ORIGINAL ARTICLE

Establishment of a library having 204 effective clones of recombinant single chain fragment of variable region (hScFv) of IgG for vasculitis treatment

Yosuke Kameoka[1*], Minako Koura[2], Junichiro Matsuda[2], Osamu Suzuki[2], Naohito Ohno[3], Ryuichi Sugamata[4#], Toshinori Nakayama[4], and Kazuo Suzuki[4#]

[1]National Institutes of Biomedical Innovation, Health and Nutrition, Laboratory of Rare Disease Biospecimen, Saitoasagi 7-6-8, Ibaraki-Shi, 567-0085, Osaka, Japan
[2]National Institutes of Biomedical Innovation, Health and Nutrition, Laboratory of Animal Models for Human Diseases, Saitoasagi 7-6-8, Ibaraki-Shi, 567-0085, Osaka, Japan
[3]Laboratory for Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy & Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo, 192-0392, Japan
[4]Department of Immunology, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba-City, Chiba, 260-8640, Japan

*Present address: A-CLIP Institute, Ltd. Inohana 1-8-15, Chuo-ku, Chiba-City, Chiba, 260-0856, Japan
#Present address: Asia International Institute of Infectious Disease Control, Teikyo University, Kaga 2-11-1 Itabashi-ku, Tokyo, 173-8605, Japan

Correspondence
Yosuke Kameoka: A-CLIP Institute, Ltd. Inohana 1-8-15, Chuo-ku, Chiba-City, Chiba, 260-0856, Japan

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Abstract

Purpose Vasculitis is a refractory disease with no established treatment. Therapeutic effect of gamma globulin (IgG) in high dose therapy (IVIg) has been reported[1]. IgG preparation which depends on human-derived material, is not completely safety and supply amount of it is finite. Development of recombinant gamma globulin having the same effect has been requested.

Methods We constructed a library of recombinant single chain fragment of variable region (hScFv) of IgG from the constitution of VH-CH1-hinge from peripheral blood lymphocytes of healthy volunteers. One thousand recombinant hScFv clones were analyzed in base sequence, and selected by having the correct structure of hScFv. Selected clones were mixed and cultured, and induced hScFv proteins were administered to spontaneous vasculitis mouse model SCG/Kj to evaluate therapeutic effects.

Results From the base sequence analysis of 1,000 clones, we obtained 245 clones having VH-CH1-hinge structure, then a library having 204 clones was established. All 204 clones were mixed and cultured to induce hScFv proteins. A purified preparation was administered to SCG/Kj mice. The hScFv administration at a concentration of 1/20 of the large amount of IgGs preparation showed same effect for suppression of glomerular crescent formation and a refinement of the peripheral blood cell was observed in hScFv administration groups. Also, a biomarker MPO-ANCA titer remarkably decreased administration dose of IVIg in 1/10

Conclusions The hScFv protein mixture showed therapeutic effects with 1/10-1/40 of IVIg in administration to SCG/Kj mouse. It seems to be a development possibility to actual preparation as recombinant IgG.

Key words: Single chain antibody fragment of variable region (hScFv), Immunoglobulin preparation, Vasculitis, antibody drug, spontaneous vasculitis mouse model SCG/Kj

Introduction

Immunoglobulin preparation therapy is an effective treatment for patients with vasculitis[1]. Microscopic polyangiitis and anti-nuclear cytoplasmic antibody (ANCA)-associated vasculitis (AAV)[2], are an intractable vasculitis. These have been frequently seen in Japanese people as approximately 10,000 registered cases in medical expense support by the Japanese government, especially in seniors[3]. Its underlying lesion is a systemic inflammation of small vessels. A high percentage of patients have auto-antibody against myeloperoxidase (MPO-ANCA). Most of patients with rapidly progressive glomerulonephritis have MPO-ANCA-associated vasculitis (MAAV), alveolar hemorrhage, and interstitial pneumonia with a poor prognosis. Although immunosuppressive agents including high dose steroids are used for AAV treatments, some patients require dialysis because of a rapid decline of renal function. Other vasculitis-related diseases are children's vasculitis Kawasaki disease[4], which are treated with immunoglobulin preparation (IVIg). Also, IVIg treatement has been used for infectious diseases[5], and has been applied to MAAV[6]. However, IVIg treatment requires high cost and short supply and has risk of unknown causes of infection due to human blood preparations. Therefore, pediatricians and patient's parents are expecting the advent of recombinant IgGs as therapeutic drugs to evade such risks.

We established a recombinant human single-chain IgG library consisting of 204 clones as a therapeutic drug for intractable vasculitis. The hScFv library was established from peripheral blood mono-nuclear cells of the several ten healthy donors. Therapeutic effect of the combined recombinant hScFv clones from the library is evaluated by using a spontaneous vasculitis mouse model SCG/Kj[7].

