Hypoxic Cervical Cancers with Low Apoptotic Index Are Highly Aggressive

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Abstract

There is evidence from experimental work that hypoxia induces apoptosis in apoptosis-sensitive neoplastic cells and that this apoptotic sensitivity is lost during malignant progression. Oxygenation profiles and apoptotic indices in human squamous cell cancer of the uterine cervix have been determined, and a subgroup of tumors has been identified with low apoptotic index despite pronounced hypoxia representing carcinomas that consist of neoplastic cells with diminished apoptotic potential. These hypoxic low-apoptotic tumors show a high probability for lymphatic spread and for recurrence despite adjuvant treatment with radiation or chemotherapy in addition to radical surgery. The clinical results presented strongly support the hypothesis derived from experimental studies that the selection of apoptosis-insensitive neoplastic cell phenotypes in a hypoxic microenvironment is an important mechanism for malignant progression in solid tumors.

Introduction

Until recently, tumor hypoxia has been regarded as a radiobiological phenomenon with uncertain clinical relevance (1, 2). In the first systematic measurements of tumor oxygen profiles in cancer of the uterine cervix, we found a strong association between tumor hypoxia (defined as median \( pO_2 < 10 \) mm Hg) and poor clinical outcome independent of the treatment mode (3, 4). Moreover, recurrent cervical cancer proved to be generally less oxygenated than primary tumors of comparable sizes (5). Thus far, all of the clinical observations with regard to tumor oxygenation in cervical carcinoma are consistent with the hypothesis that tumor hypoxia—as a consequence of nutritive deprivation due to inadequate tumor microcirculation (6)—is involved in the malignant progression of the disease. The association between tumor hypoxia and malignant progression has also been demonstrated for soft tissue sarcoma (7) and for head and neck cancer (8). Experimental evidence for various biological mechanisms linking tumor hypoxia to increased aggressiveness of the neoplasms in terms of invasiveness, metastatic potential, and resistance toward therapy has been found (9–12). The experimental demonstration that hypoxia promotes the selection of tumor cell phenotypes with diminished apoptotic potential (13, 14) prompted this investigation of the relationship between tumor cell proliferation, apoptosis, and oxygenation in our patient cohort.

Materials and Methods

Patients and Treatment. Consecutive patients who had squamous cell cancer of the uterine cervix \( \geq 2 \) cm in size [defined clinically and with imaging (sonography, computed tomography, or magnetic resonance imaging)] and in whom tumor oxygenation measurements were performed before surgical treatment at the Department of Obstetrics and Gynecology, University of Mainz, Mainz, Germany, from 1993 to 1996, formed the study group. The investigation of tumor oxygenation measurements had been approved by a medical ethics committee, and all of the patients gave informed consent. Within 1 week after tumor oxygenation measurements, patients underwent abdominal radical hysterectomy and pelvic/paraortic lymph node dissection. In two patients, supravacuclar exenteration had to be performed instead of radical hysterectomy.

Adjuvant postoperative treatment was administered in cases with lymph node metastases and/or parametrial spread. From 1993 until 1994 adjuvant treatment consisted of three to six cycles of chemotherapy with carboplatin (300 mg/m\(^2\)) and ifosfamid (1.5 g/m\(^2\) for 3 days). Thereafter, whole pelvis irradiation using the standard four-field box technique with 45–50 Gy was applied in these high-risk cases. Follow-up appointments were made at 3-month intervals over the first 2 years after treatment and later at 6-month intervals.

Tumor Oxygenation Measurements. Tumor oxygenation was measured with the Eppendorf histography system (Eppendorf, Hamburg, Germany) adhering to the standard procedure as developed and validated previously (3, 4). \( pO_2 \) readings were performed in the conscious patient along linear tracks, first in the s.c. fat of the mons pubis followed by cervical measurements at the 12 and 6 o’clock sites of the macroscopically vital tumor tissue. Within the tumor tissue, up to 35 \( pO_2 \) measurements were obtained in each tumor track starting at a tissue depth of 5 mm. The measuring points were placed 0.7 mm apart, which resulted in an overall measurement track length of approximately 2.5 cm.

Intravaginal temperature, blood pressure, heart rate, hemoglobin concentration, hematocrit and arterial oxyhemooglobin saturation were monitored continuously with the \( pO_2 \) determinations. The pretherapy \( pO_2 \) measurements were usually performed 1–5 days before oncological treatment.

