Disease Control of Uterine Cervical Cancer: Relationships to Tumor Oxygen Tension, Vascular Density, Cell Density, and Frequency of Mitosis and Apoptosis Measured before Treatment and during Radiotherapy[1]

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ABSTRACT

Identification of biological parameters of major importance for the control of malignant diseases can be useful for the design of optimal treatment regimes for individual patients. Tumor oxygen tension (pO_2), vascular density, cell density, and frequency of mitosis and apoptosis were measured before treatment (40 patients) and after 2 weeks of radiotherapy (22 patients) in patients with uterine cervical cancer. The aim was to investigate whether one of the parameters was more important for disease control than the others. Three sets of data were considered; the pretreatment parameters, the parameters measured after 2 weeks of radiation, and the changes in the parameters during this time. The pO_2 was measured polarographically; the other parameters were determined by histological analyses of tumor biopsies. Hypoxic subvolume (HSV_5), *i.e.*, the fraction of pO_2 readings <5 mm Hg multiplied with tumor volume, showed the strongest correlation to control. Patients with a small HSV_5 before treatment had a higher control probability after a median follow-up time of 50 months than patients with a large HSV_5 ($P < 0.001$). All other parameters or changes in parameters showed impaired correlation to control compared with pretreatment HSV_5. The present results suggest that pretreatment oxygenation is more important for disease control of cervical cancer than the oxygenation after 2 weeks of radiotherapy or the changes in oxygenation during this time. Moreover, vascular density, cell density, and frequency of mitosis and apoptosis before treatment or after 2 weeks of therapy are probably not as important as pretreatment oxygenation as well. Although significant correlations between disease control and some of the parameters other than pretreatment oxygenation can occur in studies based on a large number of patients, the specificity of these parameters in the prediction of control is probably not as high as for oxygenation.

INTRODUCTION

Several tumor biological parameters, such as oxygenation and activity of angiogenesis, proliferation, and apoptosis, can be important for treatment response and formation of metastases in cancer patients and hence for the control of malignant diseases. Thus, tumor hypoxia can cause increased resistance to radiation and increased expression of genes encoding for metastasis-promoting proteins, whereas the resistance to some cytotoxic agents may decrease (1, 2). Increased angiogenic activity can also enhance the metastatic process; the escape of tumor cells into the blood circulation can be facilitated in highly vascularized tumors, and the growth probability of tumor cells trapped in secondary organs can increase with elevated capacity to induce neovascularization (3). High proliferation activity may reflect increased malignancy because of rapid tumor progression (4) and can lead to local failure after fractionated treatment because of significant repopulation of surviving tumor cells during therapy (5). Finally, decreased apoptotic activity can cause local failure because of survival, rather than apoptosis, of tumor cells after treatment (6). Low apoptotic activity can also indicate increased metastatic capacity because of a highly malignant phenotype of cells adapted to adverse conditions (7). Pretreatment values of these parameters have been shown to correlate with control probability of several types of cancers, including uterine cervical cancer (8–15). However, a large number of patients are often needed to obtain significant results, and several studies report no correlations at all (8, 16, 17). The clinical usefulness of the parameters in the prediction of disease control is therefore not clear. Moreover, it is not known whether changes in the parameters during therapy are more important for control than the pretreatment values.

Identification of the biological parameter most important for disease control can be useful for selection of patients for different treatment regimes and for development of efficient strategies to influence the control probability by changing the biological parameter. Such identification should be based on studies of several parameters in the same population of patients. Comparison of results from different studies is complicated because studies often differ with respect to number of patients, stage of disease, treatment regime, and follow-up time. In the present work, tumor oxygen tension (pO_2), vascular density, cell density, and frequency of mitosis and apoptosis were measured before the start of treatment (40 patients) and after 2 weeks of

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radiotherapy (22 patients) in patients with carcinoma of the uterine cervix. The aim was to investigate whether one of the parameters was more important for disease control than the others. Three sets of data were considered: the pretreatment parameters, the parameters measured after 2 weeks of radiation, and the changes in the parameters during this time. The measurements after 2 weeks were performed during the early phase of radiotherapy, *i.e.*, before significant tumor shrinkage had occurred. Knowledge of possible relationships between disease control and biological parameters of this phase would be particularly useful because strategies for selecting patients to adjuvant treatments and/or for changing the biological parameters should be initiated as early as possible during therapy. The pO_2 was measured by use of polarographic needle electrodes. Vascular density, cell density, and frequency of mitosis and apoptosis were determined by histological analysis of biopsies taken after each pO_2 measurement.

PATIENTS AND METHODS

Patients, Treatment, and Follow-Up Schedule. Forty patients with primary squamous cell carcinoma of the uterine cervix were included in the study. Patients' ages were 27–69 years (median, 46 years). The Fédération Internationale des Gynaecologistes et Obstetristes (3) stages were Ib (7 patients), IIa (1 patient), IIb (23 patients), IIIb (7 patients), and IVa (2 patients). The largest tumor diameter was 2.7–9.1 cm (median, 5.8 cm), as determined from pretreatment MR[3] images. A subgroup of 22 patients, representative of the whole group of patients, was subjected to measurement of biological parameters after 2 weeks of therapy. Twenty-two rather than all patients were included in this part of the project because it was initiated after the study had started. Patients' ages of this group were 27–64 years (median, 46 years); the Fédération Internationale des Gynaecologistes et Obstetristes stages were Ib (2 patients), IIb (15 patients), IIIb (3 patients), and IVa (2 patients); and the largest tumor diameter was 2.7–9.1 cm (median, 6.3 cm). The study was approved by the local ethical committee, and informed consent was obtained from all patients.

