Effects of metal ions on the structure and activity of a human anti-cyclin D1 single-chain variable fragment AD5

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Abstract. Cyclin D1 has become a potential target for anti-tumor therapy. Recently, a novel human anti-cyclin D1 single-chain variable fragment (AD5) was identified, which demonstrated specific binding activity to cyclin D1 and exhibited anti-tumor effects. However, the detailed characteristics of AD5 remain unclear. In the present study, the structure and activity of AD5 in the presence of copper II (Cu^{2+}) or iron III (Fe^{3+}) metal ions was investigated by fluorescence spectroscopy, synchronous fluorescence and enzyme-linked immunosorbent assay. Cu^{2+} and Fe^{3+} were able to bind to AD5 and quench the fluorescence intensity of AD5 primarily by static quenching, which slightly altered the conformation of AD5 at temperatures of 293, 298 and 303 K; however, these temperatures demonstrated different effects on the activity of AD5. These results may be of value for the clinical application of anti-cyclin D1 single chain antibodies in the future.

Introduction

As an important regulator of cell cycle, cyclin D1 is frequently associated with tumor occurrence and development (1,2). The gene encoding cyclin D1 is an oncogene that represents the second most frequently amplified locus in a diverse range of human cancers (3). Overexpression of the cyclin D1 protein has been described in a variety of human cancers (1,4-6). Previous studies have demonstrated that the expression of cyclin D1 is an important indicator of therapeutic efficacy for a number of therapeutic agents, including all-trans retinoic acid, which is closely associated with prognosis (7). Due to its frequent deregulation and role in cancer development, cyclin D1 has become a potential target for anti-tumor therapy.

Antibodies have become powerful therapeutic and diagnostic tools, as they bind target antigens with a high degree of specificity and affinity. An antibody in a single-chain fragment variable (scFv) format is constructed by combining the heavy and light chain variable region via a flexible linker peptide. It has a variety of applications in biotechnology and clinical medicine, particularly in the field of oncology, due to its specific binding affinity to target antigens, its small size and ease of engineering (8-12). In a previous study, a novel human scFv antibody (AD5) was prepared, which demonstrated specific binding affinity to cyclin D1 (13). Intracellular AD5 was observed to significantly inhibit tumor cell growth and proliferation, which provided a novel potential tool for targeting cyclin D1 for the treatment of cancer (14,15). However, the mechanism of action and biological characteristics of AD5 remain unclear.

Metal ions in the blood and aqueous solution may affect protein interactions, as metal ions serve a role in protein folding, assembly, stability, conformation and activity (16,17). Trisler *et al* (18) designed an antibody that forms an irreversible complex with a protein antigen in a metal-dependent reaction. Such irreversibly binding antibodies may facilitate the development of next generation reactive antibody therapeutics and diagnostics. Iverson *et al* (19) constructed a catalytic metalloantibody (QM212) with a coordinate site for metals in the antigen-binding pocket. They utilized fluorescence spectroscopy to clarify the binding affinity between the antibody and different metal ions. Copper II (Cu^{2+}) and iron III (Fe^{3+}) are important trace elements in the human body, and affect the structure and function of a variety of proteins (20). Therefore, to improve the use of these antibodies in a clinical setting, it is imperative to investigate the effects of metal ions on the structure and activity of antibodies.

In the present study, the structure and activity of AD5 in the presence of Cu^{2+} or Fe^{3+} was investigated by fluorescence spectroscopy and synchronous fluorescence. The quenching constants were obtained at various temperatures. The number of binding sites for the metal ions was determined, as were

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the binding constants and the effect of different conditions. In addition, the effects of Cu^{2+} and Fe^{3+} on the biological activity of AD5 were investigated using enzyme-linked immunosorbent assay analysis (ELISA). The results verified the biochemical and biophysical characteristics of AD5, and supported the use of an anti-cyclin D1 single chain antibody in future clinical applications.

Materials and methods

Materials. AD5 and cyclin D1 were purified as previously described (13,21). A spectroscopic sample of AD5 was prepared in phosphate-buffered solution (PBS) at pH 7.4. The concentration of purified AD5 and cyclin D1 was determined using a Bradford assay (Sangon Biotech Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The anti-V5-tag antibody (cat. no. M1008-2) was purchased from Hangzhou HuaAn Biotechnology Co., Ltd. (Hangzhou, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (cat. no. SA00001-1) was purchased from the ProteinTech Group, Inc. (Chicago, IL, USA). Bovine serum albumin (BSA) and o-phenylenediamine (OPD) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). $CuCl_2$ and $FeCl_3$ were purchased from Beijing Chemical Works (Beijing, China). All other chemicals were of analytical grade.

