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ScienceDirect
Vaccine 25 (2007) 1409–1415
Vaccine
www.elsevier.com/locate/vaccine

FLK-1-based minigene vaccines induce T cell-mediated suppression of angiogenesis and tumor protective immunity in syngeneic BALB/c mice

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Received 4 August 2006; received in revised form 17 October 2006; accepted 24 October 2006
Available online 7 November 2006

Abstract

Angiogenesis is a rate-limiting step in the development of tumors. Here, we demonstrate that oral minigene DNA vaccines against murine vascular endothelial growth factor receptor-2 (FLK-1), a self-antigen overexpressed on proliferating endothelial cells in the tumor vasculature, induced protection against tumors of different origin in syngeneic BALB/c mice. This protection is mediated by CD8 T cells, which specifically kill FLK-1$^+$ endothelial cells, resulting in marked suppression of tumor angiogenesis. More importantly, the minigene vaccine proved to be of similar efficacy as a vaccine encoding the whole FLK-1 gene. These data suggest a FLK-1 minigene vaccine provides a more flexible alternative to the whole gene vaccine and will facilitate their future design and clinical applications in cancer therapy and prevention.

Keywords: Anti-angiogenesis; Tumor vaccine; CTLs

1. Introduction

Anti-angiogenic intervention, which inhibits tumor growth by attacking the tumor's vascular supply, was pioneered by Folkman et al. [1–3] who established that angiogenesis plays a central role in the invasion, growth and metastasis of solid tumors [2,4]. In fact, angiogenesis is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply [5] and beyond this minimum size, tumors often become necrotic and apoptotic [6].

Vascular endothelial growth factor (VEGF) and its receptor tyrosine kinases play vital roles in angiogenesis [7,8]. Murine VEGF receptor-2 (VEGFR2, also known as FLK-1), binds the five isomers of murine VEGF, shows a restricted expression on endothelial cells and is upregulated once these cells proliferate during angiogenesis in the tumor vasculature [4,7,8]. In fact, several strategies have been used to block FLK-1, including dominant-negative receptor mutants, germ-line disruption of VEGFR genes, monoclonal antibodies against VEGF and a series of synthetic receptor tyrosine kinase inhibitors [9,10].

We first reported on an alternative strategy, namely an oral DNA vaccine against the entire FLK-1 gene, which prevented effective angiogenesis and inhibited tumor growth largely by CD8 T cell-mediated immune responses [11]. CD8$^+$ CTLs have the ability to specifically detect and kill tumor cells, as they recognize antigens (Ag) in the form of 8–10 amino acid long peptides, presented to T cell receptors (TCR) on the cell surface as complexes with MHC class I molecules. These peptides, usually referred to as CTL epitopes, are generated in the cytosol of cells after proteolytic processing of antigens by the proteasome of antigen-presenting cells [12]. One of the primary aims of tumor vaccines is to induce specific anti-tumor CD8$^+$ CTL responses against such epitopes that can eradicate tumors and prevent their recurrence. The application of minigene vaccines provides an attractive alternative approach to achieve this objective because of their ease of synthesis and manipulation. Moreover, in contrast to vaccines encoding entire genes, minigene vaccines can induce immune responses directed against specific antigen epitopes while avoiding the interference of non-relevant Ag epitopes.

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doi:10.1016/j.vaccine.2006.10.043

Consequently, such vaccines lend themselves to in depth studies of immunological mechanism far more readily than DNA vaccines encoding entire genes.

In previous studies, we used a minigene approach to successfully induce a FLK-1-speicific CTL responses that protected C57BL/6 mice from tumor challenges and identified the first H-2 D^b-restricted FLK-1 epitope [13]. Here, we used similar approaches in syngeneic BALB/c mice, in an effort to prove the feasibility of such strategy for mice with different genetic background and to establish the proof of concept that a FLK-1 minigene vaccine provides a more flexible alternative to the whole gene vaccine, thus facilitate their future design and clinical applications in cancer therapy and prevention.

2. Methods

2.1. Animals, bacterial strains and cell lines

Female BALB/c mice were purchased from the Jackson Laboratory. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The murine D2F2 breast cancer cell line was kindly provided by Dr. W-Z. Wei (Karmanos Cancer Institute, Detroit, MI, USA). The murine colon carcinoma cell line CT-26 was provided by Dr. I.J. Fidler (MD Anderson Cancer Center, Houston, TX, USA). The murine high endothelial venule cell line (HEVc) was a gift from Dr. J.M. Cook-Mills (University of Cincinnati, OH, USA). The HEVc-FLK-1 cell line was established by retroviral transduction with the FLK-1 gene by Drs. Harald Wodrich and Andreas G. Niethammer (formerly of our institute).

