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Isolation and characterization of human anti-$VEGF_{165}$ monoclonal antibody with anti-tumor efficacy from transgenic mice expressing human immunoglobulin loci ☆

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ABSTRACT

The purpose of this study was to prepare a fully human anti-VEGF (vascular endothelial growth factor) monoclonal antibody with anti-tumor activity from five-feature mice which express human immunoglobin loci. Four hybridomas secreting mAb stably were isolated successfully. Some characters such as isotypes, cross-reactivity, inhibition on the binding of hVEGF to VEGFR-2, dissociation constants and the idiotypic characteristic were determined. Proliferation of T24 and Ls-174-T cell line and nude mice bearing human colorectal cancer were used to evaluate therapeutic effects and safety of this mAb. Pharmacokinetics data shows the half life of this mAb was about 5 days after a single intravenous injection. These results suggest the fully human anti-VEGF mAb maybe safe and efficient for cancer treatment.

1. Introduction

The development of new blood vessels (angiogenesis) is important in the pathogenesis of many disorders, particularly rapid growth and metastasis of solid tumors [1,2]. Vascular endothelial growth factor (VEGF) is an endothelial cell specific mitogen and an angiogenesis inducer released by a variety of tumor cells and expressed in human tumors in situ. K. Jin Kim demonstrated that inhibition of the action of VEGF (mAb specific for VEGF was used) spontaneously produced by tumor cells may suppress tumor growth *in vivo* [3].

Anti-angiogenesis therapy is unique in targeting tumor vasculature, but not tumor cells themselves, and therefore it is broadly applicable for most solid tumors. However, the immunogenicity of rodent antibodies in humans prevents their application in clinical procedures. To overcome this problem, the humanized and fully human antibodies were constructed. Fully human antibodies could be produced by technologies such as phage display libraries and transgenic mice expressing human Ig genes. It is known that Bevacizumab is a successful humanized anti-VEGF mAb, approved by FDA in 2004, which has been most extensively investigated in a variety of tumors, including non-small cell lung, breast, prostate, renal and colorectal cancers. Bevacizumab in combination with bolus IFL (irinotecan, 5-fluorouracil (5-Fu), leucovorin (LV)) become the first-line therapy of metastatic colorectal cancer [4].

In this study, we presented a successful isolation of fully human anti-VEGF IgM mAb from transgenic humanized mice, i.e., five-feature mice, which were produced by Babraham Institute, Cambridge, England [5]. We immunized several five-feature mice with the recombinant human VEGF expressed by *Pichia pastoris*. Four of nine specific mAb were isolated and analyzed. The data presented here suggested that anti-VEGF human IgM mAb can be produced in five-feature mice, enabling the generation of a panel of antibodies with therapeutic potential.

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2. Materials and methods

2.1. Translocus mouse strains and immunogens

The translocus five-feature mouse strains, which carry human IgM + Igκ, λ transloci [5], was provided by Babraham Institute (Cambridge). The recombinant human vascular endothelial growth factor ($hVEGF_{165}$) was expressed in *P. pastoris* and purified as previously described [6].

2.2. Immunizations and fusions

Groups of 6- to 8-week-old mice were immunized subcutaneously with 100 μg rVEGF165 expressed in *P. pastoris*. In the primary boost, rVEGF165 was emulsified with Complete Freund's Adjuvant (CFA) (Sigma). Each mouse was boosted four times at 2-week intervals with the protein in Incomplete Freund's Adjuvant (IFA) (Sigma). Animals with an anti-$hVEGF_{165}$ serum response were sacrificed, and their splenocytes were fused with SP2/O myeloma cells in the presence of 50% PEG1500, HAT (Sigma), and HFCS (Hybridoma fusion and Cloning Supplement, Sigma) using standard techniques [7].

2.3. mAbs purification

Stable, positive clones secreting hVEGF-specific IgM mAbs were identified by screening supernatants from hypoxanthine–aminopterin–thymidine (HAT)-selected hybridomas by ELISA on microtiter plates coated with 1 μg of $hVEGF_{165}$ per ml and developed with horseradish peroxidase (HRP)-labeled goat anti-human IgM (μ chain, Sigma). Stable, positive clones were selected by subcloning twice by limiting dilution.