Radiotherapy was given as combined external irradiation and brachytherapy with curative intent to all but four patients. External irradiation was delivered with 10 MV or 16 MV photons by use of a linear accelerator. A total dose of 50 Gy in fractions of 2 Gy per day five times/week was given to the pelvic region with a four-field box technique. Endocavitary brachytherapy was delivered by use of a high dose rate ^{192}Ir afterloading machine. A total dose of 29–34 Gy was given in seven to eight fractions to point A. Adjuvant chemotherapy was not used.

The patients were followed up with clinical examinations every third month for the first 2 years and thereafter twice a year. MR imaging of retroperitoneum and X-ray of the thorax were performed during the first follow-up examination, after 2 months, after 1 year, and thereafter when symptoms of recurrent disease were seen. Three different end points were used in evaluation of disease control: overall survival, disease-free survival, and locoregional control. Locoregional control was defined as complete and persistent regression of tumor within the irradiated field.

Oxygen Tension. Oxygen tension was measured in the tumors before treatment (40 patients) and after about 2 weeks of radiotherapy (22 patients), *i.e.*, after a median radiation dose of 16 Gy was achieved by external irradiation and before brachytherapy was initiated. General anesthesia (Propofol i.v.) was used in nine patients subjected to a single pO_2 measurement, otherwise no anesthetic was used. The anesthetic Propofol has no significant influence on body temperature or tumor pO_2 in cervical cancer patients (18). The measurements were performed by use of polarographic needle electrodes with a shaft diameter of 300 μm (Eppendorf pO_2 histograph 6650; Ref. 19). The same electrode was generally used in the two measurements of each patient. The electrode was moved automatically through the tumor in two to six different tracks. The number of pO_2 readings in each tumor was 57–317 (median, 168). Five pO_2 parameters were calculated for each tumor: median pO_2, fractions of pO_2 readings <2.5 mm Hg, 5 mm Hg, and 10 mm Hg ($HF_{2.5}$, HF_5, and HF_{10}), and the tumor subvolume with pO_2 readings <5 mm Hg (HSV_5, where $HSV_5 = HF_5 \times$ tumor volume). Tumor volume was calculated as $V = \pi/6 \cdot a \cdot b \cdot c$, where a, b, and c are three orthogonal diameters determined from MR images.

Biopsies. A needle biopsy (1 × 18 mm) was taken from each measurement track immediately after the pO_2 electrode was withdrawn from the track (19). Consequently, two to six biopsies were achieved from each tumor before treatment (40 patients) and after 2 weeks of radiotherapy (22 patients). This procedure ensured that possible heterogeneity in the histological parameters within the tumors was taken into account. The biopsies were fixed in phosphate-buffered 4% paraformaldehyde, embedded in paraffin casts, and cut in the length direction to 5-μm-thick sections. The sections were prepared as described below and analyzed by one person (H. L.) in a light microscope with an eyepiece grid for determination of vascular density, cell density, and frequency of mitosis and apoptosis. The reproducibility of the histological analyses was assessed by performing repeated analyses of 10 sections. The first and second determinations of the histological parameters were significantly correlated to each other ($P < 0.001$), and there was no difference between these two determinations, regardless of which parameter that was considered ($P < 0.85$). All histological parameters were therefore determined with satisfactory reproducibility.

Vascular Density. Vascular density was determined in sections immunostained for factor VIII-related antigen. A rabbit polyclonal antibody, Dako A0082 (Dako Corp., Santa Barbara, CA), applied at a dilution of 1:500 at 20°C for 30 min, was used as primary antibody. Immunoperoxidase staining was performed by using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) with goat-antirabbit IgG as biotinylated secondary antibody and diaminobenzidine as chromogen. Brown-stained endothelial cell clusters were identified as vessels. All sections of each tumor were scanned at ×100. The three areas (25 mm^2) of highest vascular density were selected, and all vessels within these areas were counted at ×200. Vascular density was calculated as number of vessels per mm^2 of

[3] The abbreviations used are: MR, magnetic resonance; Tdt, terminal deoxynucleotidyl transferase; HSV, hypoxic subvolume.

tissue. The mean value based on the three selected areas was used to represent vascular density of each tumor.

Cell Density. Sections stained with H&E, using standard procedure, were used to determine tumor cell density. Stroma, carcinoma tissue, and a negligible amount of necrosis were seen in the sections (19). Five fields, each generally including 50–150 cells, were selected within the carcinoma tissue of each section. Carcinoma cell nuclei were identified based on a blue color and a spherical shape. All nuclei within the fields were counted at ×400, and the number of nuclei per mm^2 of carcinoma tissue, D_c, was determined. The area fraction of carcinoma tissue, F_c, was determined by point-counting at ×100. Tumor cell density was defined as number of carcinoma cell nuclei per mm^2 of tissue (including stroma and carcinoma tissue) and was calculated as $D_c \times F_c$.

