A Clinical Study of Hypoxia and Metallothionein Protein Expression in Squamous Cell Carcinomas

James A. Raleigh, Shu-Chuan Chou, Dennise P. Calkins-Adams, Cynthia A. Ballenger, Debra B. Novotny, and Mahesh A. Varia

Departments of Radiation Oncology [J. A. R., S-C. C., D. P. C-A., C. A. B., M. A. V.] and Pathology [D. B. N.], University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

ABSTRACT

The objective was to discover whether the oxygen-regulated protein, metallothionein, is expressed in the hypoxic cells of squamous cell carcinomas. Twenty patients with squamous cell carcinoma of the uterine cervix or head and neck were infused with a solution of the hypoxia marker, pimonidazole hydrochloride, at a dose of 0.5 g/m². The following day, biopsies were collected, formalin fixed, paraffin embedded, and sectioned at 4 μm. Sections from each biopsy were immunostained for pimonidazole binding, metallothioneins I and II, involucrin, and proliferating cell nuclear antigen. A total of 84 biopsies were analyzed. Sixty-four of 84 biopsy sections contained hypoxia. Of the hypoxia-containing sections, 43 of 64 or 67% showed no microregional overlap between hypoxia and metallothionein; 7 of 64 showed overlap; and 14 of 64 showed a combination of overlap and no overlap. On a tumor-by-tumor basis, 5 of 7 head and neck and 7 of 13 cervix tumors showed no overlap between metallothionein and hypoxia at the microregional level. Ranges for the percentage of the area of hypoxia in head and neck (<0.9 to 17%) and cervix (<0.1 to 14%) tumors were similar. In the hypoxia-containing sections, immunostaining for involucrin, a molecular marker for differentiation, overlapped with that for hypoxia in 82% of the cases. The majority of hypoxic cells in squamous cell carcinomas do not express metallothionein protein, although metallothionein is induced by hypoxia in human tumor cells in vitro. Hypoxic cells in human tumors tend to be in regions immunostaining for involucrin, and it seems possible that differentiation of hypoxic cells in squamous cell carcinomas might affect metallothionein I and II expression.

INTRODUCTION

Human tumor hypoxia is associated with poor prognosis independent of therapy modality for squamous cell carcinomas and soft tissue sarcomas (1, 2). It has been suggested (3) that oxygen-regulated proteins (4) might be involved. In support of this hypothesis, proteins such as VEGF (5) are known to be induced by hypoxia (5) and to be associated with poor prognoses (6, 7). In human gliomas, VEGF mRNA is expressed in the vicinity of necrosis (8, 9) and immunostaining for HIF-1 is intense in pseudopalisading tumor cells surrounding areas of necrosis (10). From these microregional distributions, it was deduced that VEGF mRNA and HIF-1 are induced by hypoxia in human gliomas. In contrast, a clinical study with the hypoxia marker, pimonidazole, showed that VEGF protein, although present in tumors, was not expressed in the hypoxic cells of squamous cell carcinomas (11). This finding was unexpected on the basis of in vitro glioma data and has led us to test the result in squamous cell carcinomas by examining a second oxygen-regulated protein, MT, which is regulated by a hypoxia-sensitive transcription factor very different from that for VEGF.

MTs are a family of M, 6000 proteins comprised of MT-I, MT-II, MT-III, and MT-IV classes with multiple isoforms within each class (12–15). MT-I and MT-II are ubiquitously expressed and are stress inducible (13, 15). MT-I isoform inducibility is reported to depend on the embryonic germ layer from which a tumor is derived. For example, tumors originating from intermediate mesoderm, such as cervical carcinomas, exhibit MT-II and MT-II genes in an inducible form, whereas the MT-Ic gene is refractory to induction. MT-II inducibility is associated with poor prognosis, whereas MT-Ic inducibility is associated with better prognostic features in human tumors (13). MTs regulate intracellular concentrations of zinc and other metal ions. MT overexpression, therefore, can influence transcription, replication, and protein synthesis and might explain why MT overexpression is associated with high-grade tumors (16–20), including carcinomas of the head and neck (21).

