Neovascularization Induced Growth of Implanted C6 Glioma Multicellular Spheroids: Magnetic Resonance Microimaging

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ABSTRACT

Magnetic resonance imaging has been used to follow noninvasively tumor neovascularization and tumor growth in a model system of multicellular C6 rat glioma spheroids implanted s.c. in nude mice. By positioning a single spheroid approximately 1 cm from the site of incision both the vascularization of the tumor and the wound healing processes were spatially separated and could be simultaneously followed. The model proposed here provides defined initial conditions of tumor geometry and cell proliferative status and separation of initial tumor growth from neovascularization. Magnetic susceptibility relaxation provided an intrinsic marker for blood containing vessels. The implanted spheroid induced vessel growth within 4 days after implantation that was geometrically oriented toward the spheroid and distinct from wound healing at the site of incision. Volume measurements showed a corresponding 4-day lag in growth followed by Gompertz progression. Sham implantation of agarose beads of similar diameter showed no induction of vessel growth, ruling out a direct effect of wound healing. The new vessels penetrating the tumor were highly permeable to the contrast reagent gadolinium-diethylenetriaminepentaacetic acid. This permeability may be due to the action of vascular endothelial growth factor, a major angiogenic growth factor in this system, and a potent permeability factor.

INTRODUCTION

A major concept in solid tumor physiology states that tumor growth is angiogenesis dependent (1). Without penetration of blood vessels tumors can grow only to a size of approximately 1 mm due to limited nutrient and oxygen supply (1, 2). While supporting evidence for this hypothesis has accumulated, there was no direct way to noninvasively detect these early stages in vivo (1, 3). Nearly all solid tumors evolve through two phases, avascular and vascular. Cells of avascular tumors usually do not invade or violate the integrity of their host. In contrast vascularized tumors appear to compress, invade, and destroy the neighboring tissue (4, 5). This critical point of tumor vascularization may thus prove to become a favorable target for therapeutic intervention (for example Refs. 6–8).

The microvascular system in the adult remains quiescent without capillary growth for prolonged periods (9). However, within a short time the microvascular system appears to be capable of responding to physiological demands such as the development of the corpus luteum following ovulation or wound healing, as well as to pathological conditions such as chronic inflammation and tumors. Induction of tumor angiogenesis can be triggered by genetic transformation—"angiogenic switch" (10, 11) but can also be mediated by microenvironmental stress (12–14). A large number of angiogenic factors were identified (6), some of which are continuously overexpressed by tumors, in agreement with the angiogenic switch model. In addition, it has been reported recently that VEGF2 is highly expressed in tumors in vivo in regions adjacent to necrotic zones and can be induced by hypoxia in vitro (12, 13). Inner cell layers of C6 glioma spheroids showed hypoxia induced VEGF expression, while outer proliferating cells could be induced to express VEGF by acute glucose deficiency (14).

The critical role of this growth factor has been implied by the inhibition of tumor growth following treatment with either anti-VEGF neutralizing antibodies (15) or dominant-negative Flk-1 VEGF receptors (16).

Solid tumors are characterized by heterogeneity in the microenvironments, induced by cells outgrowing their nutrient supply. This results in the development of hypoxic zones with limited nutrient supply, in which cells become quiescent and eventually necrotic (2, 17, 18). Quiescent cells are more resistant to most therapy modalities due to their altered physiological and metabolic state rather than because of genetic factors. They appear also to be an important source for stress induced angiogenic growth factors (12, 13). The multicellular spheroid provides in vitro simulation of tumor microenvironments at the critical stage when the tumor nutrient supply cannot satisfy the metabolic needs of the newly generated cells (2, 17). Spheroids have been used extensively in studies of the resistance of inner cell layers to radiation treatments and to chemotherapy (2). Inner cell layers of multicellular spheroids simulate many aspects of regions remote from blood vessels in tumors, including low oxygen and glucose concentrations and high levels of waste products from the necrotic region. Neovascularization of implanted spheroids was used as an assay for the evaluation of antiangiogenic treatment (19).

