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Journal of Hepatology 49 (2008) 977–984

Journal of Hepatology

www.elsevier.com/locate/jhep

siRNA targeting VEGF inhibits hepatocellular carcinoma growth and tumor angiogenesis *in vivo*☆

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***Background/Aims*: We have investigated whether siRNA targeted against VEGF inhibits functional properties of endothelial cells *in vitro* and HCC tumor growth and blood vessel formation *in vivo*.**

***Methods*: The influence of siRNA-VEGF on endothelial cell proliferation, apoptosis and tube formation were analyzed *in vitro*. Antitumoral effects were examined in an orthotopic tumor model after *ex vivo* transfer or intraperitoneal treatment of siRNA, respectively. Intratumoral microvessel density was assessed by CD31 staining.**

***Results*: VEGF expression was inhibited in Hepa129 by 70% and in SVEC4-10 by 48% within two days after transfection. *In vitro*, endothelial cell proliferation and tube formation was reduced by 23% and 38%, respectively. Interference with VEGF signaling was demonstrated by reduced pAKT in hepatoma cells. Tumor growth was inhibited by *ex vivo* transfer or intraperitoneal application of siRNA-VEGF by 83% or 63% in orthotopic tumors within 14 days. VEGF protein was reduced in both models by 29% and 44%. Microvessel density dropped to 34% for tumors from *ex vivo* transfected cells and 39% for systemic treated tumors.**

***Conclusions*: The results show that VEGF knockdown can be associated with reduced endothelial cell proliferation and tube formation *in vitro* and decreased tumor growth and microvessel density *in vivo*.**

***Keywords*: HCC; RNA interference; Endothelial cell functions; Anti-angiogenesis; Tumor growth**

1. Introduction

It has been acknowledged that hepatocellular carcinoma (HCC) progression is associated with tumor angiogenesis and upregulation of VEGF (vascular endothelial growth factor) [1,2]. In HCC, increased VEGF levels correspond to increased tumor sizes [3]. Due to limited therapeutic options for HCC patients [4,5], new strategies are needed to control HCC disease. VEGF, mainly VEGF-A, is known to be secreted by tumor cells, thereby activating quiescent endothelial cells (EC) in a paracrine manner [6,7]. VEGF-A (referred here as VEGF) is bound by VEGF receptor 1 (VEGFR1), VEGFR2 and the novel VEGF receptor neuropilin-1 (Nrp1) mainly on the cell surface of endothelial cells.

Binding of VEGF to its receptor and subsequent receptor dimerization activates transduction pathways

Received 15 February 2008; received in revised form 2 July 2008; accepted 2 July 2008; available online 21 September 2008
Associate Editor: J.M. Llovet

☆ The authors who have taken part in the research of this paper declared that they do not have a relationship with the manufacturers of the materials involved either in the past or present and they did not receive funding from the manufacturers to carry out their research.

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Abbreviations: AKT, protein kinase B; EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal regulated kinase; HE, hematoxyline eosine; HCC, hepatocellular carcinoma; LDH, lactat dehydrogenase; p38 MAPK, mitogen activated protein kinase; MTT, tetrazolium; Nrp1, Neuropilin 1; PCR, polymerase chain reaction; PI3K, phosphatidyl inositol 3 kinase; siRNA, small interfering RNA; VEGF, Vascular endothelial growth factor; VEGFR1, VEGF receptor 1; VEGFR2, VEGF receptor 2.

0168-8278/$34.00
doi:10.1016/j.jhep.2008.07.022

which promote migration, proliferation and prolongs cell survival [8,9]. These effects of VEGF on EC proliferation, differentiation, migration and cell survival predispose VEGF for potential therapeutic antitumor strategies [8,10].

Inhibition of tumor growth on the molecular level can be achieved by affecting pro-tumoral and pro-angiogenic factors, like VEGF or VEGF receptors. There are several options of inhibiting VEGF binding to its receptors, such as soluble VEGF receptors [11], or anti VEGF antibodies [12]. The disadvantage of these approaches is that already circulating VEGF has to be captured and neutralised to block VEGF mediated downstream effects. In our opinion a more appropriate approach is to knock down VEGF protein synthesis and secretion at the generation site by small interfering RNA (siRNA). A variety of studies has checked antitumor effects of siRNA-VEGF, but surprisingly, there is little data about VEGF knockdown and paracrine functional effects on EC [13,14]. *In vitro* effects were mainly analyzed in the corresponding tumor cells used for the in *vivo models* [15–20].

In this study, siRNA targeted against VEGF was used to reduce VEGF expression in hepatoma and endothelial cells to alter AKT signaling and to investigate functional endothelial cell specific effects *in vitro*. These results were transferred to an *in vivo* orthotopic hepatoma model to analyze antitumoral and angiostatic effects.

