Original

Construction of an Expression Vector Containing Mouse-Rat Chimeric Genes Encoding a Therapeutic Antibody against CD81

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Abstract: CD81 belongs to the tetraspanin family of cell-surface proteins, which contain four transmembrane domains and two outer-membrane loops. CD81 has been shown to be up-regulated in synovocytes in rheumatoid arthritis (RA) and to promote synoviolin expression in the progression of RA. Recently, we showed that a small interfering RNA (CD81 siRNA) targeting the gene encoding CD81 ameliorates arthritis in collagen-induced arthritic (CIA) rats. CD81 siRNA also decreased the expression of TNF-α in SW982 cells. Additionally, we established hybridoma cell lines producing monoclonal antibodies (MAbs) against CD81 and demonstrated that some of these MAbs impaired the proliferation of C6 glioma cells. Here, we report cloning of the cDNA encoding one such MAb (H chain and L chain) from hybridoma cells and construction of mouse-rat chimeric genes for expression of the chimeric antibody. This chimeric antibody, as expressed by CHO cells, bound to CD81 at the target’s outer-membrane loop (LEL).

Key words: Rheumatoid arthritis, CD81, Monoclonal antibody, Chimeric gene, Enzyme-linked immunosorbent assay (ELISA)

Introduction

CD81 belongs to the tetraspanin family of membrane proteins, all of which consist of four transmembrane domains and two outer-membrane loops. CD81 has been shown to influence the adhesion, morphology, activation, proliferation, and differentiation of B cells and T cells. Our previous study using DNA microarrays showed that the expression of the gene encoding CD81 was significantly up-regulated in synovocytes in rheumatoid arthritis (RA) synoviocytes. Immunostaining using anti-CD81 antibodies also showed that the distribution of CD81 protein was up-regulated in RA synoviocytes. Furthermore, stimulation of CD81 was shown to promote the expression of synoviolin in SW982 cells. Synoviolin has been identified as a rheumatoid promoter that induces abnormal growth of synovial cells in RA patients. Overexpression of synoviolin in mice causes spontaneous arthropathy, and synoviolin/hrd1-/- mice exhibit enhanced apoptosis of synovial cells, resulting in resistance to collagen-induced arthritis (CIA). Recently, we showed that a small interfering RNA targeting the gene encoding CD81 (CD81 siRNA) decreased not only the expression of synoviolin but also the expression of TNF-α in SW982 cells. We also showed that CD81 siRNA ameliorates arthritis of CIA rats, indicating the potential therapeutic importance of anti-CD81 monoclonal antibodies (MAbs), which can inhibit the function of CD81, in the treatment of RA. Most conventional anti-rheumatic drugs target inflammatory cytokines or cell proliferation systems, and the anti-CD81 antibodies in our research can be a novel effective therapeutic drug of a different category. In the present paper, we report the establishment of hybridoma cell lines producing mouse MAbs against CD81, and the construction of genes encoding a mouse-rat chimeric antibody that has mouse V regions and rat C regions in both the H and L chains.

Materials and Methods

Cell culture

Hybridoma cells and myeloma cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO2 at 37°C.

Establishment of hybridoma cell lines

BALB/c mice were immunized with a recombinant protein corresponding to the outer membrane region (LEL) of CD81 to stimulate antibody production. Spleen cells from immunized mice were isolated and 1x10^7 cells were fused with 1x10^5 myeloma cells using polyethylene glycol (PEG). Fused cells were distributed into 96-well plates and hybridoma cells were selected using HAT medium. Enzyme-linked immunosorbent assay (ELISA) was used to identify hybridoma wells that showed the highest titer against the antigen. Hybridoma cells in several wells that showed the highest titer were subcloned, and several hybridoma subclones that showed the highest titer were identified by ELISA.

