Effect of disruption of 3D8 complementarity-determining regions on properties of 3D8 antibody

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A Dissertation Submitted to The Graduate School of Ajou University in Partial Fulfillment of the Requirements for the Degree of Master of Biomedical Sciences

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August, 2012
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The Graduate School, Ajou University
June, 22nd, 2012
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Modifications such as substitution, insertion, or deletion of amino acids within or near CDRs (complementarity-determining regions) in antibodies have been utilized to improve antigen-binding affinity and/or stability. However, little is reported about functional consequence by the disruption of whole amino acid sequence in CDRs of an antibody with multiple activities. Here, we dissected influence of each whole CDR-disruption on the function of an antibody, 3D8 scFv (single chain variable fragment; variable region of heavy chain connected to variable region of light chain by (G4S1)3 linker) which has DNA-binding/-hydrolyzing, heparin-binding and cell-penetrating activities. We generated single CDR-disrupted antibodies in the formats of scFv (sc-H/LCDRi; inactivated-CDR of variable domain of heavy or light chain in scFv format), VH (H-CDRi; inactivated-CDR of variable domain of heavy chain single domain format), and VL (L-CDRi; inactivated-CDR of variable domain of light chain single domain format) by replacing the amino acid sequence of each CDRs with (GlySer)n of same length. DNA-binding activity of sc-variants except sc-L1i was similar to 3D8 scFv, and VH variants had higher affinity to DNA than VH wild type. Therefore, L-CDR1 in 3D8 scFv format is likely to be responsible for DNA-binding activity.
Sc-H3i and sc-L3i still retained the heparin-binding activity similar to 3D8 scFv. Moreover, sc-H3i, sc-L3i and H-3i entered the HeLa cells and localized in cytosol of the HeLa cells like 3D8 scFv. It seems that at least H-CDR3 and L-CDR3 in 3D8 scFv format contribute to interaction with heparin and penetration the HeLa cells. For DNA-hydrolyzing activity, sc-L1i, sc-L3i, 3D8 VH and VH’s variants did not hydrolyze DNA. Thus L-CDR1 and L-CDR3 in 3D8 scFv format is not related with hydrolysis of DNA. Next, we examined which CDRs are responsible for the activities of 3D8 antibody using synthesized peptides corresponding to 3D8-CDRs. Only pep-L1 bound to DNA or heparin, however pep-L1 did not enter the HeLa cells. Pep-H1 and pep-H2 entered the HeLa cells, even though much lower penetration efficiency than Tat peptide. Consequently, our results show that effect of the single CDR-disruptions on activities of 3D8 antibody is different between scFv format and single domain format. Only L-CDR1 of 3D8 scFv is important region for DNA-binding/hydrolyzing and cell-penetrating activities.

Keyword: Complementarity-Determining Region (CDR), 3D8 single chain variable fragment (scFv), DNA-binding activity, DNA-hydrolyzing activity, Cell-penetrating activity
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I. INTRODUCTION

1. Antigen-binding site of anti-DNA antibody

Immunoglobulins are composed of polymorphic heavy and light chains. The idiotypic variability is related to the diversity of the antigen-binding site and in particular to the hypervariable domains called complementarity-determining regions (CDRs, Fig. 1). There are 6 CDRs in both variable regions of light (VL) and heavy chains (VH) with background variability on each side of the CDRs (Polonelli et al., 2008). CDRs are separated by longer stretches of sequence which are much more similar in different antibodies (Rahman et al., 1998). They are called the framework regions (FRs) because evidence from crystallographic studies suggests that they form a scaffold from which loops encoded by the CDRs extend to make contact with the antigen (Amzel et al., 1974). Thus the sequences of the CDRs in both variable region of light and heavy chains are particularly important in determining the antigen-binding properties of an antibody (Rahman et al., 1998).

Moreover anti-DNA antibodies include positively charged amino acids, such as arginine and lysine in the CDRs for binding to DNA (Radic and Weigert, 1994; Rahman et al., 1998). The specific binding properties of antibodies are mostly governed by VH-CDR of antibody, it is likely that the antigen-binding sites of polyreactive anti-DNA mAbs and their specific CDRs are also involved in the internalization (Avrameas et al., 2001). However, only...
a few studies characterize detailed binding kinetics and specificities of anti-DNA antibodies (Eivazova et al., 2000; Jin et al., 2004).

2. Modification of antibody by CDR-grafting

Antibodies are important for occurring recognition molecules with high-binding affinity. Most of the approaches have been used for modification of CDRs of antibody to obtain high-binding affinity to DNA, stability improvement of antibody (Jung and Pluckthun, 1997; Jung et al., 1999), formation of functional antibody (Hattori et al., 2008; Petrovskaya et al., 2012). Initially, human-mouse chimeric antibodies were generated by replacing the constant regions of the murine light and heavy chains (Morrison and Schlom, 1990) with those of the human antibodies. The development of techniques made that it is possible to develop humanized antibodies by grafting all the CDRs of a given murine antibody onto the framework of the VL and VH domain of human antibodies, while retaining those murine framework residues that may be critical for the integrity of the antigen-combining site structure (Winter and Harris, 1993). Hattori et al. are found functional antibody by grafting material-binding peptides into the CDR2 loop of the VH domain (Hattori et al., 2008). Petrovskaya et al. reported that they made TNF-binding protein, Hd3 containing sequences CDR-H1 and CDR-H2 of the antibody F10, by CDRs-grafting (Polonelli et al., 2008). In addition, CDRs-grafting can be used to improve the biophysical properties of antibodies by grafting their antigen specificities to a framework with better biophysical
properties (Jung and Pluckthun, 1997; Jung et al., 1999). Previously, we reported that $\text{H}_3\text{Tat}$-3D8, a Tat-grafted antibody ($\text{H}_3\text{Tat}$-3D8) by replacing VH-CDR3 of the 3D8 scFv with a Tat48–60 peptide (GRKKRRQRRRPPQ), retained the DNA-binding, DNA-hydrolyzing, and cell-penetrating activity of the 3D8 scFv in addition to localization into the nuclei of HeLa cells (Jeong et al., 2011). Moreover, Ying-Chyi Song et al. analyzed activity of 9D7, an anti-DNA antibody, to find amino acid in the H-CDRs that is important for the DNA-binding and cell-penetrating activities by point mutations to the arginine residues in the CDR of the recombinant 9D7 antibody (Song et al., 2008).

