**Communication to the Editor**

**A Novel Strategy for Etiologic Factor Removal: Drug-Navigated Clearance System (DNCS)**

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Here, we propose a novel therapeutic concept named drug-navigated clearance system (DNCS), in which a “navigator” decreases the concentration of a target etiologic factor in the blood by steering it to an unusual metabolic pathway. The navigator is composed of protein A (ProA) and dextran sulfate (DexS) and it successfully navigated antibodies (ABs), a model etiologic factor of dilated cardiomyopathy, to hepatocytes in vitro in the presence of low-density lipoprotein (LDL). ProA captured the Fc region of the target antibody while the DexS bound to LDL via the well-known electrostatic interaction. The hepatocytes simultaneously took up LDL via the LDL-receptor and internalized the AB/ProA–DexS complex that was bound to LDL. Therefore, this process demonstrates our attempt to navigate the etiologic factor to an alternate target pathway such as the LDL salvage.

Key words metabolic abnormality; navigator; low-density lipoprotein; low-density lipoprotein receptor

Increase in blood concentration of various etiologic factors has been known to cause different diseases. An effective and direct clinical strategy for reducing the blood concentration of these factors is apheresis. Furthermore, the removal of pathological autoantibodies in the blood has demonstrated therapeutic effects in diseases such as rheumatoid arthritis (RA) and dilated cardiomyopathy (DCM). Many groups reported that DCM is caused by accumulation of several autoantibodies including cardiac myosin and troponin-I. The use of an absorption column in the extracorporeal circulation for the treatment of DCM is currently attracting considerable attention. The navigator, composed of protein A (ProA) and dextran sulfate (DexS), was designed and synthesized. ProA is expected to capture the Fc region of the target antibody while the DexS plays the important role of binding to LDL via the well-known electrostatic interaction. Then, the hepatocytes are expected to take up the AB/ProA–DexS/LDL complexes through their LDLRs, which constitutes our strategy for leading the auto-antibody from the blood stream to the liver. In summary, in this scenario, LDL acts as the mediator between the navigator and LDLR, and the purpose of the present study is to provide the proof of concept evidence for the feasibility of DNCS in an in vitro model. In this study, we chose DCM as the model disease and attempted to navigate the associated antibody to the hepatocytes in vitro using the ProA–DexS DNCS navigator drug.

**Experimental**

**Chemicals** DexS (molecular weight (MW), 36–50kDa) was purchased from MP Biomedical (Aurora, OH, U.S.A.). ProA derived from *Staphylococcus aureus* was purchased from EMD Bioscience, Inc. (La Jolla, CA, U.S.A.). Fluorescein-conjugated rabbit anti-mouse immunoglobulin G (IgG) (F-AB) was purchased from Invitrogen (Carlsbad, CA, U.S.A.). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Conjugation of ProA to DexS** Synthesis of ProA–DexS is depicted in Fig. 1. The hydroxyl group of DexS (125 mg, 0.5 mmol/unit) was activated with N,N’-carbonyldimidazole (CDI, 78 mg, 484 µmol) for 2 h at 37°C in 25 mL of anhydrous dioxane. Hexamethylenediamine (67 µL, 581 µmol) was continuously added to the solutions, which was stirred for 12 h at 37°C and dialyzed against distilled water for 72 h using a membrane filter (10-kDa MW cut-off, CO, Spectrum Laboratories, Inc., Rancho Dominguez, CA, U.S.A.). The chemical structure of compound 2 was determined using proton nuclear magnetic resonance spectroscopy (1H-NMR) with a Bruker NMR spectrometer (400 MHz). The 1H-NMR characteristics were: (400 MHz, D2O, δ ppm): 5.10 (s, 1H of DexS), 3.8–5.0 (m, 2H of DexS), 3.8–5.0 (m, 2H of DexS), 3.8–5.0 (m, 2H of DexS), and 4.8–5.0 ppm (m, 2H of amino methylene linker).

Compound 2 (2 mg) was dissolved in 180 µL of 0.2 M sodium carbonate buffer (pH 9.4) and 4.4 mg of disuccinimidyl tartrate (DST, 12.8 µmol) was added. The reaction mixture was stirred for 1 h at room temperature and filtered with an Amicon membrane ultrafilter (MWCO, 30-kDa, Millipore, Billerica, MA, U.S.A.) to remove the unreacted DST. The ProA solution (100 µL, 1 mg/0.1 mL) was mixed with compound 3, incubated for 40 h at 37°C, and purified using an Amicon
Cellular Uptake of F-AB

The HepG2 cells were seeded in a 96-well multi-plate at a density of 5 × 10³ cells/well and cultured in 100 µL Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, MB Biomedicals, Inc., Eschwege, Germany), 25 U/mL penicillin, and 25 µg/mL streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h. The culture medium was changed with an equal volume of DMEM containing 10% lipoprotein-depleted human serum (LPDS), and the cells were further incubated for 12 h. For the cellular uptake study, 5 ng of LDL, 1.37 µg of F-AB, and 1.88 µg of ProA–DexS were added to the wells and incubated at 37°C for 3 or 24 h. Then, the cells were washed with the 100 µL of phosphate-buffered saline (PBS) and lysed in 50 µL of cell lysis buffer (Cell culture lysis reagent, Promega Corp., Madison, WI, U.S.A.). The fluorescence intensity of the cell lysate was measured using a multiplate reader (1420 Multilabel counter ARVO SX, Wallac, Waltham, MA, U.S.A.).

