Method for Delivering Radiolabeled Single-Chain Fv Antibody to the Brain

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Radiolabeled antibody has attractive features as a therapeutic agent or diagnostic reagent. However, it is difficult for radiolabeled antibody to enter the central nervous system (CNS). The purpose of this study was to develop a method of delivering radiolabeled single-chain Fv (scFv) antibody into the CNS with the transactivator of transcription (TAT), one of the protein transduction domains. Oligonucleotide encoding TAT was linked to the 5′-terminal of the scFv gene. The construct was subcloned into the pET-23b vector, and the recombinant protein was expressed as an inclusion body in *Escherichia coli*. After solubilization and purification, the recombinant protein was oxidatively labeled with ^{125}I, and the radiolabeled recombinant protein was injected intraperitoneally into mice. Six hours later the brains were collected and homogenized, and the protein fractions were prepared by acetone precipitation. The radioactivity in the cerebrum was about 1.6-fold higher in mice administered TAT-scFv than in those administered scFv alone. The radiolabeled TAT-scFv antibody was delivered into the cerebrum more efficiently than radiolabeled scFv antibody without TAT, suggesting that TAT peptide could be a candidate tool for delivering radiolabeled scFv antibody into the CNS.

Key words —— drug delivery, blood-brain barrier, single-chain Fv, transactivator of transcription

INTRODUCTION

Antibodies with high affinity and specificity would be attractive therapeutic agents or diagnostic reagents. For example, radiolabeled antibody is now being evaluated for the treatment of cancer in advanced stages.

The blood-brain barrier (BBB) is present between the blood circulation and brain, and prevents various substances from freely entering the central nervous system (CNS). The BBB enables the CNS to acquire only the substances it needs and to exclude other materials. This mechanism allows the CNS to maintain fine chemical homeostasis. However, since radiolabeled antibody is hydrophobic and has a large molecular weight, it cannot cross the BBB and enter the CNS, and delivering radiolabeled antibody for the treatment or diagnosis of CNS diseases is difficult. The purpose of our study was to develop a method of delivering radiolabeled single-chain Fv (scFv) antibody into the CNS.

The transactivator of transcription (TAT) protein transduction domain (YGRKKRRQRRR) was originally found as a motif of the TAT protein of HIV-1, which has the ability to traverse biological membranes.[1,2] TAT-linked β-galactosidase has been shown to be able to enter the murine brain.[3] In the present study, scFv antibody was genetically linked to TAT, and delivery of the radiolabeled antibody into the brain was evaluated.

MATERIALS AND METHODS

Preparation and Radiolabeling of the Recombinant Protein —— A scFv antibody was constructed from mouse anti-morphine monoclonal antibody MOR131.[4] Oligonucleotide encoding the TAT protein transduction domain was added at the 5′-terminal of the scFv gene using a three-step PCR.[5] Briefly, three sense primers were used in the process to extend the oligonucleotide sequence successively. The same anti-sense primer was used repeatedly. The TAT-scFv insert was subcloned into the pET-23b vector and used for transformation of *Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI, U.S.A.), and the recombinant protein was then expressed as an inclusion body. After washing, the inclusion body was dissolved in buffer A [30 mM Tris–HCl (pH 7.5)-30 mM NaCl] containing 8 M urea and 1 mM dithiothreitol (DTT). The urea was then diluted by dialysis, first against buffer A containing 4 M urea and 1 mM DTT, and then against buffer A containing 1 M urea. Protein concentrations were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

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Coomassie brilliant blue (CBB) staining with bovine serum alubumin (BSA) as the standard.

The recombinant protein was radiolabeled by allowing 27 μg of the recombinant protein to react with 36000 kBq of $Na^{125}I$ and IODO Beads Iodination Reagent (Pierce, Rockford, IL, U.S.A.).[6] The radiolabeled protein was then purified on a PD-10 column (Pharmacia Biotech, Tokyo, Japan) and measured for radioactivity with an ARC-370M gamma counter (ALOKA, Tokyo, Japan). Specific activity of the radiolabeled recombinant protein was estimated to be 78 kBq/μg (4.7×10^6 dpm/μg). ScFv without TAT was prepared and radiolabeled in a similar manner as a control antibody.

