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Short Communication

Antigen-binding characteristics of AbCD71 and its inhibitory effect on PHA-induced lymphoproliferation[1]

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Key words

AbCD71; chimeric human/murine antibody; lymphoproliferation; transplantation rejection

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Abstract

Aim: To investigate the anti-lymphoproliferative effect of the prepared recombinant chimeric human/murine anti-cluster of differentiation(CD) 71 monoclonal antibody (AbCD71), which is composed of mouse-derived, antigen-binding variable regions and human-derived constant regions. **Methods:** After plasmids construction and transfection, the expression of AbCD71 in the transfectoma supernatant was determined by the sandwich ELISA. Indirect immunofluorescence assay was used to measure the antigen-binding characteristic and the percent CD71-expressed peripheral blood mononuclear cells (PBMC). The antibodies were purified from the ascites via diethylaminoethyl(DEAE)-Sephadex A-50 chromatography and then identified by SDS-PAGE. At last, inhibitory effect of AbCD71 on PHA-induced PBMC proliferation was calculated by methyl thiazolyl tetrazolium (MTT) assay. **Results**: Constant domain of heavy chain(C_H) and light chain(C_L)of AbCD71 were in the human Cγ family and human Cκ family, respectively. AbCD71 could compete with its original murine mAb to bind with CD71-positive human leukemia cell line CEM cells. AbCD71 could inhibit the peripheral blood mononuclear cell proliferation induced by phytohemagglutinin(PHA) *in vitro* in a dose-dependent manner, especially at time-points 0 and 12 h after induction. There was no statistical difference when compared with original murine mAb. **Conclusion:** The AbCD71 is a promising immunosuppressant. Our approach to blocking CD71 with the chimeric human/murine mAb provides a novel strategy for prolonging allograft survival.

Introduction

Cluster of differentiation(CD) 71, human transferrin receptor, is abundantly expressed in rapidly dividing cells as well as on many tumor cells, which makes CD71 a relatively specific marker of these cells[1]. Laboratory investigations and clinical studies have demonstrated that anti-CD71 mAb or CD71 binding factors have antiproliferative effects on CD71-positive cells by blocking the engagement of transferrin with its receptor and interfering in the intake of iron into cell[2,3].

However, murine mAb can induce the production of human antimouse antibodies when administered repeatedly in the human body, which may render the antibody ineffective and may also harm patients[4]. Furthermore, the administered murine mAb could not bind to the Fc receptor expressed on human phagocytes, natural killer cells, B cells *etc*. Therefore, murine mAb could not exert opsonization and phagocytosis effects and could not mediate antibody-dependent, cell-mediated cytotoxicity[5]. Hence, murine mAb showed no effective suppression on lymphoproliferation *in vivo* and had limited effect on lessening transplantation rejection, which would restrain their further applications in clinic practice[6]. At present, efforts have been directed at the engi-

neering of antibodies to improve their utility, lessen unwanted effects, and alter the effector functions through the modification of Ig genes by using recombinant expression techniques[7]. The chimeric human/murine antibody containing murine V regions combined with human C regions retained the ability of mAb to recognize specific antigen, but had low antigenicity to humans[8].

In the present study, starting with a previously established hybridoma 7579 producing a monoclonal antibody to CD71, we prepared the chimeric human/murine anti-CD71 mAb (AbCD71), confirmed its biological characteristics, and exploited its effect on lymphoproliferation.

