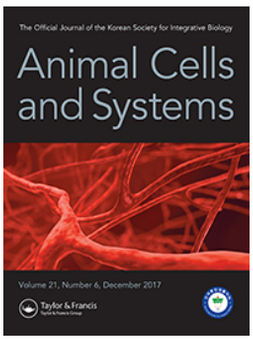

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Development of single-chain Fv of antibody to DNA as intracellular delivery vehicle

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ABSTRACT

Application of antibodies in most therapeutic area is limited to extracellular or membranous targets because of their impermeability of membrane. For the purpose of biotechnological and therapeutic application, developing intracellular localizing antibody is the invaluable research field. A new recombinant single-chain variable fragment of an anti-dsDNA monoclonal antibody G2-6, IgG of which has been previously shown to have a cell-penetrating activity, was engineered and produced for the use as a delivery vehicle of biomolecule(s). The penetrating capacity of single-chain variable fragment in three mammalian cell lines, L929, NIH/3T3, and COS-7 was analyzed using flow cytometry and confocal microscopy. The results demonstrated that the single-chain variable fragment can effectively internalize all three cell lines, although the internalization level varied. It was also shown that the internalization was time- and concentration-dependent. Moreover, the single-chain variable fragment was located in nuclei as well as cytoplasm of L929 cells. Overall, the G2-6 single-chain variable fragment might be a candidate vehicle which could be used to deliver specific genes or biomolecules for therapy or diagnosis into the cytoplasm or cell nucleus.

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Single-chain Fv; antibody to DNA; cell penetration; delivery vehicle

Introduction

Therapeutic reagents have been explored to target cells for modifying diseases. Use of antibodies (Abs) is a hopeful field in the therapy of diseases like cancer as well as biotechnology (Scott et al. 2012). In general, the cell membrane is impermeable to Ab. A subset of Abs, however, has ability to internalize living cells, and some of the internalizing Abs can be localized in cell nuclei (Alarcon-Segovia et al. 1979; Zack et al. 1996; Deng et al. 2000; Alberdi et al. 2001; Suchkov 2001; Ruiz-Argüelles et al. 2003; Jang et al. 2009; Weisbart et al. 2012; Im et al. 2015, 2017), making it possible to overcome the limitation of Ab utility only to extracellular targets. Expanding the use of therapeutic Abs to intracellular targets or antigen molecules can be an invaluable tool for modulating diseases through Ab-target interactions. The internalizing Abs can be engineered to small fragments such as variable region fragment (Fv) or single domain (Holliger and Hudson 2005) for binding to intracellular targets with high specificity, resulting in inhibiting the function of target molecules.

Autoantibodies reactive to double stranded DNA (dsDNA) are typically found in systemic lupus erythematosus and lupus nephritis (Jang and Stollar 2003; Deshmukh et al. 2006; Giles and Boackle 2013; Pisetsky 2016). We have reported that four anti-dsDNA monoclonal Ab immunoglobulin G (IgG), including G2-6 IgG produced from MRL-*lpr/lpr* mice, could internalize viable mouse kidney mesangial (MES) cells with a minimal cytotoxicity (Im et al. 2015). We have recently reported the applicability of VH domain as delivery vehicles for mammalian cells (Im et al. 2017) using the cell-penetrating VH single domain from 2C10 (Jang et al. 1998). Single-chain antibody (scFv), in which Fv is connected by a linker peptide, is also one of the smallest units of Ig molecule with function in antigen-binding activity that can be used for specific drug delivery (Jang et al. 1996; Monnier et al. 2013; Safdari et al. 2016). The scFv can be applied as tools for *in vivo* loss-of-function studies, inactivation of specific protein domains, diagnostic imaging, tumor therapy, treatment for neurodegenerative and infectious diseases (Ahmad et al. 2012; Monnier et al. 2013). The scFv could be easily expressed as a recombinant protein in *E. coli* and also conjugated with a specific molecule due to their considerably reduced size. In this work, we engineered the G2-6 IgG to produce a recombinant scFv connected with a short linker peptide of $(Gly–Gly-Gly–Gly-Ser)_3$

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having tag of six histidines, $(His)_6$. We have explored the possibility to develop the recombinant G2-6 scFv as an intracellular delivery vehicle in mammalian cells.

Materials and methods

Cloning of codon-optimized gene for scFv of G2-6

Genes encoding the VH and VL regions of G2-6 were codon-optimized and synthesized as scFv containing additional phoA leader peptide in N-terminal, and $(His)_6$ tag in C-terminal (878 bases) for detection and purification purposes, and fifteen amino acid linker peptide between the VH and VL sequences in ampicillin-resistant pJ204 plasmid by DNA2.0 company (USA). Nde-1 and Sac-1 restriction enzyme sites were introduced for scFv directional subcloning into bacterial expression vector. The G2-6 scFv digested with Nde-1 and Sac-1 was sub-cloned into Champion pET100/D/lacZ expression vector with Amp resistance (Invitrogen, USA) (Figure 1).

