Cloning of 3H11 mAb variable region gene and expression of 3H11 human-mouse chimeric light chain

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Abstract

AIM To clone mouse anti-human gastric cancer mAb (3H11) variable genes and to construct 3H11 human-mouse chimeric antibody.

METHODS The entire VH and VL genes of anti-gastric cancer mAb 3H11 were cloned by RT-PCR method from 3H11 hybridoma cells, using 5’ primers for leader sequences. The 3H11 VL gene was then inserted into human-mouse chimeric light chain expression vector and transfected into murine Sp2/0 myeloma cells.

RESULTS DNA sequence analysis indicated that the cloned genes included the whole leader sequences and the mature Ig variable region encoding sequences. After gene transfection, transient expression of chimeric light chain protein was detected.

CONCLUSION DNA sequences and transient expression indicated that the cloned gene was functional. This work laid basis for constructing 3H11 human-mouse chimeric antibody in the future.

INTRODUCTION

The mouse mAb 3H11 was raised against human gastric cancer cells¹ ². But 3H11 mAbs were derived from mouse hybridoma and their inherent immunogenicity in patients precluded its long-term use. In an attempt to circumvent this problem, chimeric antibodies in which the antigen-specific variable (V) regions of the mouse antibodies were joined to the constant (C) regions of human antibodies. These molecules should be much less immunogenic and hence be more suitable for application. In order to obtain the correct VL and VH genes, we designed 5’ primers according to the leader sequences and cloned the entire VL and VH genes by RT-PCR method. The transient expression of chimeric light chain protein indicated that the cloned gene was functional in expression.

MATERIALS AND METHODS

Materials

Mouse 3H11 mAb hybridoma was provided by Biochemistry Department of Beijing Institute of Cancer Research. Chimeric light chain expression vector pAG4622 was a gift from S.L. Morrison (University of California, Los Angeles); γ-32P-ATP was from Beijing Ya Hui LtD. Fmol DNA sequencing system, pGEM-T vector system, Tag enzyme and restriction enzymes were purchased from Promega. Lopofectin was from Gibco BRL.

Design of PCR primers

The 5’ primers for amplification of mouse V genes were synthesized according to Coloma et al.⁴. An extra VH5’ primer was added according to newly published sequences data⁵ (VHL4 in Table 1). The 5’primers were designed to hybridize to partially conserved sequences in the leader regions of VH and VL. The 3’ primer for VL genes was located at V-C junction of VL. The 3’ primer for VH genes was located at the boundary of VH and CH1, which was effective for IgG, IgG2a, IgG2b and IgG3 subclass. Restriction sites were incorporated into the primers as shown in Table 1 (R = A/G S = C/G Y = C/T M = C/A K = T/G H = A/C/T D = G/T/A).

Cloning of VL and VH genes

Total RNA was isolated from 107 cells of 3H11 hybridoma cells using Trizol reagent from Gibco BRL. The VL and VH genes were amplified by RT-
PCR method, purified by agarose gel electrophoresis and cloned with pGEM-1 system.

**DNA sequencing**
The VL and VH genes were sequenced by dideoxy-mediated chain-termination method, using fmol DNA sequencing system (Promega).

Table 1 PCR primers for cloning variable genes of rearranged immunoglobulin light and heavy chains

| Primers for VL Signal peptide | Primers for VH signal peptide |
|-------------------------------|-------------------------------|
| LL1: GGGGATATCCACCATGGAGACAGACACTCCTGCTAT | VHL1: GGGGATATCCACCATGGAGACAGACACTCCTGCTAT |
| LL2: GGGGATATCCACCATGGATTTTCAAGTGCAGATTTTCAG | VHL2: GGGGATATCCACCATGGATTTTCAAGTGCAGATTTTCAG |
| LL3: GGGGATATCCACCATGGAGWACACAKWCTCAGGTCTTTRTA | VHL3: GGGGATATCCACCATGGAGWACACAKWCTCAGGTCTTTRTA |
| LL4: GGGGATATCCACCATGKCCCCWRCTCAGYTYCTKGT | VHL4: GGGGATATCCACCATGKCCCCWRCTCAGYTYCTKGT |
| LL5: GGGGATATCCACCATGAAGTTGCCTGTTAGGCTGTTG | VHL5: GGGGATATCCACCATGAAGTTGCCTGTTAGGCTGTTG |

**Construction of chimeric light chain expression vector**
The pGEM-T vector containing 3H11 VL gene was digested with EcoR V and SalI. The 3H11 VL gene was purified by electrophoresis and electroleution and cloned into expression vector pAG4622. Transfection of DNA into Sp2/0 cells was accomplished by two methods: Lipofectin procedure: 20 µg plasmid DNA was mixed with 40 µl lipofectin and left at room temperature for 15 minutes. The mixture was added into 1.6×10⁷ Sp2/0 cells in 2ml serum-free culture medium drop by drop. The cells were then cultured at 37°C in a CO₂ incubator for 20 hours and 2ml RPMI1640 containing 20% fetal bovin serum was added. After further 72 hours incubation, the supernatant was collected.

Electroporation[3]: 20 µg PvuII linearized plasmid DNA was added into 1.6×10⁷ Sp2/0 cells. After 10 minutes in ice bath, the cells were electroporated at 960µF, 2kV/cm by GENE PULSER (Bio-RAD). After another 10 minutes in ice bath, the cells were transferred into culture flask that contained 10 ml RPMI1640 containing 20% fetal bovin serum. The cells were cultured at 37°C in a CO₂ incubator for 72 hours and the supernatant was collected.

