Biomarkers, 2010; 15(7): 619–624

informa
healthcare

ORIGINAL ARTICLE

Development of a new sensitive ELISA for the determination of uteroglobin-related protein 1, a new potential biomarker

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Abstract

Uteroglobin-related protein 1 (UGRP-1) is a protein specifically secreted in airways, where it could play an anti-inflammatory role. We developed a sandwich enzyme-linked immunosorbent assay (ELISA) allowing the detection of UGRP-1 in serum, urine, and amniotic and pulmonary fluids. Concentrations of UGRP-1 determined by ELISA and latex immunoassay were correlated in sputum and bronchoalveolar lavage fluid (BALF). The pattern of UGRP-1 concentration resembled that of Clara cell protein, both proteins occurring in high concentrations in amniotic fluid, sputum and BALF and in much lower concentrations in serum and urine. These findings suggest that UGRP-1 might serve as a biomarker of respiratory epithelium integrity.

Keywords: *Uteroglobin-related protein 1; Clara cell protein; lung biomarker; pneumoprotein*

Introduction

Uteroglobin-related protein-1 (UGRP-1), officially named secretoglobin B3A2 (SCGB3A2), is a highly specific protein of the airways that was discovered by studying the downstream targets of the thyroid transcription factor 1 (TTF-1) (Niimi et al. 2001). The protein is mainly expressed in epithelial cells of the trachea, bronchi and bronchioles (HELIOS 2003). These epithelial cells have not yet been identified but they appear to be distinct from the Clara cell (Reynolds et al. 2002). UGRP-1 is a homodimeric secretory protein of 17kDa, which shares a 25% gene homology with the lung secretory Clara cell protein (CC16) (Niimi et al. 2001, 2002, HELIOS 2003). By fluorescent *in situ* hybridization, the UGRP-1 human gene was localized in the 5q31-34 chromosomal region, where several genes implicated in the pathogenesis of asthma have been identified (Postma et al. 1995). In addition, a macrophage scavenger receptor with collagenous structure (MARCO) expressed in lung alveolar macrophages and involved in pulmonary inflammation was identified as one receptor for UGRP-1 (Bin et al. 2003).

The function of UGRP-1 in airways remains largely unknown. Experimental studies suggest that the protein might play a role in downregulating lung inflammation, in particular in allergic asthma. In mice, the expression of UGRP-1 is increased by interleukin (IL)-10, the most common anti-inflammatory cytokine (Srisodsai et al. 2004), while it is reduced by the proinflammatory IL-5 (Chiba et al. 2005). In accordance with these findings, the overexpression of UGRP-1 has been found to suppress airway inflammation, in particular the infiltration of eosinophils, in a murine model of allergic airway inflammation (Chiba et al. 2006). There is also some evidence that UGRP-1 might be involved in lung development during fetal life. The protein is indeed expressed in the fetal lung of the mouse (Porter et al. 2002) and a recent study has shown that the administration of UGRP-1 to embryonic lungs promotes the development of airways (Kurotani et al. 2008). In human subjects, increased levels of UGRP-1 have been found in induced sputum in both asthma and rhinitis, most strikingly in atopic individuals,

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(Received 07 May 2010; revised 07 July 2010; accepted 13 July 2010)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK, Ltd.
DOI: 10.3109/1354750X.2010.508842

supporting the hypothesis that UGRP-1 might play a role in these inflammatory diseases (de Burbure et al. 2007).

Whatever the exact biological function of UGRP-1, this lung-specific protein might present diagnostic interest as a lung epithelium biomarker. Given its high concentrations in pulmonary fluids (de Burbure et al. 2007) and its similarities with CC16, UGRP-1 should leak across the lung–blood barrier into the bloodstream where it should occur in small quantities like other lung secretory proteins (Bernard et al. 1992, Hermans& Bernard 1998, 1999, Greene et al. 2002, Janssen et al. 2003). Research in this area has so far been hampered by the lack of availability of a validated and sufficiently sensitive assay to quantify the protein in serum. In our previous study (de Burbure et al. 2007), we developed a latex immunoassay for UGRP-1, which, because of its limited sensitivity, was applicable only on pulmonary fluids such as sputum and bronchoalveolar lavage (BALF). A more sensitive assay was described by a Japanese team but this test apparently was not subjected to a careful analytical validation, nor was it evaluated to measure the protein in other biological fluids (Inoue et al. 2008). In addition, the concentrations of UGRP-1 obtained with this enzyme-linked immunosorbent assay (ELISA) in normal human plasma (0.1 $\mu g\,l^{-1}$) appeared abnormally low compared with that of CC16 and other lung-specific proteins secreted in the respiratory tract.