Figure 1. DNA (A) and amino acid (B) sequences of codon-optimized G2-6 scFv and the structure of cloning vector Champion pET100/D/lacZ (C). The sites for restriction enzymes, Nde-1 and Sac-1, for cloning, pho A leader peptide, CDRs of VH and VL, $(GGGGS)_3$ linker, $(His)_6$ tag, and start/stop codon are underlined.

Cell culture

Mouse fibrosarcoma cell line L929, monkey kidney fibroblast-like cell line COS-7, and mouse fibroblast cell line NIH/3T3 were obtained from the American Type Culture Collection (ATCC, USA). Cells were grown and maintained in Dulbecco's Modification of Eagles Medium (DMEM; Gibco®, USA) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in a 37°C humidified incubator supplied with a mixture of 95% air and 5% CO_2.

Purification of IgGs and recombinant fragments

G2-6 scFv constructed in Champion pET100/D/lacZ vector as fusion proteins was transformed into the bacterial expression host, Escherichia coli BL21 Star (DE3) (Invitrogen) for the induction of the protein expression. Isolation of soluble extract from the periplasmic space has been previously described (Jang et al. 1998). The expressed soluble scFv was purified using His-Trap column installed in an AKTA system (GE Healthcare, UK).

Direct binding ELISA

The wells of 96-well polystyrene microtiter plates (Nunc, Denmark) were coated with calf thymus dsDNA (Sigma-Aldrich, USA) at a concentration of 5 μg/ml. After blocking un-coated bottoms of plates with 3% non-fat milk in phosphate buffered saline (PBS), serial dilution of G2-6 scFv protein was added at the concentration as indicated. G2-6 scFv bound to dsDNA was detected with anti-His tag Ab (Abcam, USA) and horse radish peroxidase (HRP)-conjugated secondary Ab (Sigma-Aldrich). 3,3′,5,5′-Tetramethylbenzidine was used as the HRP substrate for color development. Absorbance was measured at 450 nm using an ELISA reader (Bio-Rad, USA).

Analysis of penetration by flow cytometry

Confluent cells were treated with 0.05% trypsin and 0.02% EDTA for 2 min at 37°C. The reaction was stopped by adding culture medium. Cells were pelleted by a 5-min centrifugation at 800 × g, re-suspended in culture medium and incubated with G2-6 scFv at 37°C for 1 h. Unbound G2-6 scFv was removed by washing with PBS and the cells were fixed with 4% paraformaldehyde (PFA). To detect G2-6 scFv that had penetrated into cells, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min followed by washing with PBS and incubation with anti-His tag Ab for 1 h and fluorescein isothiocynate (FITC)-conjugated secondary Ab for additional 1 h additionally. Finally, the cells were

washed with ice-cold PBS. FITC signal of penetrated scFv was analyzed by flow cytometry, counting 100,000 cells for each sample using a FACS Canto II apparatus (Beckton Dickinson, USA) and FlowJo-7 software.

Analysis of penetration by confocal microscopy

To analyze images of the penetrated G2-6 scFv, confocal microscopic experiments were performed. Cells were grown to confluence in 12-well plates on which 18 mm cover slips were seated. Cells were incubated with G2-6 scFv at 37°C for 1 h or indicated periods, washed in ice-cold PBS and fixed with 4% PFA. Cells were then permeabilized with 0.1% Triton-X 100 in PBS for 3 min followed by washing in PBS. The permeabilized cells were treated with anti-His tag Ab, followed by treatment with FITC-conjugated secondary Ab. Finally, cells were washed with ice-cold PBS. Nuclei were stained with Hoechst 33342 (Invitrogen) and the specimens were mounted using mounting medium. Images were acquired using an Axiovert 200 laser scanning microscope (Carl Zeiss, Germany) or model LSM 710 confocal microscope (Carl Zeiss).

Results

Expression and purification of G2-6 scFv

In Figure 1, codon-optimized DNA sequence (Figure 1(A)) and amino acid sequence (Figure 1(B)) with the important sites were presented. The VH (Genbank accession number KM527179) of the engineered recombinant G2-6 scFv uses IGHV1-84 (85.4% similarity with the highest similar germline sequence), IGHD4-1 (100%), and IGHJ3 (97.9%) gene segments. The VL (Genbank accession number KM527177) utilizes IGKV3-7 (95.6%) and IGKJ1 (100%) gene segments. In CDR3 of VH, there are three positively charged arginines. The structure of expression vector pET100/D/lacZ with scFv insert is presented in Figure 1(C).

