Sensitive Noninvasive Monitoring of Tumor Perfusion during Antiangiogenic Therapy by Intermittent Bolus-Contrast Power Doppler Sonography

Martin Krix,¹ Fabian Kiessling,¹ Silvia Vosseler,² Nabeel Farhan,¹ Margareta M. Mueller,² Peter Bohlen,³ Norbert E. Fusenig,² and Stefan Delorme¹

¹Departments of Radiological Diagnostics and Therapy and ²Department of Carcinogenesis and Differentiation, German Cancer Research Center, Heidelberg, Germany, and ³ImClone Systems, New York, New York

ABSTRACT

Intermittent bolus-contrast power Doppler ultrasound was used for noninvasive, quantitative monitoring of tumor perfusion during antiangiogenic therapy. Subcutaneous heterotransplants of human squamous cell carcinoma cells in nude mice were treated with a blocking antibody to vascular endothelial growth factor receptor 2 (DC101) and repeatedly examined at weekly intervals. Using replenishment kinetics of microbubbles (Leqvist) tumor vascularization, including capillary blood flow, was clearly visualized by this dynamic ultrasound method allowing the determination of a comprehensive functional status of tumor vascularization (blood volume, blood flow, perfusion, and mean blood velocity) in all examined tumors. DC101 treatment decreased tumor blood flow (~64%) and volume (~73%) compared with untreated controls (+409% and +185%, respectively). Regression of functional vessel parameters was observed early well before reduction of tumor size. The treatment-related amount of reduction in tumor volume was directly correlated for the initial tumor blood flow before start of therapy and the perfusion calculated at the preceding examination. The vessel density (immunofluorescence staining with CD31 antibody at different time points) showed an excellent correlation with the calculated relative blood volume (k = 0.84), P < 0.01, thereby validating intermittent sonography as a useful monitoring method. We conclude that intermittent sonography is a promising tool for comprehensive monitoring of antiangiogenic or proangiogenic therapies, especially during early stages of treatment, thus yielding information regarding a prospective evaluation of therapy effects beyond the follow up of tumor size.

INTRODUCTION

Insights into angiogenesis in cancer and various ischemic and inflammatory diseases have gained importance during the past years (1, 2). There is intense biomedical research on antiangiogenic or proangiogenic therapy, and various approaches are already in clinical trials (3, 4). In preclinical studies, therapy effects are mainly assessed by histological or in vitro examinations (5–8). Experimental approaches in vivo or clinical trials use as criteria for therapeutic efficacy usually reduction of tumor size or progression-free survival. However, to assess early and specific vasculature-related alterations, improved methods for noninvasive monitoring of blood flow and tissue perfusion are of critical importance.

In this respect, radiological studies provide an ideal basis because they are entirely noninvasive and therefore almost universally applicable. However, measuring perfusion (blood flow/tissue unit) quantitatively is a considerable problem with all diagnostic imaging modalities, even including MRI.4 T2*-weighted dynamic susceptibility imaging is suitable to measure perfusion (9) but only where an arterial input function can be obtained and as long as contrast media remain in the intravascular space. The use of experimental magnetic resonance blood pool agents has shown encouraging results to improve blood volume measurement (10). With T2*-weighted dynamic MRI, suitable parameters of blood volume can be assessed in experimental tumors (11); however, these are only indirect measures based on the extravasation of contrast agent into the interstitial space.

In contrast, US techniques show encouraging possibilities to directly evaluate vessel perfusion during angiogenesis (12). Power Doppler sonography can detect reductions of larger tumor vessels during antivascular therapy (13–16). However, conventional and even contrast-enhanced Doppler US is not capable of visualizing capillary blood flow (17), an essential precondition for quantifying tissue perfusion. Therefore, monitoring therapies that mainly affect the microvascular compartment may suffer limitations with these US methods. Potentially as a consequence of this lack of microvessel detection, in most previous studies, no correlation was observed between US parameters and the histologically determined vessel density (16, 18–21). There are, however, newly developed US methods that might be suitable to circumvent these limitations. High-frequency US is a novel technique, capable of detecting therapy-induced changes in small vessels with a diameter down to ~50 μm. However, its use is restricted to superficial tissue (<10 mm), malignant melanoma; Ref. 22).

