varied depending on the size of the section, but on average, 67 fields/section were analyzed (range, 5–262). Mean percentage area immunostained in each field was calculated as a percentage of the total field area minus areas of acellularity, necrosis, and stroma.

Pattern Recognition. All 34 tumors were classified according to four basic patterns of immunostaining for involucrin and hypoxia (Fig. 1). In addition to pattern classification, the extent of immunostaining for involucrin and pimonidazole adducts was semiquantitatively assessed according to the extent of immunostaining for each marker: greatest extent (+ + +); low extent (+ +); and little or no staining (+). The scoring was done on whole tissue sections at low magnification and differed, therefore, from the semiquantitative, calibrated scoring system used in earlier studies to score hypoxia on a field-by-field basis at high magnification (20). Although the extent and intensity of immunostaining generally went hand-in-hand, the assessment was based on extent and not intensity of immunostaining for reasons discussed above. The extent of immunostaining for involucrin was, in general, greater than that for pimonidazole binding. Therefore, a score of (+ + +) for involucrin staining, for example, represents a larger area of immunostaining than the same score for pimonidazole binding. All biopsies from each tumor were used in arriving at pattern classification and extent of immunostaining for the two markers.

Qualitative pattern classification and semiquantitative scoring of the extent of immunostaining was done in a blinded fashion with respect to tumor grade. Each tumor was categorized with respect to immunostaining pattern and then matched to its tumor grade. Comparisons of immunostaining patterns and tumor grade are summarized in Tables 1 and 2. A single pathologist (R. A. L.) assessed grade in all 34 tumors at one time. These tumor grades were compared with grade assigned by a variety of pathologists at original diagnosis. A difference in tumor grade occurred for only 4 tumors: 1 tumor was upgraded from grade 1 to grade 2–3, and 3 tumors were upgraded from grade 2 to grade 3. These latter data were used in Tables 1 and 2 on the basis that internal consistency would be greatest for a single observer who assessed grade for all tumors at one sitting. The limited number of grade reassignments did not affect the overall conclusions of the study.

RESULTS

Image Analysis. At the cell level, immunostaining for pimonidazole was nuclear and cytoplasmic. At the tissue level, immunostaining was zonal, appearing toward the center of tumor cords and tumor nests. Pimonidazole staining appeared around areas of necrosis, but the necrotic areas themselves are not immunostained. Immunostaining was both light and heavy.

At the cell level, immunostaining for involucrin was cytoplasmic. At the tissue level, immunostaining could be categorized as zonal or diffuse. Zonal immunostaining tended to be in the center of tumor nests, whereas diffuse or irregular immunostaining was spread throughout tumor nests. Image analysis was carried out in a way that did not distinguish between zonal and diffuse immunostaining.

A subset of tumors from the first 20 patients entered in the study was subjected to quantitative image analysis for involucrin expression and pimonidazole binding. The data are summarized in Table 3 and plotted in Fig. 2. The area percentage of tumor regions that immunostained for involucrin ranged from 3.7 to 54.4% on a tumor-by-tumor basis. The areas labeled with pimonidazole binding were less extensive ranging from <0.1 to 14.4%. In each case, the values for area percentage immunostained were averaged over all biopsies from each tumor. There was no overall, quantitative correlation between pimonidazole binding and involucrin expression (Fig. 2), and for this reason, image analysis was halted after the first 20 tumors had been analyzed. The lack of correlation was because of heterogeneous staining patterns for involucrin. An examination of these heterogeneous patterns of immunostaining was carried out.

Pattern Recognition. Four immunostaining patterns were observed (Fig. 1). In pattern I, involucrin is strongly expressed but little or no pimonidazole binding is observed (Fig. 1, A and B).

In pattern II, extensive colocalization of involucrin expression and pimonidazole binding occurs, but it is mixed with...
diffuse involucrin expression unrelated to pimonidazole binding (Fig. 1, C and D). In pattern III, strong, colocalized immunostaining predominates (Fig. 1, E and F). Patterns II and III are similar, but pattern III lacks the diffuse involucrin expression seen in pattern II. In those microregions where colocalization occurs, the edge of immunostaining for involucrin generally extends beyond the edge of immunostaining for pimonidazole binding.

