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Clara cell secretory protein: determination of serum levels by an enzyme immunoassay and its importance as an indicator of bronchial asthma in children

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Abstract

Clara cell secretory protein (CC16) is a 16 kDa protein secreted by Clara cells in the lining fluid of bronchiolar and bronchial epithelium. CC16 presents several biologic properties, and has been shown to have immunomodulatory and anti-inflammatory activity. It may play a role in controlling inflammation in the airway. There is some evidence that the CC16 level is primarily lower in adult individuals with bronchial asthma, thus contributing to its pathophysiology. This study was designed to examine CC16 serum levels of children, healthy and with asthma. An enzyme solid phase immunoassay utilizing monoclonal antibody to CC16 was the analytical method to determine the protein concentration in blood sera. The method showed excellent linearity, high sensitivity (detection limit: <50 ng/l) and precision. It was found that asthmatic children appear significantly lower levels ($P < 0.001$) of CC16 in serum as compared to healthy ones. It is, therefore, concluded that CC16 may be a useful diagnostic index of bronchial asthma in the early child-age.

Keywords: Clara cell protein CC16; Enzyme immunoassay; Bronchial asthma

1. Introduction

Clara cell secretory protein (CC16) is one of the most abundant soluble proteins within the epithelial lining fluid of the lung [1], synthesized primarily by non-ciliated bronchiolar epithelial cells, the Clara cells. From the airways, CC16 passively diffuses into plasma across the bronchoalveolar–blood barrier. Like other low-molecular size proteins, plasma CC16 is rapidly eliminated by glomelular filtration before being taken up and catabolized by renal tubules [2].

The physiological function of CC16 in vivo as well as its role in lung infection and injury is not completely understood. The localization of human CC16 gene to chromosome 11, *p12–q13*, a region occupied by genes involved in regulation of inflammation [3,4] suggests that CC16 may function as a regulator

Abbreviations: CC16, Clara cell protein 16; BALF, bronchoalveolar lavage fluid; ARDS, acute respiratory distress syndrome

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of inflammation in the lung, having immunosuppressive and anti-inflammatory properties. It has also been found that CC16 may be related to the development of postpartum depression, since it has been observed that there is a decreased anti-inflammatory capacity in the serum, in part caused by lowered serum CC16 in puerperal women [5].

It has been reported that downregulation of cytokines may account for CC16's anti-inflammatory activities [6–8]. Since these are key molecules in the production of lysophosphatidylcholine, prostaglandins and leukotrienes, their decreased synthesis and secretion may exacerbate the ensuing lung injury [9]. The need to regulate inflammation may, however, be greater in the lung due to the persistent exposure to noxious/infectious agents that may lead to destruction of lung architecture through an unwarranted inflammatory response. In accordance with this hypothesis, recent experimental data supports the anti-inflammatory role of CC16. Thus, lack of CC16 increases the host response to viral infection in the lung [10], while tolerance to chronic oxidant stress results in an increase of both the number of Clara cells and the abundance of CC16 in the conducting airways [11]. Levels of CC16 in bronchoalveolar lavage fluid (BALF) have been determined in various lung disorders. A significant reduction has been found in BALF from smokers, patients with lung cancer, chronic obstructive pulmonary disease [12,13], allergic rhinitis [14] or asthma [6,14,15]. On the other hand, increased CC16 levels have been found in BALF from patients with acute respiratory distress syndrome (ARDS).

The concentrations of CC16 in serum are significantly reduced by tobacco smoking, a situation in which the number of Clara cells and the CC16 secretion in airways are known to be diminished [16,17]. There are also documents for decreased levels of serum CC16 in patients with asthma, especially those with a long disease duration [18]. It has been shown that early stage allergic airway inflammation is associated with the reduction of pulmonary levels of CC16 in astmatics, which may cause a diminished pulmonary anti-inflammatory activity and contribute to further development of airway inflammation in asthma. Decreased CC16 serum levels in asthmatic individuals may be due to the small number of Clara cells that have been described in lungs of asthmatics [18].

It is of great clinical importance that CC16 diffuses into the serum down a concentration gradient between the surface of the respiratory tract and the bloodstream [13]. This fact renders the levels of plasma CC16 a possible marker of lung diseases.

Bronchial asthma is the most common disease in pre-junior children, its symptoms getting started very early. About 50% of pre-junior children have at least one episode of expiratory wheeze, in first years of their lives. Through these children, a significant percentage (about 65%) appears asthma symptoms because of the small diameter of airways and/or a virus infection. In this case, the loops' hyper-reactivity in these children is not permanent and does not promote a bronchial asthma. If Clara cells are really primarily reduced in asthma, then the serum CC16 concentration levels are expected to be lower in babies and children that will develop asthma in the future. The purpose of this study was, therefore, to determine the concentration of CC16 in blood serum of children with clinical proved bronchial asthma and to compare with healthy ones, using a specific solid phase assay.

2. Materials and methods

2.1. Biologic subjects

Twenty four patients (aged 0–14) that fulfill the standard clinical criteria that define the risk of asthma [19] in children, and 27 healthy individuals (aged 0–14) were examined. Control cases were children visiting the outpatient clinic for routine pediatric evaluation before orthopedic operations. Asthmatic children receiving systemic steroids were excluded.