Methods

hScFv cDNA library construction

Total RNA including IgG coding mRNA was isolated from the mononuclear cells in the peripheral blood from healthy

donors under approval of the ethical committee in National Institute of Biomedical Innovation, Health and Nutrition Japan (No. 51). The cDNA fragment coding VH-CH1-hinge region of IgG was obtained by using the reverse transcription PCR with the primer set described in Figure 1. The cDNA fragment was inserted into the correct position, which is in front of the hexahistidine tag of the plasmid vector pBAD[8]. The hScFv fragment inserted plasmid vector was chemically transfected to Top10 competent cells (Life Technologies, USA). The hScFv including recombinant library was consisted of 20,000 clones (Figure 1).

Base sequence analysis and cluster analysis of hScFv clones

One thousand recombinant clones were picked up in random from the constructed library. Inserted fragments of each clones were amplified by pBAD primer set, as described in Figure1, then the base sequence of amplified inserted fragments of each clone was analyzed by 3730 DNA analyzer (ABI, USA). Base sequence analysis of inserted fragments was carried out in both direction, forward and reverse read. The base sequence data was analyzed and edited certainly by Factura software (ABI, USA).

Deduced amino acid sequence of each clone was obtained by base sequence results. The amino acid sequence of each hScFv clone was carried out by cluster analysis with ClustalW (open source, URL http://www.clustal.org/clustal2/) software to confirm the difference of each clone[9, 10].

Purification of hScFv protein from the mixed culture cells

Ten ml of mixed clone precultured cells were inoculated to 1L of LB broth supplemented with 50 micro g of Ampiciline. Mixed culture was incubated at 37°C with shaking until OD600=0.6, then arabinose was added to 0.05% to induce hScFv protein expression. After addition of arabinose, culture was continued for 16hr. Cultured cells were collected by centrifugation, and separate cell pellet. The cell pellet was freeze-thawed three times, and suspended in 20 ml of extraction buffer (8 M urea, 10 mMTris pH 7.0), then the cells were disrupted by sonication with 80% output power of VP-050 (TITEC Gunma, Japan). The soluble fraction was separated by centrifuge at 5,000 rpm for 15 min. Soluble fraction was applied to Ni-NTA column including 1ml of resine. The column was

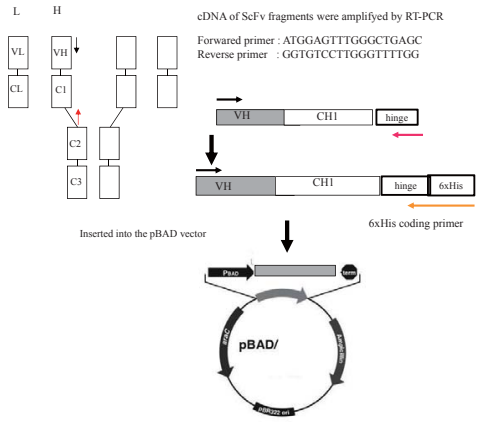

Figure 1. Construction of hScFv library
The cDNA coding VH-CH1- hinge region was amplified by RT-PCR, using primer set described in the figure. Amplified cDNA was attached to hexahistidine tag by primer extension. The fragments were cloned into the pBAD vector, which restricted transcription strictly.

washed with 10 ml of extraction buffer, then washed with extraction buffer supplemented with 5 mM glycine. The resin binding proteins was eluted with 5 ml of elution buffer (8 M urea, 10 mM TRIS pH 7.0, 0.1 M EDTA). Purified protein was examined by SDS-PAGE and western blot to confirm the purity, property and amount[11].

SDS-PAGE and western blot analysis of each clone

hScFv proteins-expressed clones were selected. hScFv expressed clones were cultured individually in mini scale (5 ml culture each), and they induced hScFv protein expression by adding 0.05% arabinose for 16 hrs. 20 micro L of cultured cells were collected by centrifuge, and then the cells were re-suspended in SDS sample buffer (0.05% SDS, 10 mM Tris pH 6.8, 2 mM DTT) and separated on the 4-20% polyacrylamide gel. The separated proteins were transferred to the PVDF membrane electrically, and hScFv on the PVDF membrane was detected by alkaline phosphatase conjugated anti-human Fab2 antibody (Rockland, USA). Isolated hScFv proteins were also applied with the same procedures.

Administration of hScFv for spontaneously vasculitis mouse model SCG/Kj

Mice care was approved by the committee in the National Institutes of Biomedical Innovation, Health and Nutrition (No. DS21-8).

Administration program was established as well as clinical administration of IVIg[4]. The administration was performed with 10-week old female spontaneous vasculitis mouse model SCG/Kj intraperitoneally at doses of 0, 10, 20, and 40 mg/Kg/day of hScFv for 5 days continuously. As a control, according to the application amount of doses, human globulin preparation (Nihon Pharmacy Company, Osaka, Japan) was administered with 400 mg/kg/day for 5 days continuously.

