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Anti-tumor efficacy and pre-clinical immunogenicity of IFNα2a-NGR

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ABSTRACT

Previously studies have shown that tumor-homing peptide NGR enhances the therapeutic efficacy of human interferon α2a (IFNα2a) against tumors. Here we investigated *in vivo* anti-tumor effect of recombinant human IFNα2a-NGR (rhIFNα2a-NGR) against human lung adenocarcinoma cell line SPC-A-1, A549 and murine Lewis lung carcinoma (LLC) subcutaneously xenografted tumors and further assessed the immunogenicity of rhIFNα2a-NGR in Sprague Dawley (SD) rats and rhesus monkeys. We found that rhIFNα2a-NGR significantly inhibited the growth of SPC-A-1, A549 and LLC cells-xenografted tumors in a dose-dependent manner. Although the antibodies to rhIFNα were detected in the serum of SD rats and rhesus monkeys treated with rhIFNα2a-NGR, these antibodies did not cause obvious pathological consequence. Taken together, these data demonstrate that rhIFNα2a-NGR has obvious anti-tumor efficacy *in vivo*, perhaps due to the tumor-homing peptide NGR. Thus rhIFNα2a-NGR represents a promising novel drug for effective treatment of cancer.

1. Introduction

IFNs, a family of multifunctional regulatory cytokines, demonstrate antiviral, antiproliferative and immunomodulatory effects (Pestka et al., 1987). IFNα has proven to be effective against hematological malignancies including hairy cell leukemia, chronic myelogenous leukemia and cutaneous T-cell lymphoma (Sliver et al., 1999; Gutterman, 1994). In addition, IFNα has been shown to have potent anti-angiogenic effects against solid tumors (Wadler, 1992; Motzer et al., 2000). Unfortunately, significant toxicity, low stability, short half-life and antigenicity of IFNα2a have hampered its wide clinical application (Caraceni et al., 1998; Yamada et al., 1996).

Administration of an exogenous protein to animals or humans has the potential to elicit an antibody response. Immunogenicity is an unique property that distinguishes biological therapeutics from traditional small-molecule drugs. An immune response to a biological drug can occur in nonclinical animal species or in clinical trial subjects and patients (Schellekens, 2000). In general, an immune response to human or humanized proteins is expected to be greater in animals than in humans due to species differences in protein structure. As a result, animal models tend to have low predictive value and often over-estimate biopharmaceutical immunogenicity and the incidence of adverse immune-mediated events in the human subjects (Wierda et al., 2001; Bugelski and Treacy, 2004). Nevertheless, immunogenicity data derived from nonclinical studies have important utility. Specifically, data from anti-drug antibody evaluations in animal studies are crucial for their adequate interpretation, especially when alterations in drug pharmacokinetic or pharmacodynamic parameters are observed (Shankar et al., 2006). Such data may reveal a potential safety hazard, particularly associated with neutralization of a critical endogenous protein.

Several tumor-homing peptides have been found *in vivo*, such as NGR (Asn–Gly–Arg), RGD (Arg–Gly–Asp) and GSL (Gly–Ser–Leu) (Arap et al., 1998). Coupling anti-cancer drugs or peptides to RGD and NGR peptides yields compounds with increased efficacy against tumors and reduced toxicity to normal tissues in mice (Sacchi et al., 2004; Hölig et al., 2004; Wang et al., 2006). Aminopeptidase N is the receptor for NGR peptides in tumors (Shipp and Look, 1993). NGR peptides bind specifically to an aminopeptidase isoform expressed in tumor vessels but not to other isoforms expressed in normal epithelia or myeloid cells (Curnis et al., 2002). To increase the anti-tumor activity of IFNα2a and reduce

Abbreviations: BAB, binding antibodies; CPE, cytopathogenic effect; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Ig G, Immunoglobulin G; Ig M, Immunoglobulin M; LLC, murine Lewis lung carcinoma; NAB, neutralizing antibodies; OD, optical density; rhIFNα2a-NGR, recombinant human interferon-α2a-NGR; VSV, vesicular stomatitis virus.

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the side-effects, we had coupled an NGR-containing peptide (GNCNGRCVSGCAGRC) to the C-terminal of IFNα2a (IFNα2a-NGR) (Meng et al., 2007). In this study, we investigated the anti-tumor activity of IFNa2a-NGR and compared it with that of IFNα2a at same dosage *in vivo*. In addition, we evaluated the immunogenicity of IFNα2a-NGR in SD rats and rhesus monkeys.

2. Materials and methods

2.1. Test article

Recombinant IFNα2a-NGR was produced with high quality and purity (>98% chromatographically pure) as described previously (Meng et al., 2007), meeting regulatory requirements for pre-clinical studies. It was formulated with mannitol, sterilized and lyophilized.

2.2. Cell culture

The human lung adenocarcinoma cell lines SPC-A-1, A549 and mouse lung carcinoma cell line Lewis were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 (GIBCO Co., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Expression and purification of GST-NGR fusion protein

The oligonucleotides encoding NGR (GNCNGRCVSGCAGRC) were synthesized with the following sequences: S1: 5′-GA TCC TGC AAC GGT CGT TGC GTG AGC GGT TGC GCG GGT CGT TGC TAG C-3′ and S2: 5′-G ACG TTG CCA GCA ACG CAC TCG CCA ACG CGC CCA GCA ACG ATC GAG CT-3′ and cloned into pGEX-4T-1 plasmid using *Bam*HI and *Xho*I sites. The recombinant plasmid was named pGEX-NGR. pGEX-NGR was transformed into BL21 cells and the induction of GST-NGR fusion protein was performed as described previously (Meng et al., 2007). GST-NGR fusion protein was purified using nickel affinity column (Qiagen) following the manufacture's protocol.

