Effects of $K_5SiW_{11}O_{39}Co$ on Mitogenic Activity of Basic Fibroblast Growth Factor

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The interaction between polyoxometalate (POM) of Keggin class ($K_5SiW_{11}O_{39}Co$) and human basic fibroblast growth factor (bFGF) was studied by fluorescence and CD spectra. The results indicate that the binding of POM to bFGF causes conformational changes of bFGF. The cell proliferation assay showed that in lower molar ratios, POM stimulates mitogenic activity of bFGF; in higher molar ratios, POM inhibits the mitogenic activity of bFGF.

Human basic fibroblast growth factor (bFGF) is a globular single chain heparin-binding polypeptide synthesized by different cell types and is involved in processes associated with proliferation and differentiation of cell.[1,2] The crystal structure studies showed that the 18 KDa bFGF consists of three copies of basic four stranded antiparalled β-sheets.[3] It was known that bFGF interacts with specific cell surface receptors.[4] In this process, heparin or heparan sulfate proteoglycans are essential for the biological activities of bFGF due to its various effects on bFGF.[5,6] Since the demonstration of the role of angiogenesis in cancer, which is fundamental in the development, progression, and metastatic spread of solid tumors,[7] the search for angiogenesis inhibitors has become the focus of interest.[8] Therefore, as an important class of angiogenesis-promoting factors, bFGF maybe become the key target prohibited by drugs such as suramin and suradista.[9,10]

Polyoxometalate, unlike chain-like organic heparin, belonging to inorganic polyanion with cage-like molecular shape and large amounts of negative charges, have potential application in medicine for their antiviral and antitumoral activity.[11] The studies for the mechanism of antiviral action have shown that POMs could inhibit HIV-1 RT, HIV-1 P or the binding of HIV-1 gp120 to CD4.[11] Based on the polyanionic POM-polycationic protein/enzyme attraction, we choose $K_5SiW_{11}O_{39}Co$ as a representative, and attempt to study the effects of POM on the structure of bFGF, and subsequent influence on the activity of bFGF.

In this study, pure recombinant human 18 KDa bFGF was generated as follows: full-length cDNA of human 18 KDa bFGF was amplified by standard PCR method and constructed into a pPIC9K yeast expression vector. The pPIC9K construct expression vector was transformed into His4 mutant of *P. pastoris* KM71, and bFGF was expressed at a high level after the transformant was induced by methanol. The bFGF was purified by heparin-Sepharose affinity chromatography. Protein purity was assessed by SDS–PAGE and Coomassie Blue staining, only one single band was detected by SDS–PAGE corresponding to the 18 KDa purified bFGF and standard bFGF (data not shown).

In characterizing the protein-drug binding interaction, the intrinsic tryptophan and tyrosine fluorescence of the protein are usually used. For native bFGF, there are seven tyrosine residues and one tryptophan residue. Figure 1 shows the concentration-dependent steady-state fluorescence quenching of bFGF by POM. The 3 μM bFGF is titrated against POM in the concentration range from 0.12 to 6 μM. The emission spectrum is monitored after incubation of bFGF with POM to reaction equilibrium. As seen from Figure 1, the tyrosine-dominated fluorescence at 307 nm can be observed alone by simultaneous excitation of the tryptophan and tyrosine fluorescence at 270 nm, the intensity of protein fluorescence decreases gradually with the addition of POM. The Stern–Volmer quenching constant (K_{sv}) calculated by linear-fitting the initiative part of values of Stern–Volmer plot using nonlinear least-square method is $\approx 4.2 \times 10^5\ mol^{-1}dm^3$ (inset in Figure 1). Because many basic amino acids residues such as lysines exist in the vicinity of tyrosine residues,[10] it is reasonable to assume that these basic residues would be attacked by POM firstly, which leads to the change of microenvironment of tyrosine residues and the quenching of its fluorescence. This phenomenon is also found in the so-called heparin-binding proteins. The basic domain including lysines is shown to play a crucial role in the affinity of bFGF towards heparin.[12] In addition, it is noteworthy that on excition at 296 nm, under which only tryptophan fluorescence can be detected, no obvious intensity changes were observed (data not shown). It suggests that tyrosine microenvironment surrounded by lysines is more sensitive to POM than tryptophan microenvironment, and the binding sites of POM in bFGF are in the vicinity of tyrosine residues.