Apparatus. Fluorescence and synchronous fluorescence measurements were performed on an RF-5301PC Spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with 1.0 cm quartz cells. Data were analyzed using Origin software (version 8.0; OriginLab Corporation, Northampton, MA, USA).

Measurement of fluorescence spectra. The concentration of AD5 was maintained at a constant level ($2x10^{-6}$ M), whereas the concentration of the metal ions in solution ($CuCl_2$ and $FeCl_3$) was varied (0.00, 0.33, 0.67, 1.00, 1.33 and $1.67x10^{-3}$ M) by adding 0, 2, 4, 6, 8 and 10 μl $CuCl_2$ or $FeCl_3$ (0.1 mM) to 600 μl PBS. In each fluorescence spectrum test, a fixed concentration of AD5 and a series of solutions were mixed in a 1 ml quartz cell, and incubated for 10 min at 293, 298 and 303 K. Fluorescence quenching spectra were recorded with emission wavelengths that ranged from 290 to 500 nm, and an excitation wavelength of 280 nm. The excitation and emission slits were set at 5 nm. The absorbance of the system was not high enough to consider inner filter effects, which are caused by the absorption of excitation and emission radiation. Therefore, inner filter effect calculations were not included in the fluorescence studies.

Measurement of synchronous fluorescence. Synchronous fluorescence spectra of AD5 were obtained by simultaneously scanning the excitation and emission spectra. The wavelength intervals ($\Delta\lambda$) between the emission and excitation wavelengths were individually fixed at 15 and 60 nm, at which the spectrum only demonstrated the spectroscopic behavior of Tyr and Trp residues in AD5, respectively. The concentration of the metal ions and AD5 were the same as the steady-state fluorescence measurement.

Formulas. The Stern-Volmer quenching constant (K_{sv}) and the bimolecular quenching rate constant (K_q) were calculated according to the following Stern-Volmer equation: $F_0/F=1+K_{sv}[Q]=1+K_q\tau_0[Q]$ (22), where [Q] is the quencher concentration, K_{sv} is the Stern-Volmer quenching constant, K_q is the bimolecular quenching rate constant and $\tau0$ is the lifetime of the fluorophore in the absence of quencher, which is of the order of 10^{-8} s. For the static quenching, the binding constant and number of binding sites were calculated according to the following equation (23): $\log[(F_0-F)/F]=\log K_b+n\log[Q]$, where n is the number of binding sites for one AD5 molecule, which can be respectively obtained from the ordinate and slope of the double logarithmic regression curve of $\log[(F_0-F)/F]$ vs. $\log[Q]$ based on the equation.

ELISA analysis. The binding activity of AD5 to cyclin D1 was evaluated using an ELISA, according to a previous study (13). A total of 100 μl purified human recombinant cyclin D1 (10 μg/ml) was coated onto the surface of wells in a 96-well microtiter plate (Thermo Fisher Scientific, Inc., Waltham, MA, USA) overnight at 4˚C. Non-specific binding sites were blocked with 0.05% Tween-20 in PBS (PBST) containing 5% non-fat milk for 1 h at room temperature. The wells were incubated with 100 μl AD5 treated with Cu^{2+} or Fe^{3+} (at the same concentration as the above fluorescence spectra analysis) for 2 h at 37˚C. Following 3 washes with PBST, anti-V5-tag antibody (1:5,000 dilution in PBST containing 3% non-fat milk) were added to each well and incubated for 2 h at 37˚C. The plate was washed with PBST followed by incubation with HRP-conjugated goat anti-mouse IgG (1:5,000 dilution in PBST containing 3% non-fat milk) for 1 h at 37˚C. The reaction was developed by adding 200 μl OPD substrate (1 mg/ml in citrate-phosphate buffer with 0.02% hydrogen peroxide) and the absorbance was measured using a microplate reader (Thermo Labsystems, Santa Rosa, CA, USA) at a wavelength of 492 nm. Microplates were incubated with the equal volume of BSA instead of cyclin D1 as a negative control.