3. Internalization of anti-DNA antibody

Most of the anti-DNA antibodies have been demonstrated to interact with negatively charged cell surface molecules, such as heparan sulfate proteoglycan (HSPG) expressed on cell surface, without cell-type specificity (Faaber et al., 1986; Zack et al., 1996; Avrameas et al., 2001; Foged and Nielsen, 2008). To inhibit the cell-penetrating activity of anti-DNA antibodies is consistent with role of CDRs in the internalization of the anti-DNA antibodies and Avrameas et al. suggested that interactions with charged molecules may control this process (Avrameas et al., 2001). Also, Song et al. reported that the positively charged amino acids, especially the arginine residues, in the VH-CDR of anti-dsDNA antibody are important site involved in the interaction with negatively charged macromolecules on the cell surface and facilitating internalization of anti-dsDNA antibody (Song et al., 2008). Ying-Chyi Song
et al. demonstrated that point mutations to the arginine residues in the VH-CDR of the recombinant 9D7 antibody significantly attenuate its cell-penetration abilities (Song et al., 2008). Some anti-DNA antibodies, for example H7, 3E10 and G1-5, penetrated the cells and localized in nucleus (Zack et al., 1996; Yanase and Madaio, 2005; Lee et al., 2007). The cell-penetrating activity of anti-DNA antibody is similar to cell-penetrating peptides (CPPs) or CPP-cargo conjugates (Said Hassane et al., 2010). It was discovered that short peptides derived from protein-transduction domains known as CPPs can internalize in most cell types and, more importantly, allow the cellular delivery of conjugated (or fused) biomolecules (Lindgren et al., 2000; Schwarze et al., 2000). A wide range of biomolecules such as antigenic peptides, peptide nucleic acids, antisense oligonucleotides, full-length proteins, or even nanoparticles and liposomes have been delivered in this way (Richard et al., 2003).

Recently studies reported that cell-penetrating anti-DNA antibody with DNA-hydrolyzing activity induces apoptotic cell death unlike CPPs which do not generally exhibit cytotoxicity (Lee et al., 2007). Even though cell-penetrating activity of anti-DNA antibody had not yet been investigated in detail, some polyclonal anti-DNA antibody had shown that their DNA-hydrolyzing activity is correlated with cytotoxicity to tumor cells (Kozyr et al., 2000; Kozyr et al., 2002).
4. DNA hydrolysis of anti-DNA antibody

Catalytic antibodies were first obtained in 1986 (Pollack et al., 1986) against transition-state analogs and are now termed “abzymes” (Abzs), derived from antibody enzyme. Anti-DNA antibodies play an important role in the pathogenesis of systemic lupus erythematosus (SLE) in humans (Shuster et al., 1992). It has been reported that some of the catalytic antibodies to DNA found in SLE patients have nuclease activity and catalyze hydrolysis of the DNA phosphodiester bond (Shuster et al., 1992). The origin of DNA-hydrolyzing catalytic antibodies mainly belonging to immunoglobulin M (IgM) or G (IgG) class has been proposed to be anti-idiotypic antibodies to active sites of nucleases, antibodies produced against DNA or nucleoprotein complexes, and/or antibodies existing in germ line cells even before somatic mutations (Baranovskii et al., 2001; Nevinsky and Buneva, 2002; Dubrovskaya et al., 2003). A number of polyclonal DNA-abzymes have been described, but the detailed biochemical, and structural basis of catalytic mechanisms of monoclonal antibodies have not been extensively characterized (Marion et al., 1997; Nevinsky and Buneva, 2002; Jang and Stollar, 2003). An exception is BV04-01, which binds and hydrolyzes both ss- and dsDNA with preferential cleavages for T-rich ssDNA and CG-rich dsDNA (Gololobov et al., 1997). In addition to intact IgG, IgM, and IgA antibodies, their fragments of Fab, single chain variable fragment (scFv), and/or light chains have been attributed to DNase activities (Kanyshkova et al., 1997; Nevinsky and Buneva, 2002; Song
et al., 2008). Sequence analysis of anti-DNA antibodies from both patients with SLE and murine models of the disease showed that high-affinity anti-dsDNA IgG contains a high proportion of somatic mutations in their VH and VL sequences (Radic and Weigert, 1994; Rahman et al., 1998). In many of these high-affinity anti-dsDNA IgG antibodies, somatic mutations lead to higher frequencies of certain amino acids, particularly arginine, asparagine, lysine, and tyrosine in the CDRs. It has been suggested that the structures of these amino acids allow them to form electrostatic interactions and hydrogen bonds with the negatively charged DNA phosphodiester backbone (Radic and Weigert, 1994).

5. Purpose of this study

The modification in variable domain of antibodies is important to improve the activities of antibodies. However, little is reported about functional consequence by the disruption of amino acid sequence in CDRs of an antibody with multiple activities. Presently, we reported that the CDR-grafting variant of 3D8 scFv still retained all activities of original 3D8 scFv. Based on these findings, we want to know about the effect of disruption of 3D8-CDRs on properties of original 3D8 antibody with multiple activities in this study.
II. MATERIALS AND METHODS

A. Peptides

All the peptides corresponding to 3D8 complementary-determining regions (CDRs) used in this study were synthesized by Peptron Inc. (Korea) with purity >95%. The peptides were labeled with Biotin or FITC at the N-terminus.

B. Plasmid constructions

All of the 3D8 variant genes were synthesized by GenScript Inc. (USA). Synthesized 3D8 variant genes were digested both XmaI and NcoI and then subcloned into the pIg20 vectors (Fig. 2).

C. Cell culture

HeLa cell line was obtained from American Type Culture Collection (ATCC). The cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen), 100 units/ml penicillin, 100 µg/ml Streptomycin (WelGENE, Korea) at 37°C in a humidified atmosphere of 5% CO₂.
D. Bacterial expression and purification of 3D8 variants

pIg20 3D8 variants and pIg20 sc-HW6as negative control were purified from bacterial expression system. The plasmids of 3D8 variant were transformed into *Escherichia coli* BL21(DE3) pLysE cells (Novagen). The transformed cells were grown at 37°C to \(A_{600}\) of ~0.8 in 1 liter Luria-Bertani (LB) medium containing ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml). The expression of 3D8 variant proteins was induced by adding isopropyl 1-thio-β-D-galactopyranoside (0.5 mM) at 23°C for 18 h. After 18 h, the culture supernatant was collected by centrifugation at 9,300 g for 30 min at 4°C. EDTA (5 mM) and tween20 (0.1%) were added to the culture supernatant. The culture supernatant was filtered through a 0.45 µm cellulose acetate filter (Sartorius-stedim biotech). The filtered culture supernatant was loaded to IgGSepharose 6 Fast Flow (GE Healthcare) column at a rate of 1 ml/min. The column was washed with 20 bed volumes of PBST (Phosphate-buffered saline, pH 7.4/0.1% Tween 20), 2 bed volumes of 5 mM ammonium acetate (pH 5.0), and then eluted with 0.1 M acetic acid (pH 3.4) in fraction each (2 ml). Immediately, Tris (1M, pH 9.0) was added to each fraction to neutralize the eluted proteins. The fractions were concentrated by centrifugation at 3,630 g at 4°C using Vivaspin 20 (molecular cut off tube 10,000 Da: Sartorius Stedim biotech), and then buffer was changed to PBS (pH 6.0).
E. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

About 5 µg of purified proteins were mixed with 5X sample buffer (60 mM Tris, pH 6.8/ 50% glycerol/ 2% SDS/ 14.4 mM 2-mercaptoethanol/ 0.1% bromophenol blue/ H₂O) and boiled for 10 min at 100°C. After 10 min, protein samples were analyzed on 12% SDS-PAGE by electrophoresis. The gel was stained with Coomassie blue for 1 h at RT. After 1 h, the stained gel was destained with desatining solution (30% methanol/ 10% acetic acid/ H₂O).