Fig. 1 Photomicrographs of immunostaining for involucrin expression (A, C, E, and G) and pimonidazole binding (B, D, F, and H) on contiguous sections from formalin-fixed, paraffin-embedded biopsies from squamous cell carcinomas of the uterine cervix. Four immunostaining patterns were observed. Pattern I (A and B); pattern II (C and D); pattern III (E and F); and pattern IV (G and H). Pattern I (A and B) shows diffuse involucrin staining in a well-differentiated tumor (grade 1) in which very little pimonidazole binding is evident. Pattern II (C and D) shows combined focal and diffuse involucrin expression in a moderately differentiated tumor (grade 2). Focal involucrin expression colocalizes with pimonidazole binding but diffuse involucrin immunostaining is largely unrelated to pimonidazole binding. Pattern III (E and F) shows focal involucrin immunostaining in a moderately differentiated tumor (grade 2) that colocalizes with pimonidazole binding. Pattern IV (G and H) shows the absence of involucrin in the presence of extensive pimonidazole binding in a poorly differentiated tumor (grade 3). Original magnification, ×25.
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In pattern IV, immunostaining for pimonidazole occurs with little or no immunostaining for involucrin (Fig. 1, G and H). Quite remarkably, in only 1 tumor of 34 did the pattern of immunostaining differ from biopsy to biopsy. The exceptional, grade 3 tumor unexpectedly expressed high levels of involucrin in all four biopsies and exhibited immunostaining patterns I and II that were generally associated with well-differentiated tumors. For simplicity of presentation, data for the exceptional tumor are not included in Tables 1 and 2.

Tumor Grade and Patterns of Immunostaining. In terms of tumor grade, well-differentiated tumors tended to express Pattern I where 4 of 6 grade 1 and grade 1–2 tumors showed this pattern. Pattern I was observed in one Grade III tumor, but as expected, involucrin expression was low.

Intermediate grade tumors (grade 2 and grade 2–3) tended to express extensive colocalization of immunostaining where 10 of 18 tumors showed either pattern II or III.

Poorly differentiated tumors tended to possess pattern IV with 5 of 9 grade 3 tumors showing this pattern. Only 1 of 17 grade 1, grade 1–2, and grade 2 tumors possessed pattern IV.

The data in Table 2 show the general trend from pattern I through patterns II and III to pattern IV as tumor grade increases.

Tumor Grade and Extent of Immunostaining. Trends linking the extent of immunostaining to pathological grade were observed (Tables 1 and 2). Decreasing involucrin staining was generally associated with increasing grade. In particular, (+/−) and (−) levels of involucrin expression were seen only in grade 2–3 and grade 3 tumors. On the other hand, a subset of grade 2–3 and grade 3 tumors (7 of 16 tumors) unexpectedly showed extensive involucrin expression.

In the case of pimonidazole binding, there was a trend to less binding in well-differentiated tumors. For example, 8 of 17 grade 1, grade 1–2, and grade 2 tumors showed (+/−) pimonidazole binding, whereas only 1 of 16 grade 2–3 and grade 3 tumors showed (+/−) pimonidazole binding.

Although various trends for immunostaining patterns were noted, sample sizes were considered to be too small for formal statistical analyses.

In situ Hybridization Analysis of hMT-IIa Expression. In situ hybridization analysis for hMT-IIa mRNA expression in the stratified epithelia of normal human tongue shows this stress-inducible isoform is constrained to basal lamina (Fig. 3B). This pattern was also observed for normal epithelium of the uterine cervix (Fig. 3C). Both patterns were similar to that for mMT-I mRNA in normal mouse tongue (Fig. 3A; Ref. 4). Visual examination of the hMT-IIa RNA antisense riboprobe in tissue sections of squamous cell carcinomas revealed that hMT-IIa mRNA tended to be in the outer rim of tumor where the corresponding protein is expressed (1). However, the staining for hMT-IIa mRNA was weaker than that in normal epithelia, and it proved impossible to capture the staining for reproduction as a photomicrograph.