G2-6 scFv retains the affinity of G2-6 IgG to dsDNA

Before large-scale induction in E. coli BL21(DE) cells, a small-scale pilot experiment of scFv induction was performed to obtain profile of expression. The scFv was continually induced in soluble extract prepared from periplasmic space after 1 h (Figure 2(A)). The soluble scFv was purified from the soluble extract by Ni-NTA affinity column chromatography and analyzed by Western blotting (Figure 2(B)). The dsDNA-binding activity of the G2-6 scFv was tested and compared to that of IgG using direct-binding ELISA (Figure 2(C)). It bound to dsDNA in a concentration-dependent manner up to 50 μg/ml. The scFv & IgG had similar affinity to dsDNA at less than 3.13 μg/ml. From concentration of 6.25 to 25 μg/ml Ab, IgG showed stronger binding affinity than scFv form. However, their affinities were similar at 50 μg/ml.

G2-6 scFv was internalized cells and cell nuclei with dose- and time-dependent manner

To analyze the internalization of the scFv, we firstly performed flow cytometry with three different mammalian cell lines, L929, COS-7, and NIH/3T3, at various concentrations (25, 50, and 100 μg/ml) of scFv (Figure 3(A)). To analyze the internalized scFv, FITC-Geo mean was measured by flow cytometry. FITC signal was increased in a dose-dependent manner in three cell lines. The scFv bound on the surface of cells were internalized. The results are consistent with those of G2-6 IgG form that we have recently obtained using flow cytometrical analysis in MES cells (Im et al. 2015).

Secondly, we observed images of the internalized G2-6 scFv in L929 cells using confocal microscope. Cells were incubated with various amounts (10, 25, 50 100, and 150 μg/ml) of scFv. The internalization level was enhanced with increased concentration (Figure 3(B)). We could observe gradual increase of cell internalization of G2-6 scFv from 10 μg/ml up to 100 μg/ml, and there was no remarkable different level of internalization by 100 and 150 μg/ml of scFv. To find out stability of the internalized scFv within cells, we investigated time-course (10, 30 min, 1, 2, and 4 h) of internalization with 100 μg/ml scFv in L929 cells. The scFv began to be detected from 10 min, with gradually increase with incubation time up to 1 h, and FITC signal was significantly decreased at 2 h and disappeared at 4 h (Figure 3(C)). Therefore, it is demonstrated that the engineered recombinant scFv of G2-6 has ability to internalize mammalian cells in a pattern similar to IgG form of G2-6. Finally, to examine intracellular localization of the scFv, we analyzed Z-stack of the image. Some of the internalized scFv in cytoplasm was translocated into the cellular nuclei (Figure 3(D)).

Discussion

Recombinant G2-6 scFv we engineered retained the affinity to dsDNA similar to that of G2-6 IgG. It internalized mammalian cells and cellular nuclei with dose- and time-dependent manner. It has been reported that positively-charged amino acid residues in CDR3-VH play an important role for the internalization of a subset of anti-dsDNA autoantibodies and their fragments (Im