Animal Experiment —— The experiment was carried out twice (experiments 1 and 2). In experiment 1, TAT-scFv or scFv with radioactivity of 3×10^6 cpm (low dose) or 9×10^6 cpm (high dose) was injected intraperitoneally into 5 female DDY mice (8 weeks old, Japan SLC). Intraperitoneal injection is technically easier than intravenous injection. It was also used in a previous study by Schwarze *et al*. to evaluate the entry of a recombinant protein into the CNS.[3] The radioactivity in the blood was measured 2, 4, and 6 hr after the injection, after which blood was removed and the mice were killed. The cerebrum (the fraction containing the telencephalon and diencephalon), cerebellum (the fraction containing the mesencephalon, pons, metencephalon, and medulla oblongata), and spleen were removed, homogenized, and centrifuged at 150 g for 10 min at 4°C. The supernatants were then centrifuged at 100000 g for 60 min at 4°C. From the supernatants, proteins were precipitated with 80% acetone and dissolved in SDS gel-loading buffer. The protein fractions were measured for radioactivity. The proteins were then separated by SDS-PAGE, and intensities of the bands corresponding to the recombinant proteins were calculated radioluminographically with BAS1500 (FUJIFILM).

The radioactivity in the protein fractions and the band intensities of the radiolabeled recombinant proteins [photostimulated luminescence (PSL)] were normalized by organ weight (g) and by average radioactivity in blood (cpm/10 μl) at 2, 4, and 6 hr. Then, data were subjected to Student's *t*-test. After normalization, the values from low-dose and high-dose administration were combined and analyzed together in the combined analysis.

In experiment 2, TAT-scFv or scFv with radioactivity of 3×10^6 cpm (low dose) was injected into 8 mice. The data were analyzed in a similar manner as in experiment 1.

RESULTS

In a preliminary experiment, the blood radioactivity levels were measured after intraperitoneal injection of mice. The blood radioactivity peaked at 2 hr, and then decreased to 60% of the maximum at 6 hr. Radioactivity was still detected in blood after 24 hr. In the present experiment, mice were killed at 6 hr. The following percentages of the injected radioactivity were recovered: 0.073% in the cerebrum, 0.03% in the cerebellum, and 0.10% in spleen. The radioactivity in the protein fractions from the cerebrum and cerebellum was normalized by organ weight and by blood radioactivity levels and used as an indicator of delivery efficiency of the radiolabeled scFv antibody. As for blood radioactivity levels, the average radioactivity obtained at 2, 4, and 6 hr was used.

The normalized radioactivity of the protein fractions from the cerebrum, cerebellum, and spleen were measured (Table 1). In experiment 1, Student's *t*-test showed when TAT was added to scFv, the radioactivity was significantly higher in the cerebrum and cerebellum for low-dose administration and combined analysis. In experiment 2, a significant difference was again obtained for the cerebrum. Even when scFv without TAT was injected, radioactivity was detected in the cerebrum and cerebellum. This indicates that a certain amount of scFv alone can enter the CNS and is consistent with the report by Banks *et al*.[7]

Next, the proteins were separated by SDS-PAGE to determine the integrity of the radiolabeled recombinant proteins and to confirm their amounts (Fig. 1). Bands corresponding to the monomeric proteins (molecular weight 33 kDa) and dimeric proteins (molecular weight 66 kDa) were detected. The radioactivity of these bands was measured as PSL for quantitative analysis, and the total band intensities for the monomeric and dimeric proteins were normalized as described above. The normalized band intensities of TAT-scFv in the cerebrum and cerebellum were stronger than those of scFv (Table 2). The radioactivity in the cerebrum was about 1.6-fold higher with low-dose TAT-scFv administration than with scFv administration. Student's *t*-test showed a significant difference for the cerebrum in combined analysis, and for the cerebellum with low-dose administration and in combined analysis. In experi-