Among the models developed for the study of tumor angiogenesis the most popular ones include implantation of tumor cells into the cornea of a rabbit (20), into the chorioallantoic membrane of the chick (21), or into transparent chambers implanted in animals (22). Most of these models either are invasive or fail to reproduce the three-dimensional nature of tumor growth required for reproduction of stress induced angiogenesis. We report here an in vivo model that enables continuous and simultaneous noninvasive monitoring of a developing tumor and of its vasculature. The goal of this work was to establish the link between the rate of three dimensional tumor growth and primary angiogenesis. The relationship between the kinetics of tumor growth and neovascularization is deduced here by noninvasive high resolution NMR imaging of both processes in C6 rat glioma multicellular spheroids implanted in nude mice.

MATERIALS AND METHODS

Cell Culture and Spheroid Preparation. C6 rat glioma cells were cultured in DMEM supplemented with 5% FCS (Biological Industries Israel), 50 units/ml penicillin, 50 μg/ml streptomycin, and 125 μg/ml Fungizone (Biolab, Ltd.). Aggregation of cells into small spheroids of about 150 μm was initiated by replating of cells from confluent cultures onto bacteriological plates. After 4–5 days in culture the spheroid suspension was transferred to a 250-ml spinner flask (Belco) and the medium was changed every other day for approximately 6 weeks. Spheroids were sorted after 10 days of culture to uniform size by sedimentation rate in a 10-ml pipet. All culture operations

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were at 37°C and 5% CO₂. Other parameters of spheroid culture were as reported previously (14).

Spheroid Implantation in Nude Mice. Spheroids, manually sorted for uniform size [970 ± 120 (SD) μm], were suspended in PBS. Male CD1 nude mice (2 months old, 30 g body weight) were anesthetized by injection of pentobarbital (Nembutal; Abbott; 50 μg/g weight i.p.). A single spheroid per mouse was implanted s.c. in the lower back, approximately 1 cm away from the site of a 2-mm incision.

Microimaging of the Implanted Spheroids. NMR experiments were performed on a horizontal 4.7-T Bruker-Biospec spectrometer using a 2-cm surface coil. Mice were anesthetized with a single i.p. dose of 75 μg/g ketamine plus 3 μg/g xylazine and placed supine with the tumor located at the center of the surface coil. Gradient echo images (slice thickness, 0.5–0.6 mm; repetition time, 100 ms; 256 × 256 pixels; in plane resolution of 110 μm) were acquired with echo time of 10.5 and 20 ms. The ratio of images at the two echo times provided good T₂* susceptibility contrast that highlighted the s.c. blood vessels. Vessel permeability studies included serial T₂ weighted gradient echo images (repetition time, 30 ms; echo time, 10.5 ms; slice thickness, 0.6 mm; 256 × 256 pixels; in plane resolution of 110 μm) acquired over 20 min after i.v. injection of 0.5 μmol/g Gd-DTPA into the tail vein. Data were analyzed on a Personal Iris workstation (Silicon Graphics) with software from NMRi (TRIPOS).

RESULTS

C6 Spheroid Growth Kinetics in Vitro. The growth of C6 glioma spheroids in vitro was monitored by light microscopy. The change in average spheroid volume was calculated by measurements of the diameter of 50 spheroids every 48 h during continuous culture. The spheroids were nearly spherical, with less than 5% difference between two perpendicular measurements of the diameter. The calculated growth of spheroid volume, assuming spherical symmetry, showed Gompertz kinetics (23) (Fig. 1). Nonlinear least square fit of the data to a Gompertz line shape,

\[ V(t) = V_0 \exp\left(\frac{A}{B}(1 - \exp(-B(t - D)))\right) \]

where \( V(t) \) is tumor volume at time \( t \), \( V_0 \) is tumor volume at time zero, and \( A \) and \( B \) are Gompertz kinetics parameters as described in Ref. 23, yielded values of \( A = 0.315 \) day⁻¹ and \( B = 0.022 \) day⁻¹. Beyond 7–8 weeks the spheroids tended to fall apart.

