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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

(ELISA) kits (R & D Systems, MN) according to instructions from the manufacturer.

2.8. TUNEL staining

To evaluate apoptotic tumor cells, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining was performed *in situ* according to a protocol (Roche Applied Science, Mannheim, Germany) to detect DNA breakage in day 9.

Sections were incubated with TdT, a fluorescein-labeled nucleotide mixture, in a wet chamber at 37 °C for 1 h. The sections were then washed with PBS and incubated with anti-fluorescein antibody conjugated with horseradish peroxidase (HRP) for 30 min.

3. Results

3.1. Growth inhibition of S180 tumors

The VEGF–SEA proteins were purified and refolded, and the molecular weight of VEGF–SEA was close to 46 kDa with a coomassie staining (Fig. 1A). S180 cells were subcutaneously inoculated into the right axilla of ICR mice, followed by i.v. injections of VEGF–SEA (5, 10, or 15 μg), saline (control) or free SEA, respectively. The solid tumors occurred on day 5 and quickly expanded in the control mice, while the tumor growth was largely delayed in mice treated with 15 μg injection of VEGF–SEA (Fig. 1B and C) with great statistical significance ($p < 0.01$). VEGF–SEA strongly suppressed solid tumor growth (control versus VEGF–SEA treated with 15 μg, mean tumor weight: 1.128 g versus 0.252 g, difference = 0.876 g, 95% confidence interval [CI] = 0.63 to 1.12, $p < 0.001$). The effects of tumor inhibition increased with adding of proteins (Fig. 1D and E). While SEA suppressed solid tumor growth with statistical significance ($p < 0.05$) in 15 μg group, similar as the previously reported data [27–31] (Fig. 1D and F).

3.2. Interaction between VEGF–SEA proteins and S180 cells

S180 tumors showed high levels of VEGFR expression (Fig. 2A), and interacted with VEGF–SEA (Fig. 2B and C) compared with saline or SEA groups after its injection. When the VEGF–SEA solution was incubated with tumor sections, S180 cells overlaid by VEGF–SEA molecules were confirmed by using anti-E-tag antibody. The brown stained areas (Fig. 2B) in tumors treated with VEGF–SEA were T cells that were reacted with SEA, the moiety of VEGF–SEA. The ability of VEGF–SEA to bind S180 cells was directly demonstrated using fluorescence-activated cell sorter (FACS) analysis. S180 cells bound labeled VEGF–SEA decreased with reduction in the protein concentration. Almost all S180 cells bound labeled proteins when 10 μg of VEGF–SEA was added.

3.3. Labeled VEGF–SEA targeting to solid tumors in vivo by imaging analysis

LSS 670-labeled VEGF–SEA was injected i.v. via the tail vein to avoid dye-labeling background and showed a clear route of VEGF–SEA movement in mice. The proteins diffused quickly to the abdomen and bosom, reached the tumor 10 h late after the injection, and gradually accumulated in the tumor (Fig. 3A and B, red arrow) and spleen (Fig. 3C and D, blue arrow). Finally there was negligibly visible deposition in other organs except bladder containing excreted proteins.

