ISSN 1022-7954, Russian Journal of Genetics, 2008, Vol. 44, No. 8, pp. 890–894. © Pleiades Publishing, Inc., 2008.
Original Russian Text © O.L. Polanovsky, S.V. Lukash, O.A. Stremovskiy, D.V. Karpenko, S.M. Deyev, 2008, published in Genetika, 2008, Vol. 44, No. 8, pp. 1023–1028.

MOLECULAR GENETICS

Expression of Anti-Tumor Recombinant IgG- and IgE-Like Genes in Eukaryotic Cells

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Received September 6, 2007

Abstract—The tandem of humanized variable VL and VH genes (ScFv fragment 4D5) possessing a high affinity to the HER-2/neu oncogene (the epidermal growth factor receptor expressed in many types of human tumors) was attached through a flexible linker to the second exon of human antibodies of IgG or IgE isotypes constant gene. The humanized construct of IgE isotype was generated for the first time. Genes of the recombinant antibodies were cloned into the pCl-neo vector under the control of universal cytomegalovirus (CMV) promoter. Transfected HEK-293 cells efficiently produced antibodies of the corresponding isotypes IgE and IgG1. The results of Western blotting confirmed homogeneity of the expressed antibodies, which had the predicted molecular weight and specifically interacted with the HER-2/neu. The attachment of leader peptide to the 5'-end of the gene resulted in the preferential accumulation of recombinant antibodies in the cultural medium. These results indicate that de novo constructed humanized immunoglobulin genes express functionally active, single-chain recombinant antibodies in eukaryotic cells.

DOI: 10.1134/S1022795408080024

INTRODUCTION

Advanced technologies for obtaining chimerical and "humanized" antibodies, which are widely adopted in clinical practice for in situ diagnostics and therapy, have been actively developed in the past years [1–3]. These approaches are underlain by antibody recognition of the markers overexpressed in cancer cells. To be clinically applicable, these antibodies must meet several requirements, including high affinity to the target, low immunogenicity and high selectivity of binding. Thus, the strategy for design and construction of monoclonal humanized antibodies, selectively targeting cancer cell markers, has emerged [4–7].

The fundamentally different approach is the design and use of vector systems carrying constructs for in situ expression of full-length antibodies intended for target cells recognition. The HER-2/neu cell surface oncogene inducing the uncontrolled cell proliferation is overexpressed by many carcinomas. Humanized anti-HER-2/neu antibodies are used to treat patients with metastatic breast cancer [8].

In this study, we developed recombinant genes expressing full-length single-chain humanized antibodies of IgE and IgG1 isotypes capable of cancer cell HER-2/neu receptor selective recognition. It is noteworthy that IgE isotype in some cases is more effective than IgG for the treatment of cancer [9]. Previously, we have analyzed the expression of the chimeric IgE gene in different mouse tissues using ballistic transfection [10]. The Humanized single-chain tandem VL-VH (ScFv fragment 4D5) [11, 12] capable of HER-2/neu receptor recognition was used to generate the expression constructs. The tandem was linked to human IgE or IgG constant genes through the linker peptide-encoding oligonucleotide. The primary objectives of the study were to estimate the efficiency of expression of recombinant genes, to identify the antibodies produced and to detect an interaction between these antibodies and antigen.

MATERIALS AND METHODS

DNA amplification primers and templates. Two-step overlap extension PCR was used for generating the full-length humanized genes of IgE and IgG isotypes. The following primers were used: 1) forward, 5'-taagaatgcggccgcgccatggatatcgttatgacccagtctc-3'; 2) reverse, 5'-cctggagcagacgccaccactgctcccggg-3'; 3) forward, 5'-gcagtggtggcgtctgctccagggacttca-3'; 4) reverse, 5'-caggtcgcggcgctcagtgatggtgatggtggtgtttaccgggattta-cagacacc-3'; 5) reverse, 5'-gtcaggagatttgggctcggaagaaacggtaacggtggta-3'; 6) forward, 5'-cgtttcttccgagcccaaatctcctgacaaaactcacacatgccc-3'; 7) reverse, 5'-caggtgcggccgctcagtgatggtgatggtggtgtttacccggagacagggagagg-3'.

The plasmids 4D5 scFv-barnase-His_5 encoding for the mini-antibody 4D5 [2], pUC.CMV.V_HC_E containing the entire constant region (including introns) of IgE [13] and pUHWγ1 [14] carrying the human IgG1 constant region genes were used as PCR templates.