Materials and methods

Plasmid construction and transfection Total RNA prepared from the anti-CD71 mAb-secreting hybridoma cell line 7579 (preserved by our laboratory) was reverse-transcripted into cDNA. Then PCR was performed using the following primers: variable domain of light chain(V_L) sense P1:5'-GGGGTCGACCTCACCAT GGATTTTCAAGTGCAGATTTTCAG-3'; V_L antisense P2:5'-GGCCTGCGGCCGCTTTAAATTCTACTCACGTTTGATTTCCAGCTTGGT-3'; variable domain of heavy chain(V_H) sense P1:5'- GGGGTCGACCTCACCATGGAATGCAGCTGTGTAATCCTCTT-3'; and V_H antisense P2:5'-GGCCTGCGGCCGCAGTAGAGCAGACTCACCTGAGGAGACAGTGACC-3'. After being digested with *Sal* I and *Not I* the V_L and V_H PCR products were subcloned into pκ-Expr and pγ-Expr vectors (preserved by our laboratory), respectively. Following linearization by *Pvu* I, Chi7579–pκ-Expr and Chi7579–pγ-Expr were cotransfected into immunoglobulin non-producing mouse myeloma cell line SP2/0-Ag14 by electroporation (Gene pulser transfection apparatus 165-2078 Bio-Rad, Richmond, CA, USA). G418 (500 μg/mL, Promega, Madison, WI, USA)-resistant clones were analyzed for the following assays.

ELISA The sandwich ELISA was used to determine the expression of AbCD71 in the transfectoma supernatant. The plate was coated with 10 μg/mL goat antihuman immunoglobulin G (IgG, γ chain specific, Sigma, St Louis, MO, USA). The secondary antibody was 1:800 diluted mouse antihuman κ chain mAb (Sigma, USA). 1:1000 diluted horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Kirkegaard Perry Labs Inc, Gaithersburg MD, USA) served as the third antibody. After exposure to the HRP substrate (Pierce, Rockford, IL, USA), optical density (OD) values were read at a 490 nm wavelength.

Indirect immunofluorescence assay The antigen-binding characteristic of AbCD71 was measured by indirect immunofluorescence assay. CD71-positive CEM cells were cocultured with original murine anti-CD71 mAb hybridoma supernatant at different dilutions (undiluted, 1:10, 1:20, 1:40, and 1:80) or PBS. Then the transfectoma supernatant was added. After goat antihuman IgG–fluorescent isothiocyanate (FITC) (BD Biosciences, San Diego, CA, USA) was supplemented, the percentage of the FITC-positive cells was evaluated by FACSCalibur flow cytometer (BD Biosciences, USA).

This methodology was also used to analyze the percentage of CD71-expressed PBMC activated by phytohemagglutinin (PHA). After being isolated by Ficoll–Hypaque density centrifugation, the PBMC were cocultured with PHA (final concentration, 25 μg/mL; Sigma, USA). At different time-points (12, 36, and 60 h) after activation, the cells were collected in order to analyze the CD71 expression by flow cytometer (FCM).

Preparation and purification of antibodies The transfectoma was intraperitoneally inoculated into Balb/c (nu/nu) nude mice to induce the ascites for the preparation of AbCD71. Hybridoma 7579 was inoculated into Balb/c mice to induce the ascites for the preparation of anti-CD71 mAb. The antibodies were purified from the ascites via diethylaminoethyl (DEAE)-Sephadex A-50 chromatography (Pharmacia, Piscataway, NJ, USA). Then the purified antibody was identified by SDS–PAGE and indirectly elevated in the immunofluorescence assay as mentioned before.

Inhibitory effect of AbCD71 on PHA-induced PBMC proliferation The PBMC were cocultured with PHA at a final concentration of 0 and 25 μg/mL, respectively, and AbCD71 or murine anti-CD71 mAb (final concentration, 1, 10, and 100 μg/mL, respectively) for 5 d. Then PBMC proliferation was measured by methyl thiazolyl tetrazolium(MTT) method. The untreated PBMC group was set as negative control and the isotype antibody-treated group was set as another negative control. The PBMC group, induced with 25 μg/mL PHA, but untreated with antibodies, was set as the positive control. Triplicates were set for each group. After reading the OD values (A) at 570 nm, the inhibitory rates were calculated as the following formula: the inhibitory rate (%) of cell proliferation=(1–[the mean A of the experimental group/the mean A of the positive control group])×100.