Administration dose was set in five groups, 1) 0 mg/kg/day (solvent), 2) 10 mg/kg/day, 3) 20 mg/kg/day, 4) 40 mg/kg/day, 5) 400 mg/kg/day which was the same dose to IVIg. 10-week old female SCG/Kj mice were administered for 5 days continuously with same dose for each group.

Evaluation of hScFv after administration into SCG/Kj mice

After observation for 3 weeks since administration, blood, kidney, lung, heart, spleen were sampled and weighed. Blood component analysis was performed with blooding and scarified under anesthesia, and excised spleens were weighted. Peripheral blood was examined by a VetScan HMII (Abaxis, USA) to analyze peripheral blood cell counts. Kidney was fixed with 10% formalin and embedded in paraffin block and stained with Hematoxilin and Eosin, then approximately 50 of glomerular crescents in the kidney were counted under a microscope. MPO-ANCA was performed with ELISA methods.

Results

hScFv of VH-CH1-hinge fragment, originated from healthy donor peripheral blood lymphocytes and inserted in the expression plasmid pBAD library, was constructed. One thousand clones were picked up in random. The clones were analyzed for base sequence of inserted VH-CH1-hinge coding region. 245 clones in one thousand could express the protein correctly jointed to histidine tag. 224 clones in 245 were

certainly coding the VH-CH1-hinge. 46 clones were redundant clones. Finally, 204 clones coding unique VH-CH1-hinge-6xHis structure were obtained.

Cluster analysis of deduced amino acid sequence of hScFv

The deduced amino acid sequence of hScFv clones were analyzed by using the clustal W software. 21 clones revealed broken structure of hScFv. Other 223 clones were ordered by resemblance of the sequence, departed in several groups (Figure 2).

Confirm the Expression of hScFv each clone by SDS-PAGE and western blot

hScFv expressed selected clones were cultured individually and they induced hScFv protein expression. After SDS-PAGE separation of each clone, we examined them by western blot with alkaline phosphatase conjugated anti-human IgG and anti-human Fab2 antibody. Each clone was confirmed as a human IgG molecule and Fab2 fragment in correct size of 28 kDa (data not shown).

hScFv protein extraction and purification

Expressed protein in *E. coli* cells from total 30 L culture was extracted by 8 M urea, and purified by Ni-NTA chromatography. Obtained hScFv protein showed over 90% purity by SDS-PAGE analysis, confirmed by western blot analysis by using anti-human IgG Fab2 antibody. Finally, 3 mg of hScFv mixed protein were recovered from 30 L culture. Obtained hScFv eliminated endotoxin by alkaline hydrolysis in the level of under 10 unit/ml.

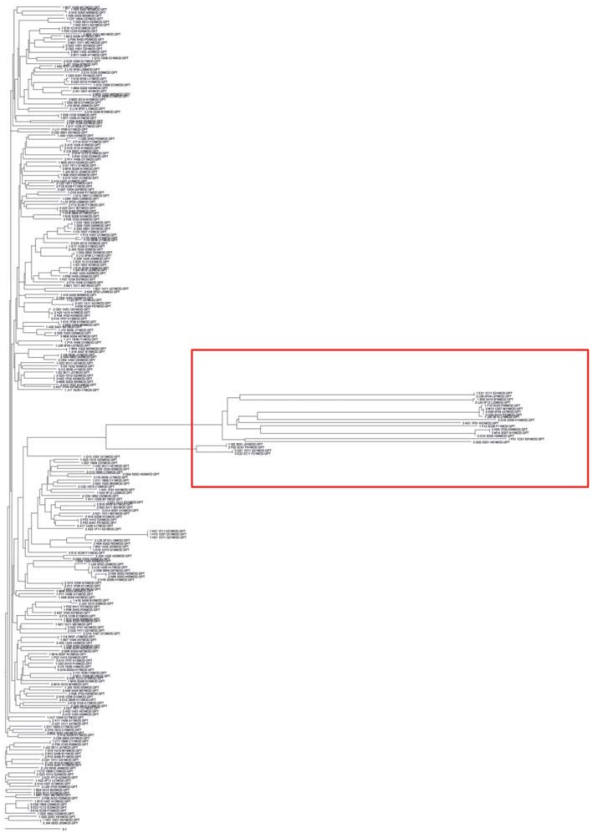

Figure 2. Cluster analysis of hScFv clones
Difference of amino acid sequence of each hScFv clone was analyzed by using Clustal W software. hScFv clones made several clusters by resemblance of amino acid sequence of VH region. The clones with broken structure made separate group in a rectangular of red line.

Final solution of hScFv protein was solved in 0.9 M Arginine, 0.9 %NaCl pH7.4 (Figure 3).