2. Materials and methods

2.1. Animals and cell lines

Hepa129 cells (Hepatoma 129, obtained from NCI-Frederick Cancer Research and Development Center (DCT Tumor Repository)) were maintained in RPMI1640 supplemented with 10% FBS, 200 mM glutamine. The murine endothelial cell line SVEC4-10 (ATCC CRL-2181) was obtained from LGC Promochem (Wesel, Germany) and cultured in DMEM supplemented with 10% FBS, 200 mM glutamine.

Eight-week old male C3H mice were supplied by Charles River (Sulzfeld, Germany) and kept in the local central animal facility of the University Hospital Bonn. The mice were housed under standard conditions and had free access to water and food. Animal procedures were performed in accordance with approved protocols and followed recommendations for proper care and use of laboratory animals.

2.2. Analysis of VEGF and VEGFR expression

Hepa129 hepatoma cells and SVEC4-10 endothelial cells were analyzed regarding expression of VEGF, VEGFR1, VEGFR2 and Nrp1. RNA was isolated from Hepa129 cells and SVEC4-10 and reverse transcribed as described previously [11]. PCR was performed using specific primers for VEGF [11], VEGFR1 (R&D Systems, Wiesbaden, Germany) VEGFR2 (R&D Systems), Nrp1 (forward: 5′-actcaacccacatttcgat-3′; reverse: 5′-acctggatccactccttgta-3′; 700 bp fragment) and murine beta-actin (Stratagene, La Jolla, CA) as control. PCR products were separated with agarose gel electrophoresis. LightCycler analysis was performed as described previously [21].

2.3. Transfection of siRNA

siRNA-VEGF and a non-sense siRNA (siRNA-CONT) were purchased from Dharmacon (Munich, Germany). The sequences had been selected according to a recent publication [15]. Transfection of Hepa129 cells and SVEC4-10 was performed with Lipofectamine2000 (Invitrogen) in OptiMEM (Invitrogen). Cells were assayed for alterations in VEGF expression 2 and 3 days post-transfection using a VEGF-specific ELISA (R&D Systems, Wiesbaden, Germany): Transfected Hepa129 and SVEC4-10 were resuspended in PBS and ruptured with three freeze–thaw cycles. Protein concentration was determined using the DC protein assay (Biorad, Munich, Germany) and 50 μg whole protein was analyzed according to the ELISA manual. Cell supernatant from Hepa129 was collected to determine LDH levels for analysis of toxic side effects.

2.4. Analysis of cell viability and tube formation after siRNA-treatment

SVEC4-10 were transfected with siRNA-VEGF or siRNA-CONT as described above.

Cell viability was examined using tetrazolium (MTT): cells were washed with PBS and MTT was added for 45 min. MTT was solubilized with DMSO for 15 min and optical density was determined at 550 nm [22].

The ability of siRNA-transfected endothelial cells to form tube like structures on matrigel was analyzed according to a previous publication [23].

2.5. Influence of siRNA treatment on intracellular signal transduction molecules

For analysis of intracellular signal transduction molecules, phosphorylated PI3K, MAPK-p38, AKT and ERK1/2 were analyzed with western blot. 50 μg of cell lysates were separated on a 10% SDS polyacrylamide gel and transferred on nitrocellulose membrane via semi dry blot. Detection of pPI3K, pMAPK-p38, pAKT and pERK1/2 was performed with specific primary (sc-1637, sc-17852-R, sc-7985-R, sc-16981-R, Santa Cruz) and secondary antibodies (Santa Cruz) and chemiluminescence on X-ray film.

2.6. Orthotopic tumor models

Orthotopic tumors were established as described previously [11]. For the *ex vivo* model, siRNA-VEGF and siRNA-CONT transfected Hepa129 (each $n = 8$) were implanted. For the intraperitoneal treatment group, siRNA (200 μg/kg bodyweight) was injected 24 h prior to tumor cell inoculations with repetitive dosages every 2 days. Fourteen days after tumor cell inoculation, animals were sacrificed and livers explanted. Tumor growth was determined by measurement of the visible tumor in length and width. Tumor bearing liver lobes were frozen in liquid nitrogen or fixed in formalin (4%) for further analysis. Serum was collected to determine interferon-beta levels.

2.7. Histological sections and staining

Formalin fixed tumor-bearing liver samples were H.E. stained according to standard procedures in order to prove tumor take, evaluate liver toxicity and cell infiltration, respectively.

Cryo-preserved subcutaneous tumor sample sections were immuno-stained with rat anti-mouse CD31 (Dako, Hamburg, Germany) according to a previous publication [23]. Intratumoral CD31 positive vessels were counted when showing a red-brownish, cytoplasmatic staining.