Growth Kinetics of Implanted C6 Glioma Spheroids. The growth and neovascularization of C6 glioma spheroids in vivo were monitored by NMR imaging. Consecutive gradient echo images obtained every 48 h through 17 days postimplantation showed well defined contrast between the host mouse tissue and the implanted tumor, thus enabling concomitant follow-up of tumor growth and development of vascularity. Tumor volume was calculated by integration of tumor ROIs from consecutive slices, in two orthogonal orientations, coronal and transverse (in plane resolution, 110 μm; slice thickness, 0.6 mm). Representative images obtained from one mouse at four time points and the calculated tumor volume show the progression of tumor growth (Figs. 2 and 3, respectively). A fit of tumor growth to Gompertz kinetics failed to reproduce a consistent lag of about 4 days observed in all tumors (Fig. 3, dashed line). A significantly better fit was obtained by adding an adjustable lag period (\( D \)) to the model (Fig. 3, solid line). The data were therefore analyzed by nonlinear least square fit to a delayed Gompertz line shape

\[ V(t) = V_0 \exp\left(\frac{A/B}{(1 - \exp(-B(t - D)))}\right) \]

This procedure yielded values of \( A = 0.364 \) day⁻¹, \( B = 0.072 \) day⁻¹, and \( D = 4.8 \) day for the data set shown in Figs. 2 and 3.

Noninvasive NMR Imaging of Directional Growth of Blood Vessels. The coupling of angiogenesis and tumor growth was observed for large vessels as well as for the capillary bed. Growth of large vessels could be followed directly by rapid T₂* weighted gradient echo imaging. The lower back is a region of skin with relatively low blood vessel density and thus provides a low background for the detection of newly formed vessels. The distribution of new vessels outlined noninvasively by NMR imaging (Fig. 4A) and those observed in the excised skin of the same tumor (Fig. 4B) seem to match very well. The radial pattern of vessel growth implies that the spheroid constitutes a source of angiogenic growth factors. While vessels proximal to the spheroid showed rapid growth, blood vessels remote from the implanted spheroid remained unchanged (data not shown).

NMR Detection of Angiogenic Contrast Due to Neovascularization. Growth of the capillary bed was reflected in a reduction of the ratio between the mean intensity at a ROI of 1 mm surrounding the spheroid, to the intensity of a distant tissue (see ROIs marked in Fig. 5). The reduction of signal intensity reflects the increased content of paramagnetic deoxyhemoglobin that shortens the T₂* relaxation of water in the proximity of capillaries. Thus it is an intrinsic marker of the change in capillary density. This angiogenic contrast was apparent within 3–4 days after implantation and reached a steady state level that was maintained in the following days (Figs. 6, B, D). Spheroids that showed angiogenic contrast (\( n = 4 \)) also showed delayed Gompertz growth kinetics (Fig. 6, D and C, respectively). Spheroids that for some reasons did not grow (\( n = 2 \)) also did not show angiogenic contrast (Fig. 6, E and F, respectively). A summary of all experiments for tumor growth kinetics and angiogenic contrast is given in Table 1.

In all models of tumor angiogenesis the accompanying wound healing following injection/implantation may result in a false positive response (24). Indeed the formation of blood vessels in the incision site is apparent in the images at the early time points but disappear upon healing of the wound (3–5 days) (data not shown). The control system we used for isolating the effect of wound healing on angiogenesis contrast was done by implantation of an agarose bead instead of a spheroid. Spherical agarose beads 1 mm in diameter were formed from 3% low temperature agarose (Sigma) and implanted 1 cm from the incision site as done with the spheroids. No angiogenesis contrast (Fig. 6D) or vessel growth in direction of the agarose beads was observed either in NMR images or in histological sections (data not shown).

The Newly Formed Vessels Show Increased Permeability. Actual tumor perfusion was followed by i.v. injection of the contrast
Fig. 2. In vivo MRI detection of growth and neovascularization of an implanted C6 glioma spheroid. The growth kinetics of an implanted C6 glioma spheroid was followed by consecutive gradient echo images of the same tumor. The mouse was placed supine with the spheroid positioned at the center of the 2-cm surface coil. Representative coronal images obtained at 1, 11, 13, and 17 days after implantation are shown (arrows), with the tail direction at the top of the images (S, spine; P, pelvis). Data were acquired on a 4.7-T Bruker Biospec spectrometer [echo time, 10.5 ms; repetition time, 100 ms; 3 slices (slice thickness, 0.6 mm); and a field of view of 3 cm resulting in 110 μm in plane resolution].

reagent Gd-DTPA to tumor bearing mice 15 days after spheroid implantation. The change in signal intensity was apparent in difference maps obtained by subtracting a T₁-weighted image acquired before Gd-DTPA administration from images accumulated after Gd-DTPA administration (Fig. 7, A, B). A transient 2-min rise in signal intensity was observed for the large blood vessels remote from the tumor, followed by quick clearance (Fig. 7D, △). Signal enhancement in peripheral regions of the tumor due to vessels that have already penetrated the tumor was slower and also the rate of clearance was slower (Fig. 7D, ◇). The enhancement gradually extended to inner necrotic regions of the tumor by diffusion (Fig. 7D, △). Signal intensity in a 1-mm ROI surrounding the tumor also enhanced gradually (Fig. 7D, ◇), implying that blood vessels in close proximity to the tumor were more permeable.

