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Citation for published version (APA):

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Chapter 2

Noninvasive analysis of growth kinetics of tumors of colon cancer in rat liver as determined with magnetic resonance imaging

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In preparation
Abstract

Quantification of tumor growth is a major tool to determine efficacy of therapy. There are several ways to quantify tumor growth. During the last decade, the use of magnetic resonance (MR) imaging in diagnostics and experimental models has evolved tremendously. MR imaging offers the opportunity to quantify tumor growth with several advantages over other means of quantification i.e. MR imaging is noninvasive and can therefore be applied multiple times on the same animal and provides 3-dimensional information that allows calculation of tumor volumes. In the present study, we applied high resolution MR imaging to study the development of colon cancer tumors in rat liver. As a model system, we used control diet fed and fish oil fed rats because it has been previously shown that the latter diet affects tumor growth in the liver.

MR imaging triggered to the breathing together with a radio frequency coil of novel design enabled detailed T1-weighted and T2-weighted imaging of the liver. T2-weighted images clearly identified hyperintense lesions representing tumors. Imaging tumors at 3, 4 and 5 weeks after their induction enabled calculation of kinetics of their development. It was found that tumor volume doubling times obtained by exponential curve fitting of individual tumor volumes at three time points was similar in control diet fed and fish oil fed rats. However, individual tumor volumes at 4 and 5 weeks after their induction were smaller in fish oil fed rats than in control rats. The interpretation of the findings that tumors in fish oil fed rats were smaller than in control rats, whereas their volume doubling time was similar suggests that growth of tumors in fish oil fed rats is delayed. Furthermore, our findings demonstrate the value of measuring noninvasively kinetics of tumor development over classical end-point measurements at the time of death of the animals because it provides relevant additional information. It is concluded that MR imaging is a useful tool to quantify tumor growth especially to establish effects of treatment or therapy.

Introduction

Tumor development is a dynamic process and depends on various factors. Important factors for secondary tumors or metastases to establish are cancer cell arrest in distant organs, immune response, altered expression of adhesion molecules and proteases and angiogenesis (1-3). These factors are important in different stages of tumor development. This implies that inhibitors of tumor growth may have their effects in specific stages only. Usually, effects of
treatment are determined at a given time after administration of the cancer cells when the animals are sacrificed and the effect of a therapy is evaluated (4). In this way, possible differences between control and treated animals can be determined but information on the kinetics of tumor growth is not obtained. To better understand tumor development and metastasis and to interpret outcomes of intervention studies, visualization of tumor growth (and the kinetics) would be of great help.

Development of subcutaneous tumors can rather easily be followed noninvasively in time (5) but often these tumors do not grow in their natural environment and therefore do not represent tumor growth as it occurs in humans. To mimic the process of tumor development and metastasis in humans as closely as possible, tumor development in target organs or orthotopic tumor growth are to be preferred (6).

There are several ways to visualize tumor development. Stable transfections of cancer cells with reporter genes like β-galactosidase (Lac-Z) (7, 8) or green fluorescent protein (GFP) (9-11) have been shown to be powerful tools in the visualization of tumor growth. However, Lac-Z cannot be imaged in live animals but only in freshly-isolated tissues. Visualization of GFP-expressing tumors is usually performed invasively and therefore measurement is limited to a few time points only for ethical reasons. Moreover, surgery involved can affect tumor growth. The first noninvasive approach of GFP imaging was shown by Yang et al. (12). Orthotopically-transplanted GFP-positive tumors and their metastases were imaged in intact mice but the limits of detection appeared to be a function of depth: tumors of minimally 1.9 mm could be imaged only up to a depth of 2.2 mm in tissues (12). Thus, the development of tumors that are growing non-superficially remained difficult.