Table 1. Normalized Radioactivity in the Protein Fraction Prepared from the Cerebrum, Cerebellum, and Spleen

Experiment 1

	TAT-scFv	scFv	p-value in t-test
Cerebrum (low dose)	1.7± 0.3	1.1± 0.1	0.009[b)]
Cerebrum (high dose)	1.6± 0.3	1.4± 0.2	0.26
Cerebrum (combined)	1.7± 0.3	1.2± 0.2	0.0034[b)]
Cerebellum (low dose)	2.6± 0.4	1.6± 0.5	0.0064[b)]
Cerebellum (high dose)	2.1± 0.6	2.1± 0.2	0.87
Cerebellum (combined)	2.4± 0.5	1.8± 0.4	0.024[a)]
Spleen (low dose)	4.6± 1.2	4.0± 0.3	0.38
Spleen (high dose)	5.3± 0.4	5.3± 1.2	0.98
Spleen (combined)	5.0± 0.9	4.6± 1.0	0.42

Experiment 2

	TAT-scFv	scFv	p-value in t-test
Cerebrum (low dose)	8.9± 0.7	7.3± 0.8	0.013[a)]
Spleen (low dose)	36 ±11	39 ±16	0.45

Radioactivity in the protein fractions was normalized by organ weight and by blood radioactivity level and analyzed as described in the MATERIALS AND METHODS section. The averages and standard deviations of the normalized radioactivity are shown ($n = 5$ for experiment 1; $n = 8$ for experiment 2). *a*) and *b*) show p-values in t-test of less than 0.05 and 0.01, respectively.

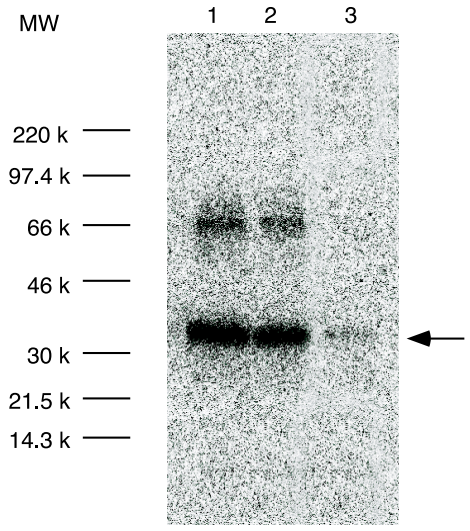

Fig. 1. SDS-PAGE Analysis of Radiolabeled scFv in the Cerebellar and Cerebral Protein Fractions

Radiolabeled TAT-scFv in the protein fractions from the cerebellum (lane 1) and cerebrum (lane 2) were separated by SDS-PAGE and visualized with BAS1500. Radiolabeled TAT-scFv administered to mice was also analyzed (lane 3). Strong bands indicated by the arrow (molecular weight of 33 kDa) are monomeric recombinant proteins. The weaker 66-kDa bands are dimeric proteins. Molecular weight markers are shown on the left side of the panel.

ment 2, a significant difference was also obtained for the cerebrum. The results show that TAT is effective in delivering scFv antibody into the CNS. The difference in normalized band intensities in the protein fractions from the spleen after TAT-scFv and scFv administration was not greater than that in the cerebrum.

DISCUSSION

Radiolabeled antibody is considered to be a clinically important reagent, but its drug delivery to the CNS is hampered by the BBB. In this study, the brain delivery of radiolabeled antibody was evaluated when scFv was linked to TAT. The results demonstrated that TAT promotes the delivery of radiolabeled scFv antibody into the CNS. Enhanced delivery was observed in the mouse cerebrum and cerebellum. In a control experiment, entry of the labeled molecule into the spleen was analyzed as a peripheral organ, although only slightly enhanced delivery was seen.