DISCUSSION

Differentiation and Stress-Induced Gene Expression. The pattern for hMT-IIa mRNA expression in human epithelia is similar to that for mMT-I mRNA in mouse epithelia (Fig. 3). In both cases, MT mRNA expression is limited to well-oxygenated basal lamina where cells are at the earliest stages of epithelial differentiation. The absence of hMT-IIa mRNA from the upper regions of human tongue and uterine cervix epithelia is consistent with the conclusion by Quaife et al. (4) that stress-inducible isoforms of MT are down-regulated by differentiation in stratified epithelia. The presence of MT-I and MT-II protein in well-oxygenated, proliferating rims of tumor nests and their general absence from hypoxic, involucrin positive regions support the notion that differentiation also regulates MT transcriptional status in squamous cell carcinomas (1). hMT-IIa mRNA expression shows a pattern similar to that for MT protein in squamous cell carcinomas, but the data are less compelling because of the weak in situ hybridization signal in the tumor tissue.

The notion that differentiation alters the transcriptional status of stress-induced genes is not limited to MT. Viac et al. (21) reported that immunostaining for VEGF, as with MT, predominates in the proliferating basal layers of human stratified epithelia and concluded that differentiation alters the transcriptional status of VEGF. In vitro studies showed that differentiation down-regulates VEGF 121 and VEGF 165 expression in keratinocytes (21). This is consistent with the lack of VEGF

<table>
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<tr>
<th>Table 1</th>
<th>Comparison of immunostaining patterns for involucrin and pimonidazole with tumor grade in 33 squamous cell carcinomas</th>
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<tbody>
<tr>
<td></td>
<td>Involucrin</td>
</tr>
<tr>
<td>Grade 1</td>
<td>+++</td>
</tr>
<tr>
<td>Grade 1–2</td>
<td>+++</td>
</tr>
<tr>
<td>Grade 2</td>
<td>+++</td>
</tr>
<tr>
<td>Grade 2–3</td>
<td>+++</td>
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<td>Grade 3</td>
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<table>
<thead>
<tr>
<th>Table 2</th>
<th>Summary of the relationship between tumor grade and immunostaining patterns for involucrin and pimonidazole binding</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pattern</td>
</tr>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Grade 1</td>
<td>2</td>
</tr>
<tr>
<td>Grade 1–2</td>
<td>2</td>
</tr>
<tr>
<td>Grade 2</td>
<td>4</td>
</tr>
<tr>
<td>Grade 2–3</td>
<td>0</td>
</tr>
<tr>
<td>Grade 3</td>
<td>1</td>
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</table>

* A general trend of change from pattern I through patterns II and III to pattern IV is observed as tumor grade increases.
expression in the majority of hypoxic cells in squamous cell
carcinomas that are, in general, involucrin positive (22). Cell-
associated VEGF 189 expression is up-regulated by differenti-
ation, but its concentration is 20 times lower than that for VEGF
121 and VEGF 165 (21), and it is possible that any increase in
VEGF 189 because of hypoxia induction would not be detected
in human tumors (22).

Patterns of Involucrin Expression. The involucrin data
are consistent with earlier reports showing that the extent of in-
volucri n expression is associated with tumor grade. For example,
Serra et al. (23) found that immunostaining for involucrin was
absent from 71% of poorly differentiated tumors and present in
75% of well-differentiated tumors. However, the more complex
relationship among tumor grade, involucrin expression, and hy-
poxia reported here is novel (Fig. 1; Tables 1 and 2).