Defined Tumor Biopsies. Core biopsies were taken from those tumor areas in which the \( pO_2 \) determination had previously been made. In lithotomy position, using a retracting speculum, the tumor was accessed transvaginally. Two 2-by-20-mm core biopsies were taken from the 12 and 6 o’clock positions of the tumor using the Biopsy device (Radioplast AB, Uppsala, Sweden). The biopsy needle was inserted into the tumor tissue to a depth of 5 mm before releasing the cut-through mechanism. If mobile, the tumors were first stabilized with a tenaculum. No analgesics were required. Biopsy-site bleeding was treated by direct application of local pressure for 3 min.

Histology. For histological examination, biopsies were fixed in neutral buffered 4% formaldehyde solution and embedded in paraffin. Sections (4-to-6-\( \mu \)m) were prepared from standard paraffin blocks. For conventional histological evaluation, sections were stained with H&E. All of the tumors were classified according to the Union Internationale Contre Le Cancer/pathological tumor-node-metastasis classification.

In parallel sections, the apoptotic cells were identified with the \textit{in situ} TUNEL assay and the cells expressing the nuclear antigen Ki-67 were immunohistochemically highlighted using the monoclonal antibody MIB1.

Immunohistochemical Detection of Ki-67 Positive Cells. Sections were stained according to Gerdes \textit{et al.} (15). Briefer, after deparaffinization and rehydration, the sections were covered with a citrate buffer and heated in a
microwave oven three times for 5 min. Endogenous peroxidase activity was blocked by covering the sections with 3% H2O2 in H2O for 5 min. The sections were then incubated with the primary antibody (MIB1, Dianova, DIA 505; dilution 1:50) for 30 min at 37°C. After PBS washing, the sections were covered with the biotin-conjugated secondary antibody (Dianova, 12 μl/ml PBS, 12 min at 37°C). The sections were again washed with PBS and incubated with the avidin-biotin-peroxidase complex (Dianova, 5 μl/ml). Diaminobenzidine was used for the color reaction. The sections were counterstained with hematoxylin.

**TUNEL Assay.** The procedure was performed according to the manufacturer’s instructions with minor modifications (ApopTag in situ apoptosis detection kit, Oncor, Gaithersburg, MD). The sections were deparaffinized with xylene, rehydrated and treated with 200 μg/ml proteinase K for 15 min at room temperature (Boehringer Mannheim, Germany, No. 745723). Endogenous peroxidase was inactivated by covering the sections with 3% H2O2 in H2O for 5 min at room temperature. End-labeling was achieved by catalytically adding residues of digoxigenin-labeled 11-dUTP and dATP to the 3'-hydroxyl ends of DNA with the enzyme TdT. The reaction buffer containing dUTP, dATP, and TdT was applied for 60 min at 37°C in a humid atmosphere. For negative controls, TdT was eliminated from the reaction buffer. The digoxigenin was detected immunohistochemically with a digoxigenin-specific peroxidase-conjugated antibody (30 min in a reaction buffer. The digoxigenin was detected immunohistochemically with a digoxigenin-specific peroxidase-conjugated antibody (30 min in a humid atmosphere. For the color reaction, metal-enhanced diaminobenzidine was used as substrate (Boehringer Mannheim, Germany, No. 1718096). The sections were counterstained with hematoxylin.

As biological-positive controls, human tonsils were used. A positive control was included in every section. For negative controls, the PI, an interactive computerized morphometry system was used (frame grabber board: ITI-MFG-3-M-V, Imaging Technology Inc. Bedford, MA; RGB camera: DXC-151P, Sony, Resolution 756 × 581 picture elements; software: Optimas, Bio Scan Inc., Edmonds WA; video monitor: FA3435KL, Mitsu-

**Determination of the PI.** For the determination of the tumor cell density and the PI, an interactive computerized morphometry system was used (frame grabber board: ITI-MFG-3-M-V, Imaging Technology Inc. Bedford, MA; RGB camera: DXC-151P, Sony, Resolution 756 × 581 picture elements; software: Optimas, Bio Scan Inc., Edmonds WA; video monitor: FA3435KL, Mitsu-

**Statistics.** Statistical analyses were performed with the SAS 6.04 software. For comparisons, the determination of Spearman rank correlation coefficient and the Mann-Whitney U test were applied. Generally, a P ≤ 0.05 was considered to indicate statistical significance.