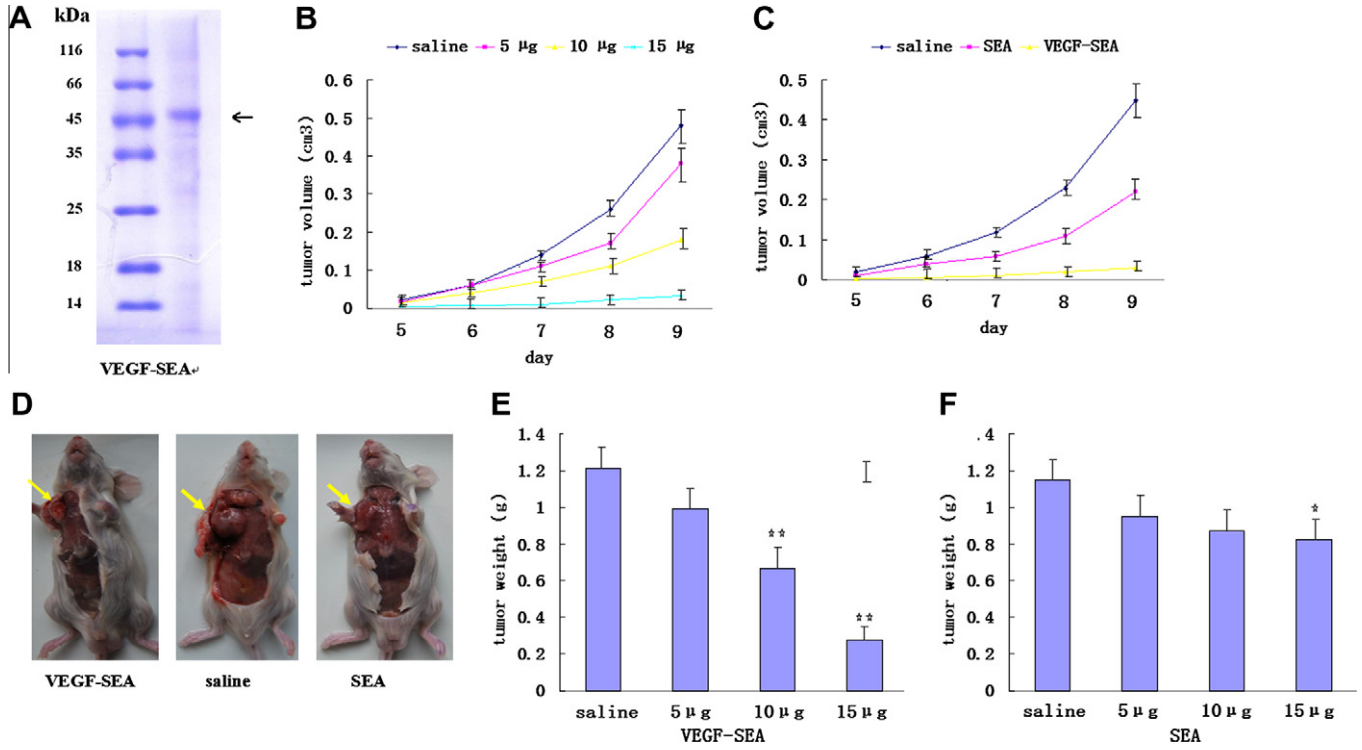

Fig. 1. Inhibition of tumor growth by VEGF–SEA. (A) SDS–PAGE identification of VEGF–SEA proteins by a coomassie staining. (B) Effect of VEGF–SEA on the growth of S180 tumors. After mice inoculated with 2×10^6 S180 cells, they were treated with four times (in day 2, 4, 6, 8, respectively) i.v. injections (0.2 ml) of 0.9% NaCl saline (control) or VEGF–SEA (5, 10, and 15 μg, respectively) starting day 2 after tumor inoculation. $n = 20$ mice per group. (C) Effect of VEGF–SEA or SEA on the growth of S180 tumors. After mice inoculated with 2×10^6 S180 cells, they were treated with four times (in day 2, 4, 6, 8, respectively) i.v. injections (0.2 ml) of 0.9% NaCl saline (control) or SEA (250 pmol) or VEGF–SEA (15 μg) starting day 2 after tumor inoculation. $n = 20$ mice per group. (D) Inhibition of S180 tumors. (E) The tumors were measured after dissection of mice on day 9 at VEGF–SEA group. (F) The tumors were measured after dissection of mice on day 9 at SEA group. Yellow arrows indicated the tumor position. $^{**}p = 0.001$. All datas are repeated three times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