DISCUSSION

The study reported here provides additional evidence for the concept that tumor growth is angiogenesis dependent. A consistent lag in tumor growth was observed for the first 4 days after the s.c. implantation of C6 glioma spheroids. Rapid tumor growth with characteristic Gompertz kinetics was observed only after the fourth day postimplantation. The delay in tumor growth was well correlated with the kinetics of neovascularization, as reflected by the appearance of an angiogenic contrast, namely, darkening of the tumor periphery in T₂* weighted NMR images. This darkening was very rapid during the first 3 days and reached a steady state 3–4 days postimplantation. The lag observed here in tumor growth is consistent with results from the chorioallantoic membrane assay (20) that shows a 72-h period of avascular phase. The observation that formation of new vessels reaches steady state 4 days postimplantation is in accord with results from the i.d. assay (25), which shows that new vessel formation was attenuated after 4–6 days. In some cases the penetrating blood vessels seemed to engulf the inner region of the spheroid, not the outer cell layers as expected from the pattern of VEGF expression (14). The penetration of blood vessels into the inner regions of the spheroid is
(c) noninvasive monitoring of the entire time course of each tumor; (d) the location of implantation is not dictated by the method; (e) the use of an agarose bead as a control system can be expanded also for evaluation of candidate angiogenic and antiangiogenic compounds.

The three dimensional configuration of the tumor is a critical variable for reproducing stress induced expression of angiogenic growth factors. In small spheroidal tumors (i.e., before the development of quiescence and necrosis) cell proliferation is proportional to the square of the radius, whereas the surface increases only with the square of the radius. Thus tumor expansion before neovascularization is limited by nutrient diffusion. By implantation of multicellular spheroids, corresponding in geometry and size with diffusion stressed small spheroidal tumors (1 mm in diameter), we obtained

also consistent with the report by Zwi et al. (19) on implanted EMT6 spheroids. The new vessels penetrating the spheroid showed enhanced permeability to Gd-DTPA. This permeability consistent with the notion that neovascularization is driven in this system by VEGF a potent vessel permeability factor. Indeed, dominant-negative Flk-1 VEGF receptors has been reported to inhibit growth of implanted C6 glioma tumors (16).

The model presented here provides a quantitative in vivo assay and could help in the study of tumor induced angiogenesis mechanisms. The technique used for monitoring angiogenesis in the present study appears to offer some significant advantages over existing methods such as the corneal micropocket grafts in rabbits or mice (20, 26), the chorioallantoic membrane grafts in fertilized chicken embryos (21), the transparent chamber in the hamster cheek pouch (22), and an i.d. inoculation of tumor cells in mice (25, 27). These include: (a) well defined initial conditions including tumor size and geometry; (b) separation of initial tumor growth and neovascularization;
MRI OF DIRECTED TUMOR ANGIOGENESIS INTO IMPLANTED SPHEROIDS

Fig. 6. Progression of tumor growth and angiogenesis contrast. A, C, E, the kinetics of tumor growth; B, D, F, the change in vessel density surrounding the spheroid manifested by the angiogenic contrast. A, B, an example of results from one animal (obtained from the images shown in Fig. 2), tumor growth fitted to delayed Gompertz kinetics (——). Angiogenic contrast shows initial loss of intensity followed by a steady state reached 4 days after spheroid implantation. C, D, mean results from 4 animals; bars, SD. The angiogenesis contrast from these 4 animals (O) shows the development of the capillary bed. In comparison no darkening associated with angiogenesis contrast could be observed for the implanted agarose bead (D, •). E, F, an example of an implanted spheroid that did not grow, in which no consistent development of angiogenesis contrast could be observed.