F. Enzyme-linked immunosorbent assay (ELISA)

1) Biotin-labeled peptides

96 well polystyrene microtiter plates (Nunc, Invitrogen) were coated with plasmid DNA (10 µg/ml, plasmid DNA mixture) or heparin (10 µg/ml) at 4°C overnight. Plates coated with plasmid DNA or heparin were washed three times with TBST (10 mM Tris-Cl, pH 7.4/ 150 mM NaCl/ 0.1% tween 20) and blocked by 3% BSA-PBS for 2 h at RT, and then incubated with the biotin-labeled peptides at the different concentrations (0, 1.25, 2.5, 5, 10 µM, diluted in 3% BSA-PBS) for 1 h at 37°C. The peptides bound to plasmid DNA or heparin were washed with TBST three times and incubated with alkaline phosphatase-conjugated streptavidin (Invitrogen, 1: 2,500 diluted in 3% BSA-PBS) for 1 h at RT. After washing three times, ρ-nitrophenyl phosphate (Sigma) solution (1 mg/ml in 0.1 M glycine/ 1 mM ZnCl₂/ 0.1 mM MgCl₂, pH 10.4) was added to each well and absorbance was read at
405 nm in a microplate reader.

2) 3D8 variants

96 well polystyrene microtiter plates (Nunc, Invitrogen) were coated with plasmid DNA (5 µg/ml, plasmid DNA mixture) or heparin (1 µg/ml) at 4°C overnight. Plates coated with plasmid DNA or heparin were washed with TBST three times and blocked by 3% BSA-PBS for 2 h at RT, and then incubated with 3D8 variant proteins at the different concentrations (0, 1.25, 2.5, 5, 10 µg/ml, diluted in 3% BSA-PBS) for 1 h at 37°C. The proteins bound to plasmid DNA antigen or heparin antigen were washed with TBST three times and incubated with rabbit IgG (1 µg/ml, Pierce, diluted in 3% BSA-PBS) for 1 h at RT. After 1 h incubation, the proteins were washed with TBST three times and incubated with alkaline phosphatase-conjugated Goat anti-rabbit IgG antibody (1: 5,000 diluted in 3% BSA-PBS, Pierce) for 1 h at RT. After washing with TBST three times, ρ-nitrophenyl phosphate (Sigma) solution (1 mg/ml in 0.1 M glycine/ 1 mM ZnCl₂/ 0.1 mM MgCl₂, pH 10.4) was added to each well and absorbance was read at 405 nm in a microplate reader.

G. Competitive ELISA

96 well polystyrene microtiter plates (Nunc, Invitrogen) were coated with DNA (5µg/ml) or heparin (1µg/ml) as antigen at 4°C overnight. Plates coated with DNA or heparin were washed with PBST three times and blocked by 3% BSA-PBS for 2 h at RT. 3D8 scFv (5µg/ml) was pre-incubated with heparin or DNA as competitor at different
concentrations (0, 0.5, 5, 50, 500 µg/ml, diluted in 3% BSA-PBS) for 30 min at RT, respectively. The pre-incubated mixtures were added to the plates coated DNA or heparin, respectively. The plates were washed with PBST three times. 3D8 scFv bound heparin or DNA was detected with anti-3D8 scFvAb (1: 5,000 diluted in 3% BSA-PBS), and then AP-conjugated goat anti-rabbit IgG (1: 5,000, diluted in 3% BSA-PBS, Pierce). After washing with PBST three times, p-nitrophenyl phosphate (Sigma) solution (1 mg/ml in 0.1 M glycine/1 mM ZnCl$_2$/ 0.1 mM MgCl$_2$, pH 10.4) was added to each well and absorbance was read at 405 nm in a microplate reader.

H. Surface plasmon resonance (SPR)

Kinetic measurements of protein-ds DNA interactions were performed at 25°C using a Biacore 2000 SPR biosensor (GE Healthcare). The following biotin-labeled ss-(dN)$_{40}$ DNA was used as substrate: ss-(dN)$_{40}$ DNA (N=5’-CCATGAGTGATAACACTGCGGCCAAC-TTACTTCTGACAAC-3’). The ss-(dN)$_{40}$ was synthesized by Integrated DNA Technologies Inc. (USA). After immobilization of 1 µM substrate onto the streptavidin surface of sensor chip SA (Amersham Biosciences) at an immobilized level of 200-800 response units, 3D8 scFv-VH (sc-VH, 0.8-25 nM), 3D8 scFv-VL (sc-VL, 0.4-200 nM), 3D8 single domain-VH (sd-VH, 3.1 nM-0.3 µM) and 3D8 single domain-VL (sd-VL, 20 nM-10 µM) which were serially diluted with HBS-EP buffer (10 mM HEPES, pH 7.4/150 mM NaCl/3 mM EDTA/0.005% (v/v) surfactant P20) were injected over the flow cells at 30 µl/min for 3 min. At the
end of each cycle, bound proteins were removed by injection of 50 mM NaOH containing 25 nM NaCl for 3 min each to regenerate the chip. All kinetic parameters were determined by nonlinear regression analysis according to a 1:1 binding model using the BIA evaluation version 3.2 software. The dissociation constant $K_D$ was calculated using the formula $K_D = \frac{k_{off}}{k_{on}}$, where $k_{off}$ and $k_{on}$ are the dissociation and association rate constants, respectively.

I. Flow cytometry

1) FITC-labeled peptides

HeLa cells were seeded at a density of $5 \times 10^5$ cells/well in 6-well plate. Cells were incubated with 10 µM FITC-labeled peptide corresponding to 3D8 CDRs in TOM TM (Transfection optimized medium, WelGene) for 8 h at 37°C. After 8 h incubation, cells were detached from 6-well plates using trypsin-EDTA, and then moved into the FACS tube (5 ml polystyrene round bottom tube, BD biosciences). For detection of peptides internalized in live cells, cells were washed with ice-cold PBS three times by centrifugation at 393 g for 3 min at 4°C, and then resuspended in PBS. For detection of peptides internalized in fixed cells, cells were washed with ice-cold PBS three times by centrifugation at 393 g for 3 min at 4°C and fixed with 4% paraformaldehyde (PFA)-PBS for 20 min at RT. Live cells and fixed cells were analyzed with flow cytometer (BD FACS Canto II).
2) 3D8 variants