Murphy et al. (22, 23) have shown that MT-I and MT-II can be induced by hypoxia and that induction in vitro is regulated by metal transcription factor-1. The possibility that the effects of tumor hypoxia are mediated by MT is supported by the report that MT is induced by hypoxia in experimental tumors (24) and that MT is overexpressed in human tumors possessing low median PO₂ (25). However, it was not known from the clinical study whether MT was induced by hypoxia. This is an important point because MT can be regulated by both hypoxia and proliferation (26–28). Under these circumstances, a technique for discriminating hypoxic from proliferating cells was not possible.

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2 To whom requests for reprints should be addressed, at Department of Radiation Oncology, University of North Carolina School of Medicine, CB# 7512, Chapel Hill, NC 27599. Phone: (919) 966-7710; Fax: (919) 966-7681; E-mail: raleigh@radonc.unc.edu.

3 The abbreviations used are: VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; MT, metallothionein; PCNA, proliferating cell nuclear antigen.
needed. The immunohistological hypoxia marker approach (29), based on pimonidazole hydrochloride combined with proliferating cell nuclear antigen as a histological marker for proliferation (11, 30–33), was considered ideal for an investigation of the relationship between hypoxia and MT expression in human tumors.

MATERIALS AND METHODS

**Immunological Reagents.** A supernatant from hybridoma clone 4.3.11.3 containing an anti-pimonidazole IgG1 monoclonal antibody was used to detect protein adducts of reductively activated pimonidazole (30). A protein blocker, liquid 3,3′-diaminobenzidine (DAB), an IgG1 mouse monoclonal antibody (clone E9) that recognizes MT-I/II, a mouse antihuman IgG2a monoclonal antibody (clone PC10) that recognizes PCNA, peroxidase-conjugated streptavidin, and goat antimouse immunoglobulins conjugated to peroxidase-labeled dextran polymer were obtained from DAKO Corp. (Carpinteria, CA). A biotin-conjugated F(ab′)2 fragment of a rabbit antimouse IgG was obtained from Accurate Chemical Scientific Corp. (Westbury, NY). An IgG1 mouse antihuman involucrin antibody clone SY5 was obtained from Sigma Chemical Co. (St. Louis, MO). Neutral buffered 10% formalin, Biomedia Pronase, enzyme grade polyoxyethylene (23) lauryl ether (Brij 35), Biomedia Crystal/Mount, ProbeOn Plus glass slides, and miscellaneous reagent-grade chemicals were obtained from Fisher Scientific Company (Norcross, GA). Clear-Rite 3, a nontoxic alternative to xylene, and antigen retrieval was achieved by incubating tissue sections with 0.01% Pronase for 25 min at 40°C in the case of cervix tumors and for 40 min at 40°C in the case of head and neck tumors. Immunohistochemical staining for pimonidazole adducts was achieved with a biotin-conjugated F(ab′)2 secondary antibody reagent. The F(ab′)2 strategy has become standard procedure in our laboratory because it provides low background and good cross species applicability.

Tumor tissue sections were immunostained for MT-I/II in a manner similar to that used for pimonidazole adducts. A 40°C Pronase antigen retrieval step was included, but the incubation time with Pronase was shortened to 20 min for both cervix and head and neck tumors. According to the manufacturer’s literature, the commercially available IgG1 mouse antihorse MT antibody (clone E9; used in 1:50 dilution) was raised against horse, self-polymerized MT-I and MT-II. The antibody binds to the 5-amino acid moietiy (AcMet-Asp-Pro-Asn-Cys-) at the end of the NH2 terminus of the B domain of equine and human MT-I and MT-II (37). The anti-MT antibody does not distinguish between MT-I and MT-II, and therefore, the designation MT-I/II is used to characterize immunostaining for MT in the present report. The NH2 terminus of MT-I (AcMet-Asp-Pro-Gly-Glu-Cys-) is essentially identical to that of MT-I/II, and it has been reported that MT-I/II antibodies do not cross-react with MT-IV (13). MT-III expression is restricted to brain tissue (12) and was not a concern for the present study.

**Immunohistochemistry.** For illustrative purposes, immunostaining for the different factors was performed on four contiguous sections from a single biopsy (Fig. 1). However, for the qualitative and quantitative analyses in Tables 1 and 2, two sets of two contiguous sections from each biopsy were prepared in which one section in each set was immunostained for pimonidazole adducts, and the second section in each set was immunostained for either MT-I/II or involucrin. There were 84 sets of contiguous sections for MT and hypoxia analysis and 83 evaluable sets for involucrin and hypoxia analysis.