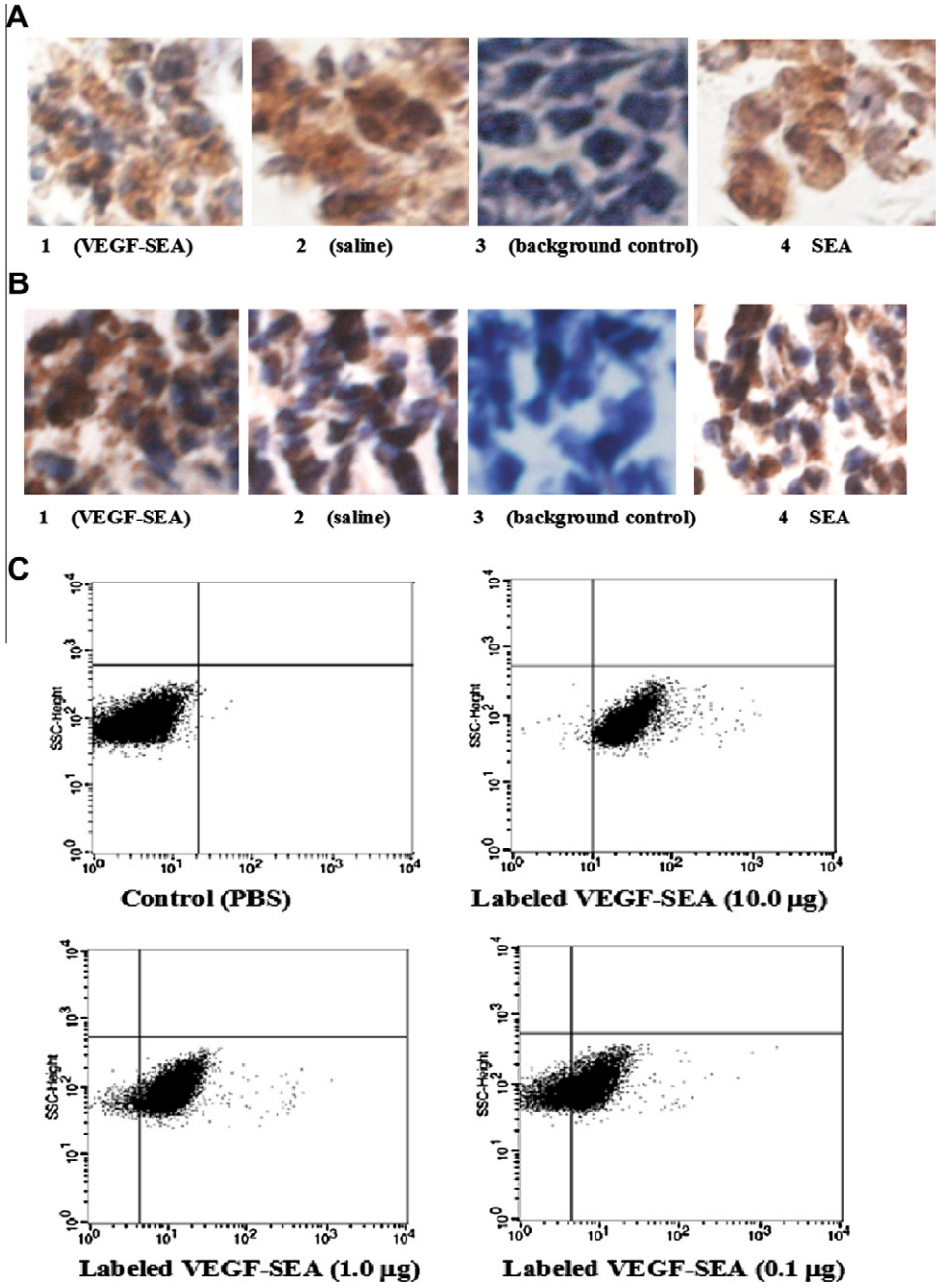

Fig. 2. Interactions between VEGF–SEA and VEGFRs on S180 carcinomas. (A) Detection of VEGFR of S180 cells. Anti VEGFR antibody was added to section 1 (VEGF–SEA), 2 (saline) and 4 (SEA) or un-added to section 3 (saline) as a background control. (B) VEGF-reactive S180 tumor cells. Mouse anti-E-tag antibody was added to section 1 (VEGF–SEA), 2 (saline) and 4 (SEA) or un-added to section 3 (saline) as a background control after the sections incubated with a VEGF–SEA solution. Anti-E-tag antibody reacted with VEGF–SEA containing E-tag on the surface of S180 tumor cells and T lymphocytes (small brown spots in the section treated with VEGF–SEA). (C) Analysis of LSS670-labeled VEGF–SEA binding to S180 cells by FACS analysis. S180 cells (2×10^6) were incubated with 10.0, 1.0 and 0.1 μg of LSS670-labeled VEGF–SEA proteins and PBS (control). Microscope magnification: 400× in A and B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. $CD4^+$ and $CD8^+$ positive T cells can be enriched around S180 tumors and secrete cytokines by VEGF–SEA

