Life Sciences 91 (2012) 1207–1215

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

# Low-molecular weight chitosan/vascular endothelial growth factor short hairpin RNA for the treatment of hepatocellular carcinoma

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ARTICLE INFO

*Article history:*
Received 9 May 2012
Accepted 19 September 2012

*Keywords:*
Vascular endothelial growth factor
Short hairpin RNA
Low molecular weight chitosan
Hepatocellular carcinoma

ABSTRACT

*Aims:* Vascular endothelial growth factor (VEGF) has been shown to be a key driving force for angiogenesis and tumor growth in hepatocellular carcinoma (HCC). As an emerging approach to block this angiogenic stimulator, the RNA interference (RNAi) technique has rapidly developed but is hindered for in vivo applications due to low cellular uptake and poor stability of small RNA. Based on low molecular weight chitosan (LMWC), a gene delivery system of short hairpin RNA (shRNA) directed against VEGF was constructed. The objective of this study was to investigate whether LMWC/shRNA nano-complexes can effectively inhibit VEGF expression in cancer cells and tumor tissues and suppress tumor growth in different HCC models.
*Main methods:* The transfection experiment and Real-time qPCR assay were used to evaluate the transfection efficiency and gene suppression activity of LMWC/shRNA complexes in Hepa 1–6 murine hepatocarcinoma cells. The therapeutic effect of LMWC/ VEGF shRNA was further tested in ectopic and orthotopic liver cancer models.
*Key findings:* LMWC/VEGF shRNA complexes significantly inhibited VEGF expression of HCC cells and liver tumor tissues. LMWC obviously enhanced and prolonged the deposition of shRNA at the tumor site when LMWC/shRNA complexes were intravenously injected into orthotopic allograft liver tumor-bearing mice. The administration of LMWC/VEGF shRNA complexes by intratumoral or intravenous injection demonstrated more effective suppression of tumor angiogenesis and tumor growth in different HCC models compared with naked shRNA.
*Significance:* This study demonstrated the feasibility of using LMWC as a potential carrier for RNA interference drugs in liver cancer therapy.



## Introduction

There is substantial evidence that pathological angiogenesis is a major factor contributing to tumor growth (Carmeliet and Jain, 2000, 2011). Tumor angiogenesis is a complex process regulated by a wide variety of angiogenic factors. Among these factors, vascular endothelial growth factor (VEGF) was found to be a key regulator of tumor-induced angiogenesis (Ferrara, 2002). Thus, anti-angiogenesis therapy through VEGF inhibition has been considered to be a promising strategy to suppress tumor growth in experimental tumor models and clinical studies (Ferrara, 2005). The FDA has approved three VEGF inhibitors, sunitinib malate, bevacizumab and sorafenib, for the treatment of patients with specific types of cancer. Clinical trials have demonstrated favorable therapeutic effects of VEGF inhibitor (Cardones and Banez, 2006). However, VEGF not only drives tumor angiogenesis but also plays a key role in normal blood vessel survival, blood pressure regulation and neurological, renal and hepatic function (Longo et al., 2002). Several adverse effects, such as vascular disturbance and even remission in normal organs following chronic and systemic administration of antibody, limit the long-term use of protein drugs in clinical applications (Kamba and McDonald, 2007).

To resolve these problems, the tumor tissue-specific suppression of VEGF was proposed as a potential strategy to ensure the therapeutic efficacy and avoid interrupting the normal physiological functions of VEGF. RNA interference (RNAi) strategies, such as small interfering RNA (siRNA) and short hairpin RNA (shRNA), are ideal for tumor-specific VEGF inhibition because these types of treatments could elicit effects only after entering tumor cells (Hannon, 2002). A VEGF silencing strategy by RNAi has been attempted, and satisfactory results have been achieved in some experimental tumor models (Kim and Rossi, 2007;

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http://dx.doi.org/10.1016/j.lfs.2012.09.015

Takei et al., 2004; Takeshita and Ochiya, 2006). However, due to poor stability of siRNA in vivo and low cellular uptake, a safe and efficient carrier was required for therapeutic RNAi applications (Whitehead et al., 2009). The two types of vectors mainly used for RNAi delivery are viral or non-viral carriers. Regarding the safety and ease of fabrication, several non-viral carriers based on cationic polymers have been successfully developed in the field of gene drug delivery. The siRNA/shRNAs associated with the cationic vehicles demonstrated an enhanced RNase resistance and an improved pharmaceutical effect compared with naked siRNA/shRNAs (Shim and Kwon, 2010).

In this study, we developed a non-viral gene delivery system based on low-molecular weight chitosan (LMWC) and VEGF shRNA in order to investigate its potential anti-angiogenesis and anti-tumor effects. The transfection efficiency of LMWC/shRNA complexes was observed in the murine hepatocarcinoma Hepa 1–6 cells. Furthermore, the therapeutic effect of LMWC /VEGF shRNA was evaluated in the mouse models with ectopic and orthotopic liver cancer.

## Materials and methods

### *Preparation and characterization of LMWC*

High-molecular weight chitosan (HMWC, from crab shells, minimum 85% deacetylated, 145 kDa, –NH2 group content: 72%) was purchased from Sigma (St Louis, MO, USA). HMWC was depolymerized into LMWC in 4 M HCl at 100 °C for 15 h as previously described (Dong et al., 2008). The molecular weight of HMWC and LMWC was determined by high-performance liquid chromatography (HPLC, LC-10A, Shimadzu, Kyoto, Japan) using a Shimadzu RID-10A refractive index detector. Two columns (Ultrahydrogel 500; Ultrahydrogel 120, 10*300 mm, Waters Corporation, Milford, MA) were connected in series. The mobile phase was double distilled water with a flow rate of 0.5 ml/min. Different molecular weight PEGs were used as standards.

