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Short Communication

Analysis of the interactions between the peptides from secreted human CKLF1 and heparin using capillary zone electrophoresis

YI LIU,[a] SHUYU ZHANG,[b] XIAOMEI LING,[b]* YUZHEN LI,[a] YINGMEI ZHANG,[c,d] WENLING HAN[c,d] and YING WANG[c,d]*

[a] Department of Pharmacy, Peking University People's Hospital, Beijing 100044, PR China
[b] Department of Pharmaceutical Analysis, School of Pharmaceutical Sciences, Peking University Beijing 100083, PR China
[c] Lab of Medical Immunology, School of Basic Medical Science, Peking University Health Science Center, Beijing 100083, PR China
[d] Center for Human Disease Genomics, Peking University, Beijing 100083, PR China

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Abstract: The Chemokine-like factor 1 (CKLF1) is a novel human cytokine and exhibits chemotactic activities on leukocytes. Two peptides named CKLF1-C27 and CKLF1-C19, were obtained from secreted CKLF1. In this study, a selective high-performance analytical method based on capillary zone electrophoresis (CZE) to investigate interactions between heparin and CKLF1-C27/CKLF1-C19 was developed. Samples containing CKLF1-C27/CKLF1-C19 and heparin at various ratios were incubated at room temperature and then separated by CZE with Tris-acetate buffer at pH 7.2. Both qualitative and quantitative characterizations of the binding were determined. The binding constants of the interactions between CKLF1-C27/CKLF1-C19 and heparin were calculated as $(3.38 \pm 0.49) \times 10^5$ M^{-1} and $(1.10 \pm 0.02) \times 10^5$ M^{-1} by Scatchard analysis. To study structural requirements, CKLF1-C19pm and CKLF1-C19km have been synthesized, and their interactions with heparin have been studied by CZE. We found that the Pro or Lys to Ala substitution within the residues of CKLF1-C19 (CKLF1-C19pm or CKLF1-C19km) strongly decreased or abolished its interaction with heparin, suggesting that the residues of Pro affect the affinity of CKLF1-C19 for heparin, and the residues of Lys of CKLF1-C19 play the important role for the interaction of CKLF1-C19 and heparin, respectively. The methodology presented should be generally applicable to study peptides and heparin interactions quantitatively and qualitatively. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: capillary zone electrophoresis; CKLF1-C19; CKLF1-C27; heparin; interaction; peptide

INTRODUCTION

The CKLF1 is a novel human cytokine isolated from PHA-stimulated U937 cells. The CKLF1 protein exhibits chemotactic activities on leukocytes [1]. The previous study showed that CKLF1 is a novel functional ligand of CCR4 [2]. Two peptide sequences from secreted CKLF1 stably expressed in *Drosophila* S2 cells were obtained, named CKLF1-C27 and CKLF1-C19. Chemically synthesized CKLF1 peptide CKLF1-C27 and CKLF1-C19 also have functional activation via CCR4, with CKLF1-C27 having a stronger effect on CCR4 mediated chemotaxis and calcium flux than CKLF1-C19. The eighth residue from the CKLF1-C27 *N*-terminus is important for receptor chemotaxis and calcium flux [3]. Chemokines bind both to soluble GAGs) [4] as well as GAGs immobilized on cell surfaces and the extracellular matrix (ECM) [5]. It has been suggested that the interaction of GAG and chemokine is critical *in vivo* and chemokine immobilization through the GAG interaction facilitates the formation of haptotactic chemokine gradients, and enhances their concentration at the site of production, particularly in the presence of shear forces in blood vessels and draining lymph nodes. Interaction with GAGs may also provide another level of specificity and control to cell migration, beyond that defined by receptor engagement, by selective binding of certain chemokines to different types of GAGs and their diverse isoforms [6–10]. Heparin is the most widely commercially available GAG. It may protect chemokines from degradation, and function as a storage mechanism, influencing the lifetime and availability of chemokines *in vivo* [11]. In common, the presence of carboxylate and sulfate groups on the GAG chains give rise to an overall negative charge, and facilitates the interaction with chemokines, which are predominantly basic proteins, and the residues of Arg and Lys are important [12,13].