**Detection of human-mouse chimeric light chain**
The expression of chimeric light chain was determined by ELISA. Goat anti-human IgG Fab (Sigma) at 2.3 mg/L in 0.05M borate buffer, was absorbed overnight in a 96-well plate. Either a control or supernatant samples was added and incubated for 1h. This was followed by a secondary antibody coupled to horseradish peroxidase (HRP) (Sigma) and developed with OPD (0.5 g/L) (Sigma) and absorbance at 492nm. Between each addition, the plate was washed with phosphate-buffered saline with 0.05% Tween. The positive control was normal human blood serum, and the negative control was the supernatant of Sp2/0.

**RESULTS**

**Cloning of 3H11 VL and VH genes**
Total RNA was isolated from 3H11 cells and covered to cDNA. PCR amplification of VL and VH genes were done separately with sets of primer pairs. Agarose gelelectrophoresis showed that 3H11 VL gene was amplified with LL2 and VH gene with VHL1 (Figure 1)

**Sequencing of 3H11 VL and VH genes**
The purified VL and VH PCR products were cloned into pGEM-T vector and sequenced with fmol DNA sequencing system. Comparison of the sequences with published antibody variable region data[4] indicated that the 3H11 VL gene contained a leader sequence of 57 nucleotides encoding leader peptide of 19 amino acid residues, while the 3H11 VH gene had a 66 bases long leader sequences that encoded a 22 residues leader peptide (Figure 2). The sequences of the mature VL and VH regions were the same as what we cloned previously using primers for framework region[5].

**Construction of chimeric light chain expression vector**
The 3H11 VL fragments in pGEM-T were digested with EcoR V and SalI and ligated into pAG4622 vector (Figure 3). The resulting expression vector pAG4622-3H11 was verified by restriction enzyme analysis.
3H11 cloning and construction of 3H11 human-mouse chimeric antibody

Figure 2  The nucleotide and amino acid sequences of 3H11 mAb VL(a) and VH(b) genes. Underlined sequences are CDR regions. Numbering according to mature protein sequences.

Expression of chimeric 3H11 light chain

The expression vector pAG4622-3H11 was transfected into murine myeloma Sp2/0 cells by lipofectin or electroporation. Culture supernatant was harvested after the transfected cells were incubated at 37°C for 72 hours. Expression of chimeric 3H11 K chain was tested by ELISA. As shown in Table 2, human Ck was expressed in the culture supernatant of transfected cells indicated that the cloned gene was functional.

DISCUSSION

Monocloned antibodies (mAbs) are well-characterized highly specific reagents, and widely applied in vitro into immunochemical characterization and quantitation of antigens. They are being used clinically for both diagnosis and therapy increasingly. Their in vivo application is limited because most available mAbs are derived from mouse hybridomas, and their inherent immunogenicity in patients precludes their long-term administration. In an attempt to circumvent this problem, chimeric antibodies have been produced. Immunoglobulin V gene used to be
obtained by genomic library which was tedious and time-consuming. Recent developments in PCR technology have greatly facilitated the cloning of variable region genes\(^6\). Two approaches have been used to design the 5' primers. In one approach, degenerated primers for the 5'end are designed to recognize relatively conserved sequences within the 5' end of framework 1. Because the primers are within the coding sequences of the mature antibody and the introduction of restriction sites, amino acid substitutions that change the Ag-binding specificity or the affinity of the cloned antibody may result\(^7\).

An alternative approach takes advantage of the conservation of the leader sequences. Since the leader is not present in the mature antibody molecules, any sequence alternations introduced by the degenerated primers will not influence antibody function. The phenomenon occurred in our work, the deduced N-terminal sequence of mature 3H11 VH protein had 4 amino acid residue discrepancies between “leader primer” amplified VH gene in this work (QUQLWQS) and previous “framework 1 primer” amplified VH gene (LLELVQS). While in 3H11 VL gene there were 2 discrepant residues, QIVLT of authentic VL versus DIVMT from “framework 1 primer” amplified VL\(^8\). Indeed the changes had profound influence on the binding of engineered 3H11 Ab molecules to gastric cancer cells (manuscript in preparation). In order to maintain the original sequences of the C terminal of the V regions, Coloma et al\(^3\) suggested that the V genes be first amplified with 3 primer located at constant regions. Then a second set of 3 primers were designed according to the authentic sequences, and the V genes were PCR amplified again. Since there were only 4J segments for murine VL and VH genes respectively, we designed 3 primers for VL and VH genes that straddled the V-C junction and might at most cause one residue substitution in VL (Leu\(^{106}\)→Ile\(^{106}\) in JK5) or VH (Ser\(^{113}\)→Ala\(^{113}\)) while the labour of cloning was greatly reduced.

Due to the diversity of Ab leader sequences, it would be more difficult to amplify V genes from leader by PCR\(^3\). There were some reports about RT-PCR method by primers for leader sequences abroad, but there is no report yet in our country. The facts that we had successfully amplified the variable genes not only of 3H11 but also of CD3 (manuscript in preparation) indicated that the primers were effective.

The amplified 3H11 VL gene was cloned into chimeric light chain expression vector and transfected into SP2/0 cells. Transient expression analysis demonstrated that the VL gene was functional, thus laid the basis for constructing 3H11 human-mouse chimeric antibody.

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