**Results**

From the 37 consecutive patients with squamous cell cancer of the uterine cervix ≥ 2 cm in whom pO2 measurements were performed before treatment with radical hysterectomy (or anterior exenteration in two cases), core biopsies were available in 29 cases for immunohistochemical evaluation of PI and AI. In the remaining eight patients, the core biopsies were too fragmented to allow morphometric assessment. Pretherapeutic staging according to the FIGO was IIB, n = 10; IIA, n = 1; IIB, n = 18. The histopathological tumor characteristics together with the results of the determination of the median pO2, Ki-67 LI and AI for each neoplasm are given in Table 1. Neither pO2

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Number of apoptotic tumor cells per section × 100

\[ LI = \frac{\text{Number of positive tumor cells}}{\text{Total number of tumor cells}} \times 100 \]

*pdmax*, maximum pathological tumor diameter [mm]; LVS, lymph-vascular space involvement; DD, dead of disease; NED, no evidence of disease.
nor tumor cell density, nor PI, nor AI showed a relationship to any of the patient and histopathological tumor characteristics. Between the AI and PI, a correlation was found with a Spearman rank correlation coefficient of \( r = 0.456 \) (\( P = 0.01 \)).

Additionally, an analysis of the dependency of tumor cell density and of PI and AI on the oxygenation status was carried out. Whereas no association could be detected for cell density and Ki-67 LI, a significant negative correlation between AI and tumor oxygenation was found (Fig. 1A; Spearman rank correlation coefficient, \( r = -0.60, P = 0.0006 \)). Although all of the tumors with a high AI had low median oxygen tensions, a significant number of tumors had a low AI despite their hypoxic state.

Hypoxic low-apoptotic tumors were defined as those with an AI \( \leq 0.5\% \) at median pO2 values < 10 mm Hg. The group of patients with hypoxic low-apoptotic cervical cancers had a very high probability of lymphatic spread and an extremely poor outcome, although they did not differ significantly in clinical and histopathological tumor size and (p)T category and in primary and adjuvant treatment from the group of patients with either hypoxic high-apoptotic or nonhypoxic low-apoptotic tumors. Hypoxic low-apoptotic tumors exhibited lymph-vascular space involvement in 100% and lymph node metastasis in 73% of the cases (Fig. 1B). These two histopathological signs of lymphatic spread that indicated an adverse prognosis were significantly less frequent with hypoxic high-apoptotic and nonhypoxic low-apoptotic tumors (\( P = 0.001 \) and \( P = 0.04 \), respectively).

Eleven of the 15 patients with hypoxic low-apoptotic tumors relapsed and died within the observation period, whereas none of the 5 patients with hypoxic high-apoptotic tumors and 4 of the 9 patients with nonhypoxic low-apoptotic tumors expired from their disease. Nine of the 11 patients with hypoxic low-apoptotic primary tumors who relapsed developed locoregional recurrences in the pelvis with or without distant metastases (Fig. 1C). The follow-up intervals were not significantly different in the three groups. Median time to recurrence was 7 months (range, 1–33 months). Survival probabilities calculated with the Kaplan-Meier method demonstrated significant differences between the group of patients with hypoxic low-apoptotic tumors compared with the patients with hypoxic high-apoptotic and nonhypoxic low-apoptotic tumors (Fig. 1D). Median observation periods for both groups were 36 and 30 months, respectively (ranges, 11–48 months and 12–48 months).

In the multivariate Cox regression analysis, the hypoxic low-apoptotic status was the most powerful predictor of recurrence-free survival (\( P = 0.02 \)) and overall survival (\( P = 0.04 \)) among the preoperative prognostic variables such as clinical tumor size and FIGO stage.