Abbreviations: CKLF1, chemokine-like factor 1; CZE, capillary zone electrophoresis; GAGs, soluble glycosaminoglycans; CCR4, CC chemokine receptor 4.

*Correspondence to: Xiaomei Ling, Department of Pharmaceutical Analysis, School of Pharmaceutical Sciences, Peking University, Beijing 100083, PR China; e-mail: pkulxm@126.com
Ying Wang, Lab of Medical Immunology, School of Basic Medical Science, Peking University Health Science Center, Beijing 100083, PR China; e-mail: yw@bjmu.edu.cn

Many methods have been applied to study the interactions between ligands and receptors, such as equilibrium dialysis, ultrafiltration, spectroscopic methods, and chromatographic methods [14–17]. Along with these powerful techniques, capillary electrophoresis (CE) has been used in a wide range of binding studies [14,18]. CE has the advantages such as lower sample consumption, easy calculation of binding constants, and simple operation procedure [19]. A few reports can be found on the use of CE to study heparin–protein interactions [20–23]. But, to the best of our knowledge, the interactions between CKLF1-C27/CKLF1-C19 and heparin have never been studied by CE. Among the various CE modes [24–26], CZE is the most appropriate for both the assay of strong binding systems in which the complex will not dissociate during the required separation time period and the assay of weak binding systems [20]. Here, CZE was first utilized to investigate the interactions between CKLF1-C27/CKLF1-C19 and heparin quantitatively and qualitatively.

MATERIALS AND METHODS

Chemicals and Reagents

According to the sequence report of peptides from secreted human CKLF1 [3], peptides (purity ≥95%): CKLF1-C27 (ALIYRKLLFNPSGPYQKKPVHEKKEVL), CKLF1-C19 (FNPSGPYQKKPVHEKKEVL), CKLF1-C19km (FNPSGPYQAAPVHEAA EVL), and CKLF1-C19pm (FNASGAYQKKAVHEKKEVL) were chemically synthesized by the Hybio Engineering Company in ShenZhen, China, and were subsequently purified and characterized by reversed-phase high-performance liquid chromatography and mass spectrometry (MS).

Heparin was purchased from GIBCO (Carlsbad, CA, USA). Tris base (ultrapure) and acetic acid used in this study were from Beijing Chemical Reagent Factory (Beijing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA). Tris-acetate was served as running buffer. All buffers and solutions used in the study were filtered through 0.45 μm membranes (Agilent, Germany) before use.

Apparatus

A Beckman P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) with a photodiode array detector and capillary tube (Yongnian Optical Fibre Corp., Hebei, China) with an internal diameter of 75 μm were used. The total and effective lengths of the capillary were 30.2 and 10.2 cm, respectively. Data were collected with 32 Karat software version 5.0 (Beckman).

Sample Preparation

To investigate interactions between CKLF1-C27/CKLF1-C19/CKLF1-C19pm/CKLF1-C19km and heparin, different concentrations of heparin were tested for the formation of the complex, achieved by mixing CKLF1-C27/CKLF1-C19/CKLF1-C19pm/CKLF1-C19km with heparin in the running buffer. Mixtures were incubated for 20 min at 37 °C before CE analysis. All solutions were prepared with deionized water.

CZE Conditions

To study the interactions between peptides and heparin, the temperature of the cartridges were kept at 30 °C. In order to reduce the adsorption of peptides to glass, all buffer reservoirs contained a plastic insert. Samples containing the mixtures of peptides and heparin were injected respectively using the pressure injection mode at 0.5 psi for 5 s (1 psi = 6894.76 Pa). The capillary electrophoresis unit was operated at 10 kV in the reverse polarity mode with the positive electrode at the outlet reservoir and the negative electrode at the inlet reservoir. The capillary was washed between runs with the running buffer (30 mM Tris-acetate, pH 7.2) for 5 min at 20 psi. Each concentration was run in duplicate.

