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Cytotoxic T lymphocyte-dependent tumor growth inhibition by a vascular endothelial growth factor–superantigen conjugate

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ABSTRACT

T cells are major lymphocytes in the blood and passengers across the tumor vasculature. If these T cells are retained in the tumor site, a therapeutic potential will be gained by turning them into tumor-reactive cytotoxic T lymphocytes (CTLs). A fusion protein composed of human vascular endothelial growth factor (VEGF) and staphylococcal enterotoxin A (SEA) with a D227A mutation strongly repressed the growth of murine solid sarcoma 180 (S180) tumors (control versus VEGF–SEA treated with 15 μg, mean tumor weight: 1.128 g versus 0.252 g, difference = 0.876 g). $CD4^+$ and $CD8^+$ T cells driven by VEGF–SEA were accumulated around VEGFR expressing tumor cells and the induced CTLs could release the tumoricidal cytokines, such as interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha). Meanwhile, intratumoral CTLs secreted cytolytic pore-forming perforin and granzyme B proteins around tumor cells, leading to the death of tumor cells. The labeled fusion proteins were gradually targeted to the tumor site in an imaging mice model. These results show that VEGF–SEA can serve as a tumor targeting agent and sequester active infiltrating CTLs into the tumor site to kill tumor cells, and could therefore be a potential therapeutical drug for a variety of cancers.

1. Introduction

Immunotherapy is an emerging treatment method for tumors, that should be recognized clearly as an abnormal tissue by self immune system. Self T or B cells are activated by cytokines, such as interleukin-2 *in vitro*, then retransported into patients themselves in some hospitals by doctors. Now this is one of the useful methods in cancer patients. However, this is just a preliminary method lacking both specificity and concentration of lymphocytes on the tumor area. Thus, how to let immune cells be enriched and activated around tumor tissues *in vivo*, then to kill tumor cells is a challenge.

Vascular endothelial growth factor (VEGF) exists as at least four isoforms, and is one of major factors for mediating tumor angiogenesis [1–5]. It stimulates endothelial cell proliferation and migration by binding to two distinct cell surface receptor tyrosine kinases: VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) [6,7]. VEGF receptors are expressed most abundantly in the tumor tissues, tumor vasculature [8], thus its high expression in tumors and their vessels provide a unique opportunity to target tumors with cytotoxic agents. VEGF fused with the translocation and enzymatic domains of bacterial toxins may cause selective toxicity to the tumor vasculature [9,10].

These chimeric VEGF proteins function as tumor-targeting molecules to kill cancer cells by toxic action, but they are not dependent on immunoregulator cytotoxicity permeated into solid tumors. The major lymphocytes in the blood are T cells that are divided into $CD4^+$ and $CD8^+$ groups. Usually, the T cell receptors (TCRs) of T cells cannot distinguish self-antigens on cancer cells. T cells passing through solid tumors via blood vessels may be reactive with carcinoma-associated antigens only if they are recruited, enriched and activated in some way, although the mechanisms of recruitment and enrichment are still unknown. The recognition of cancer by T cells has been attempted by two strategies, including the use of CD3-based bispecific antibodies [11,12], and antibodies linked with a superantigen such as staphylococcal enterotoxin A (SEA) [13–15].

SEA is a microbial superantigen that activates T-lymphocytes and induces production of various cytokines, including interferon-gamma (IFN-gamma), tumor necrosis factor-alpha (TNF-alpha), and cytolytic pore-forming perforin and/or granzyme B secreted by intratumoral CTLs. It can induce a strong cytokine

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production and CTLs in the $CD4^+$ and $CD8^+$ groups [13,14]. The SEA gene utilized here carries the D227A mutation created by Dohlsten's group, which showed a 1000-fold reduction of binding to major histocompatibility complex class (MHC) II in order to decrease systemic toxicity [15] and was consequently conjugated with antibodies as a powerful CTL inducer against cancer [15–17]. Also SEA can be genetically engineered into cancer cell lines, which can be regarded as a potential vaccine [18–20]. Mutant or nonmutant SEA also could be fused with antibody or its Fab fragment against tumor-specific antigen [13,14,16,21–26] to reduce or inhibit the growth of carcinomas and their metastases.

Furthermore, the question of how to recruit large pools of effector T cells to the intratumoral space in order to facilitate tumor infiltration is still a difficult challenge. The mechanisms underlying tumor eradication that depend on the infiltration of cytotoxic T lymphocytes (CTLs) into solid tumors remain largely unknown. The movement or diffusion of antibodies after intravenous (i.v.) injection has not yet been clearly demonstrated. Here, we constructed VEGF–SEA and examined its targeting to tumors, inducing CTLs and the tumoricidal immunostimulatory responses in sarcomas.

2. Materials and methods

2.1. Preparation of fusion protein

The fragment from pET22b plasmid (Merck, Germany) between the *Not* I and *Xho* I sites contains an E-tag sequence GAPVPYPDPLEPR from pCANTAB 5E of the antibody phage display system (GE, NJ) that can be used for protein detection by anti E-tag antibody. A synthetic DNA fragment (Takara, China) encoding 121 amino acids of human VEGF fragment [6–8], a short linker peptide VDKLGGGGSGGGGSGGGGS, and SEA with the D227A mutation was integrated into modified pET22b at the *Eco*R I and *Not* I sites to produce fusion protein VEGF–SEA. Its molecular weight including the leader and upstream sequences in pET22b was estimated as 45.9 kDa. The proteins expressed as inclusion bodies in *Escherichia coli* BL21(DE3) were purified using immobilized metal ion (Ni^{2+}) affinity chromatography, refolded using the method of GSSG and arginine dialysis.