Infiltrating T lymphocytes were abundantly found in S180 tumors treated with VEGF–SEA to be $CD4^+$ and $CD8^+$-positive (Fig. S1 in Supplementary data). Accumulated T cells (brown stained areas) were presented on the surface of and around tumor cells (large spots). Only few or negligible T cells were detected in control tumors. TNF-alpha and IFN-gamma cytokines were released at high levels around the S180 cells (Fig. 4A and B) treated with VEGF–SEA. However, there is statistical significance ($P > 0.05$) between VEGF–SEA and SEA, or VEGF–SEA and saline or SEA and saline by *t*-test (Fig. 4C).

3.5. Tumor apoptosis occurred by VEGF–SEA

Activated T cells secreted pore-forming perforin onto the target and lytic granzyme B granules are concentrated on the immune synapse between T cell and S180 cells (Fig. S2A and B in Supplementary data). The molecules judged to be perforin are assembled as the immune synapse between T cell and S180 cells according the previous published data [32]. Through the direct killing mediated

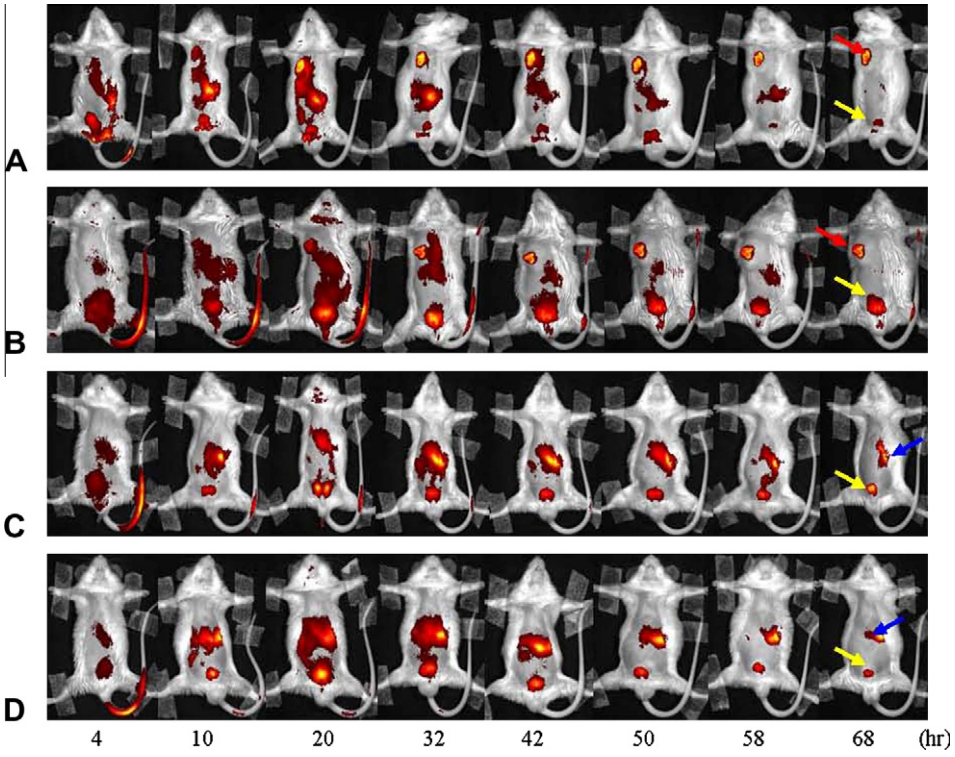

Fig. 3. VEGF–SEA targeting to solid tumors by *in vivo* imaging of mice. LSS670-labeled VEGF–SEA proteins (10 μg) were injected i.v. into mice bearing tumors (A and B) and un-inoculated mice as control (C and D). Movement of labeled VEGF–SEA proteins were observed using *in vivo* fluorescence imaging with the IVIS Kinetic imaging system. Red, yellow and blue arrows indicated the tumor, bladder and spleen positions, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by perforin and granzyme granules acting together with apoptotic cytokines such as TNF-alpha and IFN-gamma, tumor cells were eliminated in VEGF–SEA-treated mice, as demonstrated by TUNEL staining in day 9 (Fig. S2C in Supplementary data).