Pattern I predominates in well-differentiated tumors where
strong involucrin immunostaining occurs near blood vessels. In
this pattern, involucrin can be extensively expressed with little
or no pimonidazole binding (Fig. 1, A and B). In fact, the overall
level of pimonidazole binding tends to be low in well-differen-
tiated tumors (Table 2). Involucrin expression in the absence
of pimonidazole binding would appear to be inconsistent with
oxygen-regulation, but a number of biological processes are
half-maximally induced at oxygen concentrations that would

Table 3 Summary of data for cervix patients organized according to ascending order of percent area hypoxia ± SE

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Patient (no. of biopsies)</th>
<th>Stage</th>
<th>Grade</th>
<th>Hypoxia % area (range)</th>
<th>Involucrin % area (range)</th>
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<tbody>
<tr>
<td>1</td>
<td>CX014 (2)</td>
<td>IIIB</td>
<td>1–2</td>
<td>&lt;0.1</td>
<td>12.4 ± 8.5 (3.9–20.9)</td>
</tr>
<tr>
<td>2</td>
<td>CX020 (3)</td>
<td>IIIB</td>
<td>1–2</td>
<td>&lt;0.1</td>
<td>40.9 ± 15.9 (9.7–61.8)</td>
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<tr>
<td>3</td>
<td>CX060 (1)</td>
<td>IB2</td>
<td>2</td>
<td>0.34</td>
<td>50.9</td>
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<tr>
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<td>CX004 (6)</td>
<td>IIIB</td>
<td>2</td>
<td>0.7 ± 0.3 (0–1.5)</td>
<td>12.9 ± 2.7 (4.0–20.5)</td>
</tr>
<tr>
<td>5</td>
<td>CX015 (1)</td>
<td>IB</td>
<td>2</td>
<td>0.8</td>
<td>40.1</td>
</tr>
<tr>
<td>6</td>
<td>CX002 (4)</td>
<td>IB</td>
<td>2</td>
<td>0.9 ± 0.4 (0–1.5)</td>
<td>46.0 ± 7.4 (32.2–59.8)</td>
</tr>
<tr>
<td>7</td>
<td>CX001 (3)</td>
<td>IIIB</td>
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<td>0.9 ± 0.6 (0–2.4)</td>
<td>13.2 ± 4.6 (8.1–22.4)</td>
</tr>
<tr>
<td>8</td>
<td>CX012 (4)</td>
<td>IIIB</td>
<td>2–3</td>
<td>1.5 ± 0.9 (0–3.1)</td>
<td>36.6 ± 4.1 (26.1–43.8)</td>
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<td>9</td>
<td>CX013 (4)</td>
<td>IIIB</td>
<td>1</td>
<td>1.9 ± 0.4 (0.7–2.5)</td>
<td>38.7 ± 2.7 (34.1–44.7)</td>
</tr>
<tr>
<td>10</td>
<td>CX028 (5)</td>
<td>IIIB</td>
<td>1–2</td>
<td>3.7 ± 0.4 (2.4–4.9)</td>
<td>25.9 ± 2.6 (20.7–35.8)</td>
</tr>
<tr>
<td>11</td>
<td>CX045 (6)</td>
<td>IIIB</td>
<td>2</td>
<td>4.2 ± 1.1 (0.8–8.2)</td>
<td>7.3 ± 2.8 (3.2–21.2)</td>
</tr>
<tr>
<td>12</td>
<td>CX003 (4)</td>
<td>IIIB</td>
<td>2</td>
<td>4.5 ± 1.0 (1.7–8.9)</td>
<td>14.8 ± 6.0 (6.0–31.9)</td>
</tr>
<tr>
<td>13</td>
<td>CX054 (4)</td>
<td>IIIB</td>
<td>3</td>
<td>4.7 ± 2.0 (0.8–9.9)</td>
<td>38.4 ± 3.5 (33.8–48.6)</td>
</tr>
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<td>14</td>
<td>CX046 (4)</td>
<td>IIIB</td>
<td>2</td>
<td>5.2 ± 1.1 (2.6–8.2)</td>
<td>39.4 ± 6.3 (27.3–57.0)</td>
</tr>
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<td>15</td>
<td>CX005 (4)</td>
<td>IIIB</td>
<td>1–2</td>
<td>5.7 ± 2.4 (2.1–12.9)</td>
<td>54.4 ± 5.8 (42.5–68.5)</td>
</tr>
<tr>
<td>16</td>
<td>CX011 (3)</td>
<td>IIIB</td>
<td>2–3</td>
<td>6.3 ± 1.3 (3.8–8.3)</td>
<td>3.7 ± 1.0 (2.7–5.7)</td>
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<tr>
<td>17</td>
<td>CX056 (4)</td>
<td>IIIB</td>
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<td>6.4 ± 2.2 (1.6–10.9)</td>
<td>25.4 ± 5.5 (18.1–41.6)</td>
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<tr>
<td>18</td>
<td>CX039 (7)</td>
<td>IIIB</td>
<td>3</td>
<td>8.7 ± 0.9 (5.7–12.8)</td>
<td>5.2 ± 1.0 (1.8–8.5)</td>
</tr>
<tr>
<td>19</td>
<td>CX055 (2)</td>
<td>IIIB</td>
<td>2–3</td>
<td>12.1 ± 0.1 (11.9–12.2)</td>
<td>51.7 ± 1.4 (50.2–53.1)</td>
</tr>
<tr>
<td>20</td>
<td>CX030 (4)</td>
<td>IIIB</td>
<td>2</td>
<td>14.4 ± 3.3 (4.3–20.4)</td>
<td>35.0 ± 3.5 (9.8–47.2)</td>
</tr>
</tbody>
</table>