2.2. Tumor model in vivo

Male ICR mice (Experimental Animal Center, Academy of Military Medical Sciences, PR China), 4 weeks old and with weight of 18–22 g, were employed in the tumor model. All mice were maintained in a specific pathogen-free (SPF) facility at the Experimental Animal Center, Fudan University. The animal experiments were approved by the Institutional Animal Care and Use Committee at Fudan University (IACUC Protocol #2009-15). Each mouse was subcutaneously inoculated with 2×10^6 mouse sarcoma 180 (S180) cells suspended in phosphate-buffered saline (PBS) into the right axilla. Mice were divided into 4 groups ($n = 20$ per group) and given four times (in day 2, 4, 6, 8, respectively) i.v. injections (0.2 ml) via the tail vein with VEGF–SEA (5, 10, and 15 μg) in saline, 0.9% NaCl saline (control) and free SEA in saline (with same molar ratio as VEGF–SEA, about 83 pmol, 166 pmol, 250 pmol, respectively), starting on day 2 after tumor inoculation. The tumors were measured on day 9.

2.3. Immunohistochemistry assay

Tumor tissues treated with 15 μg of VEGF–SEA, free SEA or saline were fixed in 4% phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μm, and then deparaffinized in xylene and rehydrated in a graded ethanol using a general method. Sections were incubated first in a 3% hydrogen peroxide solution to block endogenous peroxidases, then with a protein-blocking solution containing preimmune rabbit serum, and finally with the following primary antibodies (1:300 dilution in PBS containing 1% bovine serum albumin) in each test: rabbit CD4-specific polyclonal antibody, rabbit CD8-specific polyclonal antibody, rabbit IFN-gamma-specific polyclonal antibody, rabbit TNF-alpha-specific polyclonal antibody, and rabbit perforin-specific polyclonal antibody from Santa Cruz Biotechnology (Cruz, CA); rabbit granzyme B-specific polyclonal antibody from Abcam (Cambridge, MA); goat anti-mouse or anti-rabbit secondary antibody from Invitrogen (Carlsbad, CA). To prepare E-tag-specific antibody, a synthetic GAPVPYPDPLEPR peptide was raised in BALB/c mice. To prepare VEGFR-specific antibody, a peptide from the extracellular part of mouse and human VEGFR, SISNLNVSLCARYPEKRFVPDGNRISWDS, was synthesized and raised against rabbits. Both IgG fractions from the antiserums with high titers against E-tag or VEGFR were purified using Hitrap protein G-Sepharose columns (GE, NJ). The bound antibody was detected by incubation with the secondary antibody for 1 h, and then avidin-biotin-peroxidase complex (Zymed, CA) for 30 min followed by diaminobenzidine staining for 8 min. The slides were rinsed with PBS and counterstained with hematoxylin for 1 min. For immunofluorescence detection of CD 8 T cells, a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Millipore, MA), was reacted with the slide in 1:100 dilution after washing the primary anti-CD8 antibody.

2.4. VEGF–SEA labeling

VEGF–SEA proteins were labeled with Kodak X-SIGHT 670 TFP ester (Carestream Health, NY) according to the protocol. Briefly, dyes were conjugated with proteins in a sodium phosphate buffer (100 mM sodium phosphate, 0.15 M NaCl, pH 7.2) and unconjugated dyes were neutralized by ammonium chloride. The labeled proteins were separated from free dyes using dialysis against PBS. The ratio of free dyes to the proteins (degree of labeling) was calculated by measuring absorbance at 280 nm and 670 nm.

2.5. Flow cytometry analysis

S180 cells were centrifuged at 1200 rpm and re-suspended in PBS. Aliquots of 2×10^6 cells were incubated with 10.0, 1.0 or 0.1 μg of LSS 670-labeled VEGF–SEA proteins, respectively, at 4 °C for 30 min, and washed three times in ice-cold PBS. The ability of labeled-VEGF–SEA to bind S180 cells was quantified by FACS analyses (BD FACS Calibur, Becton Dickinson Medical Devices, Franklin Lakes, NJ).

2.6. Mouse imaging

VEGF–SEA proteins (10 μg) labeled with LSS 670 were injected i.v. via the tail vein into mice bearing tumors of 0.5–1.0 cm in diameter or into mice without inoculation of S180 cells. Mice were anesthetized and the targeting of labeled proteins to tumors were monitored for 68 h (2 times/day, 3 h anesthesia/times) using *in vivo* fluorescence imaging IVIS Kinetic (Caliper Life Sciences, Hopkinton, MA) with an excitation bandpass filter at 710 nm and collecting emissions from 810 to 885 nm.

2.7. Cytokine detection

Blood and spleen samples were collected after the mice were dissected. All groups contained pooled sera and spleens from at least three mice. Protein levels of TNF-alpha and IFN-gamma were measured using specific enzyme-linked immunosorbent assay