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Antisense integrin αV and β3 gene therapy suppresses subcutaneously implanted hepatocellular carcinomas

J. Li [a], H. Tan [b], X. Dong [b], Z. Xu [a], C. Shi [a], X. Han [a], H. Jiang [b], G.W. Krissansen [c], X. Sun [b,c,*]

[a] *Department of General Surgery, Shandong Provincial Qianfoshan Hospital, Jinan 250014, China*
[b] *Hepatosplenic Surgery Center/Department of General Surgery, The First Clinical Medical School of Harbin Medical University, Harbin 150001, China*
[c] *Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland 1050, New Zealand*

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Abstract

Background. Integrin αVβ3 plays a critical role in tumour angiogenesis and metastasis formation, and is recognized as a key therapeutic target in the treatment of cancer.

Aim. To investigate whether antisense αV and β3 gene therapy has utility in the treatment of hepatocellular carcinomas.

Methods. Antisense expression plasmids targeting integrin αV or β3 were constructed, and examined by immunohistochemistry and Western blot analyses for their ability to inhibit αV and β3 expression. The antisense αV and β3 expression vectors, either alone or in combination, were injected into HepG2 hepatomas established subcutaneously in nude mice and tumour growth, angiogenesis and apoptosis were monitored.

Results. Antisense αV and β3 downregulated the αV and β3 subunits expressed by human umbilical vein endothelial cells, and the αV subunit expressed by HepG2 cells. Gene transfer of antisense αV and β3 expression vectors downregulated αV and β3 in HepG2 tumours established in nude mice, inhibited tumour vascularization and growth, and enhanced tumour cell apoptosis. Antisense αV suppressed tumour growth more strongly than antisense β3; however antisense therapy that simultaneously targeted both integrin subunits was more effective than the respective monotherapies. Antisense αV and β3 inhibited tumour angiogenesis to similar extents, by a process that is independent of vascular endothelial growth factor.

Conclusions. Antisense gene therapy targeting αV integrins warrants consideration as an approach to treat hepatocellular carcinomas.

Keywords: Angiogenesis; Antisense; Apoptosis; Gene therapy; Hepatocellular carcinoma; Integrin αV; Integrin β3

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an estimated incidence of over one million new cases per year [1,2]. HCC has a very poor prognosis with the overall 5-year survival rate of 3–5% [3]. Therefore, new strategies to treat HCC patients are urgently needed.

Solid tumours including HCC must establish an adequate vascular network to acquire the nutrition necessary for growth and metastasis. The latter notion provides the rationale for anti-angiogenic therapy of cancers aimed at targeting the tumour blood supply [4,5]. Given that HCC is a hypervascular tumour, anti-angiogenic therapy may offer a promising approach to treatment. Potent angiogenesis inhibitors such as angiostatin [6], endostatin [7], TNP-470 [8], and soluble

* Corresponding author at: The Hepatosplenic Surgery Center/Department of General Surgery, The First Clinical Medical School of Harbin Medical University, Harbin 150001, China. Tel.: +86 451 53643628; fax: +86 451 53643628.
E-mail addresses: kevsun88@hotmail.com, k.sun@auckland.ac.nz (X. Sun).

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vascular endothelial growth factor receptor 1 (flt-1) [7] are effective in treating HCC in experimental animal models.

Integrins are heterodimeric cell surface receptors composed of noncovalently associated transmembrane glycoproteins, which connect adhesive proteins in the extracellular matrix (ECM) to the cytoskeleton. Integrins play a central role in mediating angiogenesis, which depends largely on endothelial cell interactions with the ECM and pericytes. Integrins are involved in mediating endothelial cell migration, survival and proliferation [9]. Therefore, they contribute to angiogenesis by initiating the development of new blood vessels and the survival of the newly formed vasculature [10]. The integrin αVβ3 has proven to be critical for tumour angiogenesis [11]. Although only minimally expressed in quiescent blood vessels, αVβ3 is significantly upregulated during angiogenesis [12]. The interaction of αVβ3 with the ECM has been identified as a crucial event for endothelial cell survival in nascent vessels [13].

The αV subunit partners with additional integrin β subunits forming the integrins αVβ1, αVβ3, αVβ5, αVβ6, and αVβ8. αV integrins have been detected on the surface of neoplastic cells in a significant proportion of HCCs [14,15]. In the normal state, hepatocytes are the main cellular source of vitronectin [16], a major ligand of αV integrins [17]. The capacity to synthesize and secrete vitronectin is retained by most neoplastic hepatocytes [18]. To date, the expression of integrin β3 in HCCs has not yet been elucidated. However, the association of β3 integrins, especially αVβ3, with the aggressive and metastatic capacity of prostate [19], breast [20], cervical [21], ovarian [22], and liver [23] cancers has already been demonstrated.