HeLa cells were seeded at a density of $5 \times 10^5$ cells/well in 6-well plate. Cells were incubated with 3D8 variant proteins (5 µM) in TOM$^\text{TM}$ for 6 h at 37°C. After 6 h incubation, cells were washed with ice-cold PBS three times by centrifugation at 393 g for 3 min at 4°C. Cells were fixed with 4% PFA-PBS for 20 min at RT. For detection of internalized variant proteins, cells were permeabilized with permeabilization buffer (P.B., 1% BSA/ 0.1% saponin/ 0.1% sodium azide/ PBS) for 1 h at 4°C. The permeabilized cells were washed with PBS three times by centrifugation at 393 g for 3 min at 4°C and incubated with rabbit IgG 10 µg/ml diluted in P.B. for 1 h at 4°C. Cells were washed with PBS three times by centrifugation at 393 g for 3 min at 4°C and incubated with FITC-anti-rabbit IgG(1: 500 diluted in P.B.) for 1 h at 4°C. After washing with PBS three times by centrifugation at 393 g for 3 min at 4°C, cells were resuspended in 4% PFA and analyzed with flow cytometer (BD FACS Canto II). For detection of surface-bound variant proteins, cells were washed with PBS three times by centrifugation at 393 g for 3 min at 4°C and incubated with rabbit IgG10 µg/ml (diluted in 0.5% BSA/ 2 mM EDTA/ PBS, pH 8.5) for 1 h at 4°C. Cells were washed with PBS three times by centrifugation at 393 g for 3 min at 4°C and incubated with FITC-anti-rabbit IgG(1: 200 diluted in 0.5% BSA/ 2 mM EDTA/ PBS, pH 8.5) for 1 h at 4°C. After washing with PBS three times by centrifugation at 393 g for 3 min at 4°C, cells were resuspended in 4% PFA and analyzed with flow cytometer (BD FACS Canto II).
1. Confocal laser scanning microscopy

1) FITC-labeled peptides

HeLa cells were seeded at a density of $4 \times 10^4$ cells/well in 24-well plate over glass coverslips (12 mm, Deckglaser). Cells were washed with TOM twice and incubated with 10 µM FITC-labeled peptides corresponding to 3D8-CDRs in TOM for 8 h at 37°C. After 8 h incubation, cells treated peptides were washed with ice-cold PBS three times, and then fixed with 4% PFA-PBS for 10 min at RT. Cells on coverslip were mounted in Vectashield anti-fade mounting medium with DAPI (Vector Laboratories), and observed with Zeiss LSM 710 laser confocal microscope and analyzed with Carl Zeiss LSM Immage software.

2) 3D8 variants

HeLa cells were seeded at a density of $4 \times 10^4$ cells/well in 24-well plate over glass coverslips (12 mm, Deckglaser). Cells were washed with TOM twice and incubated with 10 µM 3D8 variant proteins in TOM for 6 h at 37°C. After 6 h incubation, cells were washed with ice-cold PBS three times, and then fixed with 4% PFA-PBS for 10 min at RT. Fixed cells were washed with ice-cold PBS three times, and permeabilized with P.B. for 10 min at RT. After washed with ice-cold PBS three times, permeabilized cells were incubated with rabbit IgG(10 µg/ml) at 4°C overnight. The cells were washed with ice-cold PBS three times and incubated with FITC-anti-rabbit IgG(1: 200, Pierce) for 1 h at RT. Cells on
coverslip were mounted in Vectashield anti-fade mounting medium with DAPI (Vector Laboratories), and observed with Zeiss LSM 710 laser confocal microscope and analyzed with Carl Zeiss LSM Immage software.

K. DNA/RNA-hydrolyzing activity

Supercoiled plasmid DNA (0.3µg) substrate was incubated with peptides (500 nM) for 2 h at 37°C. Supercoiled plasmid DNA (0.3 µg) substrate was incubated with 3D8 scFv variant proteins (800 nM) for 30 min and 2 h at 37°C or 3D8 sd variant proteins (800 nM) for 2 h and 12 h at 37°C. The reaction mixtures were analyzed on 1% agarose gel by electrophoresis. The gel was stained with ethidium bromide (EtBr).

Ribosomal RNA (0.3µg) substrate isolated from HeLa cells was incubated with peptides (500 nM) for 2 h at 37°C. The reaction mixtures were analyzed on 1% agarose gel by electrophoresis. The gel was stained with ethidium bromide (EtBr).
Fig. 1. Schematic diagrams of the basic structure of an immunoglobulin molecule. An immunoglobulin molecule (Rahman et al., 1998). FR; framework region, CDR; complementarity-determining region.
Ⅲ. RESULTS

A. Bacterial expression and purification of 3D8 variants

Synthesized 3D8 variant genes were digested both *Xma*I and *Nco*I and then subcloned into the bacterial expression pIg20 vectors (Fig. 2). pIg20 vector encodes a (G4S1)₃ flexible linker between the VH and VL sequences. All vectors encode a thrombin cleavage site followed by the Staphylococcal protein A (SPA) tag at the C terminus, and a N-terminal bacterial alkaline phosphatase signal peptide for targeting protein expression to the periplasm under control of the T7 promoter. Concentration of variant proteins was determined using extinction coefficients in units of mg ml⁻¹ cm⁻¹ at 280 nm, which were calculated from the respective amino acid sequence (1.995 for scFv, 2.325 for VH, and 1.674 for VL, Fig. 3, Table 1, Table 2).

The purity of purified each protein is ~90% of 3D8 scFv wild type, ~80% of 3D8 sc-HCDRi variants (Fig. 4A and Fig. 5A), ~70% of 3D8 sc-LCDRi variants (Fig. 4A and Fig. 5A), ~70% of 3D8 VH-CDRi (Fig. 4B and Fig. 5B), and ~70% of 3D8 VL-CDRi (Fig. 4B and Fig. 5B). When variants were purified from 1 L culture, the yields of sc-HCDRi variants, sc-LCDRi variants, VH-CDRi, and VL-CDRi were about 3-5 mg, 1-3 mg, 3-5 mg, and 0.5-1 mg, respectively. The average yield of 3D8 VH variants is better than 3D8 VL variants. However we failed to purify the 3D8 VL-1i due to its rapid degradation during purification.
Fig. 2. The bacterial expression vectors.
Fig.3. Original 3D8 scFv amino acid sequences. VH and VL of 3D8 antibody were connected using by (G4S1)₃ linker.
Fig. 4. Structure of 3D8 variants. Diagrams showing the single chain variable fragment (A) and single domain of 3D8 variants (B).
<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Amino acid sequence of original CDR</th>
<th>Amino acid sequence of inactivated CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc-H1*</td>
<td>SYVMH</td>
<td>SGSGA</td>
</tr>
<tr>
<td>sc-H2i</td>
<td>YINPYNDGTK</td>
<td>SGSGSGSGSGSG</td>
</tr>
<tr>
<td>sc-H3i</td>
<td>GAYKRGYAMDY</td>
<td>GASGSGSGSGS</td>
</tr>
<tr>
<td>sc-L1i</td>
<td>KSSQSLFNSRTKRNYLA</td>
<td>SGSGSGSGSGSGSGSGSGSGSGSGS</td>
</tr>
<tr>
<td>sc-L2i</td>
<td>WASTRES</td>
<td>SGSGSGSGSGSG</td>
</tr>
<tr>
<td>sc-L3i</td>
<td>KQSYYHYMT</td>
<td>SGSGASASGT</td>
</tr>
<tr>
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<td>SGSGA</td>
</tr>
<tr>
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* i: inactivation
Table 2. Biophysical informations of 3D8 variants

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<td>VL-3i</td>
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$^a$Unit of M$^{-1}$cm$^{-1}$ at 280 nm measured in water
Fig. 5. SDS-PAGE of purified 3D8 variants. About 5 µg of each protein was analyzed on 12% SDS-PAGE, which was stained with Coomassie Blue. (A) sc-H CDRi and -L CDRi, (B) VH- and VL-CDRi
B. DNA-binding activity of 3D8 variants

Previously we have reported 3D8 scFv that can bind to DNA without significant sequence specificities (Kim et al., 2006) and Tat-3D8 also retained DNA-binding activity, likely to 3D8 scFv (Jeong et al., 2011). We performed ELISA and SPR to quantify the interactions of 3D8 variants with DNA.