2.4. Animals

Female athymic nude mice (BALB/cA nu/nu) aged 4–5 weeks were purchased from the Shanghai Institute of Materia Medica (Shanghai, China), housed in sterile cages under laminar airflow hoods in a specific pathogen-free room with a 12 h light and 12 h dark schedule and fed autoclaved chow and water. BALB/c mice 8–12 weeks old and weighed 18–22 g were purchased from Lanzhou University (Lanzhou, China). SD rats 6 weeks old and weighed between 180 and 220 g were purchased from Shanghai SLAC laboratory Animal Co., Ltd. (Shanghai, China). Rhesus monkeys, *Macaca Speciosa Thibetanas Milne-Edwards*, 3–5 years old and weighed 4–6 kg were purchased from Medical Science Experimental Animal Institute of Sichuan (Chengdu, China). The animals were maintained in an air-conditioned barrier-system animal room with an ambient temperature of 25 ± 2 °C, a relative humidity of 50 ± 10%, and a 12-h on/off light cycle. The animals were quarantined and acclimatized for 8 days (mouse and rat) or for 2 months (monkey) and were allocated to the treatment groups. The study protocols were in accordance with the regulations of Good Laboratory Practice for Non-clinical Laboratory Studies of drug issued by the National Scientific and Technologic Committee of People's Republic of China. All experiments were done according to institutional ethical guidelines on animal care.

2.5. Production of murine monoclonal antibodies for NGR

BALB/c mice were injected with GST-NGR (0.06 mg/each mouse) emulsified in Freund's complete adjuvant (FCA) subcutaneously. Following a 1 month rest period, the animals were boosted three times per week for three consecutive weeks. Three days later, spleenocytes were fused with the SP2/0 myeloma cell line. Three highly reactive and specific mAbs (two IgG mAb, and one IgM mAb) were generated and one of them named as 6D6 had highest titer.

2.6. Tumor implantation and treatment

A total of 1×10^6 A549, SPC-A-1 or LLC cells were inoculated in 0.1 mL of serum-free medium s.c. in the right flank of 4–6-week-old healthy BALB/c nu/nu mice. When the tumors reached an average diameter of about 4 mm, the mice bearing too large or too small tumors were eliminated and the left were divided into seven treatment groups (7–10 animals per group): 0.9% sodium chloride only; 1×10^6 IU/kg IFNα2a; 3×10^6 IU/kg IFNα2a; 9×10^6 IU/kg IFNα2a; 1×10^6 IU/kg IFNα2a-NGR; 3×10^6 IU/kg IFNα2a-NGR; and 9×10^6 IU/kg IFNα2a-NGR. All drugs, diluted with 0.9% sodium chloride, were administered i.p. once a day. Tumor size in all groups was measured using a vernier calipers on alternate days, and tumor volume was calculated using a standard formula: tumor volume (mm^3) = length (mm) × width $(mm)^2 \times 0.52$. The animals bearing i.p. growing tumors were sacrificed when the control treatment groups were moribund and tumors were excised and weighed. The inhibition rates of the growth of A549, SPC-A-1 or LLC xenografted tumors were calculated according to the formula: inhibition rate (%) = (1 − mean of tumor weight in test groups/mean of tumor weight in control groups) × 100.

2.7. BAB to rhIFNα assay

Serum levels of anti-rhIFNα antibody were determined by ELISA following the manufacturer's instructions. The positive well was defined as the dilution when the positive OD_{450nm} was 2.1 times that of negative control (P/N ⩾ 2.1).

2.8. NAB to rhIFNα assay

The antiviral activity of IFNα2a-NGR was evaluated by WISH/VSV CPE assay as previously described (Meng et al., 2007). OD_{570nm} value was measured with ELISA plate reader. The result was judged as follows: (1) NAB was defined as positive if OD_{570nm} value of sera from animals in treatment group/OD_{570nm} value in normal cell control was no more than 1/2; (2) NBA was defined as weak positive if OD_{570nm} value of sera from animals in treatment group/OD_{570nm} value in normal cell control was no less than 1/2, and the difference was statistically significant ($P < 0.05$), and (3) NBA was defined as negative if OD_{570nm} value of serum samples did not meet the above two conditions.

2.9. Antibodies to NGR assay

Serum levels of anti-NGR IgG and IgM were determined by the ELISA according to standard procedures. The positive criteria of anti-NGR IgG and IgM were as follows: (1) OD_{450nm} value of the re-test samples was mainly determined by the cut-off value P/N ⩾ 2.1, and 2) the re-test samples were confirmed using an ELISA neutralization bioassay. The re-test samples of anti-NGR IgG and IgM were defined as positive if the ELISA inhibition was >50%. The percent of inhibition of binding was calculated by the following equation: percent of inhibition = [1 − (OD_{450nm} value in neutral