Establishment of an orthotopic tumour model for hepatocellular carcinoma and non-invasive in vivo tumour imaging by high resolution ultrasound in mice

Volker Schmitz1,*, Lucia Tirado-Ledo1, Klaus Tiemann2, Esther Raskopf1, Thomas Heinicke1, Carsten Ziske1, Maria A. González-Carmona1, Christian Rabe1, Nicolas Wernert3, Jesús Prieto4, Cheng Qian4, Tilman Sauerbruch1, Wolfgang H. Caselmann 1,†

1Department of Internal Medicine I, University Hospital Bonn, Bonn, Germany
2Department of Internal Medicine II, University Hospital, Bonn, Germany
3Institute of Pathology, University Hospital, Bonn, Germany
4Gene Therapy Unit, Department of Internal Medicine, Medical School, University of Navarra, Pamplona, Spain

Background/Aims: In this study we established an orthotopic tumour model for hepatocellular carcinoma and evaluated a non-invasive high resolution ultrasound technique for diagnosis and follow-up of intrahepatic HCC.

Methods: Orthotopic liver tumours were induced by intrahepatic tumour cell injection of 10^5 Hepa129 hepatoma cells. Tumour establishment and growth were assessed by explorative laparotomy, ultrasound technique and hepectectomy one and two weeks after tumour cell implantation. Tumour establishment was confirmed histologically in liver sections.

Results: Our results show that the Hepa129 hepatoma cell line is suitable for orthotopic tumour establishment and that tumours can be diagnosed correctly by ultrasound imaging in all cases as confirmed by explorative laparotomy, hepectectomy and cross-sections. Tumour diameters obtained by explorative laparotomy correlated significantly with diameters assessed by ultrasound (r = 0.7; P < 0.0001). Tumour burden was slightly overestimated (1.2-fold) by ultrasound one week after tumour induction and relative tumour extensions increased 1.7-fold and 1.8-fold within one week as determined by subsequent explorative laparotomy or ultrasound imaging, respectively.

Conclusions: These data demonstrate in a systematic study that ultrasound imaging can be used as a reliable tool to detect and to follow up orthotopic liver tumours in this tumour model in mice.

© 2004 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Keywords: Cancer; Hepatocellular carcinoma; High resolution ultrasound; Tumour model; Ultrasound imaging

1. Introduction

To detect and follow liver tumours, different imaging modalities such as computer- (CT) or magnetic resonance (MR) tomography and ultrasound techniques with or without specific contrast agents can be employed. For experiments with small animals techniques like CT and MRT are not routinely accessible. In animal models transcutaneous ultrasound is mainly applied to taller animals like pigs, baboons, dogs and rabbits and in the majority of these studies specific contrast agents were used for liver imaging or visualisation of blood vessels [1–3].

Experimental antitumour therapies are frequently tested for efficacy in subcutaneous tumour models. Tumour induction can be handled easily and tumour follow up can be done by palpation or visual control. In contrast, in orthotopic tumour models detection is complicated. Advantageously acquired data are more conclusive in the latter, because primary and secondary liver malignancies are considered to be particular since antigen presentation in the liver can result in immune tolerance [6]. The skin displays a

0168-8278/30.00 © 2004 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jhep.2004.01.010
high inherent immunogenicity due to the abundant presence of antigen presenting cells and therefore subcutaneous tumours do not mirror adequately orthotopic tumour growth [4,5]. The application of orthotopic tumour models in the liver implies a need for non-invasive techniques for tumour evaluation. But so far, determination of tumour sizes in orthotopic tumour models in mice is mainly performed through laparotomy or hepatectomy [7–10]. In this prospective study we have established an orthotopic HCC model and evaluated the value of high resolution ultrasound technique for tumour evaluation in orthotopic HCC in mice for the first time in a systematic manner.

2. Materials and methods

2.1. Animals and cell lines

C3H mice were bred in the local central animal facility of the university hospital Bonn. The mice were housed under standard conditions. Animal procedures were performed in accordance with approved protocols and followed recommendations for proper care and use of laboratory animals.