For ELISA, 96 well ELISA plate was coated with DNA (5 µg/ml). Plate coated with DNA was incubated with proteins (0-10 µg/ml). Proteins bound DNA were incubated with rabbit IgG as 1st Ab and AP-conjugated goat anti-rabbit IgG as 2nd Ab, and then p-nitrophenyl phosphate solution was added to each well. Absorbance was read at 405 nm in a microplate reader. HW6 scFv used a negative control that specifically bind to human DR5 did not bind to DNA (Fig. 6). Compared to 3D8 scFv in scFv form, sc-H1i, sc-H2i, sc-H3i, sc-L2i, and sc-L3i still bound to DNA, similar to 3D8 scFv (Fig. 6A and B). After 24 h incubation with DNA substrate, sc-H1i bound to DNA and sc-L1i did not bind to DNA (data not shown). In the single domain form, VH-3i bound to DNA, likely to 3D8 VH wt and VL-CDRi mutants, showed significantly lower DNA-binding activity than 3D8 scFv (Fig. 6C and D).

Kinetic measurements of protein-ss DNA interactions were performed at 25°C using a Biacore 2000 SPR biosensor. After immobilization of 1 µM biotin-labeled DNA onto the streptavidin surface of sensor chip SA, 3D8 sc-HCDRi (0.8-25 nM), 3D8 sc-LCDRi (0.4-200 nM), 3D8 VH-CDRi (3.1 nM-0.3 µM) and 3D8 VL-CDRi (20 nM-10 µM) that were serially diluted with HBS-EP buffer were injected, and then all kinetic parameters were determined.
In the scFv form, all scFv variants except sc-L1i showed similar DNA-binding affinity to 3D8 scFv ($4.11 \pm 0.20 \times 10^{-8}$). However, sc-L1i ($K_D = 1.05 \pm 0.03 \times 10^{-4}$) showed about 2500-fold lower DNA-binding affinity against ss-DNA substrate than 3D8 scFv (Table 3). 3D8 VL-CDR variants showed similar $K_D$ value to 3D8 VL wt ($K_D = 2.41 \pm 0.01 \times 10^{-5}$) (Table 3). Interestingly, 3D8 VH-CDR variants showed higher DNA-binding activity than 3D8 VH wt ($K_D = 5.06 \pm 0.02 \times 10^{-6}$) (Table 3). These results suggested that H-CDR1, 2, 3 and L-CDR2, 3 of 3D8 scFv except L-CDR1 do not involved in DNA-binding activity of 3D8 scFv and L-CDR1 is important for DNA-binding activity.
Fig. 6. ELISA for DNA-binding activity of 3D8 variants. DNA (5 µg/ml) were coated on the wells of ELISA plate. The wells were incubated with different concentrations of proteins (0 – 10 µg/ml of 3D8 sc-H CDRi (A), sc-L CDRi (B), VH-CDRi (C), and VL-CDRi (D)) for 1 h at RT. The proteins bound to DNA were detected with rabbit IgG, and then AP-conjugated goat anti-rabbit IgG. HW6 scFv control was used as negative. Experiments were done in triplicate.
**Fig.7. SPR for DNA-binding activity of 3D8 variants.** SPR sensorgrams were obtained from injections of different concentrations of 3D8 sc-H CDRi (0.8-25 nM, (A)), sc-L CDRi (0.4-200 nM, (A)), VH-CDRi (3.1 nM-0.3 µM, (B)), and VL-CDRi (20 nM-10 µM, (B)) variants over biotin-labeled ss-(dN)$_{40}$–immobilized streptavidin (SA) chip at a flow rate of 30 µl/min for 3 min. At the end of each cycle, bound proteins were removed by injection of NaOH (50 mM) containing NaCl (25 nM) for 3 min to regenerate the chip.
Table 3. SPR-derived\textsuperscript{a} kinetic binding parameters for the interaction between 3D8 variants and single-stranded DNA substrate.

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<th>$K_{on}$ (M$^{-1}$ S$^{-1}$)</th>
<th>$K_{off}$ (S$^{-1}$)</th>
<th>$K_D$ (M)</th>
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<td>$(2.37 \pm 0.05) \times 10^3$</td>
<td>$(4.11 \pm 0.20) \times 10^6$</td>
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<td>sc-H1i</td>
<td>$(1.79 \pm 0.00) \times 10^3$</td>
<td>$(3.49 \pm 0.13) \times 10^4$</td>
<td>$(1.95 \pm 0.07) \times 10^7$</td>
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<tr>
<td>sc-H2i</td>
<td>$(2.63 \pm 0.07) \times 10^5$</td>
<td>$(4.75 \pm 0.23) \times 10^3$</td>
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<tr>
<td>sc-H3i</td>
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<td>$(1.03 \pm 0.04) \times 10^7$</td>
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<tr>
<td>sc-L1i</td>
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<td>$(1.14 \pm 0.02) \times 10^2$</td>
<td>$(1.05 \pm 0.03) \times 10^4$</td>
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<tr>
<td>sc-L2i</td>
<td>$(1.82 \pm 0.09) \times 10^4$</td>
<td>$(2.43 \pm 0.09) \times 10^3$</td>
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<td>sc-L3i</td>
<td>$(6.44 \pm 0.20) \times 10^5$</td>
<td>$(7.23 \pm 0.30) \times 10^3$</td>
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<tr>
<td>3D8 VH wt</td>
<td>$(2.18 \pm 0.03) \times 10^3$</td>
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<td>VH-3i</td>
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<td>3D8 VL wt</td>
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<tr>
<td>VL-3i</td>
<td>$(6.16 \pm 0.30) \times 10^1$</td>
<td>$(2.38 \pm 0.01) \times 10^3$</td>
<td>$(3.86 \pm 0.04) \times 10^5$</td>
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\textsuperscript{a}At least five data sets were analyzed using different protein concentrations to determine the $K_D$ values.

\textsuperscript{b}Not determined.
C. Heparin-binding activity of 3D8 variants

Our previous result has shown that 3D8 scFv bind to negatively charged HSPG by electrostatic interaction before internalization into the HeLa cells (Jang et al., 2009). We first investigated whether 3D8 variants bind to heparin, as soluble competitor of HSPG.