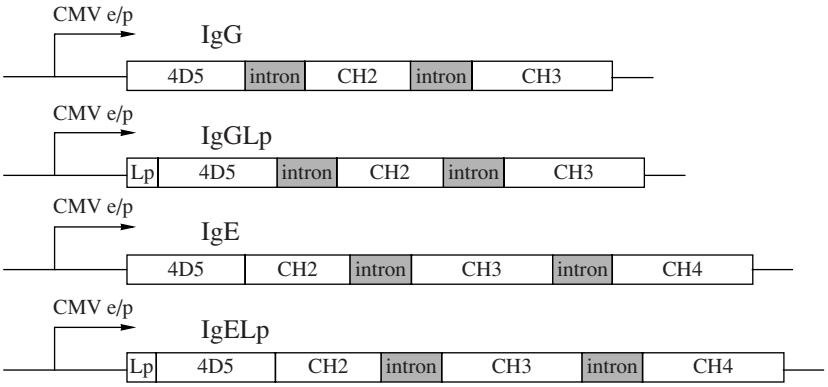

Fig. 1. Full-length genes encoding the recombinant IgG1 and IgE-like antibodies. Abbreviations: Lp, leader peptide of the light kappa chain; 4D5, heavy and light chain antigen-binding variable domains with the C-terminal hinge-peptide (ScFv fragments 4D5); CH, human heavy chain constant domains of IgE and IgG1 isotypes.

The DNA fragment corresponding to the leader peptide of mouse kappa chain VL domain was attached to the 5'-end of the recombinant constructs. An oligonucleotide encoding for the His_6 peptide was linked to the 3' ends of IgE and IgG1 recombinant genes.

All the constructs obtained were verified using standard dideoxy sequencing method.

Construction of plasmids. All DNA manipulations were performed as described [16]. The genes of recombinant antibodies were cloned into *Not*I site of the pCl-neo shuttle vector (Promega) under the control of the CMV promoter/enhancer driving gene expression in eukaryotic cells.

Transfection of cells. The expression of genes of recombinant antibodies was studied in HEK-293 cells transfected with recombinant plasmids carrying the cloned genes in the presence of Unifectin-R56 using the standard protocol (http://www.rusbiolink.com). Transfected cells were cultured in 90-mm Petri dishes for 60 hours at 37°C in a CO_2 incubator in DMEM supplemented with 10% FCS (Gibco BRL).

Gel electrophoresis and Western blot analysis. Proteins were analyzed in 12% SDS–PAGE according to the standard protocol [16]. Protein transfer from gel to membrane (Millipore) was performed according to manufacturer's protocol. One membrane was stained with anti-His alkaline phosphatase-conjugated Abs [17], while the other one was visualized with an anti-4D5 rabbit antibody obtained in our laboratory.

Immunoreactivity of recombinant antibodies. The efficiency of recombinant single-chain antibodies production by the transfected HEK-293 cells was determined by ELISA using plastic-immobilized antibodies against human IgG or IgE and horseradish peroxidase-conjugated goat antibodies against human antibodies of the corresponding isotype. The interaction with HER-2/neu antigen was also detected by ELISA. In this case the HER-2/neu antigen was adsorbed on plastic as a primary layer.

RESULTS

Construction of Single-Chain Antibody Genes

Constructs encoding recombinant antibodies of two isotypes IgE and IgG1 (Fig. 1) were generated using the templates and corresponding primers (see Materials and Methods) for PCR. In both cases, VL and VH exons (scFv fragment 4D5) [11] connected through a linker were utilized. These exons encode for mini-antibodies against the HER-2/neu oncogene expressed at a high level on the surface of many cancer cells, including breast and ovarian cancer. To construct the single-chain humanized IgE gene the 3'-end of variable genes tandem was attached to the CH2 exon through the primer, comprising the 22-mer hinge-like GTTVTVSSEFPKPSTPPGSSGG peptide-encoding DNA fragment.

Same fragment, by means of PCR amplification linked to the CH2 exon through the primer, encoding the 23-mer GTTVTVSSEPKSPDKTHTCPPCP linker, was also exploited to generate the humanized single-chain IgG1 gene. In this construct, Cys226 and Cys229 forming inter-heavy chain disulfide bonds were retained, while Cys220 capable of forming disulfide bond with Cys214 residue of C-kappa chain was replaced by a proline residue.

In both single-chain constructs (Fig. 1), the original intron–exon structure was retained unchanged, thus enabling the splicing and, in the end, synthesis of functionally active single-chain antibodies in eukaryotic cells.

A fragment encoding the leader peptide required for the secretion of synthesized proteins into the culture medium was attached to the 5'-end of the single-chain antibody VL-genes.