Statistical Analysis

The data shown in Fig. 4 are presented as means plus or minus standard derivation of the mean. An one way ANOVA was used to determine difference between cellular uptake values of fluorescein-conjugated IgG mediated by ProA–DexS and LDL. The statistical significance of the data was also analyzed by the Student’s t-test.

Results and Discussion

Characterization of ProA–DexS

The chemical structure of compound 2 and the degree of substitution of aminohexyl groups was measured using NMR spectroscopic analysis. The substitution degree was approximately 4 mol% relative to the sugar unit of DexS. To confirm the conjugation of ProA to DexS, a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed (Fig. 2). Under non-reducing condition, the distance that the final product migrated was less than that of the original ProA and its band was broader than the single band of ProA. The evidence of broadening was similar to previously reported results.13) Compound 3 was not stained with Coomassie brilliant blue CBB on the SDS-PAGE (data not shown). Although we could determine the substitution degree with amino group to the sugar unit of DexS using NMR spectroscopic analysis, substitution ratio with Protein A could not be determined due to the technical limitation directly. To make the efforts for eliminating the contamination of unreacted DST, sample was purified using ultrafiltration membrane before the coupling with Protein A. When protein A was added into compound 3 at the molar ration of 1 : 1, unreacted ProA was not detected on SDS-PAGE (Fig. 2). From these results, we considered that there are few unreacted DexS, and almost all of DexS reacted with Protein A.

Cellular Uptake of F-AB by ProA–DexS

To evaluate the efficiency of the ProA–DexS navigator, the cellular uptake of F-AB mediated by the ligand–receptor interaction of ProA–DexS binding to LDL was analyzed in the HepG2 cells (Fig. 3). After the LPDS stimulation,14) LDL, F-AB, and ProA–DexS were added to the culture medium, and the cells were incubated at 37°C for 3 or 24 h. The fluorescence intensity of the lysate is shown in Fig. 4. When the F-AB was incubated with the LDL or ProA–DexS for 3 h, weak fluorescence intensity was observed, which was likely derived from non-specific absorption onto the cell surface. A 3-h incubation of ProA–DexS with LDL and F-AB on HepG2 cells induced a fluorescence intensity that was approximately 3-fold higher than that of the other conditions. Furthermore, the 24-h incubation showed results that were similar to those at 3 h although the background increased slightly.
increase may be attributable to the non-specific interaction between the F-AB and the cell surface because it was observed in all conditions. These results indicate that ProA–DexS mediated the specific uptake of F-AB into the HepG2 cells, and its incorporation was saturated within 3 h. Therefore, the interaction between ProA–DexS and F-AB enabled its navigation to the LDL receptor binding site.

Although this is a preliminary result, for the first time, we have obtained evidence indicating the possible feasibility of our novel DNCS concept. ProA captured the target antibody while the DexS facilitated the binding of the complex to LDL via electrostatic interaction. In addition, we succeeded in navigating the AB to the hepatocytes using the LDL molecule as the “mediator,” which enabled us to design a simpler and smaller navigator molecule. This was in contrast to the considerably larger ProA–LDL navigator molecule that would have been generated in the absence of this current mediator strategy.

ProA binds to all of IgG through Fc region. In clinical site, Protein A and tryptophan conjugated columns are used in immunoabsorption therapy.5,6 Even when these columns remove the IgG molecules in a nonselective manner, therapeutic effects are confirmed clinically. Therefore, therapeutic outcome for DCM can be expected by using ProA conjugated molecules as a navigator. If autoantibody is completely identified, specific navigator could be designed as the situation demands. In that case, we could expect high therapeutic effect based on the DNCS concept.

Here, we navigated the AB to the LDL-R and interestingly, LDL itself can be a target molecule. Cholesterolemia is caused by an accumulation of LDL in the blood, which may be clinically removed by apheresis or medication. The statins are the most commonly used drugs for the treatment of metabolic abnormalities in internal medicine.15,16 They act by suppressing the synthetic pathway of cholesterol and lead to an increase in LDLR expression in liver.15,16 Our current DNCS system enables the navigation of LDL to an alternate metabolic pathway, mediated by a navigator that contains an LDL-capturing moiety.

Conclusion
We developed a novel drug designing concept for treatment of metabolic abnormalities, by synthesizing the ProA–DexS complex, which bound to the pathoetiologial factor, F-AB. Then, the navigating moiety of the complex subsequently facilitated the specific uptake of F-AB into HepG2 cells using LDL as a mediator. We are confident that the DNCS system can be easily adapted for application in other diseases. Moreover, the application of this concept to any metabolic abnormalities and disease can be easily achieved using DNCS-based drug for the diseases of interest including hypercholesterolemia, dialysis amyloidosis, and RA. Finally, therapeutic outcomes of the treatment of metabolic diseases have the potential to be improved by this novel DNCS strategy.

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Conflict of Interest
The authors declare no conflict of interest.
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