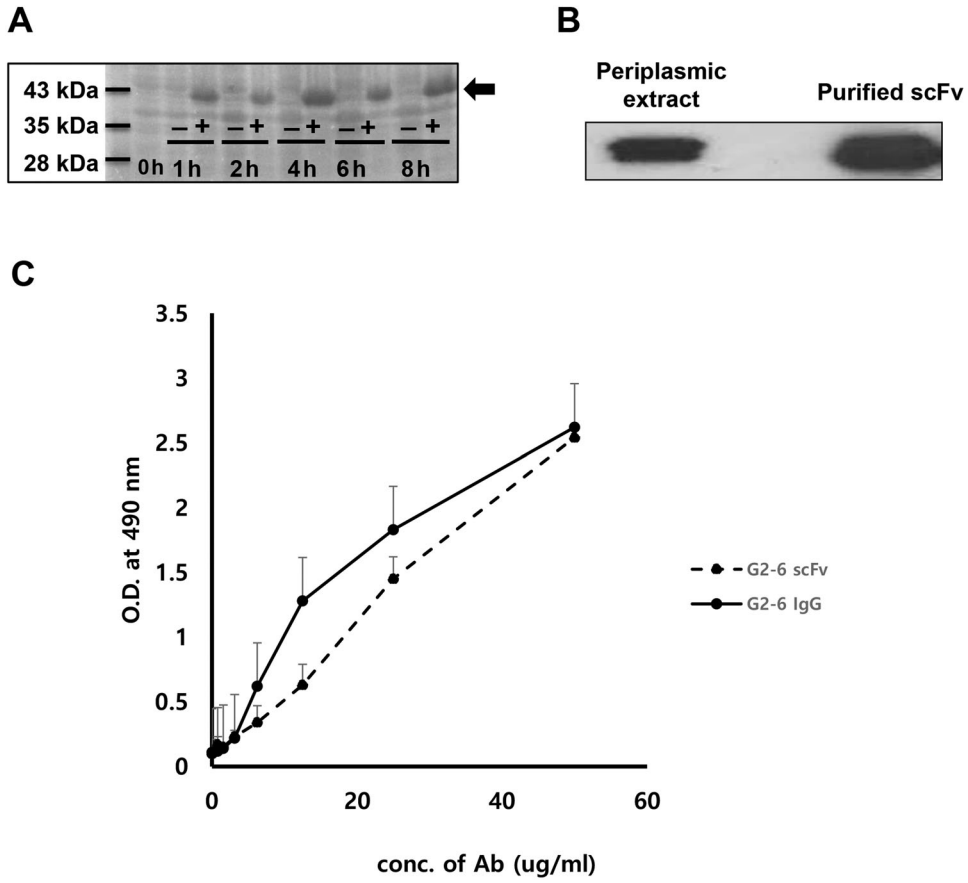

Figure 2. Expression level at various induction time (A) and analysis of G2-6 scFv by Western blotting (B) and DNA-binding ELISA (C). A. Cells with or without IPTG collected at 1, 2, 4, 6, and 8 h were lysed in a small volume of sample buffer and analyzed by 12% SDS-PAGE followed by staining with Coomassie brilliant blue G-250. B. Soluble extract and scFv purified using the histidine affinity column for His-tag were visualized by Western blotting. C. The binding affinity of scFv and IgG of G2-6 were analyzed by dsDNA-binding ELISA. Data are presented as the mean ± S.D. from triplicate experiments.

et al. 2015, 2017; Avrameas et al. 1998; Song et al. 2008). The electrostatic interaction of anti-dsDNA autoantibodies with the negatively charged proteoglycans and phospholipids on the cell surface can be the first step for the intracellular entry of Ab. G2-6 has three arginines in CDR3-VH and adjacent to CDR3 (Im et al. 2015). Therefore, its positively-charged residues could contribute to binding and internalization of cells. The sequence including basic residues (RRELGRGSWFAY) in CDR3-VH may be similar to the nuclear localization sequences (NLS) found in various nuclear localizing proteins (Sperandio et al. 2015; Kataoka et al. 2017). The NLS-like structure might play a role as a possible mechanism of the nuclear translocation of G2-6. A NLS-grafted anti-DNA Ab which internalize only to cytoplasm acquired nuclear localization activity (Jeong et al. 2011). Utilization of internalizing antibody in the form of a recombinant Ab fragment for therapeutic purpose has several advantages; IgG molecule can be engineered to be a reduced-sized; Ab fragment can be produced with tag proteins for the efficient detection and purification; it can be cloned with various molecules; it can be conjugated with various targeted molecules; it can be produced easily in *E. Coli*.

The internalization activity and pattern of the G2-6 scFv were similar to that of IgG. The scFv could enter various types of mammalian cells (mouse fibrosarcoma cells, monkey kidney cells, and mouse embryo fibroblast cells). It is suggested that the crystallizable fragment (Fc) of G2-6 IgG may not mainly be involved in its internalizing cells. The scFv was detected in 10 min and disappeared within a few hours. We have recently reported the cell-internalizing 2C10 VH single domain was shown to be applied as delivery a specific siRNA for mammalian cells (Im et al. 2017). Although the possibility with G2-6 scFv has not been tested yet, it is suggested that like 2C10 VH domain the G2-6 scFv could be linked to valuable biomolecules and may be another candidate for the medical diagnosis and therapy via cellular or nuclear delivery of the molecules. Using G2-6 scFv, a cell-penetrating bispecific Ab for targeting intracellular molecules can be designed for therapeutic purpose, as previously shown (Weisbart et al. 2012). G2-6 scFv