Hepa129 cells (Hepatoma 129 originating from C3H mice, obtained from NCI-Frederick Cancer Research And Development Center (DCT Tumour Repository)) were maintained in RPMI 1640 supplemented with 10% FCS, 200 mM glutamine and penicillin/streptomycin.

2.2. Tumour induction

Mice were anaesthetised (Ketamin 0.1 mg/g bodyweight and Rompun 2% 0.01 mg/g bodyweight) and laparotomy was performed. After preparation of the liver, orthotopic HCC were established by subcapsular intrahepatic injection of 10⁵ HCC cells (Hepa129) suspended in 50 μl RPMI into the left liver lobe in a total of 26 mice (Fig. 1). Since two mice did not develop a tumour within two weeks observation time these ones were excluded from the comparative study. Post injection bleeding and tumour cell escape were avoided by short time local compression. Laparotomy was performed one and also two weeks after tumour induction. In order to confirm correctness of tumour measurement by exploration in five of eight mice of this subgroup tumour sizes were also determined after hepatectomy and liver dissection (data not shown).

Ultrasound examination was performed blinded, meaning that it was performed without knowledge of the results obtained by subsequent explorative laparotomy or hepatectomy, respectively. For ultrasound imaging a commercially available ultrasound machine was used (HDI 5000, Philips-Ultrasound, Bothell, WA) equipped with a high frequency linear array transducer designed for intraoperative use (CL 15-7). Imaging was performed in a real-time compound imaging mode (SonoCT™) operating at 15 MHz. In the SonoCT™ mode the digital beamformer electronically steers the transducer array about nine steering angles [11]. For artefact reduction frames acquired at each angle were averaged on-line allowing real-time imaging at frame rates up to 176 Hz. Line-density was set to high. Persistence was disabled. The dynamic range (display) was set to 50 dB. Mice were placed in a supine position. Abdominal skin was carefully shaved and for acoustic coupling ultrasound-gel (Maas, Vermold, Germany) centrifuged for 2 min at 2000 g was used.

According to most experimental studies, tumour width (a) and length (b) were determined by caliper measurement during explorative laparotomy and arithmetic mean tumour diameters were calculated. Since tumour depth is not regularly accessible by explorative laparotomy we refrained from estimating tumour volumes, although three diameters were assessed in ultrasound and hepatectomy. To allow comparative, statistical analysis of the data arithmetic mean tumour diameters were calculated from width and length.

2.4. Histological sections

To verify tumour establishment liver cross-sections were checked for tumour tissue in a subgroup of animals. Livers were formalin fixed, paraffin embedded and standard HE staining was performed for histological examination.

2.5. Statistical analysis

Data are given as mean tumour diameters with standard error of the mean (SEM). Significance of the correlation between data derived from explorative laparotomy and ultrasound or liver dissection was estimated by Pearson’s test, P < 0.05 was considered to indicate significance.

3. Results

3.1. Tumour establishment

Orthotopic hepatocellular carcinomas were established by intrahepatic injection of Hepa129 hepatoma cells injected into the left liver lobe in a total of 26 C3H mice, one animal succumbed to perioperative complications. Only two (8%) out of 25 mice did not develop a tumour during the observation time. It can be concluded that the Hepa129 hepatoma cell line is suitable for the establishment of an experimental orthotopic HCC model in C3H mice.