Statistical analysis. Experiments were performed in triplicate and the reported values are representative of three independent experiments. Data are expressed as the mean ± standard deviation of three parallel measurements within the same experiment. Comparisons among multiple groups were analyzed by one-way analysis of variance with a Bonferroni post hoc test. Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cu^{2+} and Fe^{3+} induce the fluorescence quenching of AD5. Fluorescence spectroscopy is considered to be a comprehensive method for determining protein-ligand interactions. In the present study, fluorescence spectroscopy was utilized to investigate the interaction between AD5 and Cu^{2+} or Fe^{3+}. When small molecules interact with AD5, the intrinsic fluorescence fluorophores (Trp, Tyr and Phe) may be altered depending on the impact of such interaction on the protein conformation (24,25). As demonstrated in Fig. 1, as the metal

ion concentration increased, the fluorescence intensity of AD5 markedly decreased at temperatures of 293, 298 and 303 K. Therefore, Cu^{2+} and Fe^{3+} quench the fluorescence intensity of AD5 at different temperatures, and may alter the microenvironment and conformation of AD5.

Quenching mechanism analysis. The two types of fluorescence quenching mechanisms, including the dynamic quenching mechanism and the static quenching mechanism, differ depending on temperature. The dynamic quenching constant increases as the temperature rises, while the static quenching constant is reduced accordingly. To verify the quenching mechanism of Cu^{2+}-AD5 and Fe^{3+}-AD5 complexes, the fluorescence intensities in the absence or presence of a quencher (F_0/F) was plotted against the concentration of Cu^{2+} or Fe^{3+} (Fig. 2).

The results deduced by the Stern-Volmer equation are shown in Table I. The standard deviation provides a measure of how much the observed values differ from the values provided by the regression line. A low standard deviation indicates that the data points were similar to the values obtained from the regression line. For dynamic quenching, the maximum K_q of various quenchers with a biopolymer is $2.00x10^{10}$ M^{-1} s^{-1} (26). As these K_q values are markedly greater than the maximum collisional quenching constant ($2.00x10^{10}$ M^{-1} s^{-1}), it was concluded that the static quenching mechanism served a dominant role in the Cu^{2+}-AD5 and Fe^{3+}-AD5 interaction (26).

Evaluation of the binding constant (K_b) and the number of binding sites. For the static quenching, the number of binding sites for one AD5 molecule can be respectively obtained from the ordinate and slope of the double logarithmic regression curve (Fig. 3) of log [(F_0-F)/F] vs. log [Q] based on the equation. The number of binding sites and K_b values were calculated and presented in Table II. The number of binding sites (n) was approximately 1, indicating that there was one binding site in AD5 for Cu^{2+} or Fe^{3+} (Table II). In addition, K_b values calculated for the Fe^{3+}-AD5 and Cu^{2+}-AD5 system suggested that the Fe^{3+}-AD5 system binding affinity increased with increasing temperature, whereas Cu^{2+}-AD5 system binding affinity was greatest at 298 K (Table II).

Analysis of AD5 conformational alterations. Synchronous fluorescence spectroscopy is used to analyze the microenvironment of amino acid residues and to evaluate protein conformation by measurement of the emission wavelength shift, as the maximum emission wavelength of Trp or Tyr residues is associated with the polarity of their environment (27-29). The synchronous fluorescence spectra exhibit the spectral character of Tyr and Trp residues when the $\Delta\lambda$ is 15 and 60 nm, respectively (27).

The synchronous fluorescence spectra of AD5 upon the addition of Cu^{2+} or Fe^{3+} at $\Delta\lambda$=60 nm and $\Delta\lambda$=15 nm are indicated in Fig. 4. The quenching of the fluorescence intensity with Cu^{2+} was stronger when compared with Fe^{3+}, suggesting that Cu^{2+} contributes to the quenching of the intrinsic fluorescence of AD5 to a greater extent than Fe^{3+}.

Effect of Cu^{2+} and Fe^{3+} on the biological activity of AD5. To verify the effects of metal ions on the biological activity of AD5, ELISA assays were performed with various concentrations of Cu^{2+} or Fe^{3+} at room temperature (298 K). As demonstrated in Fig. 5, the effects of Cu^{2+} on the binding activity of AD5 to cyclin D1 was different from that of Fe^{3+}. Although low concentrations of Cu^{2+} reduced the binding activity of AD5 to cyclin D1 and relatively high concentrations of Cu^{2+} enhanced the binding activity of AD5 to cyclin D1, the results did not reach statistical significance when

Table I. Stern-Volmer K_{sv} and K_q of the Cu^{2+}/Fe^{3+}-AD5 interaction system.