Quantitative Model for the Binding Study

In the binding studies, the binding constant and stoichiometry are important parameters to be determined. Scatchard analysis is a common way to linearize the binding data, as expressed in Eqn (1):

$$r/C_f = -Kr + nK \quad (1)$$

where r is the ratio of the concentrations of the bound ligand (or receptor) to the total receptor (or ligand) and C_f is the unbound ligand (or receptor) concentration. K is the apparent binding constant, and n is the number of binding sites [27].

RESULTS AND DISSCUSSION

Optimization of Separation Conditions

Adsorption to the capillary wall is usually considered as a troublesome effect in CE, especially for the separation of peptides and proteins. In general, this phenomenon is unfavorable, or even fatal for the analysis of macrobiomolecules. In our experiment, electrophoretic conditions were optimized, in particular with regard to choice of buffer type, concentration of the buffer used, buffer pH value, applied voltage, and the length of the capillary. First, we investigated the effect of phosphate buffer and Tris-acetate. Using phosphate buffer, there was a peak broadening. For best results, Tris-acetate provided the better separation efficiency. Thus, Tris-acetate solution was selected as the running buffer. The electrostatic attraction between proteins and the silica surface may be reduced by increasing the ionic strength of electrolyte solutions. However, the high ionic strength limits the applied voltage, consequently decreasing efficiency [28]. We selected 30 mM Tris-acetate as the running buffer ascribing its better peak shape. pH was another important factor for evaluating the interactions in CZE. In general, the binding constant was determined in physiological condition or near-physiological condition, 30 mM pH 7.2 Tris-acetate was selected as the running buffer. Then the applied voltage and the length of the capillary were chosen. The applied voltage was chosen as high

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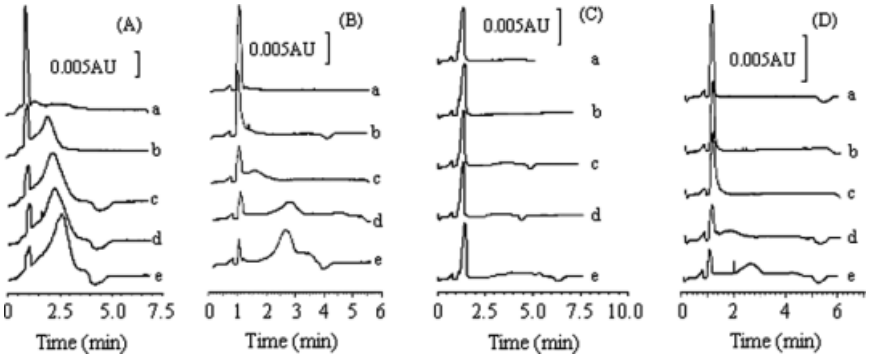

Figure 1 Electropherograms of CKLF1–heparin interactions. (A) CKLF1-C27 : heparin **a** 1 : 0, **b** 1 : 0.08, **c** 1 : 0.80, **d** 1 : 1.20, **e** 1 : 2.40. (B) CKLF1-C19 : heparin **a** 1 : 0, **b** 1 : 0.11, **c** 1 : 1.11, **d** 1 : 3.34, **e** 1 : 6.68. (C) CKLF1-C19km : heparin **a** 1 : 0, **b** 1 : 0.58, **c** 1 : 1.16, **d** 1 : 2.32, **e** 1 : 3.48. (D) CKLF1-C19pm : heparin **a** 1 : 0, **b** 1 : 0.11, **c** 1 : 0.21, **d** 1 : 1.07, **e** 1 : 2.15. Conditions used were as follows: Beckman P/ACE MDQ CE system with DAD, 214 nm. Injection: 0.5 psi for 5 s. Applied voltage: −10 kV. Capillary: capillary of 30.2 cm × 75 μm id. Running buffer: 30 mM Tris-acetate, pH 7.2.

as possible, but limited by the heating of the capillary. In protein/peptide analysis, the length of the capillary affects the interaction between protein/peptide and the capillary inner surface. In our experiment, the applied voltage was set at −10 kV, and the effective length of the capillary was 10.2 cm to obtain high resolution.