The recombinant genes of single-chain antibodies of both isotypes were cloned into *Not*I site of the pCl-neo shuttle vector under the control of the CMV promoter/enhancer. Constructs pCI.IgE.4D5 and pCI.IgG.4D5 (see Fig. 1) were used for transient

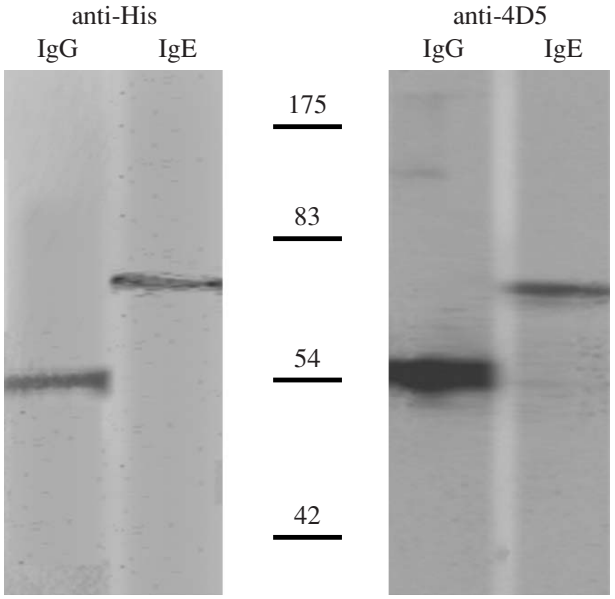

Fig. 2. Detection of the expressed proteins by immunoblotting. The culture media from cells transfected with pCI.IgG.4D5 (IgG) and pCI. IgE.4D5 (IgE) plasmids were enriched on Ni-NTA Sepharose, subjected to SDS–PAGE and probed with the recombinant anti-His-tag antibodies or rabbit anti-4D5 antibodies. Positions of molecular weight markers are indicated at the middle lane.

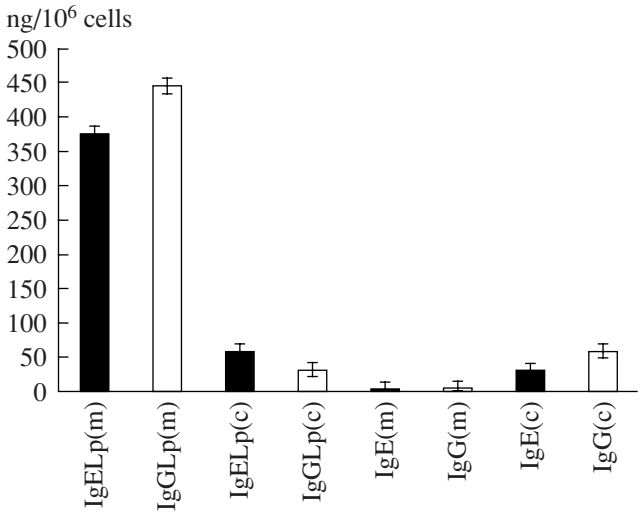

Fig. 3. Leader peptide-mediated secretion of the recombinant antibodies into a cell-free medium. Accumulation of the antibodies with (Lp) or without leader peptide in a culture medium (m) and cells (c). IgE and IgG constructs are shown as dark and light rectangles, respectively. The amount of protein expressed was determined in 60 hours after the transfection and recalculated per 10^6 cells.

expression experiments as well as for generation of CHO cell lines constitutively expressing target genes.

The Humanized Antibodies Expression in Eukaryotic Cells

Expression of recombinant antibody genes was studied in HEK-293 cells transfected with recombinant plasmids carrying the cloned genes in the presence of Unifectin-R56 using the standard protocol (http://www.rusbiolink.com). In the first stage, the of the study main parameters of the synthesized recombinant antibodies were determined. Toward this end, the expressed proteins were concentrated on Ni-NTA Sepharose, then the resulting eluates were subjected to denaturing gel electrophoresis with subsequent Western blotting. Proteins of interest were detected using alkaline phosphatase-conjugated anti-His-tag antibodies [16] as well as with rabbit antibodies against the variable domains.

This analysis revealed homogeneity of proteins produced by transfected cells (Fig. 2) and confirmed the presence of variable domains and oligohistidine tags at the N- and C-termini of antibody polypeptide chains, respectively.