The heparin-binding activity of 3D8 variants was similar to DNA-binding activity. Sc-H2i, H3i and L2i bound to heparin less than 3D8 scFv and sc-L3i bound to heparin more than 3D8 scFv (Fig. 8A and 8B). Sc-L1i did not increase the heparin-binding activity despite 24 h incubation with substrate, likely to ELISA results for DNA-binding activity (Fig. 8B). These findings suggested that VH CDR1, 2, 3, and VL CDR2, 3 of 3D8 scFv are not relationships with heparin-binding activity and VL CDR1 in scFv format of 3D8 is likely to be responsible for heparin-binding activity in common with DNA-binding activity (Fig. 8C and D).
Fig. 8. ELISA for heparin-binding activity of 3D8 variants. Heparin (1 µg/ml) was coated on ELISA plate. The wells were incubated with different concentrations of proteins (0 – 10 µg/ml, 3D8 sc-Hi (A), 3D8 sc-Li (B), VH-CDRi (C), and VL-CDRi (D)) for 1 h at RT. The proteins bound to heparin were detected with rabbit IgG, and then AP-conjugated goat anti-rabbit IgG. As negative control, HW6 scFv was used. Experiments were done in triplicate.
D. Competitive ELISA of 3D8 scFv.

We performed competitive ELISA to examine whether 3D8 scFv interacted with DNA can compete to bind to heparin or 3D8 scFv interacted with heparin can compete to bind to DNA. Heparin (1µg/ml) was coated on ELISA plate, and 3D8 scFv was pre-incubated with DNA. The pre-incubated mixture was added to plate, and then 3D8 scFv was detected. Also, DNA (5µg/ml) was coated on ELISA plate, and 3D8 scFv was pre-incubated with heparin. The pre-incubated mixture was added to plate, and then 3D8 scFv was detected. 3D8 scFv bound to DNA decreased in a concentration of heparin as competing antigen dependent manner reaching their maxima at 500 µg/ml of concentration of heparin (Fig. 9A). 3D8 scFv bound to heparin decreased in a concentration of DNA as competing antigen dependent manner reaching their maxima at 500 µg/ml of concentration of DNA (Fig. 9B).
Fig. 9. Competitive ELISA of 3D8 scFv. (A) DNA (5 µg/ml) antigen was coated ELISA plate. 3D8 scFv (5 µg/ml) was pre-incubated with different concentrations of heparin for 30 min at RT. The 3D8 scFv bound heparin was detected with anti-3D8 scFvAb, and then AP-conjugated goat anti-rabbit IgG. (B) Heparin (1 µg/ml) antigen was coated ELISA plate. 3D8 scFv (5 µg/ml) was pre-incubated with different concentrations of DNA for 30 min at RT. The 3D8 scFv bound DNA was detected with anti-3D8 scFvAb, and then AP-conjugated goat anti-rabbit IgG. Experiments were done in triplicate.
E. Cellular internalization of 3D8 variants

Many natural anti-DNA antibodies (Abs) have the ability to translocate across the plasma membrane and localize in the nucleus of mammalian cells, similar to cell-penetrating peptides (CPPs). CPPs are short peptides derived from protein-transduction domains and can be internalized in most cell types and more importantly, allow the cellular delivery of conjugated biomolecules (Pollack et al., 1986; Lindgren et al., 2000). In the previous work, we demonstrated that 3D8 scFv internalize into HeLa cells through initial interactions with HSPGs on cell surface accumulated in the cytosol without translocation to nucleus (Jang et al., 2009).

To confirm the ability to internalize of 3D8 variants and localize in the cytosol of HeLa cells, flow cytometry and confocal laser scanning microscopy were performed. In contrast with 3D8 scFv, sc-H1i, H2i, and H3i still remained near cell membrane (Fig. 10A and 11C). 3D8 scFv bound to cell membrane, and then immediately entered cells (Fig. 8 and 10). But, sc-H1i and sc-H3i bound partly to cell membrane or partly entered cells (Fig. 10A and 11C). Sc-H2i showed stronger binding to cell membrane than internalization (Fig. 10A and 11C). Sc-L1i and sc-L2i neither bind to cell membrane nor localize into the cytosol of HeLa cells (Fig. 10B and 11C). Like 3D8 scFv, sc-L3i bound to cell membrane (Fig. 8B), and then immediately entered cells and localized into the cytosol (Fig. 10B and 11C). These findings demonstrated that cell-penetrating activity of 3D8 scFv was tolerated for disruption to H-CDR3 and L-CDR3 of 3D8 scFv. Likewise with 3D8 scFv variants, VH-1i, VH-2i, and
VH-3i still remained near cell membrane after entered HeLa cells (Fig. 10C, D and 11D). In contrast with 3D8 scFv variants, VH-1i and VH-2i showed stronger binding to cell membrane than internalization (Fig. 10C and 11D). Only VH-3i in the single domain variants entered HeLa cells (Fig. 10C and 11D). VL-2i and VL-3i did not enter the cells, probably because of no binding to cell membrane (Fig. 10D and 11D). These results demonstrated that cell-penetrating activity of 3D8 VH was tolerated for disruption to H-CDR3 of 3D8 single domain.
Fig. 10. Flow cytometry for cell-penetrating activity of 3D8 variants. 3D8 sc-H CDRi (A), sc-L CDRi (B), VH-CDRi (C) and VL-CDRi (D) were incubated for 6 h at 37°C. For detection of internalized variant proteins, cells were fixed and permeabilized for 1 h at 4°C, respectively. The permeabilized cells were incubated with rabbit IgG and FITC-anti-rabbit IgG for 1 h at 4°C, respectively. For detection of surface-bound variant proteins, cells were fixed and incubated with rabbit IgG for 1 h at 4°C, respectively. The cells were incubated with FITC-anti-rabbit IgG for 1 h at 4°C. The cells were resuspended in 4% PFA and analyzed with flow cytometer.
Fig. 11. Confocal laser scanning microscopy for endocytosis of 3D8 variants. No protein and sc-HW6 as negative control (A), sc-wt, 3D8 VH, and 3D8 VL as positive control (B), sc-H and-L CDRi (C), and VH- and VL-CDRi (D) were incubated at 37°C for 6 h. After 6 h, cells were fixed and permeabilized. Internalized proteins were detected with rabbit IgG and then FITC-mouse-anti-rabbit IgG. Scale bar, 10 µM.
F. DNA-hydrolyzing activity of 3D8 variants

Some anti-DNA antibodies derived from the sera of human and mice with autoimmune diseases have shown DNase activities to hydrolyze the phosphodiester bond of DNA (Shuster et al., 1992; Nevinsky and Buneva, 2002). We have reported that anti-DNA antibodies, 3D8 scFv and VL, hydrolyzed both ss- and ds-DNAs in presence of Mg\(^{2+}\) without significant sequence specificities (Kim et al., 2006).