A, Cu^{2+}-AD5

T (K)	K_{sv} (M^{-1})	K_q (M^{-1} s^{-1})	R	SD
293	$1.98x10^3$	$1.98x10^{11}$	0.978	0.133
298	$2.38x10^3$	$2.38x10^{11}$	0.996	0.069
303	$2.96x10^3$	$2.96x10^{11}$	0.973	0.218

B, Fe^{3+}-AD5

T (K)	K_{sv} (M^{-1})	K_q (M^{-1} s^{-1})	R	SD
293	$6.54x10^5$	$6.54x10^{13}$	0.996	0.196
298	$6.56x10^5$	$6.56x10^{13}$	0.993	0.240
303	$9.44x10^5$	$9.43x10^{13}$	0.973	0.697

The experimental conditions were as follows: pH 7.4; λex, 280 nm; λem, 290-500 nm. T, temperature; K_{sv}, quenching constant; K_q, bimolecular quenching rate constant; R, linear correlated coefficient; SD, standard deviation; AD5, anti-cyclin D1 single-chain variable fragment.

Table II. K_b and the number of binding sites of the Cu^{2+}-AD5 or Fe^{3+}-AD5 interaction at different temperatures.

A, Cu^{2+}-AD5

T (K)	K_b (M^{-1})	n	R	SD
293	95.504	1.776	0.988	0.025
298	110.943	1.276	0.964	0.031
303	104.002	1.243	0.963	0.031

B, Fe^{3+}-AD5

T (K)	K_b (M^{-1})	n	R	SD
293	61.916	1.102	0.995	0.010
298	64.055	1.053	0.992	0.012
303	72.033	1.304	0.800	0.081

The pH of both systems was 7.4. T, temperature; K_b, binding constant; n, number of binding sites; R, linear correlated coefficient; SD, standard deviation; AD5, anti-cyclin D1 single-chain variable fragment.

