Angiogenic Activity of Cervical Carcinoma: Assessment by Functional Magnetic Resonance Imaging-based Parameters and a Histomorphological Approach in Correlation with Disease Outcome

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ABSTRACT

Angiogenesis plays a fundamental role in tumor growth and metastasis. What is needed is a quantitative, noninvasive, and repeatable assay to estimate functional angiogenic activity of the entire tumor. The aims of the present study were to: (a) examine the relationship between functional magnetic resonance imaging (MRI)-based parameters with established histomorphological markers of tumor angiogenesis [histological microvessel density (HMVD) and vascular endothelial growth factor expression (VEGF)]; and (b) determine the ultimate value of both approaches to assess functional angiogenic active hotspots as markers of disease outcome in patients with cancer of the uterine cervix. Pharmacokinetic parameters (amplitude $A$, tissue exchange rate constant $k_{21}$) were calculated from contrast-enhanced dynamic MRI series in 57 patients (mean age, 49 ± 14 years) with biopsy proven uterine cervical cancer. Both pharmacokinetic parameters were correlated to histomorphologically determined areas of high HMVD and VEGF expression obtained from the operative specimens after radical surgery. In addition, the functional MRI and histomorphological data were used to assess disease outcome. A significant association was found between HMVD and the amplitude $A$ ($P < 0.001$) and a less pronounced association with $k_{21}$, ($P < 0.05$), respectively. No significant associations were found between the pharmacokinetic parameters ($A$, $k_{21}$) and VEGF expression. When stratified into high and low median $k_{21}$ groups, median $k_{21}$ values $>5.4$ min$^{-1}$ were the only significant ($P < 0.05$) factors in predicting poor patient survival. None of the histomorphological markers of angiogenesis (HMVD or VEGF expression) showed any predictive power. We have found that: (a) focal hotspots of HMVD are the pathophysiological basis for differences in functional MRI; (b) areas of high microvessel density and microvessel permeability do not necessarily coincide, as demonstrated by the histomorphological and functional MRI findings; (c) the functional angiogenic activity of a tumor may not be sufficiently characterized by a histomorphological approach but rather by a functional MRI-based approach; and (d) functional MRI-based analysis may assess tumor angiogenic activity in terms of disease outcome more comprehensively than the histomorphological approach.

INTRODUCTION

Neovascularization is a fundamental prerequisite for expansive and clinically relevant growth of solid tumors (1). Various experimental models as well as clinicopathological observations have shown that solid tumors [e.g., breast (2), lung (3), and cervical carcinoma (4, 5)] cannot attain diameters $>2$–$3$ mm without their own vascular supply. Moreover, new vessels are essential for the shedding of tumor cells into the circulation, and subsequently, for the formation of metastases (1).

Although the HMVD$^2$ technique is the current “gold standard” for characterization of tumor angiogenesis, it may not be an ideal tool for clinical purposes because it is invasive, prone to sampling errors, and does not assess the functional angiogenic activity. Furthermore, it is well established that the vascular system of tumors is very inefficient. Great variations in the spatial and temporal distribution of perfusion are reported to be present in a variety of tumors (6). These regional in vivo variabilities of angiogenic activity cannot be sufficiently assessed by the purely morphological approach of counting the microvessels in tumor areas with the highest microvessel density. In addition, there is strong evidence that angiogenic activity is also reflected in the vascular permeability of tumors. VEGF

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2 The abbreviations used are: HMVD, histological microvessel density; VEGF, vascular endothelial growth factor; MRI, magnetic resonance imaging; ROI, region of interest.
was identified as a powerful enhancer of endothelial hyperpermeability (7), and the leakage of serum proteins is considered an essential step in the angiogenic process (8). Clearly, an in vivo assay for estimating angiogenic activity would be desirable and clinically powerful, particularly if the method were quantitative, noninvasive, able to sample the entire tumor, and repeatable at frequent intervals.

Dynamic contrast-enhanced MRI is proving to be a powerful new tool to assess the spatial and temporal variations of tumor microcirculation (9-11). In addition, functional imaging is likely to be further enhanced by the development of quantitative MRI methods in conjunction with a suitable pharmacokinetic model, allowing a more detailed parametrization of contrast enhancement with respect to tumor microcirculation (12, 13). However, despite the enormous clinical potential of evaluating angiogenic activity, detailed MRI correlations with present gold standards of tumor angiogenesis, HMVD and VEGF expression, and disease outcome are not available. One major obstacle is that accurate correlations necessitate comparison of anatomical MRI maps with whole-mount specimens rather than with unrepresentative biopsy specimens so that maximum values of ROIs can be compared.