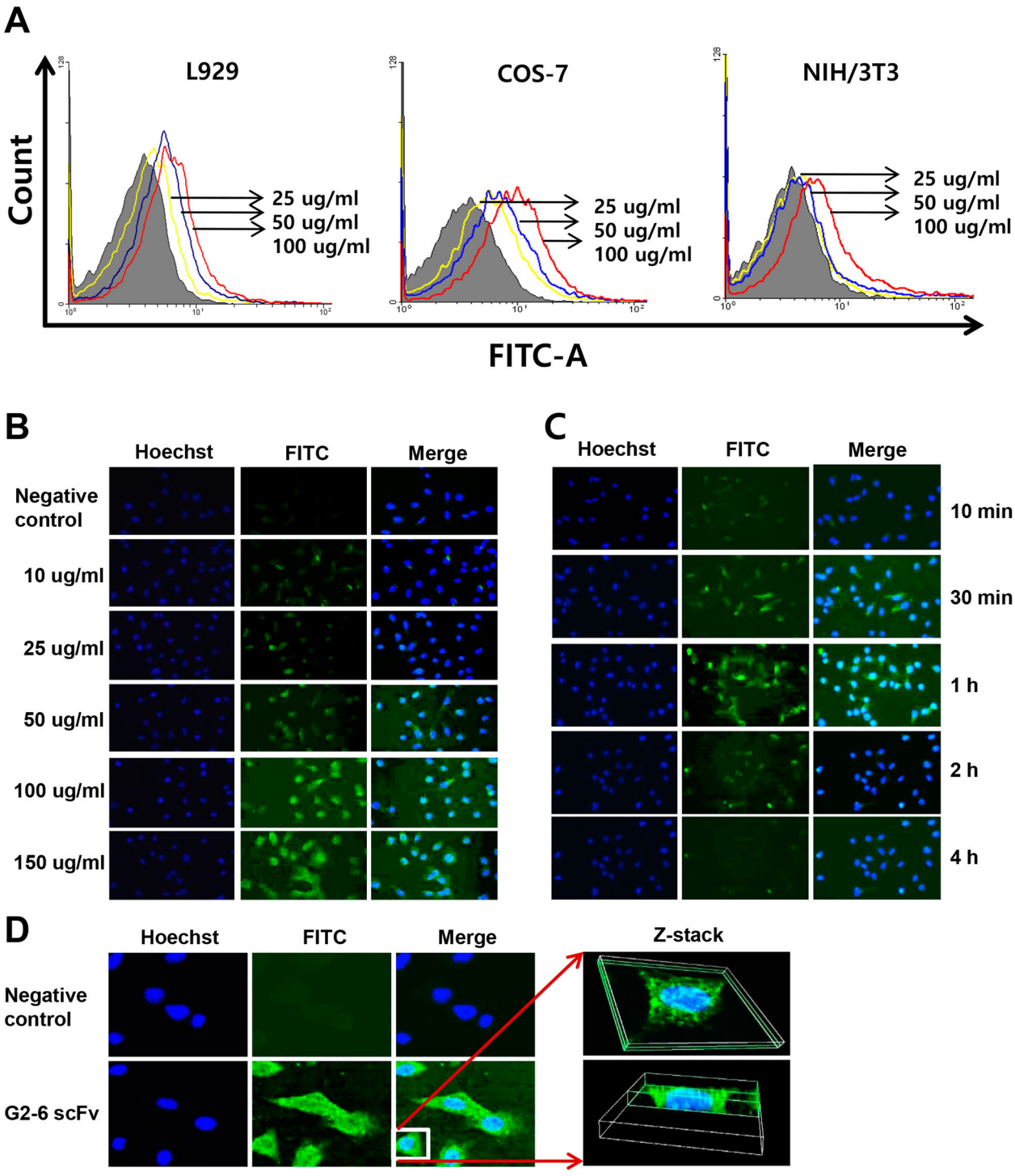

Figure 3. Internalization of G2-6 scFv was analyzed by flow cytometry (A) and confocal microscope (B-D). A. L929, COS-7, and NIH/3T3 cell lines were incubated with various concentrations of scFv for 1 h, and analyzed by flow cytometry. L929 cells were incubated with various concentrations (B) of scFv at for 1 h or with 100 μg/ml scFv for various period of time (C) and analyzed by confocal microscope. (D) L929 cells were incubated with 150 ug/ml scFv for 1 h, and Z-stack analysis of the image was performed.

would also have several advantage for being utilized *in vivo*. It has a short half-lives in circulating blood and low immunogenicity for the lack of an Fc domain. Evaluation of G2-6 scFv as an efficient vehicle for carrying therapeutic cargo molecules such as the conjugated siRNAs and proteins into the intracellular space is critical for its possible use, and now being processed. Cell-penetrating peptides have been also developed and utilized in the fields of therapy and molecular imaging (Kersemans and Cornelissen 2010; Kristensen et al. 2016).

Disclosure statement

No potential conflict of interest was reported by the authors.

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