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Vascular remodeling and antitumoral effects of mTOR inhibition in a rat model of hepatocellular carcinoma☆

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***Background/Aims*: Hepatocellular carcinoma (HCC) is amenable to only few treatments. Inhibitors of the kinase mTOR are a new class of immunosuppressors already in use after liver transplantation. Their antiproliferative and antiangiogenic properties suggest that these drugs could be considered to treat HCC. We investigated the antitumoral effects of mTOR inhibition in a HCC model.**

***Methods*: Hepatoma cells were implanted into livers of syngeneic rats. Animals were treated with the mTOR inhibitor sirolimus for 4 weeks. Tumor growth was monitored by MR imaging. Antiangiogenic effects were assessed *in vivo* by microvessel density and corrosion casts and *in vitro* by cell proliferation, tube formation and aortic ring assays.**

***Results*: Treated rats had significantly longer survival and developed smaller tumors, fewer extrahepatic metastases and less ascites than controls. Sirolimus decreased intratumoral microvessel density resulting in extensive necrosis. Endothelial cell proliferation was inhibited at lower drug concentrations than hepatoma cells. Tube formation and vascular sprouting of aortic rings were significantly impaired by mTOR inhibition. Casts revealed that in tumors treated with sirolimus vascular sprouting was absent, whereas intussusception was observed.**

***Conclusions*: mTOR inhibition significantly reduces HCC growth and improves survival primarily via antiangiogenic effects. Inhibitors of mTOR may have a role in HCC treatment.**

***Keywords*: Angiogenesis; Intussusception; Liver; Mammalian target of rapamycin; Sirolimus**

1. Introduction

There are more than half a million new cases of hepatocellular carcinoma (HCC) each year making it the fifth most common tumor worldwide and the third cause of cancer-related deaths [1]. The incidence of HCC is increasing and mortality has nearly doubled over the last 20 years [2]. Curative treatments such as liver resection and transplantation are only possible if the tumor is detected at an early stage [3]. Prognosis of HCC remains poor since only a minority of patients qualifies for these treatments. Systemic chemotherapy is not effective in HCC and local treatments such as chemoembolization have shown only limited survival benefit in selected patients [3]. Innovative therapeutic approaches are urgently needed.

HCC displays a characteristic hypervascularity and depends on angiogenesis for tumor growth [4]. Intratumoral microvessel density (MVD) is a predicting factor of disease-free survival after curative resection

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of HCC [5]. A prospective study with HCC patients found that high serum levels of vascular endothelial growth factor (VEGF), the most important growth factor for endothelial cells, are associated with absence of a tumor capsule, with tumor invasion and postoperative recurrence [6].

Recently, Geissler and coworkers reported that pharmacological inhibition of the kinase mammalian target of rapamycin (mTOR) with sirolimus (rapamycin) impairs tumor growth by an antiangiogenic mechanism. Sirolimus was shown to inhibit VEGF secretion and VEGF signaling in endothelial cells [7]. mTOR is a highly conserved serine–threonine kinase and plays a central role in modulating cell growth and proliferation [8]. In response to cellular nutritional status and activation of the upstream PI-3 kinase/Akt pathway, mTOR enhances mRNA translation and protein synthesis by phosphorylating its downstream targets p70 S6 kinase (S6K) and the translation initiation factor 4E-binding protein 1 (4E-BP1) [9]. Inhibition of mTOR induces arrest of the cell cycle in G1 phase [10] and interrupts downstream propagation of PI-3 kinase/Akt-mediated proliferative signals [7]. mTOR-dependent signaling was found to be activated in several tumor types including HCC [11,12]. Immunohistochemical studies revealed that 45% of HCC displayed an increased expression of S6K which correlates with tumor nuclear grade whereas hepatic adenomas did not have increased S6K expression [11].

The immunosuppressive drugs, sirolimus (rapamycin), temsirolimus (CCI-779) and everolimus, are inhibitors of mTOR and possess antiproliferative and antiangiogenic properties [12]. These drugs are currently being evaluated in clinical trials for various malignancies [12], but not yet for HCC. We tested the antitumoral properties of mTOR inhibition in a HCC model and investigated the antiangiogenic effects of this treatment. Our results provide the first *in vivo* data in HCC and a rationale to test pharmacological mTOR inhibition in patients with HCC.