In this study, we validated a sensitive ELISA for measuring UGRP-1 in human biological fluids including serum. We also compared the UGRP-1 distribution with that of CC16 in order to gain insight into its potential applications as a biomarker.

Methods

ELISA assay for UGRP-1

White polystyrene microtitre plates were coated with rabbit antihuman UGRP-1 polyclonal antibody. We applied 100µl of coating antibody solution (500mM bicarbonate buffer, pH 9.6, 750$ngml^{-1}$) to each well and plates were incubated at 4°C overnight. The plates were washed four times with 250µl of phosphate-buffered saline (PBS 1mM, pH 7.4 containing 0.5% of Tween). We then added to each well 250µl of blocking buffer (PBS 1mM, pH 7.4 containing 5% of gelatin). After an incubation of 2 h at 37°C, the plates were washed four times with the washing buffer. We constructed calibration curves with ten points by serially diluting a solution of recombinant protein containing 25µg protein ml^{-1} (H00117156-P01;Abnova, Taipei City, Taiwan). One hundred microlitres of calibrators or samples diluted with a diluting buffer (PBS 1mM, pH 7.4 containing 0.5% of Tween and 5% of gelatin) were applied and the plates were incubated at 4°C overnight. We washed plates six times with the washing solution. Then, 100µl of mouse antihuman recombinant UGRP-1 monoclonal antibody (H00117156-M01;Abnova; 50$ngml^{-1}$ in the diluting buffer) was applied to each well and the plates were incubated for 4 h at 37°C. The plates were washed seven times with the washing buffer. We applied 100µl of rabbit antimouse IgG2A horseradish peroxidase-labelled (LO-MG2a-9 HRPO; purchased from the laboratory of Experimental Immunology of the Catholic University of Louvain, Belgium; 500μgml^{-1} in PBS–glycerol 50% kept at -20°C) to each well and the plated were placed at 37°C for 2 h. Further we washed the plates as above. We added 250µl of acetate solution (100mM, pH 5.5) to each well and we incubated the plates at room temperature for 5 min. After removing the acetate solution, we added 100µl of substrate (BM Blue POD substrate, soluble 11484281001; Roche, Basel, Switzerland) to each well. The reaction was stopped with the addition of 100µl of H_2SO_4 (1M) and we measured endpoint absorbance at 450nm on a microplate colorimetric reader (Infinite F200;Tecan, Männedorf, Switzerland).

Latex immunoassay for CC16 and UGRP-1

CC16 and UGRP-1 were quantified by a semiautomated non-isotopic immunoassay based on the agglutination of latex particles coated with a polyclonal antibody (Bernard &Lauwerys 1983, Bernard et al. 1992, de Burbure et al. 2007). For both proteins, matrix interferences were eliminated by a polyethylene glycol treatment (7% final concentration) followed by a 20 and 40 times dilution of the serum sample. We used a rabbit antihuman CC16 polyclonal antibody (A0257;Dako, Glostrup, Denmark) and a combination of two inhouse polyclonal antibodies for UGRP-1.

Collection of biological fluids

Samples of serum, urine, nasal lavage fluid (NALF) and exhaled breath condensate (EBC) were collectedfrom healthy male and female adolescents and young adults examined in the framework of epidemiological studies on the respiratory effects of air pollution (Bernard et al. 2009).The protocol of these studies was approved by the ethics committee of the Faculty of Medicine of the Catholic University of Louvain. NALF samples were collected from both nostrils according to the procedure described by Tossa et al. (2009). Briefly, participants were asked to sit down, bend forward and put their heads down. Two and a half millilitres of sterile physiological water at 37°C was instilled into each nostril by a disposable tip connected to a peristaltic pump. After 10 s, students lifted their heads and the NALF was collected using a small funnel. EBC was collected using a portable condenser (TURBO-DECCS; ItalChill, Parma, Italy) as

described previously (Mutti et al. 2006). The subjects were asked to breathe tidally through the mouthpiece for 20 min while sitting comfortably. Each subject also provided an untimed urine sample and a blood sample that was collected on a dry tube after application of an anaesthetic cream (Emla; AstraZeneca, Karlskargo, Sweden). Samples of BALF, induced sputum and amniotic liquid were obtained from patients. All samples were kept at -20°C until analysis. To measure UGRP-1 in EBC and NALF, the samples were not diluted. Samples of serum, BALF, induced sputum and amniotic liquid were diluted 2, 10, 100 and 500 times, respectively. All measurements were carried out in duplicate.