The molecular weight of the synthesized IgG and IgE isotypes, as determined by comparison with the mobility of markers (Fig. 2), was in good agreement with the expected sizes of full-length single-chain antibodies (54 and 65.5 kDa respectively). No other products of greater or smaller molecular mass were detected, thus confirming the high selectivity of expression. The results of the SDS-PAGE indicated that under non-reducing conditions single-chain IgG1 formed dimers.

Antibody Production Level and Antibody Secretion into the medium. The level of antibody production strongly depended on presence of the N-terminal leader peptide (Fig. 3). The peptide provided the level of recombinant antibodies of IgE and IgG isotypes up to 450 and 380 ng in the medium and up to 30 and 55 ng in cells, respectively (per 10^6 cells). In absence of the leader peptide, the antibodies had accumulated in cells at a level of about 40 ng per 10^6 cells; no antibodies were detected in the medium.

Interactions between Recombinant Antibodies and HER-2/neu

The final stage of the study was to evaluate the functional activity of recombinant antibodies, i.e., their ability to bind the HER-2/neu antigen. In this case, the antigen was adsorbed on plastic, then the studied antibodies were applied and the efficiency in their binding to the antigen was determined (Fig. 4).

DISCUSSION

During the past few years, new approaches for recombinant antibody design have been developed, with particular emphasis on enhancement of their affinity and stability, as well as on the delay of antibody excretion from the organism. Earlier we have reported

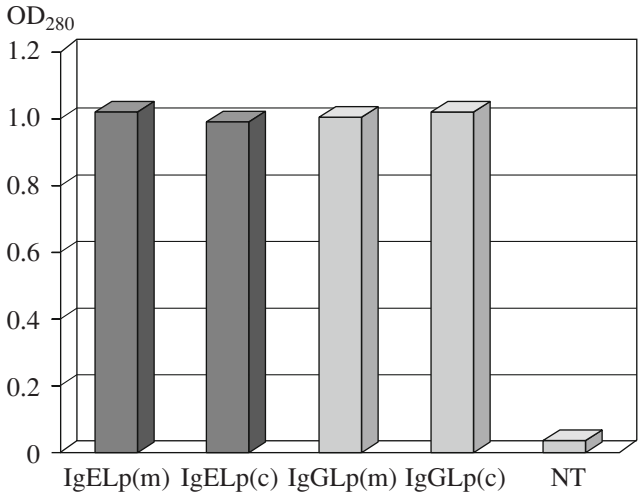

Fig. 4. Binding of the HER-2/neu antigen by the recombinant IgE and IgG antibodies. Interaction is shown for a cell free medium (m) and for the cellular proteins (c). Recombinant IgE and IgG antibodies are shown as dark and light rectangles, respectively. NT, binding of the antigen by an extract of nontransfected cells.

the production of genes encoding new, functionally active anticancer antibodies expressing in bacteria [2, 17, 18] and plant cells [19]. The fundamentally different approach is the design of genetic constructs capable for the expression of full-length recombinant antibodies intended for target in situ (in the organism) recognition. In this study we developed the genes expressing recombinant single-chain antibodies of IgE and IgG1 isotypes in eukaryotic cells.

Two vector DNA molecules are usually exploited to produce artificial antibodies: one possesses gene for the immunoglobulin light chain and the other that carries the heavy-chain gene. This approach, however, has drawbacks, caused by imbalance in the heavy and light chain biosynthesis. There is also the risk of one of the vector plasmids loss that may lead to the expression of one residual chain only. To avoid these complications, we constructed the vector molecule, possessing both full-length chimerical genes under the control of strong T7 RNA polymerase promoter [20, 21].

To finally resolve this problem in the present study, we constructed recombinant genes expressing both variable domains and a constant region of immunoglobulin heavy chain as a single polypeptide. An additional advantage of this construct is that there is no more need to analyze light and heave chains separately, as it occurs in case of the natural antibodies, expressed from different chromosomes.

It has been shown previously that the IgE antibodies are more effective than IgG ones in killing carcinoma cells [9]. It has been also demonstrated that IgE antibodies might be used for anti-tumor vaccine development [22, 23].

The data obtained in the present study indicate that the de novo constructed humanized genes of IgE and IgG1 isotypes express in eukaryotic cells full-length single-chain humanized antibodies, efficiently interacting with the cancer HER-2/neu antigen.

These results provide a basis for further investigation of the recombinant antibody functional properties, as well as for the animal tests in situ, that is an essential step of preclinical trials of anticancer drugs [24–27].

ACKNOWLEDGMENTS.

This study was supported by the Russian Foundation for Fundamental Research (grants nos. 05-04-48542a, 04-04-49024a, 06-04-49686a) and SNSF (project no. IB73AO-110842/1).

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