4. Discussion

Enhanced VEGFR expression has been developing as a target for tumor therapy. Our constructed VEGF–SEA fusion protein has similar ability as the previously reported [33,34]. The amount of VEGFR expressed on S180 cells is abundant demonstrated in the tumor site, peaking 68 h late after i.v. injection. The spleen was another target for the proteins because T cells were sequestered there. Obviously, there were two receptors for VEGF–SEA. One was VEGFR on S180 cells binding the VEGF moiety and another was TCR on T cells interacting with the SEA moiety. In other words, the tumor can gather VEGF–SEA molecules and arrested T cells based on the protein responding both to VEGFR and TCR. If there were no elevated VEGFRs, T cells carrying VEGF–SEA proteins returned to the spleen.

Once inoculated S180 cells were developed into a colonial and growing tumor, it was possible for tumor-unspecific T lymphocytes including $CD4^+$ and $CD8^+$ T cells to be retained, when the VEGF–SEA protein reached the tumor site. More carcinoma-associated VEGF–SEA molecules resulted in more captured T cells neighboring to or anchored on the surface of S180 tumor cells. After accumulation, T cells stimulated by VEGF–SEA became tumor-sensitive CTLs that would secrete the tumoricidal cytokines TNF-alpha and IFN-gamma, the granulolytic proteins perforin and granzyme B to induce tumor apoptosis [35–37].

Localization of lymphocytes to tumor tissues requires specific recognition of tumor antigens in the vascular compartment or tumor tissues. Generally, the ability of the leukocyte population to identify tumor-associated antigens and kill the tumor cells that express them is not sufficient to give an antitumor response because tumor antigen is a self-antigen, and the bypassing T cells may not generate antibody against itself. A possible explanation for this could be deficient homing of cells with necessary specificity to the tumor site [38]. Failure in repressing tumor growth was quietly in shortage of recruitment of antitumor effector cells. Targeting CTLs to the tumor site is of great potential therapeutic interest because the concentration of tumor-specific effector T lymphocytes is generally insufficient to arrest progressive tumor growth, while the majority of T cells are trapped in the liver, spleen or lungs, generating low assembly of tumor-sensitive T lymphocytes in the tumor tissue [39,40].

SEA alone cannot enrich T lymphocytes into the tumor site and show less effect on tumor inhibition, indicating that a tumor targeting agent such as EGF, a moiety of EGF-SEA, is required for powerful attacking against tumor expressing EGFR [27–31]. Here, S180 cells colonized and formed a tumor after inoculation, following administering of VEGF–SEA via i.v. injections. During a period of random diffusion, VEGF–SEA molecules concentrated selectively in developing tumors with relatively high expression of VEGFRs, and then arrested the passage of T lymphocytes. This retention of T cells effected their deep infiltration into solid tumors, and turned into tumor-challenging CTL, even though the lymphocytes were unable to recognize tumor-autologous antigens specifically. Once VEGF–SEA-promoting infiltrating CTLs massed in the tumor site, the tumor approached apoptosis by an *in situ* attack. We hope we can do more different tumor animal models to gain more results.

VEGF–SEA established linkage with CTLs and tumors, in which VEGF targeted the fusion protein to tumors expressing abundant VEGF receptors and SEA evoked the infiltrating CTLs into lysing solid tumors by granule exocytosis pathway or other unknown