Magnetic resonance (MR) imaging allows detection of tumors throughout the entire body of rats and mice. High magnetic fields enable a resolution in the xy-plane of approximately 0.2 x 0.2 mm (13). Due to the noninvasive character of MR imaging, the same animal can be imaged multiple times without harmful effects. Moreover, MR imaging allows optical sectioning through the specimen, providing 3-dimensional information which allows more precise calculation of tumor volumes. So far, the use of MR imaging of tumors in livers of rats has been applied to study the effects of somatostatin on the development of liver tumors (14) and growth characteristics of experimentally-induced hepatocellular carcinoma (15). Tumor development of colon cancer in the liver is studied in our department in a rat model using end-point assays. For example, three weeks after administration of colon cancer cells to
the portal vein rats were sacrificed and the effect of a diet enriched with fish oil on tumor growth was evaluated (16). Recently, we studied early events in invasion and metastasis of colon cancer in rat liver using GFP-expressing cancer cells but analysis of tumor development could be performed only invasively (17). In the present study, we have evaluated the use of MR imaging to quantify tumor load and to determine kinetics of tumor growth by sequential imaging of tumor development in individual rats. We compared tumor development in livers of rats that received a fish oil enriched diet starting at two weeks after administration of the cancer cells with that in rats receiving a normal diet. We used this model because it was shown previously that a fish oil diet which was started at three weeks before administration of the cancer cells strongly increased numbers and size of tumors in the liver as compared with rats receiving a normal diet (16).

Material and methods

Animals

Adult male Wag-Rij rats (n = 6) (Broekman, Someren, The Netherlands) with a body weight of 200-250 g were maintained for 2 weeks under constant environmental conditions with free access to food and water.

Cancer cells

An established colon carcinoma cell line, CC531s, has been developed by Marquet et al. (18). The cells were cultured at 37°C as monolayers in RPMI-1640 Dutch Modification without L-glutamine (GIBCO/BRL, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 IU penicillin/ml and 100 mg streptomycin/ml. Cells were washed with phosphate-buffered saline (PBS) and after detachment with trypsin (0.05% w/v) and ethylenediaminetetraacetic acid (EDTA) (0.02% w/v) in PBS and centrifugation (250 g, room temp, 10 min), cell suspensions were obtained with a viability of at least 95% (19).

Surgery

A small midline incision was made in the abdominal wall of rats under anaesthesia with FFM mix (1 ml Hypnorm, 1 ml Midazolam and 2 ml water, 0.27 ml/100 g body weight, intraperitoneally). A suspension containing 5 x 10^3 cancer cells in 500 µl PBS was injected into the portal vein with a 27-gauge needle.
Diet protocol

Two weeks after administration of cancer cells, rats were divided randomly into two groups. Rats received either a control diet (n = 3) or a fish oil diet (n = 3) until sacrifice of the animals. The composition of the diets and the feeding protocol have been described previously (16).

Visualization of liver tumors

MR images of each rat were taken at 3, 4 and 5 weeks after administration of the cancer cells. The animals were anaesthetized with 1.5% isoflurane and a mixture of N₂O/O₂ (1:1). Body temperature was maintained at 37°C by covering the rats with a bed heated with circulating warm water (20). A dedicated 5 x 7 cm microstrip RF coil of novel design was used as transmitter/receiver in order to improve the quality of the images. A customized plastic cradle was built to accommodate the rat in prone position on the coil. Rats were placed in an MR spectrometer (S.M.I.S. console equipped with a Magnex Scientific 7 T/200 mm horizontal-bore magnet and a 150 mT/m gradient set). After initial monitoring of the liver with fast gradient-echo scout images, 16 contiguous coronal images (i.e. parallel to the coil) were acquired. The image acquisition protocol consisted of T1- and T2-weighted spin echos for anatomical localization of the liver and detection of tumors. Imaging parameters were: image matrix size of 256 x 256, field of view (FOV) of 14 x 14 cm, slice thickness (SLT) of 2 mm and 1 signal average per phase-encoding step. The values of repetition time (TR) and echo time (TE) were TR/TE = 500/15 ms for the T1-weighted images and TR/TE = 710/36 ms for the T2-weighted images. Image acquisition was triggered on the rats respiration rate.