The immunostaining procedure for pimonidazole adducts was essentially identical to that reported previously (36), except that tissue sections were deparaffinized with Clear-Rite 3, except that tissue sections were deparaffinized with Clear-Rite 3, a nontoxic alternative to xylene, and antigen retrieval was achieved by incubating tissue sections with 0.01% Pronase for 25 min at 40°C in the case of cervix tumors and for 40 min at 40°C in the case of head and neck tumors. Immunohistochemical staining for pimonidazole adducts was achieved with a biotin-conjugated F(ab′)2 secondary antibody reagent. The F(ab′)2 strategy has become standard procedure in our laboratory because it provides low background and good cross species applicability.

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**Immunostaining for PCNA was performed in a manner similar to that for pimonidazole adducts, except that Pronase digestion was omitted and goat antimouse immunoglobulin conjugated to a peroxidase-labeled dextran polymer (DAKO EnVision+ reagent) was used as the secondary reagent. PCNA is a Mr 36,000 nonhistone nuclear protein, the expression of which is associated with late G1, S, and early G2 phases of the cell cycle. It is an auxiliary protein to DNA polymerase γ and plays a critical role in the initiation of cell proliferation. PCNA expression has been correlated with bromodeoxyuridine uptake in human tumors, and the characteristics of PCNA immunostaining can be used to identify S-phase cells in tissue sections (33). According to the manufacturer’s literature, the IgG2a monoclonal anti-PCNA antibody (used in a 1:100 dilution) was raised against rat PCNA made in the protein A expression vector pRIT2T.

**Immunostaining for involucrin was carried out in a manner
similar to that for pimonidazole adducts, except that Pronase antigen retrieval was omitted from the immunostaining procedure. Involucrin is a cytoplasmic Mr 92,000 protein that is cross-linked to other proteins by the action of transglutaminase during terminal differentiation of keratinocytes (38). According to the manufacturer’s literature, the IgG1 mouse antihuman involucrin antibody clone SY5 (used in a 1:100 dilution) was raised against purified human involucrin.

Negative controls, in which primary antibodies were omitted from the protocols, showed no nonspecific binding attributable to secondary antibody reagents. Positive controls containing pimonidazole adducts, MT-I/II, PCNA, and involucrin showed staining patterns and intensities that were constant from staining session to staining session. Samples of normal human tongue served as a positive control for involucrin.

### Qualitative Analysis

Microregional comparisons of hypoxia with MT and involucrin expression were carried out on a section-by-section basis without respect to whether the squamous cell carcinoma occurred in the cervix or head and neck on the assumption that MT induction is independent of tumor site. Tissue sections from the biopsies, one per biopsy, were qualitatively assessed for hypoxia, MT-I/II, and involucrin expression. Each section was viewed at ×100, and individual microscopic fields (190 × 190 μm) were assessed with respect to: (a) no overlap; (b) overlap; or (c) a mix of overlap and no overlap with immunostaining for pimonidazole adducts.

### Quantitative Analysis

Quantitative image analysis was carried out for pimonidazole adducts and MT-I/II. Color detec-

![Fig. 1](image)

**Table 1** Qualitative comparison of overlap between microregional immunostaining for MT and hypoxia and for involucrin and hypoxia in all tissue sections possessing hypoxia but without respect to tumor of origin.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>% overlap</th>
<th>% mixed overlap and no overlap</th>
<th>% no overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT + hypoxia</td>
<td>11 (8)</td>
<td>22 (17)</td>
<td>67 (75)</td>
</tr>
<tr>
<td>Involucrin + hypoxia</td>
<td>82 (71)</td>
<td>14 (12)</td>
<td>4 (17)</td>
</tr>
</tbody>
</table>

*Comparisons based on all tissue sections, including those lacking hypoxia, are given in parentheses.*
Hypoxia and Metallothionein adducts irrespective of immunostaining intensity were at pO2 in pharmacokinetics, tumor cell redox properties, and time to provides the number of tumor cells that are labeled, which is of between light and heavy immunostaining. This approach pro-
tensity were scored as labeled, with no distinction being made
analysis software. Cells immunostained above a threshold in-
background by visual inspection were also scored by the image
 hue so that all cells that were identified as labeled above
chromogen staining were optimized for intensity, saturation, and 