In another experiment, at different time-points after PHA stimulation (0, 12, 36, and 60 h), AbCD71 or murine anti-CD71 mAb (final concentration, 0, 1, 10, and 100 μg/mL, respectively) was supplemented into the PBMC culture system. After 5 d of culture, the MTT assay was performed with the same controls as before. Then the inhibitory rates were calculated.

Statistical analysis The t-test was performed to compare

the inhibitory rates. Differences were regarded as statistically significant when $P<0.05$.

Results

Confirmation of AbCD71 constant domains After G418 screening, the cells in the 4 wells showed transfectoma cells growth (numbered as C1–4).

The mean OD values of the 4 G418-resistant clones were higher than those of the blank control and the negative control (Table 1). The results indicated that the heavy chain of AbCD71 in the transfectoma supernatant could bind to goat antihuman IgG (γ specific) and its light chain could bind to mouse antihuman κ chain mAb.

AbCD71 competed with its parental murine mAb to bind to CD71-positive CEM cells It was confirmed that AbCD71 contained human IgG heavy chain and light chain constant domains. Did AbCD71 still remain the murine variable domain and retain antigen-binding specificity? Indirect immunofluorescence assays showed that as the concentration of the hybridoma supernatant decreased from undiluted to a dilution of 1:80, the percentage of FITC-positive cells increased from 15.16%±0.73% to 56.22%±2.65%. When the CEM cells were precultured with PBS, the percentage rose to 71.49%±3.60% (Figure 1). These figures suggested that the less the murine mAb combined with CD71 molecules, the more AbCD71 bound to the CEM cells, hence an increase in the CEM cells emitting green fluorescence.

Table 1. OD values of transfectoma supernatants and controls.

	OD value		Mean value
Blank control	0.071	0.077	0.074
Negative control	0.086	0.095	0.091
Positive control	0.228	0.193	0.211
C1	0.166	0.166	0.166
C2	0.171	0.157	0.164
C3	0.163	0.161	0.162
C4	0.161	0.161	0.161

C1–4 represent the 4 G148-resistant clones. In the blank control, phosphate buffered saline tween instead of the transfectoma supernatant was added. In the negative control, the supernatant of no G418-screened cells was added. In the positive control, human IgG (κ) was added. Two parallel wells were set for each sample. Representative result of 2 experiments for each group is shown.

Identification of purified AbCD71 by SDS–PAGE After the transfectoma clone secreting AbCD71 was identified, the clone cells were inoculated into mice and the resultant ascites fluid was used to prepare purified AbCD71. The yield was about 4–8 mg of the antibody in 3–5 mL of the