Volume measurements

Total tumor mass per rat (n = 6) was calculated by the sum of the tumor surface area (mm²) times the slice thickness (mm). From rats 1, 2, 5 and 6 only individually-growing tumors at 3, 4 and 5 weeks after their induction were used to calculate the tumor volume doubling time. The optical slice with the largest surface area was used to determine the surface area. As all tumors were more or less globular, the radius was calculated from the largest surface area using \( r = \sqrt{\text{surfacearea}/\pi} \). The volume was calculated as \( v = 4/3 \pi r^3 \).
**Histology**

Four (n = 2) and five weeks (n = 4) after administration of the cancer cells i.e. after the second and third MR imaging session, respectively, animals were sacrificed and the livers were removed and examined macroscopically for the presence of tumors for comparison with the MR images. After examination, liver pieces with tumors were frozen in liquid nitrogen and stored at -80°C until further use. Cryostat sections (8 μm thick) of livers containing metastases were cut with a motor-driven cryostat with rotary retracting microtome (Bright, Huntingdon, UK), at a cabinet temperature of -24°C, air-dried for 1 hr and fixed in acetone at room temp for 10 min. Colon cancer cells were detected with Ulex europaeus agglutinin-1 conjugated to horseradish peroxidase (UEA–HRP; EY Laboratories, San Mateo, CA), a selective marker of colon carcinoma cells (19). Peroxidase activity was visualized by incubation for 10 min at RT with a solution containing 20 mg 3-amino-9-ethylcarbazole (Sigma), 5 ml dimethylformamide, and 0.01% hydrogen peroxide in 50 mM sodium acetate buffer (pH 4.9). After rinsing in distilled water, sections were counterstained with hematoxylin and mounted in glycerin–gelatin.

**Statistics**

Statistical analysis was performed using the unpaired Student's t test. P values < 0.05 were considered significantly different. Values are given as means ± SEM.

**Results**

The use of a breathing-triggered MR imaging protocol enabled sharp and detailed T1-weighted imaging of the liver, allowing delineation of intrahepatic structures (Fig. 1). T1-weighted imaging did not allow visualization of tumors in the liver after 3 weeks of tumor development. At 4 and 5 weeks after induction of tumors, some but not all tumors were detected as slightly hyperintense structures (Fig. 2A). However, all tumors could be identified clearly as hyperintense lesions at all time points in T2-weighted images (Fig. 2B). Imaging of the rats at 3 time points enabled us to follow tumor development in time (Fig. 3). Liver tumors developed in all 6 animals either as solitary tumors or tumor aggregates after fusion of single tumors.
Figure 1. T1-weighted MR image of the abdomen of the rat. Different organs can be clearly distinguished and even within organs such as the liver structures such as blood vessels (arrows) can clearly be distinguished from their surrounding. Bar = 2 cm.

Figure 2. T1-weighted (A) and T2-weighted (B) MR images from the same optical section of the same rat at 5 weeks after induction of colon cancer tumors. Only some tumors (arrows) are faintly visible as hyperintense structures in the T1-weighted images whereas all tumors are clearly visible as hyperintense lesions in the T2-weighted images. Bar = 2 cm.

Figure 3. T2-weighted MR images of the same colon cancer tumors (arrows) at 3 weeks (A), 4 weeks (B) and 5 weeks (C) after induction of the tumors. Bar = 2 cm.

Total tumor mass was calculated as the sum of the areas of all tumors, including solitary tumors and tumor aggregates, times the slice thickness per rat per time point. Tumor take was
similar in the control diet group and the fish oil diet group (Fig. 4); in each group, one animal with low tumor take, one animal with medium tumor take and one animal with high tumor take were found.

The two animals with high tumor take were sacrificed at four weeks after administration of the cancer cells for ethical reasons. There was no difference in the mean total tumor mass at either time point, and thus total tumor mass increased similarly in time, when comparing control rats and fish oil fed rats (Fig. 4). Rats 2 and 5 developed solitary tumors only whereas rats 1 and 6 developed both solitary tumors and tumor aggregates. Selection of individual tumors and calculation of their volumes at 3, 4 and 5 weeks after their induction allowed calculation of growth curves for each individual tumor (Fig. 5).