Statistical analyses

All statistical analyses were performed using SAS Enterprise guide software (version 8.1).The results are reported as mean with standard deviation and median with the interquartile range (IQR). After logarithmic transformation, we used linear regression analyses for the comparisons between the ELISA and latex immunoassay and between UGRP-1 and CC16.

Results

ELISA procedure and validation

During the development of our UGRP-1 sandwich ELISA, we found that the optimal conditions were obtained with a polyclonal rabbit antihuman UGRP-1 antibody for the capture and a mouse monoclonal antihuman UGRP-1 antibody for the detection. We used also a secondary polyclonal goat antimouse antibody labelled with the horseradish peroxidase. The signal was visualized after the enzymatic reaction between the peroxidase and the added substrate (steps and the reagent concentrations described in Materials and methods). Figure 1 shows a typical calibration curve obtained with standards of UGRP-1 recombinant protein. This curve is the composite of ten curves run over a period of 6 months, which illustrates the high reproducibility of the assay. The detection limit, defined as the concentration of the protein that could be distinguished from zero, was 0.6 μg l^{-1} and the working range extended from approximately 1 to 40 μg l^{-1}.

We checked the linearity of the assay by measuring UGRP-1 in five serum samples (7.5, 8.6, 9.5, 15.8 and 20.4μg l^{-1}), which were tested undiluted and diluted 2, 4 and 8 times. Expressed in percentage of the values in undiluted samples, the concentration of UGRP-1 in these five serum samples averaged over the dilutions of 2, 4 and 8 were 89% (SD 7%), 83% (SD 9%), 74% (SD 9%), 86% (SD 11%) and 80 % (SD 4%), respectively. The trueness of the assay was assessed by supplementing ten normal sera with 40 μg l^{-1} of recombinant UGRP-1. The analytical recovery averaged 98% (SD 10%). We also tested the stability of UGRP-1 by measuring the protein in a concentrated amniotic fluid sample (UGRP-1 concentration, 34 mg l^{-1}) stored at 4°C over a period of 4 months. While the concentration of UGRP-1 did not change during the first 2 weeks, it dropped to 18 mg l^{-1} (about 50% loss) after 1 month and remained at this level until the end of the experiment. Because of this limited stability, we pursued the evaluation of the assay only on samples that had been kept frozen at -20°C.

Correlation of UGRP-1 between ELISA and latex immunoassay

We compared our technique with the latex immunoassay described previously for the determination of UGRP-1 in BALF and sputum (de Burbure et al. 2007).As shown by Figure 2, there is significant although modest correlation between the concentrations of UGRP-1 measured by both methods in BALF and induced sputum samples (r=0.67, n=41, p<0.001).

UGRP-1 and CC16 in biological fluids samples

Table 1 compares the concentrations of CC16 and UGRP-1 in different biological fluids from healthy subjects (serum, NALF, EBC) or from patients (BALF and induced sputum). In healthy subjects, the concentrations of UGRP-1 and CC16 in serum were very similar with mean or median values between approximately 5 and 10 μg l^{-1}. While serum CC16 did not differ between sexes, the concentration of UGRP-1 in serum was almost twice as high in women than in men (p<0.01) Concentrations of UGRP-1 in normal urine were in the same range of values and varied little with sex in contrast to urinary CC16, which was excreted in much larger amounts by men.

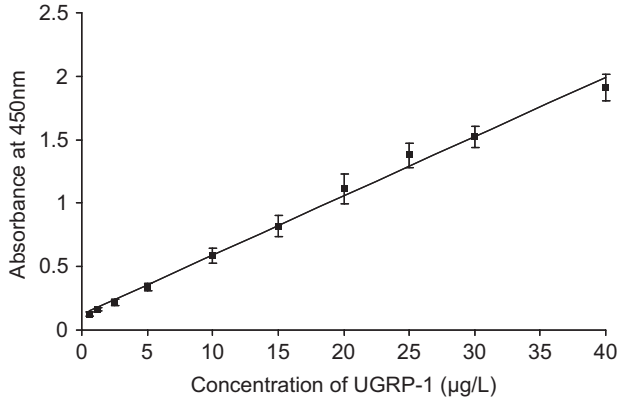

Figure 1. Standard curve for the determination of uteroglobin-related protein-1 (UGRP-1) by enzyme-linked immunosorbent assay (ELISA). The curve is a composite of ten curves run over a period of 6 months. Bars indicate the standard deviation. Slope 0.047 (SD 0.00097); intercept 0.12 (SD 0.017); r^2= 0.99. SD of residuals 0.043.