A significant body of experimental evidence has shown that αVβ3 antagonists can inhibit the angiogenesis of growing tumours including melanoma, colon carcinoma, neuroblastoma. They can suppress tumour metastasis, and improve the survival of mice that bear tumours [10,24–26]. An antisense oligonucleotide targeting the αV integrin gene inhibits the adhesion and survival of breast cancer cells [27]. Antisense integrin β3 inhibits the formation of microvascular endothelial cell capillary tubes in fibrin [28]. Here, we investigated the therapeutic efficacy of antisense αV and β3 in the treatment of HCCs in mice.

2. Materials and methods

2.1. Mice, cell lines and antibodies

Male nude BALB/c mice (H-2b), 6–8 weeks old, were obtained from the Key Laboratory of General Surgery in Shandong Province, China. The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human umbilical vein endothelial cells (HUVECs) were supplied by the Typical Animal Reserve Center of China. The cells were routinely cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum. A sheep anti-mouse mAb against the integrin αV subunit (T-20, sc-6618; cross-reacts with human), an anti-human mAb against the integrin β3 subunit (N-20, sc-6627; cross-reacts with mouse), and a mouse anti-human mAb against VEGF (C-1, sc-7269; cross-reacts with mouse) were purchased from Santa Cruz, CA, USA. The mouse anti-mouse CD31 mAb MCA1334G was purchased from Serotec, Kidlington, UK.

2.2. Construction of expression plasmids

A 281 bp DNA fragment encoding the 5′-end of the mouse integrin αV subunit (nucleotides 106–386; GenBank NM_008402) was synthesized. Four oligonucleotides (Oligo 1: aat tcg gct ctg cgc ccc gcg cgc gcc ccg gcg atg gct gct ccc ggg cgc ctg ctg cta cg; Oligo 2: agg cgc ccg gga gca gcc atc gcc ggg gcg cgc gcg ggg cgc ggg cgc aga gcc g; Oligo 3: ccc tcg ccc cgg cgg cct cct gct gct gct gct gcc ggg tct cct gct gcc cct; and Oligo 4: cta gag ggg cag cag gag acc cgg cag cag cag cag gag gcc gcc ggg gcg agg gcg tag cag c) were mixed at the final concentration of 1 μM in a total volume of 50 μL, and heated at 95 °C for 1 min followed by annealing at room temperature for 5 min to form a 112 bp DNA fragment (encoding nucleotides 190–301), which was electrophoresed on a 1.5% agarose gel. The DNA product was extracted from the gel, purified, and cloned into the pcDNA3 vector (Promega, Beijing, China), and its integrity confirmed by DNA sequence analysis. It was used as a template to generate a longer DNA fragment by PCR with the primers 5′-gcg ggc cgc ggc cac cga gca ccg gga cag gga ctg aag tct ttc ggc tct gcg ccc cgc gcg-3′ and 5′-act cgg cgg gac ttt cga cgt cca ggt tga agg cgt cgg cga ggg gca gca gga gac ccg g-3′; by employing 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. The PCR product was subsequently used as a PCR template and amplified with the two primers 5′-gga att caa gcg cac agc aca gct cgg cag ttc ggg ggc gcg cag gcg gcg ggc cgc ggc cac cga g-3′ and 5′-ggt cta gaa gtc cac ggc gaa tcc gaa gta act tcc ctc ggg acc cgc ata ctc ggc ggg act ttc g-3′ using the same PCR condition as above. The final PCR product was cloned into pGEMT (Promega, Beijing, China), and then subcloned into pcDNA3B [29] at *Eco*RI and *Xha*I sites to construct the antisense integrin αV expression plasmid (AS-αV).

A 578 bp cDNA fragment encoding the 5′-end of the mouse integrin β3 subunit (nucleotides 30–607; GenBank AF026510) was amplified from total RNA extracted from mouse EL4 cells (ATCC). Briefly, the β3 subunit cDNA (nucleotides 30–607) was amplified by PCR with two primers 5′-ggg gaa ttc cag gat gcg agc gca gtg-3′ and 5′-ggg tct aga tgt aca tgt acg gcg ata-3′ by employing 31 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. The PCR product was cloned into pGEMT, and then subcloned into pcDNA3B at *Eco*RI and *Xha*I sites to construct the antisense integrin β3 expression vector AS-β3. The integrity of the final expression plasmids was confirmed by DNA sequence analysis.