The aims of the present study were to: (a) examine the relationship between functional MRI-based parameters with recognized histomorphological markers of tumor angiogenesis (HMVD and VEGF); and (b) determine the ultimate value of both approaches to assess functional angiogenic active hotspots as markers of disease outcome in patients with cancer of the uterine cervix.

MATERIALS AND METHODS

Patients. Fifty-seven patients (mean age, 49 ± 14 years) with biopsy-proven primary (n = 45) or recurrent (n = 12) cancer of the uterine cervix were included in this study. Surgical treatment consisted of radical hysterectomy (n = 19) or pelvic exenteration (n = 38) with pelvic lymph node dissection in all cases. Patients with recurrent disease had been treated prior to MR imaging with surgery (n = 6, Wertheim-Meigs operation) or a combination of operation and postoperative radiotherapy (n = 6, Wertheim-Meigs operation and postoperative pelvic external irradiation ranging from 18 to 64 Gy). The time interval between initial treatment of the primary carcinoma and the time of suspected tumor recurrence ranged from 4 to 154 months (mean, 34 months).

MR Imaging Protocol. All MR studies were performed on a 1.5 T clinical MR system (Magnetom SP 4000 or Vision; Siemens, Erlangen, Germany). The MR examination included T2-weighted fast spin-echo images [repetition time (TR)/echo time (TE) = 2800-4200 ms/93-120 ms; field of view (FOV) = 180-350 mm; section thickness (TH) = 5-7 mm; 3-15 echos] as well as pre- and postcontrast T1-weighted SE images (600/15 ms; FOV, 180-350 mm; TH, 5-7 mm).

A strict dynamic MRI protocol was adhered to minimize errors derived from the MRI data acquisition. Therefore, the following points were taken into account: (a) the entire cervical tumor was scanned by 8-10 sections with 22 repetitions; (b) an ultrafast and linearly T1-weighted saturation-recovery, turbo fast low-angle shot (SRTF) [TR/TE, 10/4 ms; flip angle α, 12°; recovery time (TRRC), 200 ms; Matrix (MA), 256 × 128 interpolated to 256 × 256] sequence with a high temporal resolution time of 1.4 s/section was used (12); (c) the effect of operator dependency on injection times was reduced by a short constant rate infusion within 30 s using a variable-speed infusion pump (CAI 626P; Doltron AG, Uster, Switzerland); (d) the lag-time between injection of contrast material and the individual arrival at the tumor was estimated from the signal-time course measured in the common iliac arteries; (e) quantitative data analysis was performed at a high in-plane resolution (.9-1.4 × .9-1.4 mm) of a dynamic time course by a nonlinear least-squares fitting (13); and (f) to compare maximum values in the ROIs on the whole-mount specimens and correlating pharmacokinetic MRI maps, all MRI-histopathological correlations were performed by matching whole-mount specimens with MRI sections cut in a similar plane (with an approximate accuracy of 3-5 mm).

Data Analysis. Gadolinium-DTPA (Magnevist, Schering, Berlin) was used at a total dose of 0.1 mmol Gd-DTPA/kg body weight. The automated processing of the dynamic images on a pixel-by-pixel basis for each section was performed on a Dec-3000 work station (model 500; Digital Equipment Co., Maynard, MA). In addition, the calculated MR images were color coded to allow identification of intratumoral differences of areas with a fast and/or high contrast uptake. On the basis of the color-coded maps, similarly sized and positioned ROIs, as guided on the whole-mount specimens, were drawn on the pharmacokinetic maps (minimum size, 4-8 mm²).

Computation of Pharmacokinetic Parameters from Dynamic MR Imaging Measurements. The data analysis is based on the pharmacokinetic open two-compartment model as proposed by Brix et al. (13). According to this model, the kinetics of the relative signal enhancement can be described by the model equation:

\[
\frac{S_{CM}(t)}{S_0} = 1 + A\left[e^{k_2 t} - 1\right]e^{-k_1 t} - b\left[e^{k_2 t} - 1\right]e^{-k_1 t}
\]

with

\[
a = \frac{k_{21}}{k_{21} + k_{20}} \quad \text{and} \quad b = \frac{1}{(k_{31} - k_{30})}
\]