cDNA cloning of immunoglobulin genes

Total RNA was isolated and purified from hybridoma cells using TRIzol reagent (Life Technologies Co., Carlsbad, CA) and RNasey Mini Kit (QIAGEN N.V., Venlo, Netherlands). Immunoglobulin cDNAs (en-
coding both the H and L chains) were synthesized by using SMARTer RACE 5’/3’ Kit (Takara Bio Inc., Shiga, Japan). In the case of the H chain-encoding sequence, a primer pair consisting of UPM and VH-IgG-R was used to amplify the cDNA. In the case of L chain-encoding sequence, primer pairs consisting of UPM primer and VL-κ-R or VL-λ-R were used to amplify the cDNA. The primer sequences of UPM are described in the manufacture’s protocol. The primer sequences of VH-IgG-R, VL-κ-R and VL-λ-R are as follows: 5’ AAAAAAGCT-TATCGATCAGGGGCCAGTGGATAGAC 3’ (VH-IgG-R), 5’ AAAAAAGCTTATCGATTGTCGTTCACTGCCATCAAT 3’ (VL-κ-R), 5’ AAAAAAGCTTATCGATTACACACCAGTGTGGCCTTG 3’ (VL-λ-R). PCR products were purified using NucleoSpin Gel and PCR Clean-up (Takara Bio), and nucleotide sequences were analyzed using the same primers used for amplification.

**Construction of mouse-rat chimeric genes**

Rat immunoglobulin-encoding cDNAs (encoding the constant regions of the H and L chains) were synthesized following the information of published data, and mouse-rat chimeric gene was constructed with sequences in the following order:

CMV promoter - Kozak sequence – H chain cDNA (mouse variable region – rat constant region) – BGH polyA - CMV promoter - Kozak sequence – L chain cDNA (mouse variable region – rat constant region) – BGH polyA. These constructs were cloned into the pBSIISK+ vector.
Production of chimeric antibodies

The recombinant vector constructed as described above was introduced into CHO-K1 cells to estimate antibody production. CHO-K1 cells were grown in DMEM/Ham’s F12 medium (Wako Pure Chemical Corp., Osaka, Japan) supplemented with 10% FBS (Gibco, Dublin, Ireland). For transfection, FuGENE 6 (Promega Corp., Fitchburg, WI) or Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) was used. Specifically cells were seeded at approximately 4 x 10^5 cells/well in a 6-well plate, and transfection reagent (5-10 μl/well) and recombinant vector (2-4 μg/well) were added on the following day. After incubation of 2-5 days, culture medium was collected from the transfected cells and the expressed chimeric antibodies were purified from the culture medium using Protein G Sepharose 4 Fast Flow (GE Healthcare, Chicago, IL). Purified chimeric antibodies were detected by western blotting using alkaline phosphatase-conjugated anti-rat IgG (Promega Corp.).

Enzyme-linked immunosorbent assay (ELISA) for evaluation of the activity of chimeric antibodies

A recombinant protein corresponding to the outer membrane region (LEL) of CD81 was diluted in 50 mM carbonate buffer (pH 9.6) and distributed at 100 µl/well in 96-well plates. After 1 hr at room temperature, 3% bovine serum albumin (BSA) (150 µl/well) was added to the wells and the plates were incubated for another hour. The wells then were washed with phosphate buffered saline (PBS), and the culture medium or control rat IgG (total 100 µl/well) was added. After 1 hr at room temperature, each well was washed with PBS and 1,000-fold diluted peroxidase-conjugated anti-rat IgG was distributed at 100 µl/well. After 1 hr at room temperature, each well was washed with PBS and 100 µl of POD Substrate TMB kit solution (Nakarai Tesque Inc., Kyoto, Japan) were added to the wells. Absorbance of each well was measured using a Varioskan LUX (Thermo Fisher Scientific).