Additionally, intermittent sonography is a novel approach (23) that uses replenishment kinetics of a US contrast agent. Other than conventional US, it allows to indirectly assess capillary blood flow and is thus well suited to quantify functional parameters of tumor vascularization, including perfusion. The ability of this technique to quantify blood flow has been documented in previous studies where parameters derived by intermittent sonography were correlated with various measurements ex vivo [explanted veins (23), kidney perfusion model (24)], in vivo [myocardial blood flow measurement with radiolabeled microspheres (23, 25, 26)], and in phantoms (23, 27). Several clinical studies on quantifying tissue perfusion of organs have been described in cardiology, neurology, or nephrology (28–30). Limitations are related to general restrictions for US examinations (e.g., motion artifacts and energy loss in deeper tissue regions). Recently, we have further developed IBS and, for the first time, demonstrated that this method enables the quantification of microvascular perfusion in small animals (31). The calculated mean blood velocity in tumors of 0.3 mm/s was within the expected magnitude.

In this study, we used this newly developed IBS in small experimental animals to successfully monitor quantitative alterations in the vascularization induced by systemic antiangiogenic therapy.

MATERIALS AND METHODS

Tumor Model and Antiangiogenic Therapy. We used a highly malignant human keratinocyte cell line, transformed in vitro by Harvey ras transfection and selected by repeated in vivo passage as nude mouse heterotransplant HaCaT-ras-A-SRT3 (32, 33). Tumors were induced by s.c. injection of 2 × 10⁵ cells into the back of 10 nude mice.
Five of the mice were treated with 800 µg of a monoclonal antibody to VEGF receptor 2 (DC101; ImClone Systems, New York, NY) every other day by i.p. injection, starting at day 21 postimplantation when tumors had reached a size of ~0.3 ml (after the first US examination). This antiangiogenic therapy had successfully been tested previously in a comparable model (34).

**Examination Method: Intermittent Sonography.** Intermittent sonography followed a single bolus injection of 100 µl of galactose-based US contrast agent (300 mg/ml Levovist; Schering, Berlin, Germany) injected within 5 s into a tail vein. The principles and theoretical background of the IBS have been described in detail elsewhere (23, 31). After destruction of the intravascular contrast agent (microbubbles) by a high-energy US pulse, it is possible to derive replenishment kinetics of the microbubbles in an individually chosen ROI, as long as microbubbles are delivered from outside the ROI by the systemic circulation. As high US signals can be measured with high sensitivity (already one microbubble can be detected even from stationary microbubbles), capillary blood flow can be measured when microbubbles are given enough time to fill the small vessels (long pulsing interval between US pulses).

According to the model of Wei et al. (23), the replenishment curve (US signal intensity over time after destruction of microbubbles) describes an exponential increase, followed by a saturation behavior. The plateau A of this curve indicates the increase of the curve and allows the approximation of mean blood velocity v in mm/s if the US beam width d is known (v = β × d). In the used setting (see “Examination Protocol”), the beam width had been previously determined to be 0.78 mm (31).

For examining small animals with intermittent sonography, a single bolus injection is needed instead of the usual continuous infusion because reliable continuous infusions of the required small volumes and flows of contrast agent are hardly ever achievable. Consequently, the additional time-dependent decrease of the systemic microbubble concentration after the initial peak has to be considered. Nevertheless, it is possible to calculate replenishment parameters by calculating relative values of A dependent on a main pulsing interval (Fig. 1) and including the maximum (max) of the US signal intensity-time curve after contrast agent injection (31). Thus, according to the model of intermittent sonography, parameters can be derived, which are proportional to blood volume (B = max × A, blood flow [f = A × max × β, (−ml × s−1)], and perfusion [blood flow/tissue unit, P = A × max × β × V−1, (−ml × s−1 × mg−1)]) of a tumor with the volume V.

To compare the blood volume calculated from these data with the histologically derived vessel density, the proportion of blood volume over volume inside the ROI was calculated as BD = B(ROI)/size of the ROI (blood volume density). An overview of the calculated US parameter is shown in Table 1.

**In Vivo Studies.** To evaluate whether IBS is able to provide valid functional parameters of tumor vascularization for monitoring antiangiogenic therapy, complementary US and immunohistochemical analyses were performed. Therefore, the same tumors were examined noninvasively with US, and subsequently, immunofluorescence labeling was performed on frozen tumor sections of sacrificed animals at individual time points (Fig. 2). Three weeks after tumor induction (20 ± 1 days), IBS examinations of 10 nude mice were performed, and each animal was repeatedly examined every week, throughout 1 month. Five were treated with the VEGF receptor antibody, and 5 were kept as control animals.