During the infusion (0 < t < T) of the contrast material, the identity \( r' = T \) has to be used, afterward the identity \( r' = \tau \). A noteworthy property of this equation is that apart from multiplication by the multidimensional amplitude A, the shape of the temporal response \( S_{CM}(t)/S_0 \) is determined in the approximation described only by the kinetic parameters \( k_{21} \) and \( k_{20} \). Three transport parameters, defined in Fig. 1, describe the velocity of the contrast material between the central and peripheral compartments \( [k_{12}, \ k_{21} \ \text{min}^{-1}] \) and the elimination \( (k_{30}) \) of the contrast material from the central compartment. Assuming that the contrast material is not hampered in one direction, the exchange parameters \( k_{12} \) and \( k_{21} \) are equal (12, 13). The three parameters \( k_{31} \), \( k_{20} \), and \( A \) were determined by least-squares fitting of the measured data. By this approach, the complete tissue-specific information contained in a signal-time curve is condensed into two parameters: the rate constant \( k_{21} \) characterizing the velocity of the tissue specific signal enhancement; and
the amplitude $A$ reflecting the degree of MR signal enhancement. The amplitude $A$, which can be understood as an asymptotic degree of relative signal enhancement if there is no contrast material elimination, depends on three tissue-specific parameters: on the precontrast tissue relaxation time $T1$; on the tissue-specific relaxivity; and on the fraction of the extracellular volume. In addition, the amplitude $A$ depends on the sequence used ($T_{REC}$) and on the infusion rate (12, 13). As proven by computer simulations, $k_{21}$ can be determined reliably in the range $k_{21} < 13$ min$^{-1}$. This limit corresponds to a distribution time of $t_{21} = \ln(2)/k_{21}$, which is on the order of one-quarter of the temporal sampling interval (13 s; Ref. 12).

**MRI-based Histopathological Correlation.** All surgical specimens were cut in a mediasittal or axial plane resembling (with an approximate accuracy of 3–5 mm) the sections obtained by functional MRI. The operative specimens were processed using macroslide techniques to verify histopathological tumor extension. Histology was based on H&E staining and immunohistochemical staining of endothelial cells described previously (5, 16, 20). Briefly, the whole-mount sections of 5 μm were stained with immunohistochemical factor VIII-related antigen. The criteria used for vessel counting were those established by Weidner et al. (2), and each ROI was counted twice by two independent pathologists. The mean counts in eight areas with the highest density (hot spots) of microvessels (HMVD) were recorded at ×100. These sections were marked with a 2 × 2-mm rectangle and correlated with the maximal pharmacokinetic parameters ($A$, $k_{21}$).

Staining for VEGF was performed using a commercially available polyclonal anti-VEGF antibody AB-2. For evaluation of VEGF expression, immunoreactivities were graded on a 4-point scale (1, none; 2, slight; 3, moderate; and 4, strong) according to the staining intensity. A tumor with a score of 1 or 2 was classified as negative, and a tumor with 3 or 4 was regarded as positive.

**Statistical Analysis.** The statistical analyses of the histopathological and dynamic MR data were performed by using SAS software (SAS for Windows, version 6.10; SAS, Cary, NC). To test the strength of association between the various histomorphological markers and the MRI parameters, the unpaired $\chi^2$ test was used. All cases were stratified into high and low pharmacokinetic MR parameters and histomorphological data according to the median value. For both groups, Kaplan-Meier survival curves were calculated and compared using log-rank statistics. A $P$ value of $<0.05$ was considered as being statistically significant.

**RESULTS**

After a median follow-up time of 24 months (range, 3–39 months), 23 (40%) of 57 patients died of relapsed disease. Median HMVD, VEGF score, and dynamic MRI parameters are summarized in Table 1.

Correlations of maximal values of ROIs between the histopathological and the MRI data were possible in all tumors. The median (range, 7–45) HMVD in a ×100 field for all tumors was 25. The intratumoral HMVD and VEGF heterogeneity from microregion to microregion was moderate, and the mean HMVD/VEGFs on the macroslide specimens obtained in eight different areas exhibited a significant association ($P < 0.05$).

For individual tumors, two main patterns of HMVD expression were noted: (a) low values for central and high values for peripheral microvessel density were observed, although no significant differences in the mean HMVD between central and peripheral regions could be found; and (b) a homogeneous distribution of HMVD was recorded.