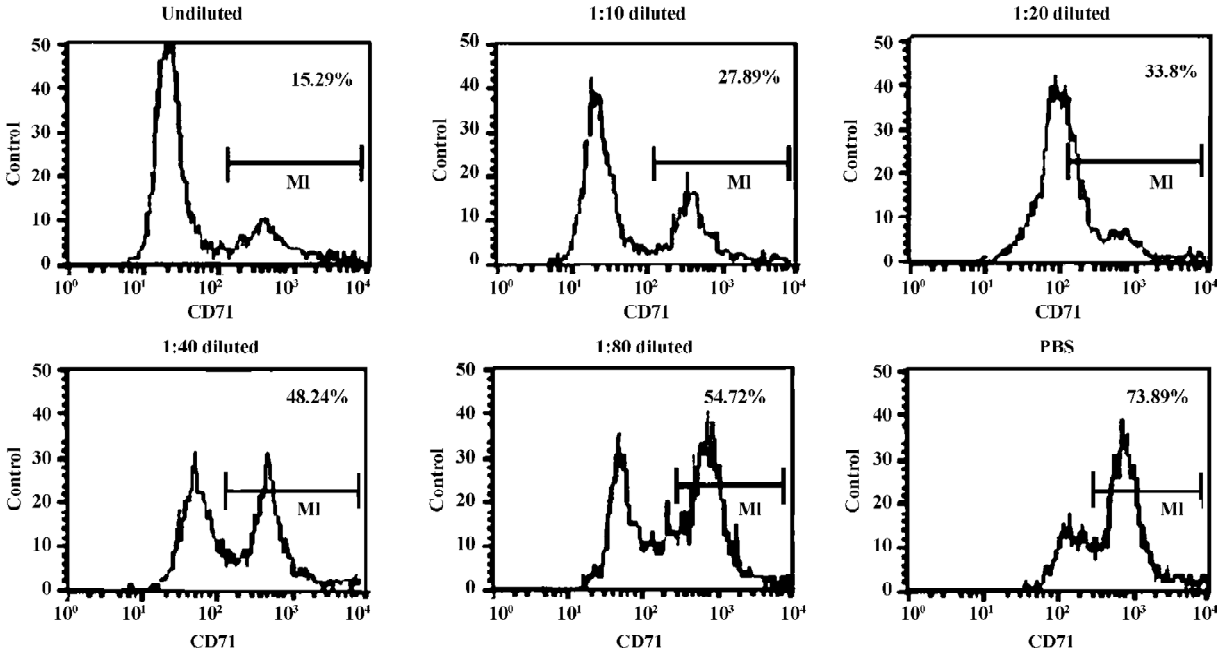

Figure 1. AbCD71 competed with its original murine mAb for binding to CD71 molecules. 1×10^6 CEM cells were cocultured with 200 μL hybridoma supernatant with different dilutions at 37 °C for 30 min. After washing 3 times, 200 μL transfectoma supernatant was added. Then the cells were incubated with 100 μL goat antihuman IgG-FITC at a dilution of 1:10 for 60 min at 37 °C. The M1 gate demarcates the FITC-positive populations. Percentage of gated cells is indicated. Results are representative of 3 experiments.

ascites. SDS–PAGE displayed 2 specific protein bands with molecular weights of about 55 and 25 kDa, which presumably represented the heavy chain and light chain of AbCD71, respectively (Figure 2). The purity of the antibody was more than 95%. The specificity of purified AbCD71 was identified by indirect immunofluorescence assay. The same results (data not shown) were obtained as those of the transfectoma supernatant.

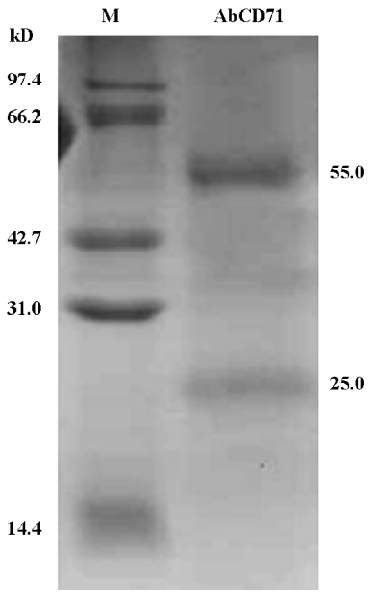

Figure 2. SDS-PAGE analysis of purified AbCD71. Molecular weight of the obtained AbCD71 was determined by SDS-PAGE. Acrylamide gel (10%) was used and the gel was stained with Coomassie brilliant blue R250. Molecular weight was estimated by comparison with molecular weight markers (M).

AbCD71 could inhibit PHA-induced lymphoproliferation Data showed that AbCD71 could inhibit PHA-induced lymphocyte proliferation obviously (Figure 3A). When the concentration of the antibody varied between 1–100 μg/mL, the inhibitory rate increased as the concentration of AbCD71 rose. There was no statistical difference in the inhibitory rates between AbCD71 and original murine anti-CD71 mAb at the corresponding concentrations (P>0.05).