Frequency of Mitosis. H&E-stained sections were used to determine mitotic frequency. Carcinoma cell nuclei with morphological changes caused by chromosome segregation were identified as mitotic cells. All mitotic cells were counted by careful examination of the whole sections at ×400. Mitotic frequency (%) was calculated as the number of mitotic carcinoma cells per number of all carcinoma cells.

Frequency of Apoptosis. Apoptotic frequency was determined in sections stained by use of the Apotag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD). The staining was based on the TdT-mediated dUTP-biotin nick end labeling method (20). Shortly, a solution of 30% TdT was applied at 37°C for 60 min to link dUTP-digoxigenin to the 3′-hydroxy ends of fragmented DNA. Anti-digoxigenin peroxidase conjugate was applied for 30 min to detect labeled nucleotides. Diaminobenzidine was used as chromogen. A biopsy from a neoplastic lymph node of a patient with B-cell non-Hodgkin's lymphoma served as a positive control. Apoptotic frequency of this lymph node was ~20%, as determined by flow cytometry earlier in our institution. Negative controls received no TdT. To avoid erroneous identification of apoptotic cells because of light staining of necrotic cells, only brown-stained carcinoma nuclei with morphological characteristics associated with apoptosis were identified as apoptotic cells. Such characteristics include overall shrinkage, homogeneous dark basophilia, a generally round or crescent shape, and a narrow empty space often surrounding the nucleus. All apoptotic cells were counted by careful examination of the whole sections at ×400. Apoptotic frequency (%) was calculated as number of apoptotic carcinoma cells per number of all carcinoma cells.

Statistical Analysis. The patients were divided into two groups based on high (above median) and low (below median) value of the biological parameters. The control probability was compared between the groups by actuarial analysis, using a log-rank test in Kaplan-Meier estimates. Univariate and multivariate Cox regression analyses of continuous data were used to search for correlations between disease control and biological parameters. A two-tailed *t* test or a Mann-Whitney rank sum test was used, depending on whether the data were normally distributed, to search for differences in biological parameters between two groups of patients. A significance level of $P = 0.05$ was used throughout.

RESULTS

Biological Parameters before Treatment. After a follow-up time of 31–69 months (median, 50 months), 18 of 40 patients progressed or relapsed; 17 patients had metastases outside the radiation field with or without locoregional recurrence, and 1 patient had locoregional recurrence without metastases. Fourteen of these patients died from cervical cancer during the follow-up period. Locoregional recurrence was observed in seven patients. There were, therefore, 14, 18, and 7 failures, using overall survival, disease-free survival, and locoregional control as end points, respectively. Tumor stage was significantly correlated to overall and disease-free survival ($P = 0.01$; Table 1).

The pO_2 distributions measured before treatment differed considerably among the patients. Relationships were found between pretreatment pO_2 and disease control. HSV_5 was the pO_2 parameter that showed the strongest correlation to control. Cumulative frequency diagrams of pretreatment HSV_5 are shown in Fig. 1, *A*, *C*, and *E*). The failures are indicated by black symbols, using overall survival (*A*), disease-free survival (*C*), and locoregional control (*E*) as end point. The HSV_5 of all patients ranged from 3.3 to 184.5 cm^3, with a median of 33.9 cm^3. Failure occurred most often among patients with a large HSV_5. Patients with a HSV_5 above median had significantly lower control probability than those with a HSV_5 below median, regardless of whether overall survival, disease-free survival, or locoregional control was used as an end point ($P < 0.001$; Fig. 1, *B*, *D*, and *F*). Moreover, there was a significant correlation between survival and HSV_5 ($P < 0.001$, overall and disease-free survival; $P = 0.004$, locoregional control; Table 1). The HF_5 was also related to control. Patients with a HF_5 above median had significantly lower control probability than those with a HF_5 below median ($P = 0.03$, overall survival; $P = 0.006$, disease-free survival; $P = 0.02$, locoregional control). The correlation between HF_5 and control was significant when disease-free survival was used as end point ($P = 0.05$) but on the borderline of significance when overall survival ($P = 0.08$) or locoregional control ($P = 0.18$) was considered in the analyses (Table 1).

Pretreatment vascular density, cell density, and frequency of mitosis and apoptosis also differed among the patients. The figures presenting the histological parameters refer to disease-free survival as an end point; however, similar results were achieved, regardless of which end point was considered. The histological parameters showed no correlation to disease control (Table 1). Moreover, there was no difference in control probability between patients with a high value and patients with a low value of these parameters (Fig. 2). Pretreatment vascular density, cell density, and frequency of mitosis and apoptosis were therefore probably of minor importance for disease control compared with pretreatment HSV_5.