3.2. Tumour detection and time course of intrahepatic tumour growth

During explorative laparotomy Hepa129 tumours are easily detectable as whitish, nodular irregularity on the otherwise smooth liver surface (Fig. 5A/B). Histologically, HE-stained cross-sections of the liver and the tumour, showed tumour infiltration into the liver without a capsule (Fig. 2). In ultrasound the tumour nodules contrasted with
surrounding liver tissue as heterogeneous hypodense areas sometimes affecting and altering the liver surface thereby facilitating tumour detection (Fig. 3). In vivo ultrasound was able to distinguish clearly structures as the abdominal artery and the caval vein with such small diameters as about 2 and 0.7 mm, respectively (Figs. 3, 5 and 8). Explorative laparotomy was used in this study as standard method. Tumours ranging from 2 mm up to 15.5 and 3.75 mm up to 16.75 mm in diameter as determined by exploration and ultrasound, respectively, were detected and followed up in this study. Tumour extensions were overestimated by ultrasound technique 1.15-fold as compared to exploration. Mean tumour diameters as determined by ultrasound and explorative laparotomy one week after tumour induction were 5.3 and 6.1 mm, respectively (n = 23; Fig. 4). Correlation of all data pairs of tumour extensions obtained by ultrasound and exploration one or two weeks after tumour induction reached high significance (38 data pairs of n = 23 at week one, n = 15 at week two; r = 0.7; P < 0.0001; Fig. 6). When the relative tumour growth was determined, tumour growth was very similar with a 1.8-fold and 1.7-fold increase as determined by ultrasound and exploration, respectively, within one week time (subgroup n = 15; Fig. 7). Two weeks after tumour cell implantation
some of the animals had developed ascites. This could also be detected by ultrasound (Fig. 8). To confirm the correspondence of results between exploration and hepatectomy, in a subgroup of five out of these eight mice hepatectomy was performed additionally. Tumour extensions were determined in the dissected liver sample in this subgroup. Here, mean tumour diameters were almost identical (8 mm vs. 8.2 mm), respectively, and correlation was high ($n=5; r=1; P=0.017$; data not shown).

4. Discussion

Here, we used Hepa129 hepatoma cells for the establishment of orthotopic liver tumours. Tumour diagnosis and tumour extensions were assessed by high resolution ultrasound imaging and explorative laparotomy in a prospective, comparative study design.

Antitumour therapeutical strategies are mainly tested in subcutaneous experimental animal studies in mice or rats. As in the case of HCC, orthotopic tumour models are strongly desirable in order to approach as close as possible the pathological tumour disease course. Yet existing murine HCC models using e.g. Hepa1-6 or BNL cells have some disadvantages like high rate of spontaneous tumour regression, low tumourigenicity or slow tumour progression. In order to overcome these obstacles we established a tumour model employing the Hepa129 hepatoma cell line originating from C3H mice. Intrahepatic, subcapsular injection of $10^5$ Hepa129 cells led to progressive, lethal tumour disease in almost all cases in this model. In contrast to subcutaneous tumour models, orthotopic tumour detection and follow up are complicated and frequently done by (repetitive) explorative laparotomy [7,10]. Non-invasive methods are highly desirable and nowadays MRI examinations became feasible [12–15] but are frequently not accessible. Recently, the technical advances allowed the development of high resolution ultrasound devices that open the horizon for application to smaller animals like mice with a spatial resolution as high as 100 μm [16]. Decent overestimation of tumour sizes by ultrasound measurement (1.15-fold) compared to data obtained by explorative laparotomy can probably be attributed to different aspects in this study: peritumoural edema together with (peri-)tumoural hypervascularisation, and infiltrative tumour growth. Additionally, deeper tumour areas might escape visual detection by exploration, but might be detectable by ultrasound technique. Since the tumour extensions, that were obtained by explorative laparotomy, correlated closely and significantly with tumour extensions measured by liver dissection, explorative laparotomy was considered to be the appropriate reference method for the evaluation of tumour burden in this model. In this experimental setting, tumour diagnosis was principally facilitated, because tumours were induced as single tumour nodules by tumour cell implantation at a known, selected, subcapsular site. Evidently, ultrasound examination will be complicated in more authentic models like a multiple metastases model and depends highly on the individual examiner’s skills.
Our results show that Hepa129 hepatoma cells are suitable for the establishment of orthotopic liver tumours in C3H mice and that ultrasound imaging represents a reliable tool for the non-invasive assessment of tumour diagnosis and tumour burden in this model.

Acknowledgements

We gratefully thank Philips-Ultrasound, namely Dr Jeff Powers and Matt Bruce for their valuable technical support. VS and KT were supported by a university research fund (BONFOR). Part of the work was supported by Instituto Carlos III C02/03.

References