The PBMC failed to proliferate in the absent of PHA. Hence, AbCD71 or the murine antibody showed no effect on PBMC proliferation (inhibitory rate <1%).

It was demonstrated that when AbCD71 and PHA were added together, proliferation of resting lymphocytes could be inhibited by AbCD71. It remained unclear whether stimulated lymphocyte proliferation could be suppressed. For that reason, first, the percentage of activated PMBC, on which membrane CD71 expression was upregulated, was measured. We found that 38.6%±1.91%, 89.6%±4.38%, and 90.8%±4.12% PBMC were activated and expressed CD71 on their membranes after 12, 36, and 60 h of PHA stimulation, respectively (Figure 3B). Second, following stimulation for 12, 36, and 60 h, the PBMC were incubated with AbCD71. The results showed that AbCD71 inhibited proliferation of induced PBMC in a dose-dependent manner when the concentration of the antibody varied between 1 and 100 μg/mL. Proliferations were suppressed strongly at the time-points of 0 and 12 h. PBMC proliferation at 60 h was not evidently inhibited. There were statistical differences (P<0.01) between the groups treated with the same concentration, but induced for different time periods (Figure 3C). All these data showed no statistical difference (P>0.05) when compared with the corresponding concentration of murine mAb-treated groups.

Discussion

In the present study, the light chain gene and the heavy chain gene of AbCD71 were constructed into 2 expression vectors, respectively. Only when those 2 vectors were cotransfected into the same recipient cell could an intact antibody be produced. Using ELISA, the coated goat anti-human IgG γ could be combined when the transfectoma supernatant contained the human IgG heavy chain. Subsequently, the mouse antihuman Ig κ was supplemented. After exposure to the substrate, only those wells containing intact human IgG (κ specific) could be developed into yellow. Hence, the species specificity of AbCD71 constant regions was confirmed. The following indirect immunofluorescence assay demonstrated that the chimeric Ab could compete with their original murine mAb to bind to the target antigen. This means that AbCD71 retained the affinity and antigen-binding specificity of its parental murine mAb. The above 2 assays verified that AbCD71 not only reserved the variable region of its original murine mAb, but also possessed of the constant region of human IgG.

CD71 expression is essential for continued growth and is closely linked to the proliferative status of a cell. In addition, the CD71 molecule is recognized as a lymphocyte activation marker[9,10]. Therefore, CD71 may be a potential therapeutic target for transplantation rejection. After the biological characteristics of AbCD71 were identified, its antiproliferative effect on human PBMC was investigated. PBMC could proliferate and express CD71 on their membranes when induced by mitogen[11], which could mimic post-transplantation lymphoproliferative responses. Our experiment also verified