Biological Parameters after 2 Weeks of Radiotherapy. The 22 patients subjected to a second measurement of biological parameters after 2 weeks of radiotherapy had a follow-up time of 51–64 months (median, 45 months). Twelve patients progressed or relapsed, and 10 of these patients died from cervical cancer during the follow-up period. There were 6 patients with locoregional recurrence. Consequently, 10, 12, and 6 failures occurred, using overall survival, disease-free survival, and lo-

Table 1 Univariate Cox regression analysis of stage, volume, and biological parameters *versus* disease control for patients with uterine cervical cancer

End point	Pretreatment[a] *P*	Pretreatment[b] *P*	After 2 weeks of radiotherapy[b] *P*	Changes during radiotherapy[b] *P*
Overall survival				
Stage[c]	0.01			
Volume	0.0002		0.07	0.03
HSV_5[d]	0.0001	0.02	0.13	0.09
HF_5[e]	0.08		0.95	0.48
Vascular density	0.80		0.74	0.59
Cell density	0.70		0.40	0.41
Mitotic frequency	0.34		0.84	0.69
Apoptotic frequency	0.72		0.80	0.85
Disease-free survival				
Stage[c]	0.01			
Volume	<0.0001		0.01	0.03
HSV_5[d]	<0.0001	0.004	0.007	0.24
HF_5[e]	0.05		0.50	0.76
Vascular density	0.51		0.43	0.51
Cell density	0.18		0.90	0.29
Mitotic frequency	0.26		0.43	0.20
Apoptotic frequency	0.46		0.40	0.43
Locoregional control				
Stage[c]	0.07			
Volume	0.004		0.21	0.03
HSV_5[d]	0.004	0.06	0.33	0.09
HF_5[e]	0.18		0.84	0.61
Vascular density	0.32		0.74	0.71
Cell density	0.14		0.74	0.27
Mitotic frequency	0.49		0.80	0.45
Apoptotic frequency	0.35		0.94	0.89

[a] Based on 40 patients.
[b] Based on a subgroup of 22 patients subjected to measurement of biological parameters after 2 weeks of radiotherapy.
[c] Stages I and II *versus* stages III and IV.
[d] Fraction of pO_2 readings <5 mm Hg × tumor volume.
[e] Fraction of pO_2 readings <5 mm Hg.

coregional control as end points, respectively. Tumor volume showed no significant change during 2 weeks of radiotherapy. Statistical analyses of the biological parameters after 2 weeks of radiation and the changes in the parameters during this time were performed. The results were compared with results from corresponding analyses of pretreatment HSV_5 based on the same subgroup of 22 patients, assuming that the subgroup was representative of the whole group of patients. Thus, the relationship between HSV_5 and control persisted in analyses, including only the subgroup rather than the whole group of patients (Fig. 3, *A, C,* and *E*; Table 1). It was concluded that a parameter was of minor importance for disease control compared with pretreatment HSV_5 if the correlation between control and the parameter was weaker than the correlation between control and pretreatment HSV_5.

The pO_2 changed significantly during radiotherapy for most patients; 10 patients had an increase, 10 patients had a decrease, and 2 patients had no change in pO_2. Median HSV_5 was 57.0 cm^3 before treatment and 45.5 cm^3 after 2 weeks of therapy. Analyses of the data after 2 weeks of therapy showed a reduced difference in control probability between patients with a large (above median) and patients with a small (below median) HSV_5 (Fig. 3). Thus, HSV_5 after 2 weeks of therapy showed a weaker correlation to control ($P = 0.13$, overall survival; $P = 0.007$, disease-free survival; $P = 0.33$, locoregional control) than pretreatment HSV_5 ($P = 0.02$, overall survival; $P = 0.004$, disease-free survival; $P = 0.06$, locoregional control; Table 1). Similar results were achieved when the other pO_2 parameters after 2 weeks of radiotherapy were considered (HF_5; Table 1). The pO_2 after 2 weeks of radiotherapy was, therefore, probably not as important as pretreatment HSV_5 for disease control.

Vascular density, cell density, and frequency of mitosis and apoptosis after 2 weeks of therapy were not related to control either. Failure occurred about equally as frequent, regardless of whether patients with a high (above median) or a low (below median) value of these parameters were considered (Fig. 4). All parameters showed an impaired correlation to control ($P > 0.4$) compared with pretreatment HSV_5 (Table 1). The vascular density, cell density, and frequency of mitosis and apoptosis after 2 weeks of radiotherapy were, therefore, probably also of minor importance for disease control compared with pretreatment HSV_5.

Changes in Biological Parameters during Radiotherapy. Changes in HSV_5 during 2 weeks of therapy are shown in Fig. 5 for patients with failure and patients with control, using overall survival (*A*), disease-free survival (*B*), and locoregional control (*C*) as end points. HSV_5 decreased in 13 patients and increased in 9 patients. There were no more failures among the patients with decreased HSV_5 than among those with increased HSV_5. The control probability was, therefore, not increased for