### *Tumors and mice*

The mouse hepatoma cell line Hepa 1–6 was obtained from ATCC (Manassas, VA, USA). Hepa 1–6 cells were cultured in DMEM containing 10% FCS. Female ICR mice (18–20 g) were purchased from the experimental animal center of Nanjing Medical University (Nanjing, China). All animals received humane care according to the Chinese legal requirements.

### *shRNA preparation*

The sequences of the three shRNAs targeting mouse VEGF and one mismatch shRNA (used as the control) were as follows: VEGF shRNA no. 1: 5′- GGGAUUGCACGGAAACUUUguaaucuucAAAGUUUCCGUGCAA UCCCUU-3′; VEGF shRNA no. 2: 5′- AGGCUGCUGUAACGAUGAAguaa ucuucUUCAUCGUUACAGCAGCCUUU-3′; VEGF shRNA no. 3: 5′-GCGG AUCAAACCUCACCAAguaaucuucUUGGUGAGGUUUGAUCCGCUU-3′; and mismatch VEGF shRNA (m-shRNA): 5′- GACUUCAUAAGGCGCAUGCgu aaucuucGCAUGCGCCUUAUGAAGUCUU-3′ (Invitrogen, Carlsbad, CA, USA). To verify the gene suppression activity, different VEGF shRNAs were transfected into Hepa 1–6 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 h post-transfection, the cells were collected for VEGF mRNA quantification, and the supernatants were used to determine the VEGF120 and VEGF164 concentrations with an ELISA assay (R&D Systems, Minneapolis, MN, USA). n = 5 for each sample, and the experiments were performed in triplicate. Fluorescein isothiocyanate-labeled shRNA (FITC-shRNA, Invitrogen) was used for the tissue distribution studies.

### *Characterization and preparation of the LMWC/shRNA complexes and the gel retardation assay*

LMWC/shRNA complexes were prepared by mixing an aqueous solution of LMWC with shRNA according to specific N/P ratios. Briefly, various amounts of LMWC were dissolved in 500 μl PBS, mixed with 500 μl PBS containing 1 mg shRNA and incubated for 60 min at room temperature to obtain various polyionic LMWC and shRNA complexes. The diameters of the LMWC/shRNA complexes were analyzed by a 90 Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY, USA). n = 5 for each sample, and the experiments performed in triplicate. After incubation at room temperature, the complex solution was applied to a 4% agarose gel containing 0.1 mg/ml ethidium bromide, and the gel was visualized with a UV transilluminator (Gel Doc, 2000, BioRad Laboratories, Hercules, CA, USA).

### *Transfection experiment*

Hepa 1–6 cells were cultured in 6-well plates for the transfection experiments. Before transfection, the complete medium was removed, and the cells were rinsed with PBS. Naked shRNA or LMWC/shRNA complexes containing 10 μg shRNA was diluted with 1 ml medium and subsequently used to refill each well. After incubation at 37 °C for 6 h, the medium was removed, and fresh growth medium was added. A transfection with lipofectamine/shRNA complexes was used as a positive control. At 24 h post-transfection, the cells and supernatants were

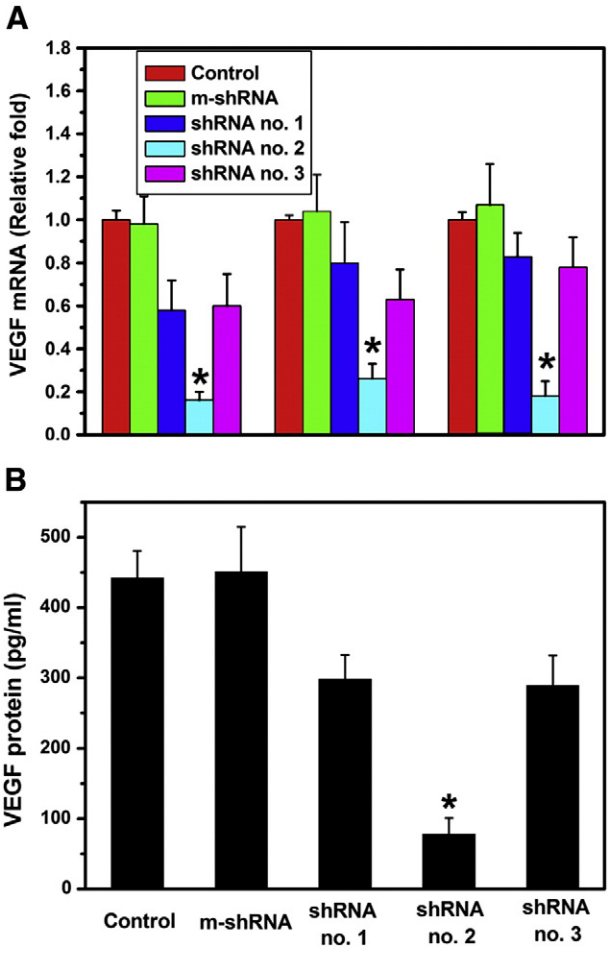


**Fig. 1.** Efficiency of VEGF shRNA inhibition. The VEGF mRNA levels (A) and protein expression profiles (B) of Hepa 1–6 cells transfected with VEGF shRNA nos. 1, 2, and 3 and a mismatch shRNA (m-shRNA) by lipofectamine. Values are expressed as the means ± SEM of five samples per condition of experiments performed in triplicate. *, $p<0.05$ compared with the control group.