Exponential fitting of the growth curves enabled the calculation of tumor volume doubling time as a measure of tumor growth rate. The average tumor volume doubling time was $0.59 \pm 0.11$ weeks in 9 tumors in control animals and $0.58 \pm 0.04$ weeks in 16 tumors in fish oil fed animals (Fig. 6A), which means that growth characteristics of solitary tumors were identical in the 2 groups (approx. 4 days). However, the mean tumor volume was significantly different at 4 and 5 weeks but not at 3 weeks of tumor development in fish oil fed rats and control rats (Fig. 6B). These measurements show that the tumor volume doubling time was similar in both groups whereas the average volume of solitary tumors was significantly lower in fish oil fed rats. Similar tumor volume doubling times and smaller tumor volumes in fish oil fed rats suggest that tumor development is only temporarily inhibited by treatment with a fish oil diet.
Discussion

MR imaging studies in the early nineties have shown that it is a powerful tool to study tumor development in live animals (14, 21). During the last decade, the technology in MR imaging has been improved considerably by the development of stronger magnetic fields, modified coils and improvement of imaging protocols which has resulted in improved signal-to-noise ratios and increased spatial resolution (22). Noninvasive imaging of the liver in live animals is difficult due to motion artefacts caused by respiration. However, triggering of the MR protocol to the breathing of the animal enabled us to acquire well-contrasted and detailed images of rat livers and to image tumor development of colon cancer in the liver. As an example, we have compared tumor development in livers of rats that were either fed a control
diet or a fish oil diet, which has been shown previously to facilitate tumor growth dramatically (16).

There are several ways to evaluate tumor growth in animal models as a test for efficacy of therapy or treatment. Usually, these studies are performed as end-point assays. This approach does not provide information on changes that occur during tumor development. In order to monitor tumor development without interference it should be done noninvasively. The GFP technology has proven to be a powerful tool in the visualization of tumors in live animals (9-11). Noninvasive whole body imaging has been performed on mice bearing GFP-positive tumors but sizes of tumors that can be imaged appeared to have an inverse relation with the depth of location (12). In contrast, MR imaging of tumors does not rely on transfection of cancer cells with any reporter gene, can be performed noninvasively for multiple times and tumors can be imaged in any part of the animal. Therefore, MR imaging provides a valuable tool for imaging tumor development in live animals.

In T1-weighted images of the liver, only a small part of the tumors could be detected with small differences in intensity as compared with liver tissue. However, T2-weighted imaging clearly showed hyperintense lesions in the liver. Imaging of the rats at 3, 4 and 5 weeks enabled us to visualize tumor development as it occurs in time. One way of determination of tumor growth is calculation of the total tumor mass as has been described by Qin et al. (14). In analogy, we calculated total tumor mass present in the livers in time. Total tumor mass as a measure of tumor load was similar in control rats and fish oil fed rats suggesting that in this small group of animals the tumor load was similar. Furthermore, total tumor mass at 3, 4 and 5 weeks after induction of the tumors indicated similar kinetics of tumor development in both groups.

Quantification of growth of solitary tumors appeared to be possible as well. Tumor volume doubling times obtained by exponential curve fitting of solitary tumor volumes at 3, 4 and 5 weeks showed that the tumor volume doubling time of solitary tumors was similar in both groups. However, by comparing mean tumor volumes that were used to calculate the tumor volume doubling time, it became evident that the mean tumor volume in fish oil treated rats was lower than in the control rats at 4 and 5 weeks after administration of the cancer cells. Taken into account that the tumor volume doubling times were equal, it suggests that tumors that were growing in fish oil fed rats were only temporarily delayed in growth as compared with tumors growing in control rats. After the delay, growth showed similar rates in both
groups. Proliferation of hepatocytes after partial hepatectomy has been shown to be inhibited due to a fish oil enriched diet (23). Possibly, a similar mechanism is responsible for the delay in tumor development in fish oil fed rats. Apparently, treatment of rats with a fish oil diet that was started at 2 weeks after administration of the cancer cells has a different effect on tumor development than a diet that is started before cancer cells are administered (16).

Taken together, we have shown that MR imaging is a valuable tool to study tumor development in livers of live rats. Calculation of kinetics of tumor growth provides additional information that is not obtained by end-point studies which can lead to different insights in tumor growth characteristics. For example, when only judging tumor volumes at the end-point of 5 weeks, one could conclude that tumors in fish oil fed rats grow slower than tumors in control rats. However, on the basis of additional information on the kinetics of tumor development this conclusion appeared not correct. It shows that tumors grew at a similar rate but tumors in fish oil treated rats were inhibited in their growth only temporarily. Therefore, we conclude that dynamic observations during tumor development provides additional and sometimes crucial information as compared with end-point observations only.

References