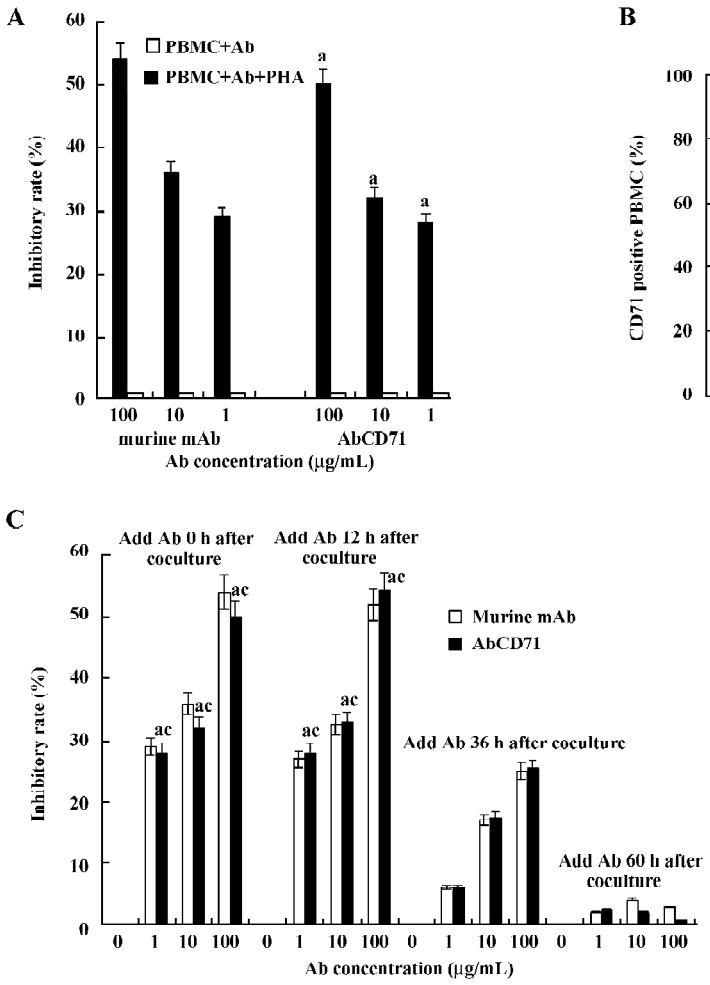

Figure 3. AbCD71 inhibited PHA-induced lymphoproliferation. (A) inhibitory rates of AbCD71 and murine mAb proliferation of PBMC when added in combination with PHA. (B) percentage of activated PBMC after different induction times. A DEAE-Sephadex A-50 column purified murine mAb was supplemented into the culture medium. FCM analysis was performed after FITC–goat antimouse IgG was added. (C) effect of timing and concentration of AbCD71 on PBMC proliferation. [a]$P>0.05$ *vs* corresponding murine mAb control, [c]$P<0.01$ *vs* corresponding 60 h time-point groups. Data are representative result of triplicate experiments.

that the expression of CD71 on PBMC increased in response to 25 μg/mL PHA stimulation. After exposure to PHA for 36 h, most PBMC were activated and proliferated efficiently because the percentage of CD71-expressed PBMC showed no evident alteration in the following hours.

A subsequent inhibitory rate analysis suggested that the administration of AbCD71 at the time of PHA stimulation could strongly inhibit proliferation and clonal expansion of PBMC *in vitro*. In evaluating the optimal timing of AbCD71 addition to the culture medium, we could see that there was a distinct inhibition when AbCD71 was added at 0 and 12 h after PHA induction, compared with 36 and 60 h timepoints. AbCD71 administration at the early stage of mitogen presentation was more effective than at the time of maximal receptor expression, suggesting an early role for AbCD71 in antirejection. The possible explanation is that AbCD71 exerts growth suppression by blocking the CD71 molecules distributed on PBMC, and thus blocking iron uptake in activated lymphocytes. Cellular iron is crucial for cell survival[1,12]. At the early stage of mitogen stimulation, PBMC needed iron for continued growth and proliferation. CD71 blockade at this period resulted in the lack of sufficient iron availability. Hence, PBMC proliferated poorly in response to mitogen stimulation[13]. At the late stage, when PBMC already underwent clonal expansion, their particular needs for high levels of iron ceased. CD71 blockade and iron starvation at this time would have had a limited effect on the total PBMC number. Hence, the inhibitory rate at 36 h decreased and the data reached nadir at 60 h. Future studies will improve our understanding of AbCD71 in the activation of lymphocytes and will provide the knowledge necessary for utilizing AbCD71 blockade as a novel therapeutic strategy for clinical transplantation.

The above data manifested that AbCD71 could specifically bind to the CD71 molecules expressed on the membranes of activated lymphocytes and could inhibit the lymphoproliferation. It seems that AbCD71 administration would lower the incidence of transplantation rejection and

not produce human antimouse antibodies. Thus, the use of AbCD71 may result in more effective immunosuppression in transplantation. AbCD71 looks to be a promising immunosuppressant. Our approach to blocking the transferrin receptor using chimeric human/murine mAb provides a novel strategy for prolonging allograft survival.

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