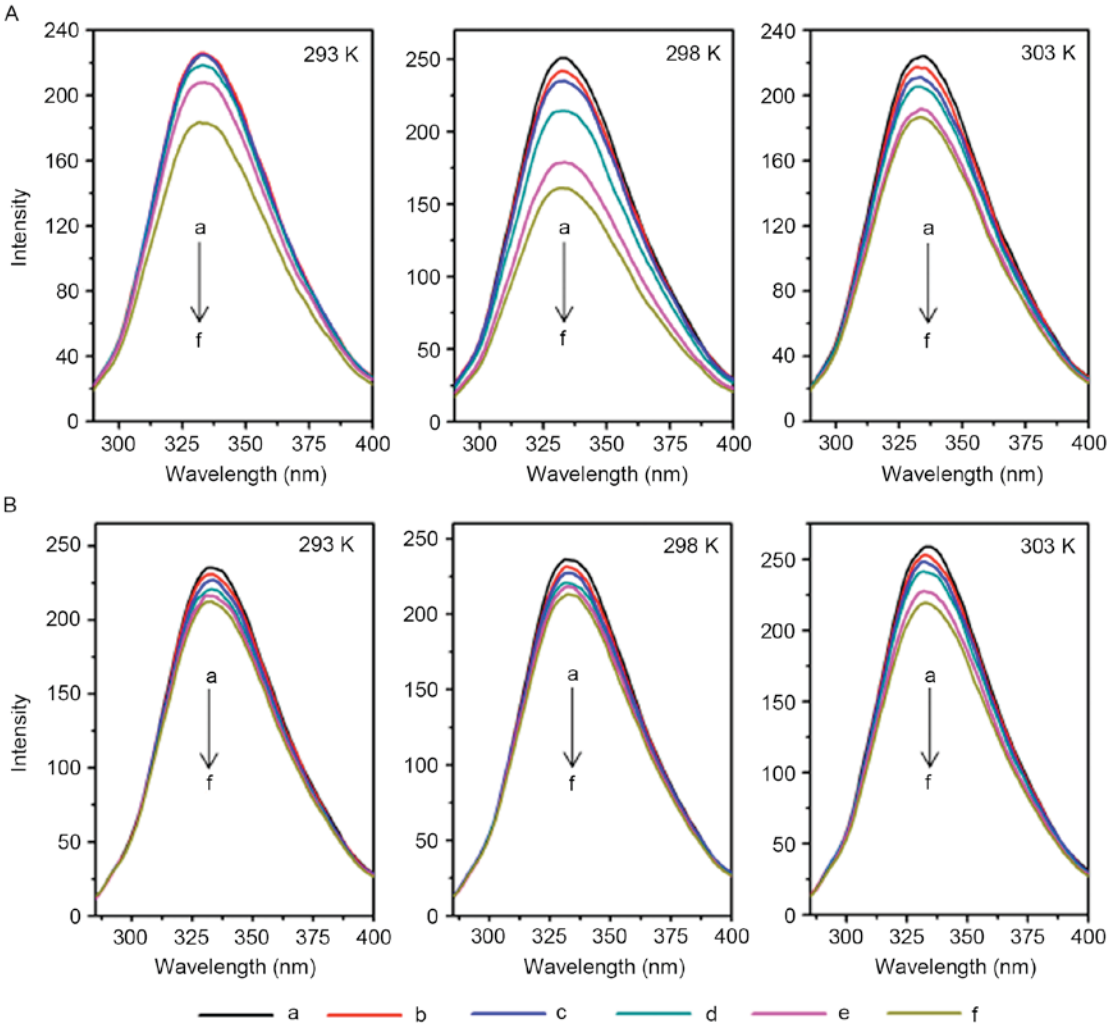

Figure 1. Fluorescence spectra of AD5 treated with various concentrations of Cu^{2+} or Fe^{3+}. AD5 ($2x10^{-6}$ M) was treated with various concentrations of (A) Cu^{2+} or (B) Fe^{3+}, and the fluorescence spectra assay was performed. The λex and λem were 280 nm and 290-500 nm, respectively (pH 7.4). a, $0.00x10^{-3}$ M; b, $0.33x10^{-3}$ M; c, $0.67x10^{-3}$ M; d, $1.00x10^{-3}$ M; e, $1.33x10^{-3}$ M; f, $1.67x10^{-3}$ M; AD5, anti-cyclin D1 single-chain variable fragment; λex, excitation wavelength; λem, emission wavelength.

compared with 0.00 M Cu^{2+} treatment (Fig. 5). However, the absorbance at 492 nm was significantly reduced following Fe^{3+} treatment when compared with the 0.00 M Fe^{3+}-treated control group ($P<0.05$; Fig. 5). This suggested that Fe^{3+} reduced the binding activity of AD5 to cyclin D1 and inhibited the biological activity of AD5.

Discussion

Cyclin D1 is the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein and promotes progression through the G_1-S phase of the cell cycle (1,2). Amplification or overexpression of cyclin D1 serves a role in the development of a subset of human cancers, including melanoma and breast, colon and prostate cancer (30). Overexpression of cyclin D1 may lead to aberrant cell growth, proliferation and tumorigenesis (31). Cyclin D1 has become a potential prognostic marker and a therapeutic target for cancer (4,32,33). The scFv may be useful for cancer prevention, diagnosis and therapy due to its specific binding affinity to its antigens, its small size, and ease of engineering to include modifications, such as the production of intrabodies (9,10,12,34). In a previous study, a novel human AD5 scFv with specific binding affinity to cyclin D1 was designed (13), and an intracellular anti-cyclin D1 scFv suppressed the growth and proliferation of HeLa and MCF-7 cells (14,15). Investigations using intracellular AD5 have suggested that scFv may be a powerful tool to inhibit the function of cyclin D1 in cancer cells (14,15). However, the mechanism of action and biological characteristics of AD5 remain to be understood. In the present study, the effects of metal ions on the structure and activity of AD5 were investigated by spectroscopy analysis and ELISA. The results revealed that the fluorescence of AD5 may be quenched by Cu^{2+} and Fe^{3+}. Quenching mechanism analysis demonstrated that static quenching was dominant in the AD5-metal ion system. In addition, the effects of Fe^{3+} on AD5 activity were stronger than that of Cu^{2+}. The ELISA results revealed that Cu^{2+} and Fe^{3+} demonstrated different effects on the biological activity of AD5, where Cu^{2+} had few effects on AD5 activity, whereas Fe^{3+} significantly reduced the biological activity of AD5 binding to cyclin D1. These results may facilitate an improved understanding of the characteristics of AD5.