**RESULTS**

**Qualitative Analysis.** The photomicrographs in Fig. 1 are representative of immunostaining patterns for pimonidazole adducts (Fig. 1A), MT-I/II (Fig. 1B), PCNA (Fig. 1C), and involucrin (Fig. 1D). A comparison of A and B shows little or no overlap between hypoxia and MT-I/II. This is most clearly seen in the upper right of the photomicrographs, where staining for pimonidazole adducts in A is in a zone separate from that for MT-I/II staining in B. The arrows in the photomicrographs identify the periphery of the tumor nest and help in comparing immunostaining patterns. A comparison of B and C in Fig. 1 reveals that MT-I/II is expressed primarily in cells in the pe-
riphery of the tumor nests that also stain for S-phase PCNA.

A comparison of A and C in Fig. 1 shows that there is little or no overlap between cells that are stained for S-phase PCNA and those stained for pimonidazole adducts. This is consistent with previous studies in which immunostaining for hypoxia markers and PCNA have been compared (32, 33). A comparison of A and D in Fig. 1 shows that involucrin staining overlaps with that for pimonidazole adducts to a remarkable extent.

Of the total number of 84 sets of tissue sections used for the analysis of hypoxia and MT expression, 64 were found to contain hypoxia. Of these, 43 of 64 (67%) possessed little or no overlap between hypoxia and MT. That is, these sections had
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<table>
<thead>
<tr>
<th>Tumor</th>
<th>Patient (no. of biopsies)</th>
<th>Stage (grade)</th>
<th>Grade</th>
<th>Hypoxia % area (range)*</th>
<th>MT % area (range)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CX14 (2)</td>
<td>IIIB</td>
<td>1–2</td>
<td>&lt;0.1</td>
<td>3.3 (1.0–5.5)</td>
</tr>
<tr>
<td>2</td>
<td>CX20 (4)</td>
<td>IIIB</td>
<td>1–2</td>
<td>&lt;0.1</td>
<td>5.8 ± 2.1 (0.9–11.2)</td>
</tr>
<tr>
<td>3</td>
<td>CX4 (6)</td>
<td>IIIB</td>
<td>2</td>
<td>0.7 ± 0.3 (0.0–1.5)</td>
<td>29.3 ± 2.3 (22.6–35.6)</td>
</tr>
<tr>
<td>4</td>
<td>CX15 (1)</td>
<td>IIIB</td>
<td>2</td>
<td>0.8</td>
<td>1.8 (1.4–2.1)</td>
</tr>
<tr>
<td>5</td>
<td>HN6 (2)</td>
<td>T,N2,M0</td>
<td>2</td>
<td>0.9 ± 0.4 (0–1.5)</td>
<td>2.7 ± 1.2 (0.4–5.8)</td>
</tr>
<tr>
<td>6</td>
<td>CX2 (4)</td>
<td>IIIB</td>
<td>2</td>
<td>0.9 ± 0.6 (0–2.4)</td>
<td>17.0 ± 5.0 (4.4–28.8)</td>
</tr>
<tr>
<td>7</td>
<td>CX1 (4)</td>
<td>IIIB</td>
<td>3</td>
<td>1.5 ± 0.9 (0–3.1)</td>
<td>17.7 ± 3.4 (10.1–24.6)</td>
</tr>
<tr>
<td>8</td>
<td>CX12 (4)</td>
<td>IIIB</td>
<td>2–3</td>
<td>1.9 ± 0.4 (0.7–2.5)</td>
<td>28.6 ± 4.6 (15.0–35.6)</td>
</tr>
<tr>
<td>9</td>
<td>CX13 (4)</td>
<td>IIIB</td>
<td>2</td>
<td>1.9 ± 0.4 (0.7–2.5)</td>
<td>28.6 ± 4.6 (15.0–35.6)</td>
</tr>
<tr>
<td>10</td>
<td>HN25 (9)</td>
<td>T,N2,M0</td>
<td>2</td>
<td>2.3 ± 0.5 (1.1–4.8)</td>
<td>&lt;0.1 (0.0–0.25)</td>
</tr>
<tr>
<td>13</td>
<td>HN14 (6)</td>
<td>T,N2,M0</td>
<td>2</td>
<td>3.1 ± 1.1 (0.7–5.7)</td>
<td>19.1 ± 1.2 (16.3–22.1)</td>
</tr>
<tr>
<td>12</td>
<td>CX28 (5)</td>
<td>IIIB</td>
<td>2–3</td>
<td>3.7 ± 0.4 (2.4–4.9)</td>
<td>22.8 ± 2.6 (12.9–27.6)</td>
</tr>
<tr>
<td>13</td>
<td>CX3 (6)</td>
<td>IIIB</td>
<td>2</td>
<td>4.5 ± 1.0 (1.7–8.9)</td>
<td>36.6 ± 2.7 (29.8–45.2)</td>
</tr>
<tr>
<td>14</td>
<td>HN17 (4)</td>
<td>T,N2,M0</td>
<td>1–2</td>
<td>4.8 ± 1.8 (2.6–10.0)</td>
<td>10.5 ± 2.9 (0.0–17.0)</td>
</tr>
<tr>
<td>15</td>
<td>CX5 (4)</td>
<td>IIIB</td>
<td>1–2</td>
<td>5.7 ± 2.4 (2.1–12.9)</td>
<td>1.1 ± 0.2 (0.5–1.5)</td>
</tr>
<tr>
<td>16</td>
<td>CX1 (3)</td>
<td>IIIB</td>
<td>2</td>
<td>6.3 ± 1.3 (3.8–8.3)</td>
<td>14.2 ± 1.9 (10.5–16.4)</td>
</tr>
<tr>
<td>17</td>
<td>HN7 (5)</td>
<td>T,N2,M0</td>
<td>1</td>
<td>6.4 ± 2.3 (1.0–12.3)</td>
<td>32.3 ± 4.4 (23.7–48.1)</td>
</tr>
<tr>
<td>18</td>
<td>HN31 (6)</td>
<td>T,N2,M0</td>
<td>2</td>
<td>9.6 ± 1.9 (5.2–14.7)</td>
<td>2.0 ± 1.9 (0.0–9.4)</td>
</tr>
<tr>
<td>19</td>
<td>CX30 (5)</td>
<td>IIIB</td>
<td>2</td>
<td>14.4 ± 3.3 (4.3–20.4)</td>
<td>25.8 ± 5.9 (7.0–38.9)</td>
</tr>
<tr>
<td>20</td>
<td>HN32 (3)</td>
<td>T,N2,M0</td>
<td>2</td>
<td>17.4 ± 4.3 (9.1–23.7)</td>
<td>6.9 ± 4.5 (0.0–15.2)</td>
</tr>
</tbody>
</table>