To examine the DNA-hydrolyzing activity of 3D8 variants, 3D8 sc-H and –L CDRi (800 nM) were incubated with supercoiled plasmid DNA at 37°C for 30 min and 2 h (Fig. 12A), and VH- and VL-CDRi (800 nM) were incubated with supercoiled plasmid DNA at 37°C for 2 h and 12 h (Fig. 12B). After incubation with DNA, mixtures of DNA with proteins were analyzed on 1% agarose gel electrophoresis. Sc-H1i (Fig. 12A (a) and (b)) and sc-L2i (Fig. 12A (c) and (d)) hydrolyzed DNA at the same period of time as 3D8 scFv. Sc-H2i and H3i slowly hydrolyzed DNA (Fig. 12A (a) and (b)). Sc-L1i and L3i did not hydrolyze DNA compared to 3D8 scFv (Fig. 12A (c) and (d)). These results showed that L-CDR1 and/or L-CDR3 of 3D8 scFv format plays an important role in DNA hydrolysis. In contrast with 3D8 scFv, H-CDRi variants of single domain did not hydrolyze DNA (Fig. 12B (a) and (b)). Sc-L2i hydrolyzed DNA, like 3D8 scFv and sc-L3i hydrolyzed DNA, like 3D8 VL (Fig. 12B (c) and (d)).
Fig. 12. DNA-hydrolyzing activity of 3D8 variants. About 800 nM of 3D8 sc-H CDRi and sc-L CDRi (A) were incubated with supercoiled plasmid DNA at 37°C for 30 min and 2 h, and 800 nM of VH- and VL-CDRi (B) were incubated with supercoiled plasmid DNA at 37°C for 2 h and 12 h. After incubation, supercoiled plasmid DNA was analyzed on 1% agarose gel electrophoresis. DNaseI was used as positive control, and buffer or sc-HW6 was used as negative control.
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**soFv**

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:- comparable to PBS, +/- 5%, +: 5-30%, ++: 30-50%, +++: 50-80%, ++++: more than 80%
G. Properties of peptides corresponding to 3D8-CDRs

We investigated the activities of the peptides in the H- and L-CDRs of 3D8 scFv, using synthesized peptides corresponding to 3D8 H- and L-CDRs. Pep-Ran used as negative control for cell-penetrating activity and pep-HFR3 used as negative control for all activities were included (Table 5). Wang et al. reported amino acid sequences of pep-Ran to use negative control for cell-penetrating activity (Wang et al., 2010). Pep-HFR3 was selected 10 amino acids in FR3 of VH of 3D8 antibody (Fig. 3). A small basic peptide, Tat, which rapidly translocates through the plasma membrane and accumulates in the nucleus (Brooks et al., 2005) was used as positive control. pI value of peptides was calculated by ExPASy program. pI value of pep-L1 was similar to pep-Tat (Table 5).

To examine the DNA- and heparin-binding activity of peptides, DNA (10 µg/ml) and heparin (10 µg/ml) were coated on ELISA plate. The biotin-labeled peptides were added to plate coated with DNA or heparin. The peptides bound to DNA or heparin were detected with AP-conjugated streptavidin. In the results, only pep-L1 in the peptides corresponding to CDRs of 3D8 antibody bound to DNA or heparin, similar to pep-Tat (Fig. 13A and B). In addition, pep-Ran also bound to DNA or heparin to a lesser extent than pep-L1 (Fig. 13A and B). These findings suggested that high pI value is involved in DNA- and heparin-binding activity.

To investigate the DNA- or RNA-hydrolyzing activities of peptides, supercoiled plasmid DNA or RNA isolated from HeLa cells was incubated with biotin-labeled peptides.
Then, DNA or RNA was analyzed on 1% agarose gel electrophoresis. In the result, all of the peptides did not hydrolyze DNA and RNA, in contrast with 3D8 scFv protein (Fig. 13C and D).

To examine the cell-penetrating activity of peptides, FITC-labeled peptides (10 µM) were incubated for 8 h. After 8 h, cells were analyzed by flow cytometer and confocal laser scanning microscopy. Interestingly, pep-L1 did not enter the cells. Pep-H1 and pep-H2 penetrated through cell membranes and localized both in cytosol and nucleus of HeLa cells, even though much lower penetration efficiency than control pep-Tat (Fig. 13E and F). These findings suggested that there are no direct relationships between heparin-binding and cell-penetrating activities of peptides corresponding to 3D8-CDRs.
Table 5. Peptides corresponding to 3D8-CDRs

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<td>YINPYNDGTK</td>
<td>10</td>
<td>5.8</td>
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<td>pep-H3</td>
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<td>8.4</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>pep-HFR3</td>
<td>KSSSTAYMEL</td>
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<tr>
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<tr>
<td>pep-Tat</td>
<td>GRKKRRQRRRPPQ</td>
<td>13</td>
<td>12.7</td>
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Fig. 13. Properties of peptides corresponding to 3D8-CDRs. About 10 µg/ml of DNA for DNA-binding activity (A) or 10 µg/ml of heparin for heparin-binding activity (B) were coated on ELISA plate. DNA or heparin coated on plates was incubated with different concentration of peptides 0-10 µM for 1 h at RT. The peptides bound DNA or heparin were detected with AP-conjugated streptavidin. Results shown are the average of triplicate measurements and are representative of at least three independent experiments. Supercoiled plasmid DNA (0.3 µg, C) substrate and ribosomal RNA (0.3 µg, D) substrate isolated from HeLa cells were incubated with peptides (500 nM) for 2 h at 37°C. The reaction mixtures were analyzed on 1% agarose gel by electrophoresis. The gel was stained with ethidium bromide (EtBr). DNaseI or RNase A was used as positive control. About 10 µM of FITC-labeled peptides were incubated at 37°C for 8 h. For detection of peptides internalized in live cells (E (a)), cells were detached, and then resuspended in PBS. For detection of peptides internalized (E (b)), cells were fixed for 20 min at RT. Live cells and fixed cells were analyzed with flow cytometer. About 10 µM of FITC-labeled peptides were incubated at 37°C for 8 h. Cells were fixed, and analyzed with confocal laser scanning microscopy (F). Scale bar, 10 µM.

IV. DISCUSSION
Previously, we have reported a Tat-grafted antibody (H$_3$Tat-3D8) in which CDR3 within the VH domain of the 3D8 scFv is replaced with a Tat48–60 peptide (GRKKRRQRRRPPQ). H$_3$Tat-3D8 retained the DNA-binding and DNA-hydrolyzing activity of the scFv, and translocated to the nuclei of HeLa cells (Jeong et al., 2011). Based on our findings, we generated 3D8 variants in this study by replacing whole 6 CDRs within the VH and VL domain of 3D8 with Gly/Ser and peptides corresponding to 3D8 CDR to investigate the influence of whole CDR disruption of 3D8 antibody on the activity of 3D8 antibody.