To gain a further insight into the conformational changes occurring in bFGF upon binding to POM, CD measurements were performed in the same molar ratios of POM to bFGF as fluores-

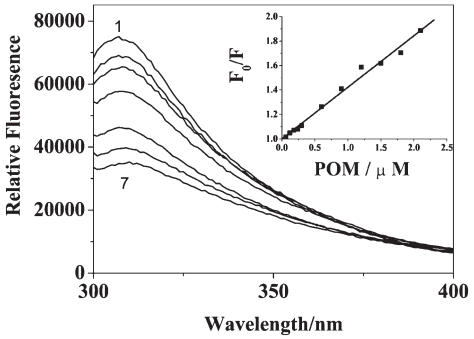

Figure 1. Fluorescence spectroscopy of bFGF titrated by POM in 20 mM PBS Buffer, pH 7.4, and the emission spectra were collected using 270 nm as an excitation wavelength. The concentrations of POM for different spectra from the top: 0, 0.12, 0.3, 0.6, 1.2, 2.4, and 6.0 μM. The bFGF concentration was 3 μM. (Inset) Stern–Volmer analysis of the fitting to the initiative part of values of Stern–Volmer plot according to the equation: $F_0/F = 1 + K_{sv}$ [POM].

cence measurement was done. As shown in Figure 2, the spectrum of bFGF alone contained a negative band near 205 nm and a positive band near 230 nm. This spectrum is representative of large contents of unordered and β-sheet secondary structure in bFGF.[13] Addition of POM caused a progressive change in the CD spectrum of bFGF, consisting mainly in a diminution of the 205 and 230 nm band intensities. It indicates that POM exerts strong effect on the conformation of bFGF. The similar cases are found in the binding of heparin or its analogues, such as dextran derivatives,[14] its inhibitors, such as suradista to bFGF,[10] as well as in the effect of increasing alkaline pH for the free protein.

In order to investigate how the binding of POM affects the bFGF activity, the mitogenic assay was performed. Briefly, the selected concentration POM and 3 μM bFGF were incubated at room temperature for 30 min, and then the mixtures were dialyzed against 2 Liters of 20 mM PBS, pH 7.4, 150 mM NaCl, 1 mM EDTA at 4 °C overnight. Then, the bFGF and bFGF-POM complexes were introduced into 96 well multiplates with 8000 3T3 cells/well respectively, and the cell proliferation was measured by the MTT method.[15]

Figure 3 shows the comparison of POM–bFGF complexes in different molar ratios with bFGF alone in mitogenic activities. It is interesting to find that in lower molar ratios (0.1:1 or 0.4:1), POM–bFGF complex exhibits higher activity than bFGF alone, stimulating the mitogenic activity of bFGF; in higher molar ratios (1:1 or 4:1), POM–bFGF complex has lower activity than bFGF alone, inhibiting the mitogenic activity of bFGF. Herein, it should be mentioned that POM alone have no obvious effect on the growth of 3T3 cells (data not shown). Obviously, stimulation or inhibition of bFGF activity depends on the concentrations of POM, which is similar to the role of exogenous heparin in modulating bFGF activity.[16] The possible reason for the results may be that: in lower molar ratios, POM maybe stabilize the conformation of bFGF, making it associate with cell surface receptors in proper way, and finally enhance the proliferation of cell as heparin does; but, in higher molar ratios, with the binding sites in bFGF saturated and the surface charges of bFGF neutralized by POM, the protein becomes much more unstable and partly denatured, not binding its receptor, which is supported by spectral analysis.

In conclusion, the addition of POM induced the conformational changes of bFGF and finally affected its activity. Furthermore, what attracted us more is the dose response to varying ratios of POM to bFGF, and the study in depth for the interesting results is proceeding. The results also render POM possible as a potential anticancer medicine.

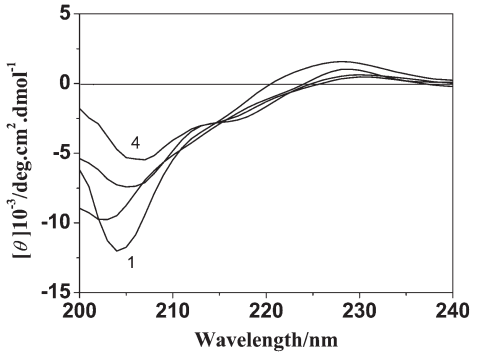

Figure 2. Far-UV CD spectra of bFGF titrated by POM in 20 mM PBS buffer, pH 7.4. The concentrations of POM for different spectra from the bottom: 0, 0.3, 3.0, and 6.0 μM. The bFGF concentration was 3 μM.

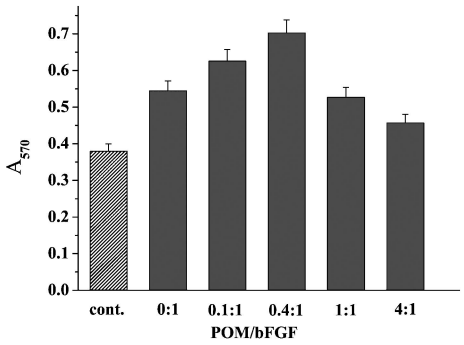

Figure 3. Effects of POM on bFGF activity. The untreated cells are used as control, and the concentration of bFGF is fixed in 3μM ($P < 0.05$).

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