**Discussion**

This study demonstrates for the first time the aggressive behavior of hypoxic tumors with a low AI in a human neoplasm. Cervical cancers have been referred to as hypoxic if their median pO2 was less than 10 mm Hg. The cutoff level of 0.5% defining “low-apoptotic” tumors was derived from the relationship between the AI and P02 of the first 29 cervical cancers investigated as reported in this study. It was given by the lowest AI value above the maximum in the group of the nonhypoxic tumors (in 0.1% scaling). Hypoxic low-apoptotic cancers of the uterine cervix of medium sizes exhibited lymphatic vascular space involvement in 100% and lymph node metastases in 73% of the cases. Median recurrence-free survival and overall survival of patients with hypoxic low-apoptotic tumors was 11 and 21 months, respectively. The clinical course of this subgroup of cervical cancers that were pretherapeutically staged as FIGO IB and IIB corresponded to FIGO stage IVA tumors.

If the prognostic information inherent in the hypoxic low-apoptotic tumor constellation that is apparent from the present study can be
confirmed in a prospective trial with a sufficiently large number of patients, this would be particularly useful clinically for medium-size and medium-stage cancer of the uterine cervix, for which reliable predictive parameters that can be obtained presurgically are lacking.

In the present study in patients treated with primary surgery, neither the AI alone, nor the mitotic index, nor the tumor cell density were of prognostic significance. In the study by Levine et al. (16) investigating patients with cervical cancer who underwent primary radiotherapy, the mitotic index was also not related to outcome, but a high AI proved to be an indicator for poor outcome (16, 17).

The differential prognostic relevance of the AI for cervical cancer treated with radiotherapy or surgery may be explained by our observation that tumors with high AI were hypoxic. Tumor hypoxia could have negatively influenced radiation treatment because of the reduced oxygen enhancement effect (1). If, therefore, seems worthwhile to investigate whether the subgroup of patients with medium-sized cancers of the uterine cervix with hypoxic high-apoptotic tumors might benefit more from primary surgical treatment rather than from radiation.

In addition to the potential clinical significance, the results presented deserve consideration from the perspective of tumor cell biology. The AI correlated positively with the PI and negatively with the oxygenation status, which might be expected for cells with an unimpaired apoptotic potential. However, a significant number of tumors had a low AI despite being severely hypoxic. The tumor cells in these neoplasms obviously had a diminished apoptotic potential; otherwise, they would have responded to hypoxia with increased apoptosis.

Giaccia and coworkers (13, 14) have provided strong experimental evidence that suggests that hypoxia mediates the selection of neoplastic cells with diminished apoptotic potential by providing a growth advantage to cells with genetic alterations that impair the process of apoptosis. Hypoxia-mediated clonal selection of tumor cells with diminished apoptotic potential has been suggested as an important biological mechanism of tumor progression. The clinical results in our present study fully support this basic concept derived from in vitro and experimental tumor investigations; hypoxic cervical cancers with a low AI representing tumors with diminished apoptotic potential exhibited a very aggressive clinical behavior in terms of a high ability of lymphatic spread. It can be assumed that pelvic lymph fluid contains low concentrations of oxygen (18), and the lymphatics do not provide an adequate matrix for proliferation, features that would promote apoptosis in tumor cells with intact apoptotic potential. However, tumor cells with diminished apoptotic potential are obviously able to proliferate in lymphatic vascular spaces and in the subcapsular sinus of lymph nodes as initial step in the formation of lymph node metastases. Because all except two patients from the hypoxic low-apoptotic tumor group who relapsed had tumor recurrences in the pelvis—despite radical tumor resection, systematic lymph node dissection, and postoperative adjuvant treatment with either pelvic radiotherapy or chemotherapy—radioresistance as well as chemoresistance of the microscopic tumor foci left behind in the pelvis after surgery must have existed. The ability of tumor cells to undergo apoptosis seems to be a prerequisite for radio- and chemoresistance (19). Therefore, the identification of hypoxic tumors with low AI as neoplasms that consist predominantly of tumor cell clones with diminished apoptotic potential in the majority of patients with lymph node metastases provides an explanation for the fact that the benefit from postoperative adjuvant radiation of nodal-positive cervical cancers could not be unequivocally demonstrated thus far (20). The underlying molecular pathology of resistance toward apoptosis in the clinically aggressive squamous cell carcinomas of the uterine cervix remains to be clarified. Insights—derived from oxygenation status and AI—are expected from the investigation of the differential expression of proapoptotic genes, such as p53 and Bax, and of antiapoptotic genes, such as bcl-2, in the three subgroups.

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References

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