2.2. Analytical methods

Samples were assayed in a blind, randomized manner. All samples were analyzed in triplicate at a dilution range 1:20 of serum. CC16 was analyzed with the DIAMED CC16-ELISA kit (made by DiaMed Eurogen Belgium). This is an enzyme immunoassay used for the quantitative determination of CC16. Analysis was performed following coating of microtiter strips with anti-CC16 monoclonal antibodies and incubation at $37 \pm 2\,^{\circ}C$ for 120 ± 10 min with standard sera and test samples. During this incubation, CC16 present

in samples is bound to the immobilized antibodies. After removal of the unbound material by a washing procedure (with a phosphate buffered washing solution for five times), CC16 was bound in a second incubation step to a second CC16 specific polyclonal antibody, which is conjugated to biotin. Following washing to remove excess of biotin-conjugate, the amount of biotin-conjugated polyclonal antibody was detected in a third reaction step by the addition of streptavidin–horse rabbit peroxidase. After the removal of unbound horse rabbit peroxidase conjugate, the microtiter strips were incubated with a substrate solution containing hydrogen peroxide and a tetramethylbenzidine buffer solution. The blue color developed is proportional to the amount of CC16 bound to the wells of the microtiter strips. The enzymic reaction was terminated by the addition of 2 N H_2SO_4 and the absorbance at 450 nm was measured, using a molecular device microtiter reader. Standard curve was constructed by plotting absorbance values versus the corresponding concentration of standard (ng/ml). The concentration of CC16 in test samples was determined using the SOFT max PRO software (version 2.4.1).

2.3. Statistical analysis

Data was expressed as means ± S.D. *t*-test statistical analysis at the 95% confidence interval was used to evaluate the serum levels of CC16 in the two groups used in this study.

3. Results

3.1. Method's quality parameters

Anti-CC16 specific monoclonal antibody is immobilized on each well of the microplate in the DiaMed CC16-ELISA. It is selective and has high affinity for the CC16 and presents negligible grade of cross-reactivity with other molecules present in serum samples. The minimum detectable concentration (mean value of: 0 standard + 3S.D.) is expected to be <50 ng/l. The intra-variation from well to well in the same ELISA microplate, as calculated by analysis of three standards with known concentrations, was very low (R.S.D. ≤3.2%). Inter-variations, examined by performing the same immunoassay on different days or by different analysts, showed excellent reproducibility (R.S.D. ≤3.8%). The low intra- and inter-assay variations demonstrated that the ELISA method used for CC16 determination has a very high degree of reproducibility, repeatability and robustness. Calibration curve used for the determination of CC16 serum levels was obtained using standards with known concentrations (Fig. 1).

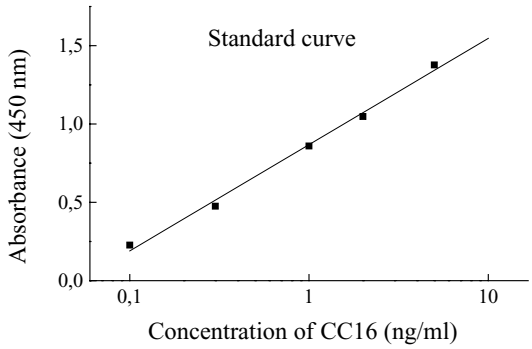

Fig. 1. Calibration graph for quantitative determination of CC16 in blood serum. The optimized curve follows the equation: $y = 0.869 + 0.678 \log x$, where y: absorbance at 450 nm and x: concentration of CC16 (ng/ml).

3.2. Application of enzymic immunoassay to the analysis of serum CC16

Serum CC16 levels were measured in both groups of children. Mean values for healthy children was 27.5 ± 17.6 ng/ml (range: 1.22–95.6 ng/ml). The respective values for children with cronchial asthma were 13.2 ± 8.4 ng/ml (range: 2.6–35.9 ng/ml) (Fig. 2). Statistical analysis showed that CC16 serum levels in asthmatic children were significantly lower as compared to control cases ($P = 0.0004$).

4. Discussion

The identification of certain CC16 levels in children by the ELISA method used suggests a possible role for the protein in airway inflammation and the effect of anti-inflammatory treatments. The difference between CC16 levels found in serum of children with asthma symptoms and healthy ones generate the pos-

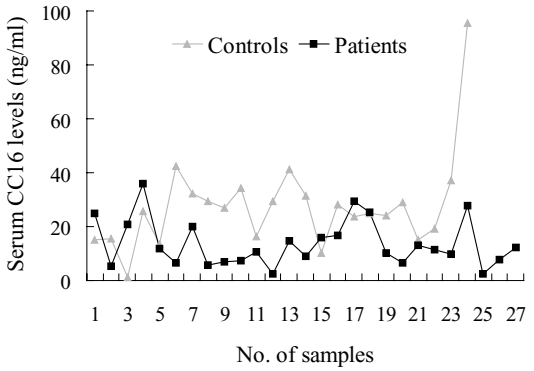

Fig. 2. Levels of CC16 in blood sera from healthy ($n = 24$) and asthmatic children ($n = 27$).

sibility of using the ELISA method for diagnostic test of bronchial asthma in early children age.

Levels of CC16 in BALF have been determined in various lung disorders. A significant reduction has been found in BALF from smokers, patients with lung cancer, chronic obstructive pulmonary disease [12,13], allergic rhinitis [14] or asthma [6,14,15], while increased CC16 levels have been found in BALF from patients with ARDS.

Previous studies showed that plasma CC16 levels were reduced in asthmatic subjects as compared to healthy non-smokers [18,20]. The investigators postulated that the primary causes of these altered levels may be the remodeling of the small airways and alteration of CC16 turnover by the respiratory epithelium.

In this study, it was demonstrated that the enzyme solid phase assay can be easily used for the determination of CC16 in sera samples. Furthermore, it was found that there is a statistically significant difference in CC16 serum concentrations between patients and healthy children. CC16 serum levels were significantly lower in children with asthma, as compared to healthy ones. The present findings support the potential usefulness of the described solid phase assay and particularly of CC16 serum levels as a marker for the in time detection of asthma in children appearing expiratory wheeze. This will give them the opportunity to have a proper corticosteroid therapy and minimize the various side effects.

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