Human $VEGF_{165}$ mAb containing ascitic fluid was prepared by injecting hybridoma cells into the peritoneal cavity of pristine (Sigma–Aldrich Co.)-primed Balb/C mice. mAb concentrations in ascetic fluid were determined by ELISA. Ascitic fluid was collected and centrifuged to remove the oil. At a temperature of 4 °C, MBP–Sepharose 2B (5 ml) was packed into a chromatographic column. Binding and washing steps are performed at 4 °C in 10 mM Tris–HCl (pH 7.4) buffer containing sodium chloride and 20 mM calcium chloride. Elution is made at room temperature in a similar Tris buffer, except that it contains EDTA and is devoid of calcium chloride. The IgM fractions were pooled, and subjected to ultrafiltration against PBS buffer and stored at −20 °C.

2.4. Human anti-VEGF mAbs characterization by SDS–PAGE and Western blotting

To further access the reactivity of antibodies to purified $hVEGF_{165}$, an immunoblotting analysis was performed. Briefly, 20 μg per slot of purified $hVEGF_{165}$ were applied to a 7.5% SDS–polyacrylamide gel. After electrophoresis, the separated proteins were transferred to PVDF membrane (Millipore) at 100 V for 18 h at 4 °C. The membrane was blocked with 3% BSA for 2 h and developed with HRP-labeled anti-human μ chain antibody (Sigma) diluted 1:10,000. After washing, the substrate 3,3-diaminobenzidine tetrahydrochloride plus hydrogen peroxide was added and the reaction was stopped by distilled water addition.

2.5. Isotypes and cross-reactivity determination

To identify potential murine-human hybrid mAbs and determine the species-specific heavy and light-chain isotypes of all mAbs isolated, two ELISAs were performed. In one ELISA, each mAb was added to microtiter plates (Costar, Corning) coated with 1 μg of recombinant human $VEGF_{165}$ per ml. Each well was then developed with HRP-labeled goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA (Southern Biotechnology Associates) or HRP-labeled anti-human IgM (μ chain, Sigma). In the second ELISA, the HRP-conjugated mouse antibodies specific for human κ, λ chain, and murine λ chain (Sigma) were used to determine the light-chain isotypes.

The specificity of these mAbs was confirmed by ELISA, using as controls several unrelated antigens such as human epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and human serum albumin.

2.6. The idiotypic characteristic

Five-feature male mice, 6-weeks-old, were obtained from Shanghai transgenic center. The mice were normally bred and maintained under specific-pathogen-free conditions, with a constant temperature ranging between 25 and 27 °C, and a constant humidity ranging between 45% and 50%. The mice were injected the MAb V75 3 times at 4 days intervals. At the end of experiment, serum after each injection was got to test its idiotypic characteristic by ELISA.

Serum (100 μL) was mixed with 20 μL V75 (500 μg/ml) at 37 °C for 2 h until the equilibrium was reached. The mixture was added to microtiter plates (Costar, Corning) coated with 1 μg of recombinant human $VEGF_{165}$ per ml. Each well was then developed with HRP-labeled anti-human IgM (μ chain, Sigma). Twenty microliters of V75 (500 μg/ml) diluted with 100 μL blank serum was determined as control. Absorbance at 490 nm was observed.

2.7. Inhibition of mAbs on the binding of hVEGF to VEGFR-2

The inhibition effect of mAbs on $hVEGF_{165}$ binding to VEGFR-2 was observed by ELISA. Microtiter plates were coated with VEGFR-2 (Sigma, diluted 10,000 times) and developed with horseradish peroxidase (HRP)-labeled human mAbs (were made as described [8]) and 1 nM $hVEGF_{165}$ mixture. Labeled human IgM was determined as control. Absorbance at 490 nm was observed.

2.8. Affinity of mAbs

The dissociation constants (K_d) of mAbs were determined by their affinity to $hVEGF_{165}$ as described by Friguet et al. [9]. mAbs were incubated in solution with different antigen concentration until the equilibrium was reached, then a classical indirect ELISA measured the proportion of mAbs that remained unsaturated at equilibrium. The K_d