According to pretherapeutic size (after the first US examination), tumors were assigned to either the treatment group or the controls, trying to keep the baseline tumor volumes in the treatment and control group as similar as possible. The size was measured with the US caliper; the volume V was approximated as $V = qw × d × m$, with $f$ being the maximal length, $w$ the width, and $d$ the depth of tumor. Because the initial blood flow in the tumors’ groups (controls and treated animals) differed, values were normalized to the median value of initial blood flow $f$ (n.a.u.) and perfusion P (n.a.u.), respectively.

Animals were anesthetized by i.p. injection of ketamin (0.1 mg/g body weight, Ketanest; Parke-Davis, Berlin, Germany) and xylazin (1.5 mg/g body weight, Rompun; Bayer Vital, Leverkusen, Germany). The animal experiments were officially approved and complied with legal requirements and institutional guidelines.

**Examination Protocol.** A Siemens-Acuson Sequoia 512 (Erlangen, Germany) US scanner was used, with a fixed linear array transducer 15L8W (power Doppler transmit frequency of 7 MHz). US device parameters were: maximum power (mechanical index, 1.9); maximum pulse repetition frequency (scale, 0.55) to reduce motion and noncontrast flow artifacts; high spatial resolution (“S2”); and standard for all other device parameters. The size (2 × 2 cm) and depth (>0.5 cm) of the color Doppler box, as well as the focus depth (tumor center), were kept constant; the main frame rate was 1.33 s−1. Intermittent imaging was started 1 min after the bolus injection of Levovist and lasted 380 s. This starting point and the following time interval had proven suitability in our previous study (31). A commercial software (Data Pro; Noesis, Courtaboeuf Cedex, France) was used for quantifying the number of color pixels (power Doppler mode), assuming these values were proportional.

### Table 1 Vascularization parameters calculated with intermittent bolus-contrast power Doppler sonography

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculation</th>
</tr>
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<tbody>
<tr>
<td>Blood volume B</td>
<td>max × A × V/ROI</td>
</tr>
<tr>
<td>Blood volume density BD</td>
<td>B/ROI</td>
</tr>
<tr>
<td>Blood velocity v</td>
<td>β × d</td>
</tr>
<tr>
<td>Blood flow f</td>
<td>A × max × β</td>
</tr>
<tr>
<td>Perfusion P</td>
<td>f/V</td>
</tr>
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</table>

* A, plateau of the replenishment curve; β, exponential coefficient of the replenishment curve; max, maximum of the US signal intensity-time curve; d, US beam width; V, tumor volume.
to the number of microbubbles inside the ROI (35). For calculating the mathematical fit of the replenishment curves, we used nonlinear least squares fitting.

In addition, the data were calculated for two ROIs. The first was drawn around the entire tumor circumference, the second ROI was positioned in the central tumor region in a distance from all tumor borders of around the entire tumor circumference, the second ROI was positioned in the central ROI from the total ROI. The periphery was defined by subtracting the central ROI from the total ROI. The central blood flow was calculated by subtracting the peripheral from the total blood flow. The periphery was defined by subtracting the central ROI from the total ROI.

All 10 mice were repeatedly examined (each animal for three to four times). In 1 animal, the first i.v. injection failed. Four untreated mice had to be excluded from the study because of large tumor size. One treated mouse died after the third examination for unexplained reasons. Thus, only results until the third of the weekly examinations were used for statistical evaluation.

Histology. For histological diagnosis and assessment of vital tissue and its vascularization, eight tumors were dissected at given intervals: three untreated tumors 2 weeks after start of treatment and four treated tumors and one untreated tumor after 3 weeks of treatment (Fig. 2). Dissected tissues were covered with tissue tek (Sacura, Zoeterwoude, the Netherlands) and frozen in liquid nitrogen vapor. The tumor slice, which had been examined with IBS a few minutes before, had been tagged with tattoos (s.c. placed ink dots) to match scanning and cutting plane as well as possible 5-μm thick sections were cut with a Reichert-Jung Frigocut 2700 microtome. For morphology, sections were stained with H&E. Blood vessels and tumor cells were visualized by double immunofluorescence staining using a rat monoclonal antibody against PECAM (anti CD 31; PharMingen, San Diego, CA) and a guinea pig pan-keratin antiserum (Progen, Heidelberg, Germany), respectively, following a standard protocol (34).