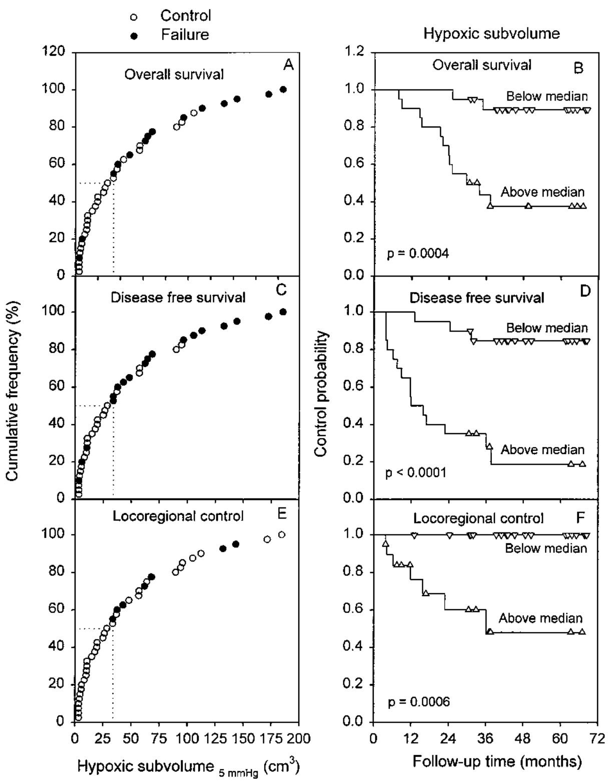

Fig. 1 Cumulative frequency diagrams of tumor HSV (fraction of pO_2 readings <5 mm Hg × tumor volume) before treatment (*A, C,* and *E*) and Kaplan-Meier estimates of control probability after radiotherapy (*B, D,* and *F*) for 40 patients with uterine cervical cancer. *A, C,* and *E,* patients with control (○) or failure (●) are indicated, using overall survival *(A)*, disease-free survival *(C)*, and locoregional control (*E*) as end points; each *symbol* represents one patient; the median value of the HSV is marked (····). *B, D,* and *F*, patients with pretreatment HSV below the median value (▽) and above the median value (△) are compared, using overall survival (*B*), disease-free survival (*D*), and locoregional control (*F*) as end points; each *symbol* represents a censored observation.

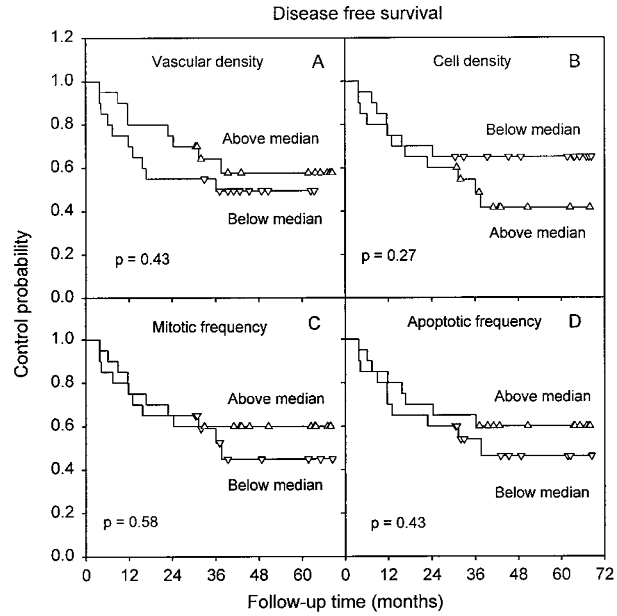

Fig. 2 Kaplan-Meier estimates of control probability after radiotherapy for 40 patients with uterine cervical cancer. Patients with pretreatment vascular density (*A*), cell density (*B*), mitotic frequency (*C*), and apoptotic frequency (*D*) below the median value (▽) and above the median value (Δ) are compared, using disease-free survival as an end point; each *symbol* represents a censored observation.

the former patients, although the HSV had decreased. The magnitude of the changes in HSV_5 was not related to control either. The changes, ranging from 38.1 cm^3 to -91.2 cm^3 for patients with failure and from + 22.9 cm^3 to -55.3 cm^3 for patients with control (Fig. 5), were comparable with the pretreatment HSV_5, suggesting that an influence of the changes on control should be detected, if present. The changes in HSV_5 showed a weaker correlation to disease control ($P = 0.09$, overall survival; $P = 0.24$, disease-free survival; $P = 0.09$, locoregional control) than the pretreatment HSV_5 (Table 1). Thus, the changes in HSV_5 during 2 weeks of radiotherapy were probably not as important as pretreatment HSV_5 for disease control. Analyses of changes in the other pO_2 parameters showed similar results (HF_5; Table 1).

Fig. 6 shows changes in vascular density, cell density, and frequency of mitosis and apoptosis during 2 weeks of therapy. The cell density decreased significantly, both for patients with failure and patients with control ($P < 0.001$; Fig. 6*B*), reflecting a marked treatment effect on the tumors. The two groups of patients had also a significant increase in apoptotic frequency ($P = 0.03$; Fig. 6*D*). Vascular density and mitotic frequency showed no changes during therapy. The changes in the histological parameters showed an impaired correlation to disease control ($P > 0.2$) compared with pretreatment HSV_5 (Table 1). The changes in these parameters were, therefore, probably also of minor importance for disease control compared with the HSV_5.