Fig. 2 Quantitative comparison between involucri n expression and pimonidazole adducts in squa-
mous cell carcinomas of the uterine cervix. Data represent mean values ± SE. The absence of error
bars indicates tumors for which fewer than three biopsies were available. The data are presented in
terms of increasing hypoxia from left to right. Tumor numbers refer to tumors described in Table 3.
strongly inhibit hypoxia marker binding. If involucrin induction had a $K_m$ similar to that for VEGF (0.8–2.2%; Refs. 24, 25), for example, it could be induced in microregions where the binding of hypoxia markers such as pimonidazole would be strongly inhibited ($K_m = 0.2–0.4%$; Ref. 11). A similar line of reasoning was followed in accounting for the extent of CA9 immunostaining exceeding that for pimonidazole binding in cervix and head and neck carcinomas (7, 8). If this is the correct explanation for pattern I, then wide areas of tumor exist at intermediate levels of hypoxia implying that gradients of oxygen in well-differentiated tumors are relatively shallow. This might also account for the relatively low levels of pimonidazole binding in well-differentiated tumors.

Patterns II and III occur primarily in moderately differentiated tumors and are characterized by strong colocalized immunostaining for involucrin and pimonidazole binding (Fig. 1, C and D, and E and F, respectively). Pattern II is distinguished by strong colocalized involucrin and pimonidazole staining mixed with diffuse involucrin expression that is not matched by pimonidazole binding. Colocalization of involucrin and pimonidazole staining in patterns II and III is generally similar to that reported for CA9 and Glut-1 expression and pimonidazole binding in squamous cell carcinomas (7, 8, 17).

Pattern IV occurs in poorly differentiated tumors where little or no involucrin is seen even in regions of extensive pimonidazole binding (Fig. 1, G and H). As was the case for
pattern I, pattern IV does not appear to be consistent with oxygen regulation. One possible interpretation is that involucrin transcriptional status is suppressed in poorly differentiated cells to the extent that it is not available for hypoxia induction.