The color-coded MR maps also exhibited two main patterns of contrast enhancement: (a) there was a homogeneously colored map encoding the entire tumor with a fast and/or high contrast medium uptake (Fig. 2); and (b) there was a spatial distribution of contrast uptake with a high uptake in the tumor periphery and a fast uptake in the tumor center (Fig. 3).

Positive VEGF expression was observed in 28 of 57 (49%) tumors; negative VEGF expression was observed in the remaining 29. Areas of high VEGF expression were commonly associated with a low HMVD, and those with a low VEGF expression with a high HMVD. This inverse association between VEGF expression and HMVD was statistically significant ($P < 0.01$).

On the basis of information from the color-coded maps, pharmacokinetic MR parameters were distributed in cervical carcinomas in two distinct patterns. The first or homogeneous pattern was present in 42% of the tumors ($n = 24$) and consisted of a homogeneous distribution of both parameters ($A$ and $k_{21}$) with a fast and/or high contrast medium uptake (Fig. 2). The second pattern ($n = 33$) was characterized by a spatial distribution of the pharmacokinetic parameters with a high amplitude.
Fig. 2  Demonstration of a Fédération Internationale des Gynaecologistes et Obstétristes IIB cervical carcinoma (A, arrow on the whole-mount specimen) with a homogeneous distribution of HMVD and VEGF expression shown by pharmacokinetic MRI characteristics (B), HMVD (C), and VEGF expression (D). B, the pharmacokinetic MRI slice (corresponding to the whole-mount specimen) shows a homogeneously colored map (arrow; pink coloring) encoding areas with a fast \( k_2 \) but low amplitude \( A \) contrast uptake. Note that the blue areas encode normal tissue of the corpus uteri. C, \( \times 100 \) (arrow) of one of the hot spots (square in A) reveals a low HMVD (seven vessels/4 mm\(^2\)), confirming that focal hotspots of HMVD colocalize with areas of high signal intensity on dynamic MRI. D, areas of high VEGF expression (arrow; tumor cells intensively brown stained; score, 4) were commonly associated with a low HMVD. However, no significant associations were observed between the pharmacokinetic MRI parameters and VEGF expression.
A in the tumor periphery and a low amplitude $A$ in the tumor center on the one hand, and a high $k_{21}$ in the tumor center with a low $k_{21}$ in the tumor periphery on the other hand (Fig. 3). However, these spatial differences in the MR parameters did not reach significance.

A significant association was found between HMVD and the amplitude $A$ ($P < 0.001$) and a less pronounced association with $k_{21}$ ($P < 0.05$), respectively (Table 2). No significant associations were found between the pharmacokinetic parameters ($A, k_{21}$) and VEGF expression ($P = 0.9$, respectively).

Univariate analysis of survival is shown in Fig. 4. When stratified into high and low median $k_{21}$ groups, median $k_{21}$ values $>5.4$ min$^{-1}$ were the only significant ($P < 0.05$) factors in predicting poor patient survival. None of the histopathological markers of angiogenesis (HMVD or VEGF expression) showed any predictive power ($P = 0.3$ and 0.4, respectively).

**DISCUSSION**

Presently, there is no single validated method that can sufficiently describe the functional status of tumor angiogenesis.

In the absence of such a standardized assay, we have chosen HMVD and VEGF expression as the best available indicators of tumor angiogenesis for comparison with contrast-enhanced dynamic MRI of microvascular characteristics.

Recent studies have shown that differences in tumor angiogenesis are correlated with different patterns of contrast enhancement observed by MRI (9–15). The regenerated signal-time curves depend on the local microvessel density, regional blood flow, microvessel permeability for the contrast agent, and on the size and physicochemical nature of the extracellular...
space accessible for gadolinium-based contrast agents (12, 13). Despite the enormous potential of MRI to assess angiogenic activity, accurate correlations of anatomical MRI maps (or pharmacokinetic maps) with histopathological markers of tumor angiogenesis, such as HMVD and VEGF expression, derived from histopathological whole mount specimens of human tumors do not exist. However, an improved understanding of the fundamental processes involved in tumor angiogenesis and metastatic spread by MRI-derived signal-time curves would have a significant clinical impact (e.g., monitoring of the action of new anti-angiogenic therapies, noninvasive prediction of tumor aggressiveness) and would improve our in vivo understanding of tumor angiogenesis.