Multivariate analysis including stage, volume, pO_2 parameters, and histological parameters showed that HSV was the only independent parameter correlated to disease control ($P = 0.0001$, overall survival; $P < 0.0001$, disease-free survival; $P = 0.004$, locoregional control; Table 2). Analysis including stage, volume, and histological parameters identified volume as an independent parameter ($P = 0.0002$, overall survival; $P < 0.0001$, disease-free survival; $P = 0.004$, locoregional control; Table 2).

DISCUSSION

Relationships between disease control of cervical cancer on the one hand and tumor pO_2, vascular density, cell density, and frequency of mitosis and apoptosis on the other were compared in the present work in search for a biological parameter of major importance for control. Parameters measured before treatment in 40 patients and after 2 weeks of therapy in 22 patients were analyzed. Analyses based on such a limited number of patients are associated with some uncertainties:

(*a*) The analyses may fail to identify true differences in control probability between patient groups (type I error). The

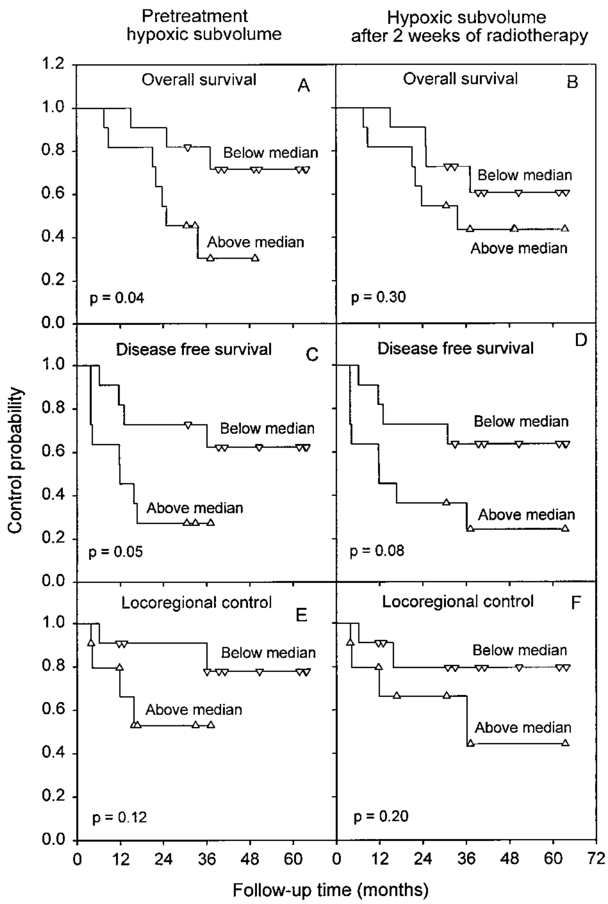

Fig. 3 Kaplan-Meier estimates of control probability after radiotherapy for 22 patients with uterine cervical cancer. *A*, *C*, and *E*, patients with pretreatment HSV below the median value (▽) and above the median value (Δ) are compared, using overall survival (*A*), disease-free survival (*C*), and locoregional control (*E*) as end point. *B*, *D*, and *F*, patients with HSV after 2 weeks of radiotherapy below the median value (▽) and above the median value (Δ) are compared, using overall survival (*B*), disease-free survival (*D*), and locoregional control (*F*) as end points. Each *symbol* represents a censored observation.

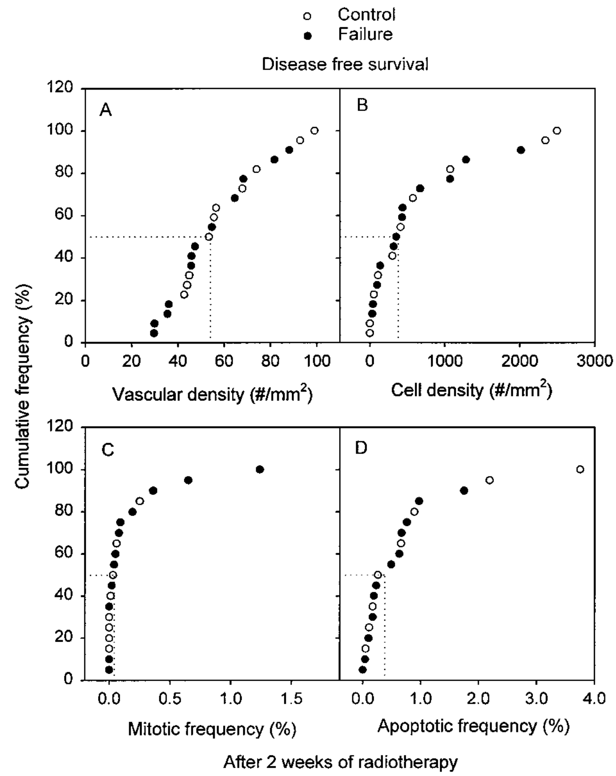

Fig. 4 Cumulative frequency diagrams of vascular density (*A*), cell density (*B*), mitotic frequency (*C*), and apoptotic frequency (*D*) after 2 weeks of radiotherapy for 22 patients with uterine cervical cancer. Patients with control (○) or failure (●) are indicated, using disease-free survival as an end point; each *symbol* represents one patient; the median value of the biological parameters is marked (····). *B*, *C*, and *D*, the biopsies of two patients with control contained no carcinoma tissue after 2 weeks of radiotherapy, although the tumors were highly palpable, leading to two points with zero cell density (*B*) and 20 rather than 22 determinations of mitotic frequency (*C*) and apoptotic frequency (*D*).

aim of our work was to find the parameter most important for control. Because significant results were achieved when one of the parameters, pretreatment pO_2, was considered in the analyses, the number of patients was large enough for our purpose. Although inclusion of more patients might lead to strong correlations between disease control and some of the other parameters also, the specificity of these parameters in prediction of control will probably not be as high as for pO_2.