Results

Establishment of hybridoma cell lines

Mice were immunized with CD81 protein (recombinant outer membrane domain, LEL) and hybridoma cells were established. ELISA screening was used to identify hybridoma cells producing anti-recombinant CD81 MAbs at high titer. Specifically, we selected five hybridoma clones that produced IgG MAbs and five hybridoma clones that produced IgM MAbs. The binding activity and cross-reactivity of these MAbs then were tested. Of the IgG MAbs (the product of the TSK8 line) showed growth inhibitory effects against tumor cells; the corresponding clone was selected for further cloning of the immunoglobulin-encoding gene.

cDNA cloning of immunoglobulin-encoding genes

Total RNA was isolated and purified from the hybridoma clone (TSK8) and immunoglobulin-encoding cDNAs (specifically, those encoding the H chain and those encoding the L chain) were synthesized using SMARTer RACE 5‘/3’kit (Takara Bio). In the case of the H chain-encoding cDNA, PCR reaction was performed using a primer set of UPM consisting of a short oligonucleotide (a 22-mer) and a long oligonucleotide (a 45-mer) and VH-IgG-R (a 35-mer). In the case of the L chain-encoding cDNA, PCR reaction was performed using a primer set of UPM and VL-κ-R (a 36-mer) or VL-λ-R (a 36-mer). Following PCR amplification, DNA products corresponding to the H chain-encoding cDNA were observed using the UPM + VH-IgG-R primer pair. Similarly, DNA products corresponding to the κ chain-encoding cDNA were observed using the UPM + VL-κ-R primer pair (Fig. 3). However, no DNA products corresponding to the λ chain-encoding cDNA were apparent using the UPM + VL-κ-R primer pair. The H chain- and κ chain-encoding cDNAs were subcloned into the T7 Blue T-vector and nucleotide sequences were determined. Sequencing detected the IgG H chain-encoding open reading frame in multiple clones obtained as UPM + VL-κ-R products. Similarly, the κ chain-encoding open reading frame was detected in multiple clones obtained as UPM + VL-κ-R products.

Construction of mouse-rat chimeric genes

A rat immunoglobulin cDNA (encoding the constant regions of both the H and L chains) was synthesized as described in the Methods; the resulting cDNA was confirmed by sequencing, and mouse-rat chimeric gene (encoding both the H and L chains) was constructed by using the synthesized cassette DNAs and appropriate restriction enzymes. Chimeric nucleotide sequences and deduced amino acid sequences corresponding to the boundary between the H and L chains are shown in Fig. 2. Two of these chimeric DNAs were subcloned into the same pBSIISK+ vector (Fig. 1) to permit production of a chimeric TSK8 IgG antibody.

Production of chimeric antibodies

The recombinant vector described above was introduced into CHO-K1 cells to produce chimeric TSK8 IgG antibody. FuGENE 6 or Lipofectamine 2000 was used as the transfection reagent, and six different transfection conditions were examined by using 6-well culture plates. The transfection reagent - cell number - Opti-MEMI volume -
vector amount - volume of the transfection reagents used for each condition were as follows:

- **FuGENE 6**
  1. $4 \times 10^5$ cells/well - $94 \mu l$ - $2 \mu g$ - $6 \mu l$
  2. $4 \times 10^5$ cells/well - $94 \mu l$ - $2 \mu g$ - $9 \mu l$

- **Lipofectamine 2000**
  3. $4 \times 10^5$ cells/well - $125 \mu l$ - $2 \mu g$ - $5 \mu l$
  4. $4 \times 10^5$ cells/well - $125 \mu l$ - $2 \mu g$ - $10 \mu l$
  5. $4 \times 10^5$ cells/well - $125 \mu l$ - $4 \mu g$ - $5 \mu l$
  6. $4 \times 10^5$ cells/well - $125 \mu l$ - $4 \mu g$ - $10 \mu l$

In the case of 10-cm dishes, the proportions were $3.6 \times 10^6$ cells/well - $557 \mu l$ - $12 \mu g$ - $36 \mu l$

Production of chimeric IgG in the culture medium at various time points (48, 72, 96, and 120 hr) after transfection was analyzed by western blotting using alkaline phosphatase-conjugated anti-rat IgG. Detectable chimeric IgG was produced under all of the indicated conditions, with the exception of condition (1) at 48 hr post-transfection. Notably, western blotting readily detected chimeric antibody in culture medium recovered from the 10-cm dish at 120 hr post-transfection (data not shown). IgG antibody was purified from the culture medium obtained from the 10-cm dish using Protein G Sepharose 4, and antibody production was estimated by western blotting (Fig. 4), demonstrating a yield of 78.8 µg of chimeric IgG from 10 ml of spent medium.