The heterogeneity of expression observed for involucrin is a potential limitation to the use of endogenous hypoxia markers in general. For example, although 60 of 92 oropharyngeal carcinomas showed focal patterns for HIF-1α immunostaining generally consistent with expected locations of tumor hypoxia, diffuse patterns of HIF-1α expression unrelated to tumor hypoxia predominated in 32 of 92 tumors. The investigators concluded that alternative regulatory pathways must override hypoxic regulation of HIF-1α in a significant number of cases (26). Janssen et al. (16) found a low degree of colocalization between HIF-1α expression and pimonidazole binding in a wider range of head and neck tumors and concluded that HIF-1α was a poor marker for chronic hypoxia. A similar conclusion was reached for squamous cell carcinomas of the uterine cervix where the majority of HIF-1α patterns was not associated with expected regions of hypoxia (27). In renal cell carcinomas, HIF-1α immunostaining patterns were, for the most part, diffuse and seemingly unrelated to hypoxia possibly because of functional inactivation of the von Hippel-Lindau gene in many of these tumors (28). Olive et al. (8) found that immunostaining for CA9 could be correlated with pimonidazole binding in cervix carcinomas, but the correlation depended on data for a small number of tumors, and Airley et al. (17) were unable to confirm a correlation. A high correlation between CA9 expression and pimonidazole binding was observed in head and neck carcinomas, but only after the data had been corrected by a factor for microregional distribution (7). A statistically significant but weak correlation between Glut-1 expression and pimonidazole binding has been reported (17). Overall, heterogeneity of expression characterizes the expression of endogenous hypoxia markers, and it appears that this will compromise their utility.

**Differentiation and Prognosis.** Hypoxia has been reported to inhibit differentiation in some cell lines (29, 30), and this could conceivably contribute to tumor aggressiveness. However, hypoxia does not appear to completely inhibit differentiation in squamous cells given that involucrin is strongly expressed in the majority of hypoxic cells in squamous cell carcinomas. It is important to emphasize, however, that involucrin is initially expressed at intermediate stages of differentiation and tumor hypoxia, although not totally inhibiting differentiation, might arrest it. For example, Auersperg et al. (31) found that cell lines derived from a poorly differentiated squamous cell carcinoma were arrested at different stages of differentiation, and importantly, proliferation was not precluded for cells that expressed involucrin. Although Auersperg et al. (31) did not invoke hypoxia, it is conceivable that cells arrested by hypoxia at a stage of differentiation where they express involucrin but retain proliferative capacity might contribute to tumor aggressiveness.

With respect to effects on therapy, it is known that differentiation increases radiosensitivity in human carcinoma cells under hypoxic conditions (32). This might be attributable to inhibited DNA repair arising from the limited access of DNA repair machinery in differentiated cells (33). To the extent that the in vitro results can be extrapolated to human tumors, the present study raises the possibility that the radiation resistance of hypoxic cells in well-differentiated and moderately differentiated tumors will be decreased relative to poorly differentiated tumors where hypoxic cells show little signs of differentiation (Table 1).

As a measure of differentiation, traditional grading of tumors is of limited prognostic value for cervix carcinomas. However, a number of investigators have observed that the details of microregional patterns of growth and differentiation might have prognostic value in gynecological malignancies (31, 34–36). Of particular interest with respect to cervix carcinomas, Okada et al. (36) have reported that tumors possessing nests of keratinizing cells are more radiosensitive than tumors that lack keratinization or glandular differentiation. The time from treatment to local recurrence is significantly longer for tumors with keratinizing cells (P < 0.0001). In multivariate analyses, the absence of histological signs of squamous differentiation was confirmed as an independent predictor of radioresistance (P = 0.0072). This is consistent with the in vitro data discussed previously but does not address the generally accepted notion that hypoxic tumors have poor prognosis irrespective of tumor grade (37). Fyles et al. (38) have reported that poor prognosis for hypoxic cervix tumors might be related to distant metastases than pelvic failure in node-negative patients who receive radiation treatment. This is compatible with tumor hypoxia as a negative prognostic factor for cervix patients treated with surgery alone (37) and with the Okada et al. finding that local control in irradiated cervix cancer patients can be related to tumor growth and differentiation without reference to hypoxia (36).

**Summary.** There is a strong association between hypoxia and involucrin expression in moderately differentiated squamous cell carcinomas. However, this strong association is not generally observed in either low-grade or high-grade tumors, and overall correlation between hypoxia and involucrin expression was not observed. This makes involucrin of limited value as a marker for tumor hypoxia. In principle, differentiation of hypoxic cells in squamous cell carcinomas might make them less resistant to radiation therapy than indicated by their hypoxic status alone.

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Hypoxia and Differentiation in Squamous Cell Carcinomas of the Uterine Cervix: Pimonidazole and Involucrin

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