Tissue sections were viewed by phase contrast and fluorescence microscopy using an Olympus AX-70 microscope. Images were captured with an analysis SIS color view 12 digital camera (Soft Imaging System), and morphometric analyses were performed at ×100 magnification using the analysis SIS (Soft Imaging System) software. To quantify vessel density, including microvascularity, the fraction of stained vessels/square millimeter was determined over the whole section, as well as in the central region.

Statistical Evaluation. Changes of tumor parameters during therapy compared with those of untreated tumors were evaluated using Mann-Whitney-Wilcoxon tests (U test) with a significance level at \( P < 0.05 \). Pearson’s correlation coefficient was used to compare vessel density with IBS parameters and changes of tumor volume with perfusion or blood flow, respectively.

RESULTS

Antiangiogenic Therapy: Reduction of Tumor Size. Tumor size varied considerable in the control and the treatment group, respectively, at the start of therapy (range, 0.004–0.47 ml), reflecting the individual tumor growth. However, tumor volume increased steadily in the control group, whereas it decreased in tumors treated with DC101 (Fig. 3). The initial median tumor volume in the control group was 0.34 ml (range, 0.004–0.42 ml) and increased to 0.63 ml (range, 0.01–0.98 ml) 2 weeks later. Conversely, the median tumor volume in the treatment group was reduced after 2 weeks under therapy from initially 0.30 ml (range, 0.01–0.47 ml) to 0.08 ml (range, 0.02–0.29; \( P < 0.05 \)).

Tumor Vascularization and Effects of Antiangiogenic Therapy: Qualitative US Results. IBS detected contrast-enhanced Doppler signals of the tumor vessels and of the vascularization in the adjacent tissue (Figs. 4 and 5). Nondynamic US pictures at the time of maximal enhancement after contrast agent injection could visualize individual variations of tumor perfusion before start of therapy (Fig. 4, A and C). Particularly in small tumors, high vascularization signals could be observed (Fig. 5A).

A reduction of the vascularization detected by IBS was observed as early as 1 week after the start of antiangiogenic therapy (Fig. 4B). Although there was some initial variation in vascularization visible.
before treatment (Fig. 4, A and C), the contrast-enhanced power Doppler signals were clearly reduced in the treated animals, whereas they additionally increased in the untreated animals (Fig. 4D). Importantly, a reduction in tumor vascularization could already be observed in tumors under therapy before a reduction in tumor size became visible (Fig. 5).

Correlation with Vessel Density. At histological examination, the calculated vessel density in the examined ROIs ranged from 0.27 to 2.60%. An untreated tumor with high vascularization signals in both the immunofluorescence staining and the corresponding US picture at the time of maximum enhancement after contrast agent injection is depicted in Fig. 6, A and C. In contrast, in the treated tumor, histology as well as US data showed clearly lower signals (Fig. 6, B and D). Quantitatively, the vessel density on the microscopic slice (whole tumor) correlated well (k = 0.84, P < 0.01; Fig. 7) with the blood volume density calculated from intermittent sonography (Table 1). When ROIs covering only the central part of the tumors were included in the analysis, this correlation was even more pronounced (k = 0.79, P < 0.001). Interestingly, treated tumors did not show significantly lower overall vessel densities than controls, whereas there was an excellent correlation between blood volume density and vessel density. Because of the large tumor size and thus development of necrotic areas at late stages, vessel density was reduced also in untreated tumors at the analyzed end points of the experiment (2 and 3 weeks after start of therapy, respectively). Dynamic parameters determined by intermittent sonography such as blood flow, blood velocity, or perfusion showed no correlation with the vessel density.

Quantitative Analysis of Functional Imaging. For the quantitative analysis, we obtained a calculated baseline blood flow before treatment that showed some variation as did perfusion (range: 0.3–26.8 a.u.) and blood volume (range, 0.1–6.2 a.u.; Table 2). Despite the randomization of the tumors according to pretherapeutic size to either the treatment group or the controls, the treatment group showed higher initial blood flow values (range, 1.6–6.4 a.u.) than the control group (range, 0.8–3.0 a.u.). However, this bias should not substantially interfere with the results of treatment effects because it were the treated tumors, which initially showed the higher vascularization.

