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The enhanced anti-angiogenic and antitumor effects of combining flk1-based DNA vaccine and IP-10

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ABSTRACT

The purpose of the present study was to evaluate the anti-vasculature effects and the anti-tumor effects of attenuated *Salmonella typhimurium* vaccine strain encoding murine vascular endothelial growth factor (VEGF) receptor-2 (flk1) in combination with plasmid DNA vector encoding the murine interferon-induced protein of 10 kDa (IP-10 or CXCL10) gene. Mouse models of malignant melanoma (B16-F10) were treated with combining orally given attenuated *S. typhimurium* vaccine strain encoding flk1 with direct intratumoral injection of a non-viral plasmid DNA vector encoding the murine IP-10 gene. The volumes of tumors and survival of mice bearing B16-F10 tumors were observed. Cytolytic T lymphocyte (CTL) response was measured by cytotoxic assay, vessel density and tumor-cell proliferation were observed by immunostaining, and tumor apoptosis was determined by TUNEL staining. The results revealed the combination therapy groups showed more significantly inhibited tumor growth, apoptosis of tumor cells, and reduced neovascularization, cell proliferation, and developed a strong CTL response in these mice. In summary, the therapy of attenuated *S. typhimurium* vaccine strain encoding flk1 combined with the IP-10 gene has significant synergistic effect against tumors.

1. Instruction

The assumption that tumor growth and metastasis are angiogenesis-dependent was proposed initially by Folkman in 1971 [1]. Its key implication is that inhibition of new vessel formation can serve as a universal strategy to interfere with tumor growth and progression. Over the past three decades, the dependence of tumor growth on neovascularization has been firmly established by extensive experimental evidence, demonstrating that tumors as small as a few cubic millimeters in size are not able to continue to grow without vigorously inducing new blood vessel formation. As a result, tumor starvation through interference with tumor blood supply has become a well-recognized approach of cancer therapy [2,3]. Vascular endothelial growth factor (VEGF) is the most potent angiogenic cytokine [4]. The expression of VEGF is stimulated by hypoxia, which is persistent and frequently found in tumor cells [5]. The VEGF receptor fetal liver kinase 1 (flk1; VEGFR-2, KDR) is an endothelial cell-specific receptor tyrosine kinase that mediates physiological and pathological angiogenesis. It was recently shown that active immunotherapy targeting endothelial products could inhibit tumor progression. Immunization of mice with paraformaldehyde-fixed xenogeneic endothelial cells [6], with flk1 protein-loaded dendritic cells (DCs) [7], or with an attenuated *Salmonella typhimurium* expressing flk1 cDNA [8], inhibited tumor growth and led to the rejection of transplanted tumors. Angiogenesis is an essential process for tumors to grow in vivo and is the result of a complex balance between angiogenic and anti-angiogenic factors [9,10]. Inhibition of the development of new blood vessels in tumors is effective in suppressing growth of these tumors.

Interferon-induced protein of 10 (IP-10) is a CXC chemokine produced by certain types of cells, including activated monocytes, fibroblasts, endothelial cells, and keratinocytes. IP-10 has been shown to display anti-tumor and anti-metastatic properties by immunological [11] as well as anti-angiogenic mechanisms [12,13] when applied (i) by genetically engineered tumor cells [11], (ii) as recombinant defective adenovirus [14], or (iii) as recombinant protein [12,14]. Its immunological properties appear to be based on the attraction of monocytes, T lymphocytes, in part neutrophils [15], and possibly NK cells [16,17], whereas its anti-angiogenic effect may rely on the suppression of both endothelial cell proliferation [18] and differentiation of endothelial cells into tubular capillary structures [19].

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In this study, we investigated the influence of a novel strategy consisting of a combined orally administered flk1-based DNA vaccine together with an immunomodulatory IP-10 gene on B16-F10 tumor and angiogenesis.

2. Materials and methods

2.1. Plasmids

Construction of the expression vector encoding flk1: DNA fragment encoding flk1 was obtained using reverse transcriptase-PCR with the primers 5′-CCGGTACCATGGAGAG CAAGGCGCTG-3′ and 5′-CCTCTAGACAGCAGCACCTCTCTC-3′. Such DNA fragment was inserted into the pcDNA3.1 vector (Invitrogen, San Diego, CA) between the restriction sites Kpnl and Xball generating pcDNA3.1-flk1, as described previously [20]. Construction of the expression vector encoding IP-10: Total RNA was extracted from IFN-γ (1000 U/ml)-stimulated L929 cell line using Trizol (Invitrogen, Carlsbad, CA). The oligonucleotides used for RT-PCR were as follows: IP-10 forward (5′-CGGAATTCATCAGCACCATGAACCCAAGT-3′), and backward (5′-GCGTGGCTT CTCTCCAGTT-3′), and GAPDH forward (5′-CTGCACCACCAACTGCTTAG-3′) and backward (5′-GTCTGGGARGGAAARRGRGA-3′). IP-10 cDNA was amplified with RT-PCR and subsequently subcloned into the EcoRI and XhoI sites of the ampicillin-selectable mammalian expression vector pcDNA3.1. Plasmid DNA was purified in the absence of ethidium bromide or penicillin derivatives by using a commercially available column chromatography method according to the manufacturer's protocol (Qiagen, Chatsworth, CA).