2. Materials and methods

2.1. Cells and culture conditions

Morris Hepatoma McA-RH7777 (MH) cells were obtained from the German Cancer Research Center (DKFZ; Heidelberg, Germany) and were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum, L-glutamine, penicillin and streptomycin. Rat aortic endothelial cells were isolated from thoracic aorta of ACI rats by collagenase and cultured in F12-K medium (Gibco, Basel, Switzerland) with 10% fetal bovine serum (Sigma–Aldrich Chemie GmbH, Munich, Germany), penicillin and streptomycin, supplemented with 10 ng/ml epidermal growth factor and 25 μg/ml heparin. Endothelial cells were cultured on fibronectin coated wells (10 μg/ml, BD Biosciences, Allschwil, Switzerland) and were used between passages 1 and 6.

2.2. Animals

Experiments were performed in 12–18 weeks old ACI rats (Harlan, Indianapolis, USA). Animals received humane care in accordance with the regulations for laboratory animals and the experiments were approved by the Local Animal Use Committee.

2.3. Tumor implantation and treatment with sirolimus

Subcutaneously injected MH cells (5×10^6) in a syngeneic ACI rat led to formation of a subcutaneous tumor within 14 days. Tumor inocula were prepared by mincing the excised subcutaneous tumor into equal cubes of 1 mm^3. One single cube per rat was then immediately surgically implanted in the liver [13]. Rats were anaesthetized by intraperitoneal injection of medetomid 0.15 mg/kg, climazolam 2 mg/kg, and fentanyl 5 ng/kg. A subxyphoid incision was performed and a small superficial incision of the liver capsule allowed placement of a single 1 mm^3 tumor cube into the parenchyma (Fig. 1). After randomization, treatment with sirolimus was started on day 5 posttumor implantation. Treated rats were separated in single cages and received 2 mg sirolimus/kg body weight per day mixed in drinking water vs. drinking water without sirolimus in controls. Sirolimus solution was kept in light protected bottles and renewed every 48 h. The amount of ingested water and sirolimus per rat was monitored and kept at 2 mg sirolimus/kg body weight/day for 4 weeks. Rats were euthanized on day 33 after tumor implantation. In a separate series for survival analysis, animals were euthanized if they became wasted and seemed to be suffering (hunch-back posture) as assessed by a person unaware of treatment assignment. Serum, whole blood, tumors and livers were harvested. Tumor volume was calculated *ex vivo* (tumor volume = $4/3 \times \pi \times r_1 \times r_2 \times r_3$).

2.4. MR imaging

MR imaging was performed on day 17 after tumor implantation and weekly thereafter with a 1.5 T Sonata MRI unit (Siemens, Erlangen, Germany) using a high-resolution wrist phased array coil. Contiguous, 1-mm thick slices in axial plane were acquired using non-enhanced 3D T1 (VIBE, TR = 10 ms, TE = 3.71 ms, voxel size $0.5 \times 0.4 \times 1$ mm) and 3D T2 (3D turbo spin echo sequence for isotope resolution TR 3200 ms, TE 113 ms, voxel size $0.4 \times 0.4 \times 1.0$ mm) weighted sequences. Tumors were seen as demarcated areas of low signal intensity in T1 and high signal intensity in T2 weighted images, compared to the normal hepatic parenchyma.

2.5. Sirolimus level

Sirolimus whole blood levels were measured at harvest by high-performance liquid chromatography.

2.6. Tumor necrosis

Central tumor necrosis, characterized by macroscopic demarcation of the necrotic areas from the surrounding viable tumor tissue, was assessed by cutting the tumor at its largest diameter in half and measuring the largest diameter and the corresponding perpendicular diameter (x, y) of the macroscopically visible central necrosis. The radius of the tumor (r_{tumor}) and of the central necrotic zone (r_{necrosis}) was calculated for a hypothetical circle with the same surface as the ellipse formed by the two measured perpendicular diameters x and y.

2.7. Microvessel density

A monoclonal antibody against the endothelial cell antigen CD31 (clone JC/70 A, Dako, Glostrup, Denmark) was used at 1:150 dilution to mark the vessels. Microvessel density was determined blindly as described by Weidner and Folkman [14].