Clinica Chimica Acta 424 (2013) 148–152

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Serum KL-6 concentrations are associated with molecular sizes and efflux behavior of KL-6/MUC1 in healthy subjects

Masahiko Shigemura [a,b], Satoshi Konno [a,*], Yasuyuki Nasuhara [a], Noriharu Shijubo [c], Chikara Shimizu [b], Masaharu Nishimura [a]

[a] First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan
[b] Division of Laboratory and Transfusion Medicine, Hokkaido University Hospital, Sapporo, Japan
[c] Department of Respiratory Medicine, JR Sapporo Hospital, Sapporo, Japan

ARTICLE INFO

Article history:
Received 23 January 2013
Received in revised form 6 June 2013
Accepted 7 June 2013
Available online 15 June 2013

Keywords:
Serum KL-6 concentrations
KL-6/MUC1 molecular size
Healthy subjects

ABSTRACT

Background: Serum KL-6, a sialylated sugar chain on human MUC1, is used as a marker of interstitial lung diseases. We recently reported that efflux behavior of KL-6/MUC1 from the alveoli into the bloodstream assessed by molecular analysis differed according to genetically determined molecular sizes and influenced serum KL-6 concentrations in sarcoidosis. This study was designed to investigate associations between molecular size and efflux behavior of KL-6/MUC1, and factors contributing to serum KL-6 concentrations in healthy subjects.
Methods: Western blot analysis using anti-KL-6 antibody was performed on serum obtained from 250 healthy subjects.
Results: The efflux behavior of KL-6/MUC1 differed according to the genetically determined molecular sizes in healthy subjects. In subjects having low molecular size, there were significant associations between smoking status, aging, renal function and serum KL-6 concentrations. However, these associations were not significant in the subjects having higher molecular size and the efflux behavior of high molecular size was the only significant determinant of serum KL-6 concentrations.
Conclusions: This study showed an association between KL-6/MUC1 efflux based on molecular size and serum KL-6 concentrations in healthy subjects. We propose that the molecular size and efflux behavior of KL-6/MUC1 should be considered when interpreting serum KL-6 concentrations.

1. Introduction

Krebs von den Lungen-6 (KL-6) is a posttranslational modification for human mucin-1 (MUC1) and a mucinous sialylated sugar chain on MUC1 is recognized by anti-KL-6 mAb [1,2]. Serum KL-6 concentrations are specifically elevated in a majority of patients with interstitial lung diseases (ILDs) [3–6]. This phenomenon is considered to reflect the increased production of KL-6/MUC1 by regenerating type II epithelial cells in the lung, and/or enhanced permeability following destruction of the alveolar–blood interface [7,8]. Therefore, measurement of serum KL-6 is widely accepted, particularly in Japan, as a diagnostic test for ILDs and is a marker of disease activity. However, in some cases, we have experienced significant limitations in the interpretation of serum KL-6 concentrations, including its low diagnostic sensitivity.

Recent reports have shown that serum KL-6 concentrations are affected by several factors other than lung diseases [9–13]. Aging and long-term smoking are associated with increased concentrations in serum KL-6 [9]. Renal function can also influence interindividual variability, as KL-6/MUC1 is renally cleared [10]. There have been several reports describing the association between serum KL-6 concentrations and the genotype of a single nucleotide polymorphism (SNP) in exon 2 (rs4072037) of the *MUC1* gene [11,12]. We recently reported a significant relationship between *MUC1* genotypes and KL-6/MUC1 molecular size in bronchoalveolar lavage fluid (BALF); the A allele was linked with the low molecular size KL-6/MUC1, while the G allele was linked with high molecular size in subjects with sarcoidosis [13]. In addition, the efflux behavior of KL-6/MUC1 from the alveoli into the bloodstream assessed by molecular analysis differed according to KL-6/MUC1 molecular sizes and influenced serum KL-6 concentrations [13].

We believe that our findings provide new insights into understanding the efflux mechanisms of KL-6/MUC1, and the limitations in interpreting serum KL-6 concentrations. However, these results were based on an inherent study in subjects with sarcoidosis [13], which may present obstacles for better understanding the efflux mechanisms of KL-6/MUC1 characterized by molecular analysis. The

Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; ILDs, interstitial lung diseases; KL-6, Krebs von den Lungen-6; MUC1, mucin-1; VNTR, variable number of tandem repeat.

* Corresponding author at: North 15, West 7, Kita-ku, Sapporo 060-8638, Japan. Tel.: +81 11 706 5911; fax: +81 11 706 7899.
E-mail address: satkonno@med.hokudai.ac.jp (S. Konno).

0009-8981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2013.06.002

presence of alveolitis and the subsequent destruction of the alveolar–blood interface related to sarcoidosis may influence KL-6/MUC1 efflux from the alveoli into the bloodstream. The effects of local production of KL-6/MUC1 by regenerating type II epithelial cells on this efflux also need to be considered in some subjects.

Thus, performing an analysis in healthy subjects is crucial for understanding the association between serum KL-6 concentration and ILDs. Therefore, in this study, we focused only on healthy subjects and evaluated the associations between *MUC1* allele-related molecular sizes and efflux behavior of KL-6/MUC1, as well as factors contributing to efflux behavior and serum KL-6 concentrations.