2.2. Mice, cells and bacterial strains

C57BL/6 (denoted B6, H-2b) female mice used in these studies were obtained from the pathogen-free animal facility of the Tongji Medical College, Huangzhong University of Science & Technology. Mice were used at the age of 8–10 weeks. H5V endothelial cell line (H-2b), which expresses both flk1 and MHC class I, was provided by Dr A. Vecchi (Istituto Mario Negri, Milan, Italy) in Ref. [21]. B16-F10 cell line (a C57BL/6 melanoma cell line) was obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cell culture media consisted of DMEM (Invitrogen, San Diego, CA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS). Attenuated *S. typhimurium* Aro/A (strain SL 7207) was kindly provided by Dr B.A. Stocker (Stanford, California, USA).

2.3. DNA delivery

The attenuated S. *typhimurium* strain SL7207 was used for vaccination experiments. A single colony was grown in LB medium since mid-log phase was reached. After the cells were washed twice with ice-cold water, 1×10^8 bacterial cells were admixed with 0.5 μg of plasmid DNA vaccine and placed on ice in a 0.2 cm-cuvette (Eppendorf, Germany). Electroporation was performed in the Eppendorf Multiporator at 2 kV, 25 μF and 200 Ω for 0.5 s. Cells were immediately covered with 600 μl SOC medium (Invitrogen, USA) and shaked for 45 min at 37 °C. A sample of 100 μl was plated onto LB plates containing 50 μg/ml ampicillin (Sigma, Germany). Resistant colonies harboring the DNA flk1 vaccine were cultured and stored after confirmation of the coding sequence.

2.4. Combined therapy with oral immunization and IP-10 gene

To establish s.c. tumors, 1×10^5 B16-F10 in 200 μl of PBS were injected into the right flank of mice. Mice were divided into four groups ($n = 10$ each). Mice receiving combined therapy were inoculated s.c. tumor on day 0, and then were immunized by oral gavage 3 times on days 1, 8 and 15 with 0.1 ml sodium bicarbonate ($NaHCO_3$) buffer containing 1×10^8 *S. typhimurium* transformed with pcDNA3.1-flk1. The pcDNA3.1-mIP-10 plasmid was given three treatments on 3, 7 and 11 days. For intratumoral injections of naked DNA, DNA (20 μg) was diluted in 100 μl of sterile PBS, left at 25 °C for 15 min, and then injected into the tumor center via insulin syringes. Mice receiving single agent therapy received either oral immunization flk1-based vaccine only (three times on days 1, 8 and 15) or the therapeutic gene-IP-10 plasmid DNA only (three treatments on 3, 7 and 11 days). Mice from the $NaHCO_3$ group received oral $NaHCO_3$ and served as the control group. Tumor volumes were calculated using Vernier calipers at 5-day intervals after tumor cells inoculation according to the formula: $d1 \times (d2)^2 \times 0.5$ (d1 = largest diameter, d2 = perpendicular diameter). Mice were monitored daily for survival. They were sacrificed when any single or combined tumor linear measurement exceeded 20 mm.

2.5. Cytotoxicity assay

Spleens were harvested from three mice/experimental groups ($NaHCO_3$ control, flk1, IP-10 and flk1 + IP-10) at 20 day after tumor-cell inoculation. Splenocytes were separated from red blood cell (RBC) using a Ficoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and cocultured with irradiated (5000 rad) B16-F10 cells or $flk1^+$ H5V endothelial cells. Five days later, the activated CTLs were harvested and tested for activity against ^{51}Cr-labeled (500 μCi) H5V or B16-F10 cells. The assay was conducted in a volume of 50 μ1 using a 250-μ1 V-bottom 96-well plate at effector: target (E: T) ratios of 100:1, 50:1, 25:1, and 12.5:1 for 4 h. The percentage of ^{51}Cr release was calculated according to the following formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

2.6. Quantification of tumor vessel counts, PCNA, and TUNEL

B16-F10 tumors in the right flanks of mice were treated with flk1-based DNA vaccine or mIP-10 in a manner identical with that described above. The tumors were removed 20 days after tumor-cell inoculation. Staining of tumor tissue for mouse vessels was performed by incubating sections with anti-CD31 antibody, overnight, at 4 °C. Sections were then incubated with biotin-labeled goat anti-rat IgG for 30 min at 37 °C and were developed with diaminobezidin and counterstained with hematoxylin. Vessel density was captured using Image software and quantified by analyzing 10 random fields per section. TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed using an *in situ* cell-death detection kit (Roche, Mannheim, Germany). Section fluorescent images of immunostained tissue were viewed and digitized using a Canon (Wuhan, Hubei, China) camera. To quantify proliferating cell nuclear antigen (PCNA) expression, the number of positive cells was counted in 5 random fields.

2.7. Statistical analysis

All data in this study was analyzed using SPSS version 10.0 software (SPSS, Chicago, USA). The statistical significance of differential findings between experimental groups and controls was determined by a student's *t* test. $P < 0.05$ was considered as statistically significant.