Evaluation of hScFv after administration into vasculitis mouse model SCG/Kj

The glomerular crescent formation in histological analysis of kidney showed significant decrease in the dose of 20 mg/kg/day administration compared to that of control (solvent administration) (Figure 4A). MPO-ANCA titer, a biomarker of vasculitis, which is auto-antibody against to myeloperoxidase showed also significant decrease at the administration dose of 40 mg/kg/day compared to that of control group (Figure 4B). The number of whole blood cell indicated significant decrease

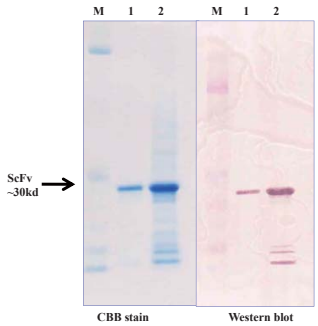

Figure 3. Purified hScFv mix clone proteins
The protein which was purified from hScFv mixed culture were examined by SDS-PAGE and stained by CBB. The separated proteins were blotted to PVDF membrane and examined by immunological detection, using anti-human IgG Fab2 antibody. Lane M: molecular marker, 1:0.1 micro g purified hScFv applied, 2: 1 micro g of hScFv applied.

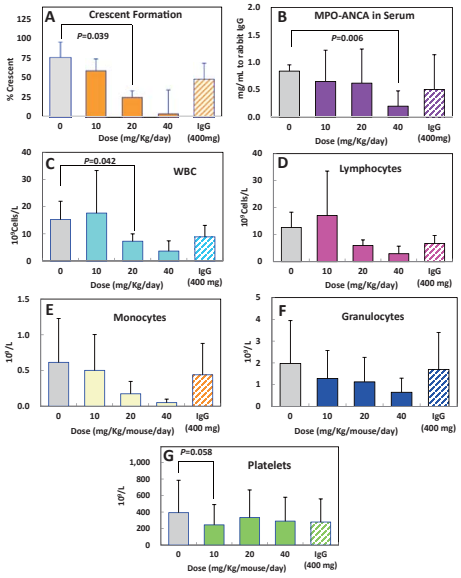

Figure 4. Evaluation of hScFv treatment with kidney histology and biomarkers
A: Crescent formation rate in kidney (orange color bar), The groups of 20 and 40 mg/kg/day administration indicated more decrease of spleen weight. B: MPO-ANCA titer (violet color bar) MPO-ANCA titer was decreased in dose dependent manner. The titer of administration dose 40 mg/kg/day showed remarkable decrease than that of IvIG administration. C: Counts of white blood cells in peripheral blood; whole blood cells (WBC: light blue color bar), D: lymphocytes (red color bar), D: monocytes (yellow color bar), E: granulocytes (dark blue color bar), F: platelets (green color bar). Controls treatment with solvent (gray color bar) and IgG 400 mg/Kg/day for 5 days (hatched bar) in each data. Statistical analysis was performed between solvent control vs treatment group by Student's *t*-test (p-value).

at the dose of 20 mg/kg/day compared to that of control (Figure 4C). The number of lymphocytes, monocytes, granurocytes and platelet in peripheral blood decreased slightly in hScFv administration group, however the deference was not significant (Figure 4 D, E, F, G).

Discussion

A recombinant human ScFv library consisting 204 clones of VH-CH1-hing was established from a 1,000 recombinant clone library. Purified hScFv was recovered from a mixed batch culture of polyclones, and 0.1 to 0.2 mg per 1 L of the final purified preparation was realized, because the production level in *E. coli* was quite low.

The purified hScFv was administered to SCG/Kj mice for examination of the therapeutic effect. After three week observation, the therapeutic results were observed in decreasing the number of the glomerular crescent formation and peripheral whole blood cell in hScFv administered groups. It has obtained comparable results at concentrations of 1/10 to 1/40 of the IgG preparations which are currently used clinically.

It has been showed the possibility for actual formulation as a recombinant gamma globulin. In addition, the solubility of the VH-CH1-hinge complex was considered to be increased to use as a therapeutic drug.

It was possible to obtain an effective soluble preparation at low concentration compared to the gamma globulin preparation. It seems to be hopeful results that we approach the actual drug preparation. Antibody drug development becomes important for the therapeutic strategy in the field of various diseases, which depend on the new resources for new antibody drugs[12]. This study gives a useful new resource for the development of next generation antibody drug.

By collecting effective clones from larger libraries, it is possible to create recombinant gamma globulin with higher effect. These 204 clones library will be useful for infectious diseases.

Conclusion

The administration of the mixed batch of recombinant gamma globulin hScFv 204 clones having the VH-CH1-hinge composition showed inflammation inhibitory activity such as MPO-ANCA.

Acknowledgements

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