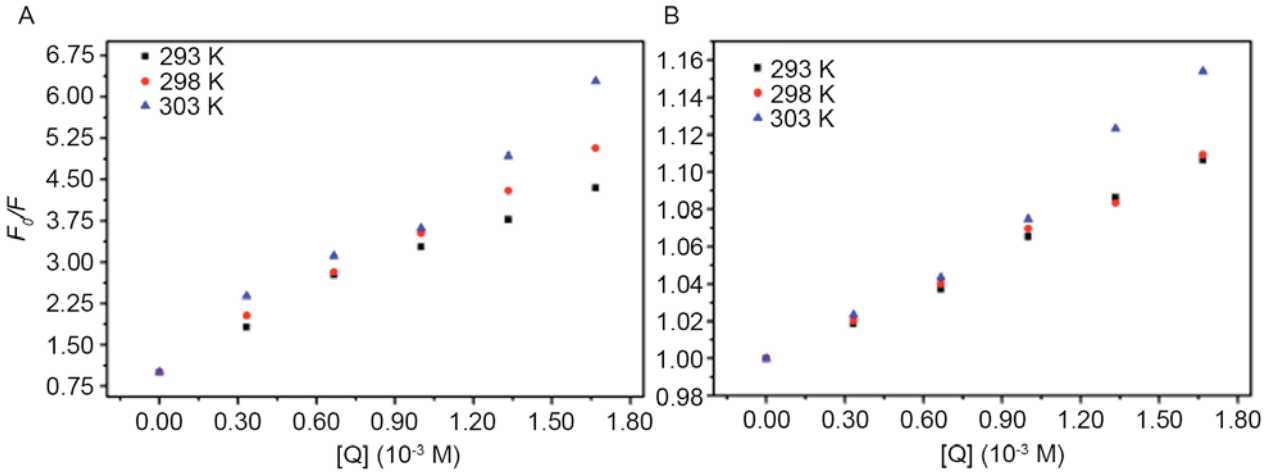

Figure 2. Stern-Volmer plots of AD5 quenching by (A) Cu^{2+} and (B) Fe^{3+} at temperatures of 293, 298 and 303 K. The concentration of AD5 was $2x10^{-6}$ M and the Cu^{2+} and Fe^{3+} concentrations were 0.00, 0.33, 0.67, 1.00, 1.33 and $1.67x10^{-3}$ M, respectively. The λex and λem were 280 nm and 290-500 nm, respectively (pH 7.4). AD5, anti-cyclin D1 single-chain variable fragment; λex, excitation wavelength; λem, emission wavelength; F_0, fluorescence intensity in the absence of a quencher; F, fluorescence intensity in the presence of a quencher; [Q], concentration of quencher.

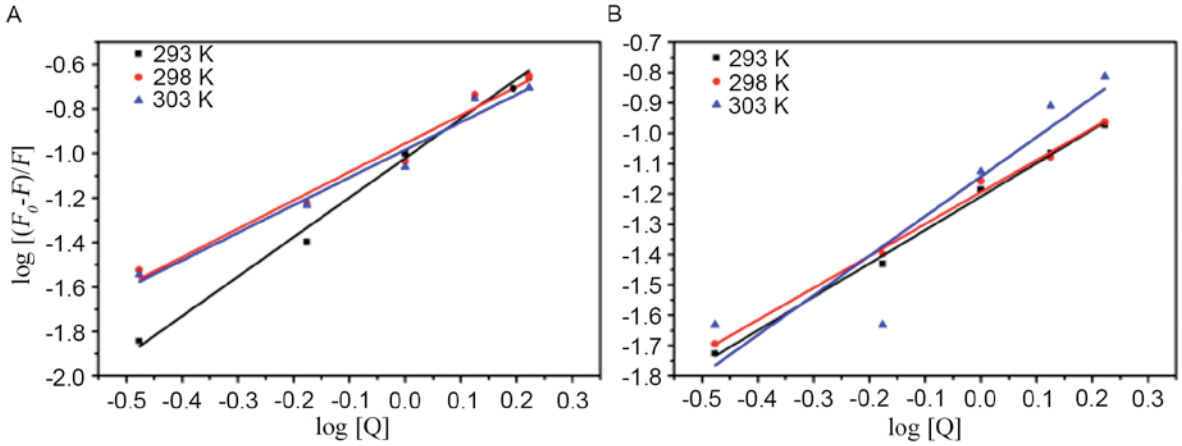

Figure 3. Quenching effects of Cu^{2+} or Fe^{3+} on AD5 fluorescence at different temperatures. (A) Cu^{2+}-AD5 system and (B) Fe^{3+}-AD5 system at temperatures of 293, 298 and 303 K. The concentration of AD5 was $2x10^{-6}$ M, and the Cu^{2+} and Fe^{3+} concentrations were 0.00, 0.33, 0.67, 1.00, 1.33 and $1.67x10^{-3}$ M. The λex and λem were 280 nm and 290-500 nm, respectively (pH 7.4). AD5, anti-cyclin D1 single-chain variable fragment; λex, excitation wavelength; λem, emission wavelength; F_0, fluorescence intensity in the absence of a quencher; F, fluorescence intensity in the presence of a quencher; [Q], concentration of quencher.