The quantitative US analysis confirmed the observation, which had already emerged from the nondynamic US pictures (Figs. 4 and 5): tumor blood flow f in tumors treated with VEGF receptor antibody...
was different from that in the controls (Fig. 8). In the control group, blood flow increased 1 week after start of observation (median increase, 409%) and then remained high. In the treated tumors, it decreased (median reduction, 64%) and remained low throughout the whole treatment and observation period \((P < 0.05)\). Tumors in the treatment group with an initially higher blood flow showed a larger relative decrease in tumor size \((\Delta V/V)\) during the following 2 weeks relative to those with a lower blood flow \((k = -0.90; P < 0.05)\).

The mean blood velocity \(v\) tended to decrease in treated tumors. Conversely, untreated tumors initially showed an increased mean blood velocity that later slowly decreased (Table 2). However, there were high interindividual differences in mean blood velocity.

Furthermore, the blood volume \(B\) increased in untreated tumors during the observation period of 2 weeks but remained low and slightly decreased in treated ones. Again, interindividual variations were considerable (Table 2).

Perfusion (i.e., blood flow normalized to tissue volume) decreased in central regions of treated tumors (Fig. 9) during the first week of therapy down to almost zero (Table 2; Fig. 4D), but it increased in central parts of untreated ones (Fig. 4D).

No significant changes of the perfusion of the whole tumor were found in both treated and untreated tumors during 2 weeks of treatment. However, from the first to the second and the second to the third examination after starting the DC101 treatment, the overall tumor perfusion was negatively correlated to the tendency of tumors to decrease, meaning that tumors, which showed high perfusion during therapy, decreased in size to a minor degree, whereas tumors with low perfusion showed a good treatment response. This was reflected by an inverse correlation of perfusion with change of tumor volume \((\Delta V/V; k = -0.69, P < 0.05)\). Accordingly, the tumor in Fig. 5 showed a marked reduction in volume after the second week of therapy (Fig. 5C) and had already been poorly vascularized the week before (Fig. 5B).

### Discussion

In light of the generally accepted importance of angiogenesis for tumor growth and progression and the increasing number of antiangiogenic therapy protocols, the noninvasive monitoring of tumor perfusion during antiangiogenic therapy has gained critical importance. In this study, we established for the first time the successful use of intermittent bolus-contrast power Doppler sonography as a sensitive tool for quantitative monitoring of antiangiogenic therapy and demonstrated an excellent correlation of the results obtained with this functional US method with histologically determined vessel density. Previous studies with intermittent imaging have proven the validity of derived parameters for monitoring tissue perfusion and blood flow (23–27). We have evaluated its use for angiogenesis research in small animals by adapting intermittent sonography to low injection volumes of contrast agent and to bolus injection rather than continuous infusion (31). With this modification, IBS can now visualize perfusion even in tumors of small animals and allows to comprehensively describe tumor vascularization by quantifying tissue perfusion, blood flow, blood volume, and mean blood velocity.

Previous studies (34) with the HaCaT-ras model have shown that suppression of ongoing angiogenesis and concomitant inhibition of tumor invasion can be achieved by a blocking antibody to VEGF receptor 2 (DC101). In our study, we used IBS to observe functional blood flow parameters and its changes under therapy in vivo. Tumor blood flow and the perfusion of the central tumor parts decreased in treated tumors as early as 1 week after the start of the treatment, whereas it increased in controls. Thus, IBS enabled us to observe tumor response on antiangiogenic therapy early and independently of a potential reduction of tumor size (Fig. 5) by visualizing a reduction of existing functional vasculature under DC101 therapy.