*a Quantitative data are presented as mean ± SE of intratumor measurements.

*b Tumors in which keratin pearls were observed.
showed no overlap at all (Table 1). The lack of overlap in individual fields was attributable entirely to microregions of hypoxia that did not express involucrin. However, when all 83 sections were analyzed including those that had no detectable hypoxia, it was found that 11 of 83 (13%) sections showed involucrin expression in the absence of hypoxia, as defined by the absence of pimonidazole binding. When examined on a tumor-by-tumor basis, 4 of 7 head and neck and 7 of 13 cervix tumors tissue sections possessed involucrin and hypoxia overlap as the only patterns of immunostaining. In the remaining tumors, sections showed mixed patterns of overlap and no overlap.

Quantitative Analysis. Patient stage and grade are summarized in Table 2. The data are organized in ascending order of tumor hypoxia. No trends between hypoxia and stage or grade were observed. Hypoxia was measurable in all except two tumors [tumors nos. 1 and 2 in Table 1 (<0.1% area hypoxic)]. The range of the percentage of area hypoxia was wide for both head and neck and cervix tumors, 0.9% to 17% (area hypoxic)]. Areas immunostained for MT-I/II were measurable in all but one tumor (tumor 10 in Table 1) and ranged widely from 0.0 to 36.6%. No correlation between overall hypoxia and MT was discernible (Fig. 2).