Spectroscopy is the favored method in the study of functional protein structure, including scFvs. Jäger and Plückthun (35) compared the equilibrium denaturation and unfolding kinetics of the variable domain light chain (VL) and variable domain heavy chains (VH) with those of the fragment variable (Fv) and scFv of an engineered variant of the McPC603 antibody, in the presence and absence of the phosphorylcholine antigen by fluorescence spectroscopy. The results demonstrated that scFv fragment is significantly more stable than the isolated constitutive domains. Paoletti *et al* (36) revealed that, in response to an excitation wavelength of 295 nm, the maximum fluorescence of native anti-nerve growth factor precursor scFv was 334 nm, whereas in denaturing conditions, the fluorescence maximum was shifted towards higher wavelengths of 353 nm (36). In the present study, the structural alterations of AD5 in the presence of metal ions were investigated by spectroscopy. The results demonstrated that AD5 fluorescence was quenched by Cu^{2+} or Fe^{3+}. Synchronous fluorescence revealed that the AD5 chromophore environment was altered slightly in the presence of Cu^{2+} or Fe^{3+}, leading to polarity, hydrophobicity and a minor alteration of AD5 conformation. The findings of the present study suggested that each AD5 molecule possessed ~1 Cu^{2+} or Fe^{3+} binding site. This may provide additional information regarding the spectroscopy characteristics of scFv.

The binding activity of an antibody to its antigen is critical in determining its function. In a previous study, the active anti-cyclin D1 single chain antibody, AD5, was designed. This antibody binds specifically to human recombinant cyclin D1 with a moderate affinity constant (13). However, increasing evidence suggests that antibodies with moderate affinity (10^{-7}-10^{-9} M) may facilitate effective penetration into tumors and to enhance anti-tumor activities in cancer cells (37-39). In addition, low binding activity may result in inefficient therapy (8). The results of the present study revealed that Fe^{3+} disturbed the binding between AD5 and cyclin D1. The biological activity of AD5 was significantly reduced by Fe^{3+}.