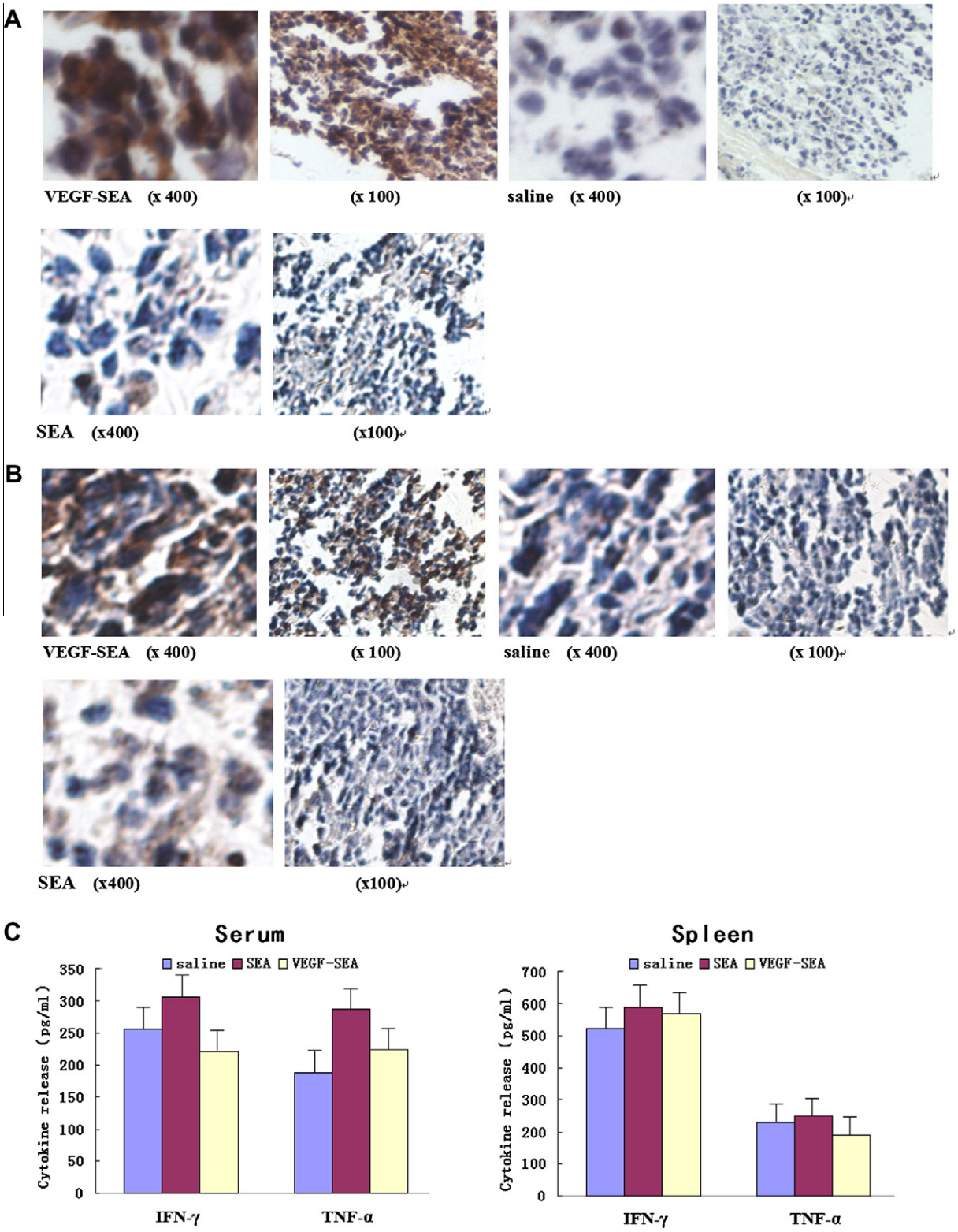

Fig. 4. Cytokine release in mice bearing S180 tumors. 60 mice were divided into 3 groups,with injection of 15 μg VEGF–SEA, SEA and saline, respectively. 9 days after injection, the sera were collected,then the mice was killed for the experiments. The experiments were done 3 times. The demonstrated figures were one of them. (A) IFN-gamma secretion in tumors. The sections were labeled with rabbit IFN-gamma-specific polyclonal antibody. (B) TNF-alpha secretion in tumors. The sections were labeled with rabbit TNF-alpha-specific polyclonal antibody. (C) Cytokine secretion in mice. The data represented cytokine levels in sera in pg/ml and in spleens and tumors in pg/g. Cytokines in blood, spleen and tumor samples were measured using specific ELISA kits.

methods. The success of immunotherapeutic strategies against cancer depends on the generation of effective tumor-specific T lymphocytes that must not only enter the tumor area but also can traverse the interstitial region and directly interact with the target cells [40]. Our studies demonstrate that VEGF–SEA-dependent accumulation of intratumoral CTLs is necessary for effective

tumor regression, and could therefore be a potential immunotherapeutical drug for a variety of tumors with highly-expressed VEGFR.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.122.

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