The present work has implications for the selection of ROI-based MRI-histopathological correlations. Maximal areas with the fastest or highest contrast medium uptake (A, $k_{21}$) colocalized significantly with focal hotspots of HMVD, as estimated on the whole-mount specimens. In particular, areas with a high contrast enhancement (amplitude $A$) coincided with areas of HMVD, especially in tumors with a rim-like pattern of enhancement. Because the gadolinium-based infusion rates were kept constant, the observed differences in the amplitude $A$ probably represent the size of the intravascular volume.

A second fundamental finding of this study is that areas of high microvessel density and high microvessel permeability do not necessarily colocalize, as demonstrated by the inverse association between HMVD and VEGF expression, on the one hand, and large spatial variations of areas with a high and fast MRI contrast uptake, on the other.

Theoretically, the velocity (upslope) of contrast media uptake is dependent on multiple factors, including perfusion rate, microvessel density and microvessel permeability, and the size of the extracellular space (10, 11, 16). Which of these pathophysiological mechanisms is the major contributor for the differences in the velocity of contrast media uptake is not clear. In a recent study using mice, it has been postulated that differences in tumor microvessel permeability can be assessed by Gd-DTPA-based contrast agents (11). The assumption that areas of high HMVD influence the velocity of the signal enhancement is partly supported by the results of the present study. However, the spatial analysis of the color-coded parameter images revealed no significant association between VEGF expression, a recognized marker for tumor angiogenesis, especially microvessel permeability, and areas of fast contrast uptake (the rate constant $k_{21}$).

The findings that VEGF expression did not colocalize with dynamic MRI parameters is not necessarily contradictory to the above-mentioned statement that contrast-enhanced MRI can assess tumor permeability. The results of this study substantiate recent findings that the overall angiogenic activity of tumor cells is adjustable and continuously modulated according to the metabolic demands (17, 18). Tumor tissue may sense insufficient nutrient supply and respond by eliciting compensatory angiogenesis, thereby matching supply to demand. Furthermore, there is a growing body of evidence for the key role of VEGF as a mediator of this feedback response. It has been shown that once the increased nutritional supply is matched by an efficient increase in microvessel density and permeability, VEGF expression is down-regulated, specifically in regions already invaded by microvessels (18, 19). For this reason, a “static” view of VEGF expression alone might not be the appropriate marker for microvascular function in all instances. Functional microvessel properties (e.g., microvessel permeability) may be present at a given time and location, whereas the inducing signal, i.e., VEGF expression, has already been down-regulated (Fig. 5). This fundamental finding would explain the inverse correlation observed between areas of high HMVD and VEGF expression on the one hand, and the missing association between VEGF expression and MRI contrast enhancement characteristics on the other. Apparently, tumors may be in a dynamic steady state in which the hypoxic regions that were recently up-regulated will later become the highly metastatic angiogenic hotspots with low VEGF expression (17–19). Such tumor dynamics and highly metastatic areas cannot be assessed sufficiently by a histopathological approach, whereas contrast-enhanced dynamic MRI can contribute a lot. This hypothesis is supported by the observation that functional MRI markers were more suited to predict patient survival.

Fig. 4 Kaplan-Meier curves. When stratified into high and low HMVD (A) and $k_{21}$ groups (B), median $k_{21}$ values $>5.4$ min$^{-1}$ were the only significant ($P < 0.05$) factors in predicting poor patient survival.
survival than histomorphological markers of tumor angiogenesis.

Apparently, the present gold standard for assessing angiogenic activity, HMVD or VEGF expression, cannot describe the functional angiogenic status as a marker for metastatic spread and patient survival sufficiently in discrete tumor areas. However, contrast-enhanced dynamic MRI uniquely enables the mapping of areas with high tumor microcirculation in the entire tumor. Furthermore, contrast-enhanced MRI reflects the functionally active tumor parts that may better mirror angiogenic activity in terms of disease outcome, as demonstrated by our results. This additional information on the location of angiogenic hotspots and the functional status of these microvessels may significantly improve our understanding of the complex processes involved in tumor angiogenesis and may influence therapeutic considerations and the ability to monitor new antiangiogenic treatment regimens.

We conclude that: (a) focal hotspots of HMVD are the pathophysiological basis for differences in functional MRI; (b) areas of HMVD and/or microvessel permeability do not necessarily coincide as demonstrated by the histomorphological and functional MRI findings; (c) the functional angiogenic activity of a tumor cannot be sufficiently encompassed by a histomorphological approach but by a functional MRI-based approach; and (d) functional MRI-based analysis may assess tumor angiogenic activity in terms of disease outcome more comprehensively than the histomorphological approach.

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