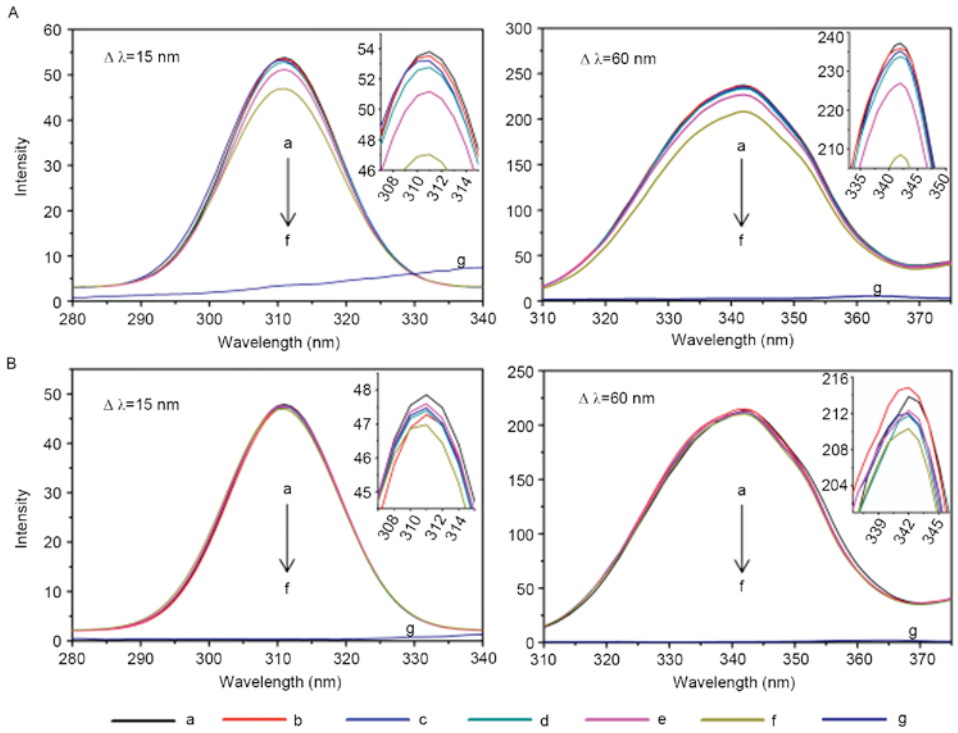

Figure 4. Synchronous fluorescence spectra of AD5 treated with various concentrations of Cu^{2+} or Fe^{3+}. AD5 ($2x10^{-6}$ M) was treated with various concentrations of Cu^{2+} or Fe^{3+}, and a synchronous fluorescence spectra assay was performed at Δλ=15 nm and Δλ=60 nm and at a temperature of 298 K (pH 7.4). Synchronous fluorescence spectra of AD5 treated with various concentrations of (A) Cu^{2+} at Δλ=15 nm (left panel) and Δλ=60 nm (right panel) or (B) Fe^{3+} at Δλ=15 nm (left panel) and Δλ=60 nm (right panel). A partially enlarged view of the peak in the synchronous emission spectra is included in the top right corner of every figure. a, $0.00x10^{-3}$ M; b, $0.33x10^{-3}$ M; c, $0.67x10^{-3}$ M; d, $1.00x10^{-3}$ M; e, $1.33x10^{-3}$ M; f, $1.67x10^{-3}$ M; AD5, anti-cyclin D1 single-chain variable fragment; Δλ, wavelength interval.

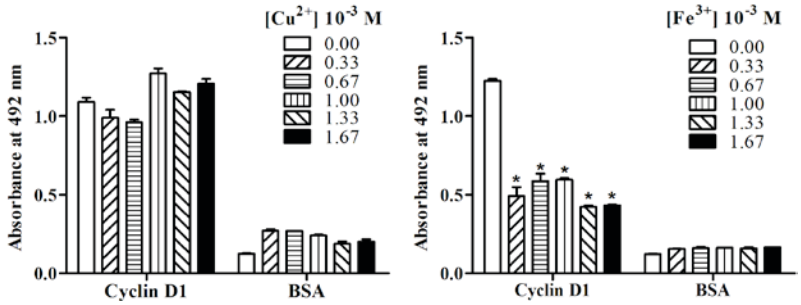

Figure 5. Effects of Cu^{2+} and Fe^{3+} on the binding activity of AD5 to cyclin D1. Wells were coated with BSA or AD5 and treated with 0.00, 0.33, 0.67, 1.00, 1.33 and $1.67x10^{-3}$ M Cu^{2+} or Fe^{3+}. An enzyme-linked immunosorbent assay was performed and the absorbance was measured at 492 nm. Data are expressed as the mean ± standard deviation of three replicates in the same experiment. *P<0.05 vs. AD5 treated with 0.00 M Fe^{3+}. AD5, anti-cyclin D1 single-chain variable fragment; BSA, bovine serum albumin.

However, Cu^{2+} had few effects on the binding activity of AD5 to cyclin D1. These results may facilitate the identification of optimal working conditions of AD5 for tumor therapy or cyclin D1 detection. The specific association between antibodies and antigens is primarily based on the interactions between the epitope of the antigen and complementary determinant regions in the VH/VL domain of the antibody molecule (40,41). Therefore, a strong antigen-antibody interaction depends on a close structural fit between antigen and antibody. Therefore, these interactions may occur if the antigen and antibody have precise conformations and are in close proximity (41,42). The results of the present study suggested that Cu^{2+} and Fe^{3+} demonstrate similar effects on AD5 conformation, as Cu^{2+} or Fe^{3+} induced slight AD5 conformation alterations. Therefore, the underlying reason why Fe^{3+} significantly inhibited the biological activity of AD5 is not explained by the Fe^{3+}-induced conformational alterations. However, it is likely that Fe^{3+} inhibits the interaction between AD5 and cyclin D1 via association with key amino acids of AD5 involved in binding to cyclin D1. Further investigation to elucidate the underlying mechanisms for the stronger effects of Fe^{3+} on AD5 activity than Cu^{2+} is warranted.

In conclusion, the results of the present study provide important information regarding the structure and biological activity of AD5 and how it is affected by metal ions. This may provide a foundation for elucidating the mechanism of action of AD5 and its potential clinical applications.

Acknowledgements

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