Table 1 Growth of implanted C6 glioma spheroids and associated angiogenic contrast

| Experiment | V₀ (mm³) | A (day⁻¹) | B (day⁻¹) | Lag (day) | Err | A₀/A₀day  
|------------|---------|-----------|-----------|-----------|-----|-----------
| 1          | 2.8463  | 0.3639    | 0.0718    | 4.8636    | 0.0308 | 0.56 ± 0.14⁴⁶⁸  
| 2          | 1.4550  | 0.5780    | 0.1350    | 4.2160    | 0.0542 | 0.63 ± 0.09  
| 3          | 0.9278  | 0.5796    | 0.1238    | 3.4530    | 0.0659 | 0.72 ± 0.05  
| 4          | 2.5772  | 0.7600    | 0.2728    | 8.1123    | 0.0370 | 0.65 ± 0.14  
| 5d         | 2.2559  | 0.0001    | -0.3913   | -1.9972   | 0.0584 | 0.84 ± 0.11  
| 6d         | 2.0550  | 0.0003    | -0.2080   | -5.0730   | 0.0506 | 1.23 ± 0.19  
| p          | 0.68    | 0.01      | 0.007     | 0.008     | 0.008  |

⁴ Mean error of the fit of data to the model of delayed Gompertz kinetics.
⁵ Mean ± SD.
⁶ Example of two spheroids that did not grow.
⁷ Statistical comparison of tumors from experiments 5 and 6 to the rest of the tumors that showed positive implantation (experiments 1–4). Analysis was done by 2 tailed Student t test.
§ Highly significant differences.

well defined and reproducible initial conditions. The angiogenic capacity could be evaluated with regard to the measured stress induced expression of angiogenic growth factors in the spheroids as reported for VEGF (14). High resolution NMR imaging provides the unique ability to obtain noninvasively a complete time course of tumor angiogenesis and tumor growth from each animal. Thus, fewer animals can be used than needed for a comparable study using invasive postmortem techniques (22, 27). Tumor location can be adjusted by biological considerations and is not dictated by the method. In order to maintain the imaging resolution and sensitivity, a surface coil can be used for s.c. tumors as shown here, or alternatively, an implanted inductively coupled coil can be used for tumors implanted on internal organs (28). The arsenal of contrast mechanisms in MRI offers a wide range of possibilities. The intrinsic susceptibility contrast of deoxyhemoglobin shown in this study enabled the in vivo follow-up of the development of directed growth of blood vessels into the implanted spheroid. Additional parameters that can be mapped by MRI include measurements of vessel permeability, by use of specific contrast reagents as shown here and mapping of cellular density and tumor necrosis by diffusion imaging (29).

The location and configuration of implantation are very important aspects in designing a model to study tumor angiogenesis. For example, the transparent chamber assay and the cornea assay at the early stage of tumor establishment. Noninvasive high resolution MRI can be used to follow spheroids implanted in different locations and to study the influence of the cellular environment on tumor angiogenesis. The ability to observe the early stages of neovascularization in vivo in a tumor with well defined initial conditions will open new possibilities for the evaluation of the role of metabolic stress in this critical stage of tumor establishment. Noninvasive high resolution MRI can be used to follow spheroids implanted in different locations and to study the influence of the cellular environment on tumor angiogenesis.
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2 minutes after injection

4 8 12 16 20

Time after Gd-DTPA injection (minutes)

Fig. 7. Tumor perfusion and vessel permeability. Difference maps obtained by subtraction of a $T_1$ weighted image acquired before Gd-DTPA injection from images acquired 2 min (A) and 6 min (B) after Gd-DTPA injection. These difference maps show the influx of Gd-DTPA into the tumor. C, a skin specimen of the same tumor excised at the end of the NMR measurement (19 days after spheroid implantation). The 2 large blood vessels apparent in A can be identified. D, the rates of Gd-DTPA entrance and clearance are different in the different regions. The change in signal intensity is presented for the ROIs marked on C. △, large remote vessels; □, necrotic center; □, 1-mm ROI surrounding the tumor as marked in Fig. 5; ○, viable vascularized rim of the tumor.

The use of contrast reagents (like Gd-DTPA) for studying permeability of the newly formed blood vessels could provide NMR markers for the action of specific angiogenic growth factors. Development of such NMR markers can be aided by the use of implanted agarose beads containing known and new angiogenic factors.

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