Next, the antigen (CD81)-binding activity of the purified chimeric IgG was estimated by ELISA. A defined amount of LEL recombinant...
CD81 was added to each well of a 96-well dish, and purified TSK8 chimeric antibody was then added. Binding activity was detected using alkaline phosphatase-conjugated anti-rat IgG. The results of the ELISA are shown in Fig. 5. TSK8 mouse MAb was used as a positive control; rat polyclonal antibody was used as a negative control. A BSA-coated plate was used to estimate background. In the case of TSK8 mouse MAb, about four times higher binding to recombinant LEL than the polyclonal control was observed. Similarly, in the case of TSK8 chimeric IgG, 1.7 times higher binding to recombinant LEL than the polyclonal control was observed comparing with that of rat polyclonal antibody.

Discussion

In this paper, we established hybridoma cell lines producing mouse MAb against CD81, and constructed mouse-rat chimeric antibodies incorporating mouse V region sequences and rat C region sequences in both the H and L chains. These chimeric antibodies were produced from CHO-K1 cells by transfection with an expression vector containing cDNA encoding both subunits of the chimeric antibody. Previously, recombinant antibodies were produced from CHO cells at approximately 10-14 µg/ml of spent medium. Under optimal conditions, recombinant antibody has been recovered from the culture medium at 21 µg/ml. The present production level of chimeric recombinant antibody is 7.9 µg/ml which was consistent with those past results. Therefore, our work suggests that construction of the chimeric genes was completed following the appropriate process and the produced antibody proteins were correctly assembled in CHO cells. Furthermore, the produced chimeric IgG antibodies showed significant binding to CD81 (LEL), again confirming that the chimeric antibody was normally processed in CHO cells. Intriguingly, the binding activity of TSK MAb to LEL (as shown in Fig. 5) appears to be higher than that of chimeric TSK antibody. This difference might be caused by (1) A difference in antibody concentration between ascites fluid (TSK MAB) and culture medium (chimeric TSK antibody), and/or (2) A difference in avidity of the enzyme-labeled secondary antibody between the anti-mouse antibody (bind to TSK MAb) and anti-rat antibody (bind to chimeric TSK antibody). In future work, we propose to produce chimeric TSK antibodies from CHO-K1 cells at larger scale and to then use those chimeric TSK8 antibodies to test the therapeutic effects in CIA rats. CD81 was originally identified as a target of the TAPA-1 MAb. We previously have reported that our anti-CD81 MAbs are functionally active, as demonstrated by their ability to inhibit the proliferation of mouse C6 glioma cells. Some anti-CD81 MAbs have been shown to be functionally active in inhibition of the binding of hepatitis C virus (HCV) to the cell surface and in inhibition of the proliferation of rat astrocytes. It is also known that E2 envelope protein of hepatitis C virus (HCV) associates with CD81 molecule and there are some other candidates of association molecules with HCV. In the case of RA, inflammation and abnormal growth of synovial tissues are the major symptoms and two important functions of anti-CD81 MAbs, inhibition of inflammatory cytokine production and down regulation of synoviolin expression could contribute to amelioration of RA. Recently, JNK inhibitors have been developed as effective anti-rheumatic drugs. But JNKs play various important roles in cells and it has found that JNK inhibitors show severe side effects in some cases. CD81 is also known to be expressed in various cells especially in B lymphocytes regulating B cell activation by composing B cell coreceptor with CD21 and CD19. In this point of view, chimeric anti-CD81 antibodies are expected to facilitate examination of therapeutic effects in both mouse and rat models of RA especially in the case using local administration in joint cavities.

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Conflict of Interest

The authors have declared that no COI exists.

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