When we purified variant proteins from bacterial expression system, all of the variant proteins were well expressed and secreted from bacterial culture supernatants, except VL-1i. The yield of sc-HCDRi and 3D8 VHCDRi variants was higher than sc-L CDRi and 3D8 VL CDRi. We were not able to purify VL-1i from bacteria expression system due to rapid degradation during purification steps. After checked purify of 3D8 variants, we performed ELISA or SPR to investigate the DNA-binding activity. The results of ELISA did not match SPR results exactly, except sc-L1i. The sc-L1i weakly bound to DNA in both ELISA and SPR (Fig. 6B, 7A, and Table 3). Seeman et al. reported that the arginine has the potential to interact with the phosphodiester backbone of dsDNA or with cytosine-guanine base pairs (Seeman et al., 1976). It seems that sc-L1i did not bind to DNA because sc-L1i has 6 arginine residues compared with 3D8 scFv that has 10 arginine residues. To know whether VL-CDR1 of 3D8 antibody involved in DNA-binding activity, we confirmed DNA-binding
activity of pep-L1. We found that pep-L1 that has 2 arginine residues bound to DNA, like Tat peptide that has 6 arginine residues (Fig. 13A). In the single domain variants, 3D8 VH variants bound to DNA with about 10-100 fold higher affinities than 3D8 VH wt (Table 3). These findings suggested that VL-CDR1 of 3D8 scFv is likely to be responsible for DNA-binding activity. Likewise, we found that sc-L1i weakly bound to heparin compared to other variants (Fig. 8B) and pep-L1 had higher affinity to heparin than other peptides (Fig. 13B).

Naparstek et al. has reported that heparin as soluble competitor of HSPG inhibits the DNA-binding of antibodies that had been eluted from the kidneys of both humans and MRL-lpr/lpr mice with systemic lupus erythematosus (Naparstek et al., 1990). We wanted to know whether heparin inhibits the DNA-binding activity of 3D8 scFv, competitive ELIAS was performed using heparin or DNA as antigens. As respected, heparin inhibited the DNA-binding activity of 3D8 scFv and DNA inhibited the heparin-binding activity of 3D8 scFv (Fig. 8).

Next, we investigated which 3D8 variants still retain cell-penetrating activity and CDRs are influence on cell-penetrating activity. In the result, sc-H3i, sc-L3i, and VH-3i still retained cell-penetrating activity, similar to 3D8 scFv (Fig. 10 and 11). Therefore, it is likely that VH-CDR3 or VL-CDR3 of 3D8 scFv is not important region for cell-penetrating activity of 3D8 scFv. It seems that pep-L1 did not enter the cells whereas pep-H1 and pep-H2 entered the cells. There is no direct relationship between heparin-binding activity and penetrating activity of peptides. Peptides may take different ways for heparin-binding and
cell-penetrating activities. Also, peptides results about internalization were different from 3D8 variants. The unexpected introduction of immunoglobulins that have a source of bioactive peptide sequences was independent of antibody specificity (Polonelli et al., 2008; Magliani et al., 2009).

We hypothesized that nucleic acid-hydrolyzing activity of 3D8 scFv could be involved in amino acid sequences in CDRs of 3D8 scFv. To confirm the hypothesis, supercoiled plasmid DNA and RNA isolated from HeLa cells were incubated with the peptides corresponding to 3D8-CDRs. All of the peptides did not hydrolyze both DNA and RNA (Fig. 13D and E). Therefore, it suggests that amino acid sequences in 3D8-CDRs are not important to hydrolyze DNA. As expected, sc-L1i that did not bind to DNA also did not hydrolyze DNA (Fig. 12A). Sc-L3i also did not hydrolyze DNA despite binding to DNA (Fig. 12A). Previously, His residues frequently play a role as key active sites for DNA-hydrolyzing activity of many DNases and DNA-abzymes (Suck and Oefner, 1986; Gololobov, 1997), and 3D8 scFv has two His residues in the CDR of VH domain (His-H35) and the CDR of VL domain (His-L94) (Kim et al., 2006). These findings suggest that His-L94 residue would be more important to hydrolyze DNA than His-H35 residue.

V. CONCLUSION
To investigate for the influence of whole CDRs-disruption of 3D8 antibody on the function of 3D8 antibody, we made variants of 3D8 antibody by replacing amino acids from whole CDRs of 3D8 with Gly/Ser. VL-CDR1 of 3D8 scFv is important region for DNA-binding/hydrolyzing and cell-penetrating activities. Effect of the single CDR-disruptions on activities of 3D8 antibody is different between scFv format and single domain format. Based on this study, we could make new functional antibody via CDR-grafting.

REFERENCES


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3D8 항체의 상보성 결정 부위 변이가 3D8 항체의 활성에 미치는 영향

이 지연

(지도교수: 권명희)

항체가 항원과의 결합력 또는 안정화를 증가시키기 위해, 항체의 상보성 결정부위 또는 그 근처의 아미노산을 교체, 삽입 또는 결실 등의 방법을 사용한다. 그러나 다기능 항체의 상보성 결정부위 변이에 의한 항체 기능을 조사한 보고는 거의 없다. 본 연구에서는 3D8 scFv, VH, VL의 각각 상보성 결정부위들을 (complementarity-determining regions; CDRs) 다른 아미노산 (글라이신/세린)으로 교체함으로써, 기존의 3D8 scFv가 가지는 DNA-가수분해능력, DNA-결합능력, 그리고 세포내 첨투능력이 유지되는지를 확인하였다. 홍미롭게도 scFv format 변이체들의 DNA-결합능력은 sc-L1i를 제외한 모든 변이체들에서 3D8 scFv와 유사한 것을 관찰하였고, VH 변이체들은 오히려 3D8 VH 보다 DNA에 대한 결합력이 뛰어남을 확인하였다. ELISA를 통해 혈관 결합능력을 확인한 결과, sc-H3i와 sc-L3i가 3D8 scFv와 유사한 정도의 혈관 결합능력을 갖는 것을 확인할 수 있었고, 이와 동시에 세포 분석과 공조점 현미경 분석을 통해 sc-H3i, sc-L3i와 VH-

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3i의 세포내로의 유입 정도가 3D8 scFv와 유사함을 확인할 수 있었다. DNA 가수분해 활성을 조사한 결과, scFv format에서 DNA에 잘 결합하는 sc-L3i와 DNA에 결합하지 않는 sc-L1i를 제외한 모든 변이체들에서 3D8 scFv와 유사한 DNA 가수분해 활성을 확인하였고, VH 변이체들은 DNA를 가수분해하지 못하며, VL-3i가 3D8 VL과 비슷한 DNA 가수분해 활성을 가지는 것을 확인하였다. 이러한 결과들은, 3D8 항체의 CDRs 전체를 다른 아미노산으로 교체하여도 3D8 scFv의 모든 활성들이 동시에 없어지지 않으며, 특히 3D8 scFv의 중쇄가변영역 CDR3와 경쇄가변영역 CDR3는 3D8 항체의 활성에 관여하지 않는다는 것을 보여주며, 이 부위에 특정 활성을 갖는 아미노산들을 첨가하여 새로운 기능성 항체를 만들 수 있는 가능성을 제시한다.

핵심어: 상보성 결정부위 (complementarity-determining region; CDR), 3D8 single chain variable fragment (scFv), DNA-결합능력, DNA-가수분해능력, 세포내침투능력