The doubly attenuated *Salmonella typhimurium AroA*$^-$ and *dam*$^-$ strain RE88 was kindly provided by Remedyne Corporation (Santa Barbara, CA, USA) and was transduced with DNA vaccine plasmids to serve as vaccine carrier as previously described [14].

2.2. Construction of expression vectors

Vector construction is illustrated schematically by Fig. 1A. The expression vectors were established based on the pcDNA/Myc/His vector (Invitrogen, Carlsbad, CA, USA) containing the ubiquitin sequence. The peptides were cloned downstream of ubiquitin, and the sequence of each peptide is indicated in Table 1. All peptides were engineered to be in-frame with the myc epitope. Constructs were confirmed by DNA sequencing at The Scripps Research Institute's Core Facility (La Jolla, CA, USA). Peptide expression was demonstrated by Western blotting analysis of transfected 293T cells with monoclonal anti-myc antibody (Invitrogen). Once peptide expression was verified, a stop codon was introduced immediately in front of the *myc* epitope sequences. The resulting vectors, pDd, pKd, pDd+Kd1, and pKd+Dd2 were each verified by nucleotide sequencing and used to transform doubly attenuated *S. typhimurium* (*dam*$^-$, *AroA*$^-$) for subsequent immunization.

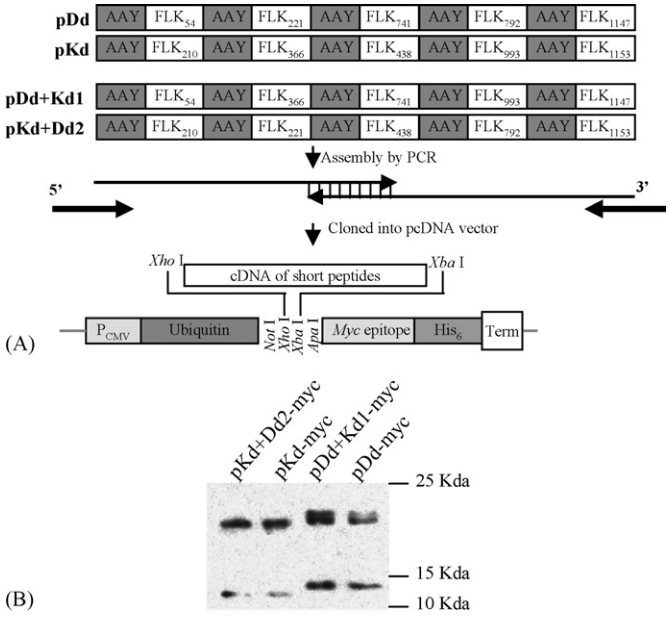

Fig. 1. Construction and expression of the FLK-1 DNA minigene vaccines. (A) Schematic map. Minigenes encoding the murine H-2 D^d- and K^d-restricted FLK-1 nonapeptides and spacers were assembled by PCR with overlapping oligonucleotides as templates. The sequence of each nonapeptide is shown in Table 1. The PCR fragments generated were cloned downstream of ubiquitin in a modified pcDNA expression vector by using *Xho* I and *Xba* I restriction sites. (B) Proteins encoded by minigenes were expressed in mammalian cells. The 293 T cells were transfected with either pDd-myc, pDd+Kd1-myc, pKd-myc or pKd+Dd2-myc for 24 h, harvested, lysed and analyzed by Western blotting with anti-myc monoclonal antibody.

2.3. Oral immunization and tumor cell challenge

Groups of BALB/c mice were immunized three times at 1-week intervals by gavage with 100 μl PBS containing approximately 5×10^8 CFU of doubly mutated *S. typhimurium* harboring either empty vector, pDd, pKd, pDd+Kd1 or pKd+Dd2 plasmids. Mice were challenged i.v. with different carcinoma cell lines 2 weeks after the last immunization.

Table 1
Peptides used in minigene vaccines

Peptide name	Sequence	Included in vaccine
FLK_{54}	RGQRDLDWL	pDd; pDd+Kd1
FLK_{210}	TYQSIMYIV	pKd; pKd+Dd2
FLK_{221}	VGYRIYDVI	pDd; pKd+Dd2
FLK_{366}	WYRNGRPIE	pKd; pDd+Kd1
FLK_{438}	QYGTMQTLT	pKd: pKd+Dd2
FLK_{741}	LGCARAETL	pDd; pDd+Kd1
FLK_{792}	EGELKTGYL	pDd; pKd+Dd2
FLK_{993}	LYKDFLYTE	pKd; pDd+Kd1
FLK_{1147}[a]	QRPSFSELV	pDd; pDd+Kd1
FLK_{1153}	ELVEHLGNL	pKd; pKd+Dd2

[a] The underlined serines (S) residues indicate potential glycosylation sites, with the one in bold print being the most likely to be glycosylated.