Determination of the Interaction Between CKLF1-C27 and Heparin

CKLF1-C27 with the concentration range of 78.25–156.5 μM was injected into the capillary column to get the calibration plot. The peak height of each sample was proportional to its concentration. The RSD of repeated injection of 99.29 μM CKLF1-C27 was 3.9% ($n = 4$). The calibration plot of CKLF1-C27 was $y = 175.64 \times -11\,729$ ($n = 5$) with a correlations coefficient of 0.9947. Figure 1(A) shows electropherograms for CKLF1-C27 alone and mixtures containing a fixed concentration of CKLF1-C27 and increasing concentrations of heparin. With increasing heparin ratios in the sample, the peak height of CKLF1-C27 decreased consecutively and regularly, which means that it is not occasional or due to other effects, but rather, the result of the interaction. The peak of the CKLF1-C27–heparin complex was clearly visible at a tenfold excess of CKLF1-C27. When a CKLF1-C27/heparin ratio increased to 1 : 6.39, almost all CKLF1-C27 was bound. The CKLF1-C27–heparin complex was stable under CZE conditions. The binding constant is an important quantitative parameter for characterizing the interaction. The peak heights of CKLF1-C27 in each sample were determined and free concentrations were calculated from the calibration curve to obtain the values of r. Figure 2 shows the Scatchard plot based on Eqn (1). From the slope of the Scatchard plot, K was calculated to be $(3.38 \pm 0.49) \times 10^5$ M^{-1}.

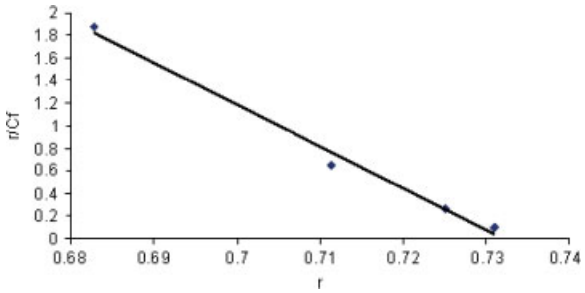

Figure 2 Scatchard plot for the interaction between CKLF1-C27 and heparin. Experimental conditions were the same as in Figure 1.

Determination of the Interactions and Structural Requirements Between CKLF1-C19 and Heparin

The mixture of the same concentration of CKLF1-C19 and an excess concentration of heparin were injected into the capillary column. The experimental results (Figure 1(B)) show that there were binding interactions between CKLF1-C19 and heparin. To obtain the binding constant, the method used was the same as CKLF1-C27, the results obtained are listed in Table 1.

A study showed that CKLF1-C19 peptide could inhibit the airway inflammation and airway hyperresponsiveness in mice, while CKLF1-C27 peptide promoted the inflammation (unpublished data), suggesting that CKLF1-C19 may be a candidate of antagonist peptide of the interaction of chemokine and their receptors. Based on the interest in CKLF1-C19, the Lys or Pro residues of CKLF1-C19 were substituted and the structural requirements were studied. First, because Arg and Lys residues of chemokines are important for GAG binding [12,13], the Lys in residues 9, 10, 15, and 16 of CKLF1-C19 were substituted to Ala and the mutant peptide, CKLF1-C19km, was synthesized, which interaction with heparin was studied by CZE. In Figure 1(C), it can be verified that CKLF1-C19km had no affinity for heparin, indicating that Lys to Ala substitution in

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Table 1 Binding constants of CKLF1-C27/CKLF1-C19/CKLF1-C19km/CKLF1-C19pm with heparin