2. Materials and methods

2.1. Subjects

A total of 250 unrelated healthy Japanese subjects, who visited for routine physical examination, were enrolled into this study. By completing a questionnaire, these subjects provided relevant background information, including medication use, and hereditary diseases. We excluded subjects who met the following conditions: (1) history of any pulmonary disease or respiratory symptoms; (2) percent of predicted vital capacity <80%; (3) ratio of forced expiratory volume in the first 1 s to the forced vital capacity of the lungs <70; and (4) estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2. eGFR was calculated using the following equation for Japanese populations [14]: $eGFR = 194 \times serum\ creatinine^{-1.094} \times Age^{-0.287}$ (for females, ×0.739). To definitively evaluate the effects of cigarette smoking on the efflux behavior of KL-6/MUC1 and serum KL-6 concentrations, we excluded former smokers from this study. The study population, gender, age, smoking history, serum KL-6 concentrations and eGFR levels are shown in Table 1.

Venous blood samples were collected into Vacutainer™ tubes containing clot activator, and were kept in the tubes for 30 min. Tubes were centrifuged at 3000 rpm at room temperature for 10 min. Serum samples were immediately frozen and stored at −80 °C until assay. The Institutional Review Board of the School of Medicine, Hokkaido University, approved the study protocols and all subjects provided formal written consent.

2.2. Genotyping of MUC1 polymorphism

The *MUC1* SNP (exon 2; rs4072037) was genotyped using the TaqMan system (Assay ID: C_27532642_10; Applied Biosystems, Foster City, CA).

2.3. Western blotting

Western blotting was performed on all serum samples as described previously [13]. Briefly, protein samples from serum were electrophoresed on 3%–8% NUPAGE Tris-acetate gels (Invitrogen, Carlsbad, CA) and were transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with PBS containing 3% skim milk. Western blot analysis was performed using anti-KL-6 antibody (anti-KL-6 antibody was kindly provided by Sanko Junyaku Co., Ltd., Tokyo, Japan) followed by alkaline phosphatase-conjugated goat anti-mouse Ig. Bands were developed using the WesternBreeze Chromogenic Immunodetection Kit (Invitrogen).

Table 1
Characteristics of the study population.

	Healthy subjects
No. of subjects	250
Men/women	113/137
Age, y	51 (24–77)
Cigarette smoking	
Never/current	157/93
MUC1 gene polymorphism	
AA/AG/GG	204/40/6
Serum KL-6 concentrations, U/ml	214 (112–759)
eGFR levels, ml/min/1.73 m^2	84.6 (60.3–268.7)

Data are presented as median (range).

2.4. Measurements of KL-6 and creatinine

KL-6 concentrations in serum were measured by electrochemiluminescent immunoassay using the PICOLUMI KL-6 kit (Sanko Junyaku). Concentrations of creatinine in serum were measured using a Hitachi 7170 automated analyzer with Serotec CRE-L (Serotec, Sapporo, Japan) standardized using the isotopic dilution mass spectrometry method.

2.5. Statistical methods

Statistical analysis was performed with SPSS for Windows (SPSS Inc., Chicago, IL). Data are expressed as medians and ranges. All data were not normally distributed on univariate analysis, and the natural logarithms of all data were used for further statistical analyses. Comparisons were performed using unpaired *t*-test. Differences between groups were evaluated by ANOVA and were assessed by Bonferroni post-hoc test. Correlations between different parameters were determined by Pearson's correlation coefficient. We used Haploview software ver 4.1 (http://www.broad.mit.edu/mpg/haploview) in order to compare the observed numbers of genotypes with the number of expected genotypes under the Hardy–Weinberg equilibrium using the χ^2-test. The relationship between efflux behavior of high molecular size KL-6/MUC1 and smoking status was assessed using χ^2-test. A $p < 0.05$ was regarded as significant.

3. Results

3.1. MUC1 genotypes and Western blot analysis

There were 448 (89.6%) sequences with A and 52 (10.4%) with G. The genotype frequencies were 204 (81.6%) for AA, 40 (16.0%) for AG, and 6 (2.4%) for GG. No significant deviation from the Hardy–Weinberg equilibrium was observed ($p > 0.05$). Western blot analysis of serum with anti-KL-6 antibody revealed 3 bands (low molecular size (L), middle molecular size (M) and high molecular size (H), at approximately 400, 450 and 500 kDa, respectively) and four band patterns (L alone, L/M, L/H and H alone) under reducing conditions, as described previously [13]. The frequency and percentage of the *MUC1* genotypes and the KL-6/MUC1 band patterns in serum are summarized in Table 2.

In our previous report, we demonstrated a significant relationship between MUC1 genotypes and KL-6/MUC1 molecular sizes in BALF in 128 subjects with sarcoidosis; the A allele was linked with the low molecular size KL-6/MUC1 and the G allele with the high molecular size [13]. Therefore, we can extrapolate the KL-6/MUC1 molecular sizes in BALF from the subjects in this study according to MUC1 genotypes. Namely, the subjects with genotype AA correspond to low molecular size band patterns (i.e. L alone) in BALF and the subjects with genotype non-AA correspond to higher molecular size band patterns (i.e. L/M, L/H and H alone). Next, we examined the association between MUC1 genotypes (molecular size of KL-6/MUC1 in BALF) and KL-6/MUC1 band pattern in serum. In the subjects with genotype AA (i.e. low molecular size band patterns in BALF), all subjects displayed L alone bands in serum. On the other hand, the subjects with genotype non-AA (i.e. higher molecular size band patterns in BALF) showed diverse patterns in serum.

Based on the presence of higher molecular size bands (M or H) in serum (i.e., the efflux of higher molecular size KL-6/MUC1 from the alveoli to the bloodstream), we classified the subjects with genotype non-AA into two groups according to the absence or presence of higher