(*b*) Differences in control probability between patient groups may be erroneously identified (type II error). We found that patients with a high pretreatment pO_2 had a higher control probability than patients with low pretreatment pO_2, regardless of whether all patients or the subgroup of 22 patients was considered. Others have reported similar differences in studies based on a larger number of patients (14), suggesting that our analyses identified true differences in control probability. Moreover, conclusions were based on the subgroup of 22 patients, assuming that the subgroup was representative of the whole group of patients. This assumption was probably fulfilled, because patient age, stage of disease, tumor volume, follow-up time, and biological parameters were about the same for the subgroup and the whole group of patients. It was, therefore, possible to draw conclusions based on the subgroup also.

The second measurement of biological parameters was performed after a treatment period of 2 weeks, *i.e.*, before significant changes in tumor volume were expected. A tumor diameter of $>$2 cm is required for reliable pO_2 measurements in cervical carcinomas with the Eppendorf pO_2 Histograph (11). Our choice of time point for the second measurement, therefore, ensured that no patients were rejected from this measurement because of comprehensive tumor shrinkage during treatment. Thus, in a previous study on cervix tumors, Fyles *et al.* (21) rejected $>$13% of the patients from the second pO_2 measurement after a treatment period of 4 weeks because of insufficient tumor remaining. It should be emphasized, however, that the use of another time point for the second measurement may lead to relationships between control and the biological parameters that differ from those presented here, because considerable changes

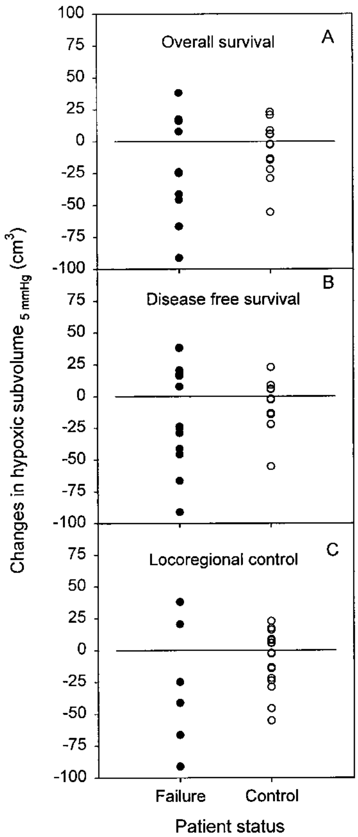

Fig. 5 Changes in tumor HSV (fraction of pO_2 readings <5 mm Hg × tumor volume) during 2 weeks of radiotherapy for 22 patients with uterine cervical cancer. The changes were calculated as the difference between the values after 2 weeks of therapy and the pretreatment values. Patients with control (○) or failure (●) are indicated, using overall survival (*A*), disease-free survival (*B*), and locoregional control (*C*) as end points; each *symbol* represents one patient.

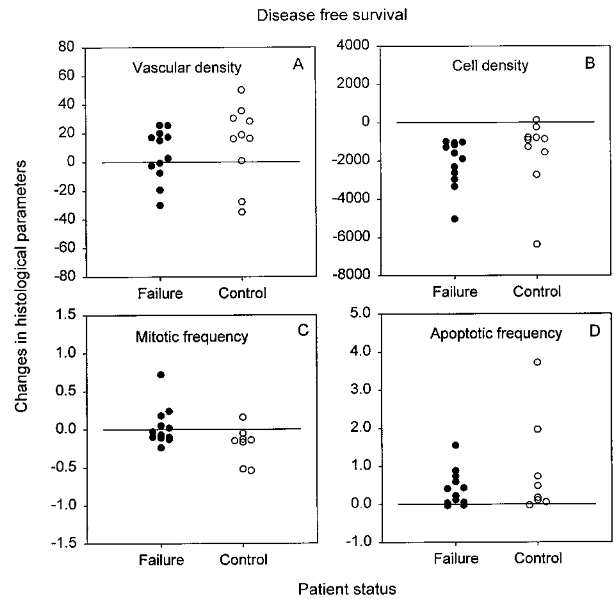

Fig. 6 Changes in vascular density (*A*), cell density (*B*), mitotic frequency (*C*), and apoptotic frequency (*D*) during 2 weeks of radiotherapy for 22 patients with uterine cervical cancer. The changes were calculated as the difference between the values after 2 weeks of therapy and the pretreatment values. Patients with control (○) or failure (●) are indicated, using disease-free survival as an end point; each *symbol* represents one patient.

in the parameters may occur during the late phase of radiotherapy (22–25). Parameters measured during this phase or the changes in these parameters during that time may therefore be of significant importance for control of cervical cancer compared with pretreatment oxygenation.