To validate the IBS measurements, we compared them with histological vessel density, a well-established method to determine tumor vascularization. In general, it is difficult to compare histological slices with noninvasively obtained images. Effort was made to match the slices as well as possible. Comparison of parameters derived from IBS with the histological findings revealed a significant correlation between vessel density with the IBS parameter blood volume density but not with other parameters. Because the calculated blood volume is a nondynamic parameter, it was expected to be the closest corresponding quantity to the static measurement of vessel density. These parameters include both the macrovessels and the microvessels. However, because only the vessel wall but not the lumen contributes to the signal in immunofluorescence images, blood volume in larger

### Table 2

**Quantitative analysis of HaCaT-ras tumors under therapy with DC101 with functional imaging using intermittent bolus-contrast sonography**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5 treated tumors</th>
<th>5 untreated tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood flow</td>
<td>1 week</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Blood flow</td>
<td>4.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Central perfusion</td>
<td>2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Total</td>
<td>1.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Blood volume</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Blood velocity</td>
<td>1.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Stated are median (bold) and ranges.
vessels will not be adequately reflected by histology. Furthermore, vessel density does not discriminate between perfused and nonperfused vessels, and CD31-positive structures found in necrotic areas may reflect nonfunctional vessels, which are not yet disintegrated, thereby giving an insufficient picture of the actual tumor perfusion. The results showed an improved linear correlation of vessel density and calculated blood volume density when ROIs covering only the central tumor parts were included. This emphasizes the good congruence of histology and intermittent sonography, which, however, may be less appropriate at the periphery of tumors, where US could not discriminate between tumor vascularization and adjacent s.c. vessels. The other derived vascularization parameters were dynamic ones and, thus by nature, not expected to correlate with histological findings. Nevertheless, the excellent correlation between histological vessel density and blood volume clearly validate IBS as a useful and sensitive method for monitoring tumor perfusion.

Furthermore, other than conventional US methods (13–15, 19), intermittent sonography can visualize capillary blood flow, thereby allowing the monitoring of the highly relevant microvessel compartment during angiogenesis. In our opinion, the lack of capillary detection might be the reason why in conventional color or power Doppler studies the derived vascularization parameters of Refs. 16, 18–20 did often not correlate with histological blood vessel counts. Thus, the IBS method developed and validated by us is clearly better suited than conventional US methods to monitor therapies which mainly affect microvascularity.

Because the number of the examined tumors was relatively low, general predictions of therapeutic effects must be discussed with caution. It is very likely that an initial high tumor blood flow and a strong reduction of tumor perfusion under therapy are correlated with the subsequent DC101-related tumor size reduction. However, previous studies (11, 31) had shown that perfusion of the HaCaT-ras tumors was inversely correlated with their volume, probably because of increasing necrotic areas that evolve as the tumors grow. As a consequence, these studies demonstrated a decrease in perfusion of untreated tumors during the later time points, a result that we could confirm in our study, where blood flow remained high but tumor volume additionally increased (Figs. 3 and 8). Thus, blood flow and perfusion of HaCaT-ras tumors showed an inverse correlation with the tumors growth. The observation that the calculated perfusion is mainly reduced in the central parts of treated tumors is in line with recently performed studies of monitoring antiangiogenic therapy (DC101) with dynamic MRI. Furthermore, in both studies, treated tumors sometimes showed a higher global perfusion value than controls because of the strong decrease in tumor volume under treatment and thus of tumor mass, to which perfusion is normalized. This complies with our suggestion that only at early time points the degree of perfusion could be of prognostic relevance for any later tumor response. Finally, these findings suggest that vascularization parameters that are normalized to tumor size, as vessel density and perfusion, may not be a general indicator of efficacy of antiangiogenic treatment (36) because they are also influenced by shrinkage of tumor size. More detailed studies, in particular, using early examination points after start of therapy, have to clarify whether the vascularization parameters derived with intermittent sonography will allow an early assessment of tumor response and may serve as prognostic parameters for therapeutic efficacy.

In summary, we have shown that intermittent sonography is a highly sensitive and promising method to measure several valuable perfusion parameters during early stages of antiangiogenic therapy, which are superior to static measurements such as vessel density or to conventional US parameters. Thus, using this novel technique will further our understanding of early alterations in tumor angiogenesis and allow a sensitive and quantitative noninvasive assessment of therapy-related changes of tumor vasculization.

ACKNOWLEDGMENTS

We thank Heinrich Steinbauer and Silke Haid for their excellent technical assistance. We also thank Professor Dr. Ivan Zuna for his help with the statistical analysis, which was greatly appreciated.

REFERENCES


Fig. 9. Calculated central perfusion (n.a.u.) of treated (●) and untreated tumors (○) during the first three examination points in time. The therapy with antibody to VEGF receptor 2 reduced the central perfusion.
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