Recently, Kilic *et al*. reported brain delivery of a recombinant glial cell line-derived neurotrophic factor (GDNF) linked to TAT.[8)] In their study, green fluorescent protein (GFP) linked to TAT was used

Table 2. Normalized SDS-PAGE Band Intensity in the Cerebrum, Cerebellum, and Spleen

Experiment 1

	TAT-scFv	scFv	*p*-value in *t*-test	TAT-scFv /scFv
Cerebrum (low dose)	0.14 ± 0.044	0.088 ± 0.032	0.058	1.6
Cerebrum (high dose)	0.12 ± 0.052	0.078 ± 0.0064	0.096	1.5
Cerebrum (combined)	0.13 ± 0.047	0.083 ± 0.022	$0.0078^{b)}$	1.6
Cerebellum (low dose)	0.67 ± 0.22	0.33 ± 0.14	$0.017^{a)}$	2.0
Cerebellum (high dose)	0.45 ± 0.21	0.36 ± 0.069	0.39	1.3
Cerebellum (combined)	0.56 ± 0.23	0.34 ± 0.11	$0.015^{a)}$	1.6
Spleen (low dose)	1.7 ± 1.6	0.90 ± 0.99	0.38	1.9
Spleen (high dose)	2.1 ± 2.6	0.86 ± 0.81	0.35	2.4
Spleen (combined)	1.9 ± 2.0	0.88 ± 0.85	0.17	2.2

Experiment 2

	TAT-scFv	scFv	*p*-value in *t*-test	TAT-scFv /scFv
Cerebrum (low dose)	0.13 ± 0.019	0.098 ± 0.023	$0.013^{a)}$	1.3
Spleen (low dose)	0.28 ± 0.068	0.19 ± 0.055	$0.011^{a)}$	1.5

The SDS-PAGE band intensity of the recombinant proteins was normalized by organ weight and by blood radioactivity level and analyzed as described in the Materials and Methods section. The averages and standard deviations of the normalized band intensities are shown ($n = 5$ for experiment 1; $n = 8$ for experiment 2). *a*) and *b*) show *p*-values in *t*-test of less than 0.05 and 0.01, respectively.

as a control. The study demonstrated delivery of GDNF into the CNS based on biochemical and neurologic markers.

In our study, radiolabeled scFv recombinant antibody was delivered into the CNS using TAT, and radiolabeled scFv antibody without TAT was used as a control. Both our study and that of Kilic *et al.* suggest that TAT can be utilized to deliver a wide range of recombinant proteins into the CNS.

Many CNS diseases, such as stroke, multiple sclerosis, and epilepsy are associated with breakdown of the BBB,[9–11] and in such cases drug delivery into the CNS might be easier. However, it is reported that the BBB is not impaired functionally to a large extent in most CNS diseases, and the methodology described here can be applied to drug delivery in those diseases.

One example of a CNS disease in which the BBB continues to function is brain tumors. The functional integrity of the BBB in patients with brain tumors has been a matter of controversy for a long time, but the nature of the BBB in brain tumors has recently been better understood and the BBB is believed to maintain its function in brain tumors, at least in some cases. For example, experimental ethylnitrosourea-induced glomas, grade 2 astrocytomas, and many oligodendrogliomas have very tight capillaries, resembling those of the normal brain.[12] 9L rat glioma exhibits increased permeability to small molecules but not to large molecules.[13] Since antibodies have high molecular weights, delivery to the brains of patients with glioma will probably be a problem. Radiolabeled tumor-specific antibodies linked with TAT will provide a new therapeutic or diagnostic tool in patients with these brain tumors.

Cationization of the molecules with hexamethylenediamine is another method of enhancing the brain delivery of antibody,[14] but a drawback of this method is that direct derivatization of crucial Asp or Glu residues in the antigen-binding site might abrogate its binding affinity.[15] Thus it is important to develop a new method of delivering antibody to the CNS. Some other protein transduction domains, such as the one found in *Drosophila* homeotic transcription factor encoded by the antenapedia gene, and Pep-1, are other candidates as methods for the brain delivery of radiolabeled antibody.[16,17]

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