Compound	Calibration plot $Y = a + bX$[a]	Correlation coefficient	Concentration range (μM)	K (M^{-1})
CKLF1-C27	$y = 175.64x - 11729$ ($n = 5$)	0.9947	78.25–156.5	$(3.38 \pm 0.49) \times 10^5$
CKLF1-C19	$y = 97.187x - 1224.5$ ($n = 7$)	0.9974	32.07–224.50	$(1.10 \pm 0.02) \times 10^5$
CKLF1-C19pm	$y = 73.956x - 1586.5$ ($n = 7$)	0.9962	38.81–232.85	$(7.28 \pm 0.80) \times 10^4$

[a] Where the Y and X are the peak height and concentration (μM) of the analytes, respectively.

residues 9, 10, 15, and 16 of the CKLF1-C19 strongly abolished the CKLF1-C19 and heparin interaction.

Another study examined the CKLF1-C27/CKLF1-C19 peptides with traditional ^{1}D and ^{2}D ^{1}H NMR methods. Results showed that both peptides adopted a flexible extended conformation in solution. Only the three proline residues seemed to mildly restrict the local region adjacent to them, which might play an important role in the recognizing and binding process of CKLF1-C19 or CKLF1-C27 (unpublished data). To investigate whether the Pro residues of CKLF1-C19 are important for heparin binding, the Pro in residues 3, 6, and 11 of CKLF1-C19 were substituted to Ala and the mutant peptide, CKLF1-C19pm, was synthesized, which interaction with heparin was studied by CZE (Figure 1(D)). The results demonstrated there were interactions between heparin and CKLF1-C19pm. The assay results of binding constant are presented in Table 1.

In this study, the present method based on CZE was shown to be capable of investigating interactions between heparin and CKLF1-C27/CKLF1-C19. Compared with CKLF1-C27, CKLF1-C19 lacks eight amino acids at its *N*-terminus. CKLF1-C19 was still able to interact with heparin but had a weaker heparin binding, suggesting that CKLF1-C27 *N*-terminus is important for heparin binding. Furthermore, we focused on the critical amino acids to CKLF1-C19-heparin recognition. CKLF1-C19pm and CKLF1-C19km have been synthesized and their interactions with heparin have been studied. We have found that the Pro or Lys to Ala substitution within the residues of CKLF1-C19 strongly decreased (CKLF1-C19pm) or abolished (CKLF1-C19km) the CKLF1-C19 and heparin interactions. According to the results, it can be concluded that the Lys residues in CKLF1-C19 play a crucial role for CKLF1-C19 and heparin recognition. Meanwhile, by comparison of the value of binding constants, we found that the residues of Pro in CKLF1-C19 affect the affinity of CKLF1-C19 for heparin. This study of CKLF1-C27/CKLF1-C19-heparin interactions could facilitate the understanding of their exact immunomodulatory and pharmacological roles, especially *in vivo*, because the interaction of GAG and chemokine is critical *in vivo* [6–9]. Compared with CKLF1-C27, the weaker ability of heparin binding of CKLF1-C19 might be a reason of CKLF1-C19 antagonizing inflammation.

CONCLUSIONS

The CZE method described here had potential as an evaluation of interactions between heparin and CKLF1-C27/CKLF1-C19 under aqueous conditions simulating the *in vivo* environment. The CZE approach is a highly efficient, fast, quantitative method for the study of biomolecular interactions, and the approach also avoids the interference when protein was immobilized on a plate. Each run was completed within 6 min and the binding constants could be determined. The binding constants of the interactions between CKLF1-C27/CKLF1-C19 and heparin were calculated as $(3.38 \pm 0.49) \times 10^5$ M^{-1} and $(1.10 \pm 0.02) \times 10^5$ M^{-1} by Scatchard analysis. The results demonstrate that Lys residues in CKLF1-C19 play a crucial role for CKLF1-C19 and heparin recognition, and Pro residues in CKLF1-C19 affect the affinity of CKLF1-C19 for heparin.

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