Pimonidazole Hydrochloride as Hypoxia Marker. Pimonidazole hydrochloride was found to have advantages as a marker for human tumor hypoxia. The solid is stable for at least 2 years at room temperature in subdued light and has a maximum solubility of 400 mM or 116 g/100 ml of 0.9% saline. The solutions used for infusion contained 34 mM pimonidazole hydrochloride in 0.9% saline and were stable for at least 1.5 years at 4°C in subdued light, as determined by high performance liquid chromatography and UV spectroscopy. The pH of the 34 mM infusion solution was 3.9 ± 0.1. When exposed to laboratory light, solid pimonidazole hydrochloride and its solutions slowly turned yellow. At a dose of 0.5 g/m², pimonidazole hydrochloride caused neither central nervous system toxicity nor sensation (e.g., flushing) in any of the 20 patients studied. Central nervous system toxicity was of particular interest because this was the dose-limiting toxicity for pimonidazole hydrochloride at the higher, multiple doses used in radiosensitizer trials (39, 40). In addition to the absence of central nervous system effects, the overall procedure from pimonidazole hydrochloride infusion to tumor biopsy was well tolerated in both inpatient and outpatient settings. The 4.3.11.3 hybridoma supernatant containing the monoclonal antibody to pimonidazole adducts was stable for at least 4 months at 4°C when supplemented with 10 mg/ml of BSA and 10 mM sodium azide.

DISCUSSION

Hypoxia and MT Expression. Hypoxic inducibility and pluripotential biological activity made metallothionein an interesting possibility as a link between hypoxia and poor prognosis in human cancer. It was known that hypoxia induces MT in human cell lines, and there was clinical data linking MT to poor prognosis. However, the present results show that MT-I/II is not overexpressed in the majority of hypoxic cells in established squamous cell carcinomas. Furthermore, there is no quantitative correlation between overall hypoxia and MT expression at the time of clinical presentation. This result was unexpected on the basis of preclinical studies, but it is consistent with clinical data for VEGF expression in squamous cell carcinomas (11). It does not appear to be consistent for VEGF mRNA expression in gliomas, where the microregional distribution of VEGF mRNA and HIF-1 protein is generally consistent with the expected location of hypoxic cells (8–10). Nevertheless, hypoxic cells were not positively identified in the gliomas, and it is known that necrosis can develop in glioma xenografts in the absence of microregional hypoxia (41, 42). Under these circumstances, necrosis might not be a reliable marker for hypoxia in gliomas. In the present study, hypoxic cells were positively identified, and it is clear that MT is not expressed in the majority of these cells in squamous cell carcinomas.

Transcriptional control for both VEGF and MT is complex and, in the case of hypoxic induction, subject to different hypoxia-sensitive transcription factors. The fact that neither VEGF nor MT is expressed in the majority of hypoxic cells in squamous cell carcinomas indicates that a generalized mechanism for the suppression of oxygen-regulated protein expression might be operating. One possibility is that hypoxic regions defined by pimonidazole binding are ischemic. Shweiki et al. (43) have shown, for example, that combined severe glucose and oxygen depletion suppresses the expression of VEGF in rat C6 glioma cells in monolayer culture. Although this mechanism cannot be ruled out, Arteel et al. (44) have shown that the binding of hypoxia markers requires energy, and it seems unlikely that pimonidazole-labeled cells are severely nutrient depleted. A second possibility is that pimonidazole binds to cysteine residues in oxygen-regulated proteins, thereby inhibiting their detection by antibodies (45). The 20-amino acid epitope at the NH₂ terminus of VEGF is cysteine free, but the 5-amino acid epitope at the NH₂ terminus of MT-I/II contains a cysteine moiety. However, this does not appear to be a problem in practice because MT-I/II protein was easily detectable in pimonidazole-labeled cells of rodent tumors (24) with the anti-
body used in the present study. Consideration of other possible global mechanisms led us to reports that keratinocyte differentiation suppresses the expression of VEGF (46) and MT (15) and that squamous carcinomas express markers for terminal differentiation (47).

During normal stratified epithelial maturation, keratinocytes move from the proliferating, basal layer into suprabasal layers, where synthesis of specific cytoskeletal proteins occurs. As keratinocyte differentiation proceeds, cells are pushed through the stratum spinosum and stratum granulosum toward the outermost stratum corneum, where they become flattened cytoskeleton-filled scales or squames. Morphological changes are accompanied by the expression of molecular markers for terminal differentiation such as involucrin, transglutaminase, and a variety of cytokeratins (48). Details of the molecular biology of these changes in squamous cells is under active investigation (49). Interestingly, molecular markers for terminal differentiation are also expressed in squamous cell carcinomas (Fig. 1), and it is generally accepted that the markers are associated with differentiation processes in the tumors (50–58).