Pretreatment pO_2 showed a stronger correlation to disease control than the other biological parameters, indicating that the oxygenation is of major importance for control after radiotherapy of patients with cervical cancer. This hypothesis is supported by results from other studies (11, 14, 26). The strong correlation between pretreatment pO_2 and control suggests that the oxygenation influences rate-limiting steps of the events, leading to failure. Regional failure is the most common cause of death from this disease (27, 28). Failure can be seen as extensive tumor infiltration and lymphogeneous spread throughout and regionally beyond the pelvis, leading to obstruction of the ureters, loss of renal function, and uremia. The main factors determining control after radiotherapy are, therefore, probably incidence of lymphogeneous metastases and tumor radioresistance (29–31). Hypoxia may induce genetic instability of tumors, leading to increased metastatic potential (32, 33). Our recent clinical studies showing that high incidence of lymph node metastases at the time of diagnosis is related to high lactate level and low oxygenation of the primary cervix tumor, suggest a significant influence of oxygenation on the metastatic process (34, 35). Increased radioresistance of hypoxic tumors is, however, also a probable factor contributing to the impaired disease control of patients with poorly oxygenated cervix tumors (36).

Although several studies indicate that pretreatment oxygenation is of major importance for disease control of cervical cancer, it is not clear whether the oxygenation during radiotherapy is important (21, 37). Our results suggest that the oxygenation after 2 weeks of radiotherapy and the changes in the parameter during this time are of minor importance for disease control compared with the pretreatment oxygenation. Similarly, Fyles *et al.* (21) reported impaired correlation between oxygenation and survival of patients with cervical cancer when hypoxic fraction after 4 weeks of radiotherapy rather than pretreatment hypoxic fraction was considered. There may be several reasons for these observations:

(*a*) Metastasis formation often occurs during an early phase of the disease, *i.e.,* before treatment is started, and may therefore depend primarily on pretreatment oxygenation and not on the oxygenation during therapy. Thus, lymphogeneous spread of cervical cancer is common, even in early-stage carcinomas, and occurs in 25–50% of patients with stages Ib and II (28, 38).

(b) The hypoxia-induced radioresistance, depending on the oxygenation and clonogenicity at each time of irradiation, may not be very well reflected by the oxygenation after a certain time of radiotherapy. Thus, the oxygenation may fluctuate between high and low values during therapy, depending on changes in

Table 2 Multivariate Cox regression analysis of stage, volume, and biological parameters *versus* disease control for patients with uterine cervical cancer

	End point					
	Overall survival		Disease-free survival		Locoregional control	
Parameter	*P*	Relative risk	*P*	Relative risk	*P*	Relative risk
I						
Pretreatment HSV_5[a]	0.0001	1.02	<0.0001	1.03	0.004	1.03
II						
Pretreatment volume	0.0002	1.01	<0.0001	1.02	0.004	1.02

[a] Fraction of pO_2 readings <5 mm Hg × tumor volume.

biological parameters important for the oxygen consumption and supply of the tumor (39, 40). An increase in oxygenation during the early phase of radiotherapy may be followed by a decrease during a later phase and *vice versa*. Pretreatment oxygenation may be a better indicator of the radioresistance because of a higher clonogenicity before treatment.

Tumor vascular density, cell density, and frequency of mitosis and apoptosis were also of minor importance for disease control compared with pretreatment oxygenation, regardless of whether the pretreatment parameters, the parameters after 2 weeks of radiotherapy, or the changes in the parameters during this time were considered. In accordance with our data, Hawighorst *et al.* (17) found no correlation between pretreatment vascular density and control in a study of 37 patients with cervical cancer. Moreover, pretreatment apoptotic frequency showed only a weak correlation to control in a study of 44 patients with this disease (8). It is possible that studies based on more patients than included here would show significant correlations between control and some of the parameters other than pretreatment oxygenation. Thus, pretreatment vascular density has been found recently to influence control of cervical cancer in studies of >100 patients (15, 41). However, the need for a large number of patients to achieve significant results suggests that these parameters are not as important as pretreatment oxygenation for disease control.

The present results indicate that pretreatment oxygenation is more important for disease control of cervical cancer than the oxygenation after 2 weeks of radiotherapy or the changes in the parameter during this time. Moreover, vascular density, cell density, and frequency of mitosis and apoptosis before treatment or after 2 weeks of therapy are probably not as important as pretreatment oxygenation for control as well. Significant correlations between disease control and the oxygenation were achieved for 40 patients, suggesting a high specificity of this parameter in prediction of control. Selection of patients to adjuvant treatments should therefore be based on pretreatment measurements of tumor oxygenation. Development of efficient strategies for influencing the control probability by changing tumor oxygenation probably necessitates knowledge of whether increased metastatic potential or increased radioresistance is the major cause of the impaired disease control of patient with hypoxic cervix tumors.

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