The remarkable colocalization of involucrin and pimodone-zole adducts raises questions of whether involucrin is an oxygen-regulated protein and whether oxygen gradients regulate differentiation. Although there are no published data on this point, there is evidence that differentiation events are initiated in the well-oxygenated basal cell layer of normal stratified epithelia and that cell migration to suprabasal layers is a consequence, and not a cause, of differentiation (48). If this is correct, then the expression of involucrin in hypoxic cells of squamous cell carcinomas is coincidental. Whether oxygen gradients play a role in intensifying the involucrin signal seen in Fig. 1D is not known. It is generally accepted that patients with hypoxic and poorly differentiated tumors, as defined pathologically, have poorer prognoses. The tight association between hypoxia and involucrin expression would seem paradoxical in this regard. However, pathological assessment of differentiation makes use of morphological clues in tissue sections, and it is possible that tumor cell differentiation can, in many cases, proceed to a point that falls short of producing changes that are visible under the microscope (50).

As reported by others and shown in Fig. 1, MT-I/II immunostaining occurs in proliferating microregions in the periphery of tumor nests (59), as might be expected of a protein that is associated with cell cycle progression (26). On the other hand, MT-I/II immunostaining is largely absent from hypoxic regions in the center of tumor nests where involucrin is strongly expressed (Fig. 1; Refs. 47, 54, and 55). Quaife et al. (15) have found that MT gene expression switches from highly inducible MT-I to noninducible MT-IV when cells transition from the basal layer to more differentiated layers in normal stratified epithelia, and it is tempting to speculate that the absence of MT-I/II protein expression in hypoxic cells in squamous cell carcinomas is attributable to the suppression of the inducible forms of MT by cell differentiation in hypoxic zones. It has been shown in studies of explanted cell lines from human cervical carcinomas that cells expressing involucrin are not necessarily undergoing a process of normal differentiation (50), and it remains to be seen whether changes in the expression of MT isoforms are correlated with the microregional expression of involucrin in squamous cell carcinomas. In situ hybridization studies of the microregional distribution of human MT-I, MT-IIa, and MT-IV mRNA expression are under way to test this.

Relevance to Cancer Therapy. The expectation that MT expression in the hypoxic cells of human tumors might add to the radioresistance (60–63) or chemo-resistance (64–66) of these cells is not supported by the present results. However, MT in proliferating compartments of tumors could account for the inverse relationship between overall MT expression and chemotheraphy response (21, 67, 68) by both protecting cells near blood vessels and limiting drug diffusion beyond perivascular regions (69). With respect to radiation therapy, it is interesting to note that differentiation sensitizes human tumor cells in vitro (70, 71). Bothoxic and hypoxic cells are sensitized, and their relative radiosensitivities are, therefore, unchanged. However, if hypoxic cells in squamous cell carcinomas were more differentiated than oxic cells, then hypoxic cells might be selectively radiosensitized and their therapeutic importance diminished. The importance of hypoxic cells in squamous cell carcinomas would be further diminished if they were differentiated to the extent that they were incapable of reentering the cell cycle. Given these observations and the conventional wisdom that differentiated tumor cells belong to the cell loss compartment in tumors (72), further investigations of the extent to which hypoxic cells in squamous cell carcinomas are differentiated appear to be warranted.

Summary. The present results combined with earlier data for VEGF indicate that the expression of oxygen-regulated genes in human squamous cell carcinomas at the time of clinical presentation is not predicted by in vitro studies with tumor cell lines. The reason for this is not clearly understood, but a possible scenario is that differentiation of the hypoxic cells exerts collateral control on gene expression whereby oxygen-regulated protein expression is suppressed. The lack of MT expression in hypoxic tumor cells indicates that this radio- and chemoprotective protein will not contribute directly to the therapy resistance of hypoxic cells in squamous cell carcinomas.

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A Clinical Study of Hypoxia and Metallothionein Protein Expression in Squamous Cell Carcinomas

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