UGRP-1 could not be detected in NALF samples in contrast to CC16 present in this material at similar concentrations as in serum. None of the 64 samples of EBC tested contained detectable amounts of UGRP-1 and CC16. As expected for these lung-specific proteins, the highest concentrations of UGRP-1 and CC16 were found in BALF, induced sputum and amniotic fluid, with however some noticeable differences between the two proteins. While median concentrations of UGRP-1 in BALF and sputum were on average 50–100 times higher than in serum, the median concentrations of CC16 showed much greater variations, being up to three orders of magnitude higher in sputum than in serum. It is interesting to note also that amniotic fluid and sputum contained rather similar quantities of UGRP-1 while concentrations of CC16 were on average more than 200 times lower in amniotic fluid than in sputum.

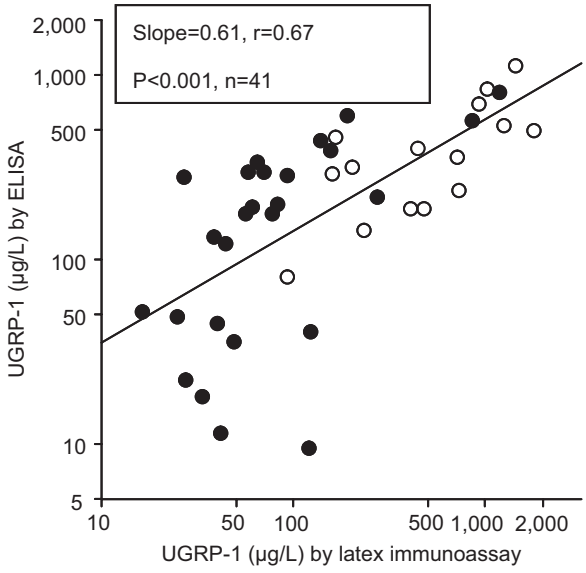

Figure 2. Comparison of enzyme-linked immunosorbent assay (ELISA) with latex immunoassay for the determination of uteroglobin-related protein-1 (UGRP-1) in samples of bronchoalveolar lavage fluid (BALF) (closed circles) and induced sputum (open circles).

Correlation between UGRP-1 and CC16

Concentrations of UGRP-1 measured by our new ELISA and of CC16 determined by the latex immunoassay were compared in induced sputum, BALF and amniotic fluid. As shown in Figure 3, the concentrations of the two proteins were significantly correlated in sputum and amniotic liquid but not in BALF.

Discussion

In this study, we developed a sensitive sandwich ELISA for the determination of UGRP-1 in human biological fluids. The assay was validated for measuring the protein in serum, urine, induced sputum and in BALF. Because in serum the assay provides linear results from a dilution of two, the quantification limit can be estimated at approximately 2 µg l^{-1}, which means that the assay can accurately measure UGRP-1 in most normal serum samples. Because of the limited stability of the protein, the assay is reliable only when used with samples and standards that have been stored frozen or at +4°C for no more than 2 weeks. The correlations between this new ELISA and latex immunoassay, although statistically significant, were relatively modest and despite adjustment of standards, values obtained by ELISA were on average about 40% lower than those determined by latex immunoassay. These variations are not really surprising when one considers the differences between the two methods. An ELISA combining a polyclonal and a

Table 1. Concentrations of uteroglobin-related protein-1 (UGRP-1) and Clara cell protein (CC16) in biological fluids from healthy subjects or patients.

		UGRP-1 (µg l^{-1})			CC16 (µg l^{-1})		
	n	Mean	Median	IQR	Mean	Median	IQR
Healthy subjects							
Serum							
Women	10	8.8	6.9	6.3–8.8	10.3	9.8	8.1–11.8
Men	10	4.8	4.6	3.9–5.5	10.1	9.7	8.2–13.9
Urine							
Women	10	8.2	7.1	0.6–13.1	2.5	2.3	1.9–2.9
Men	10	7.0	1.2	0.6–19.6	107	104	78–142
NALF	64	nd	–	–	15.5	6.7	3.0–14.9
EBC	64	nd	–	–	nd	–	–
Patients							
BALF	25	211	178	45.7–300	889	713	230–1240
Induced sputum	15	420	366	237–517	14 700	9400	5900–25000
Amniotic fluid	53	706	407	251–721	88.9	49.3	21.7–102

IQR, interquartile range; NALF, nasal lavage fluid; EBC, exhaled breath condensate; BALF, bronchoalveolar lavage fluid; nd, not detectable.

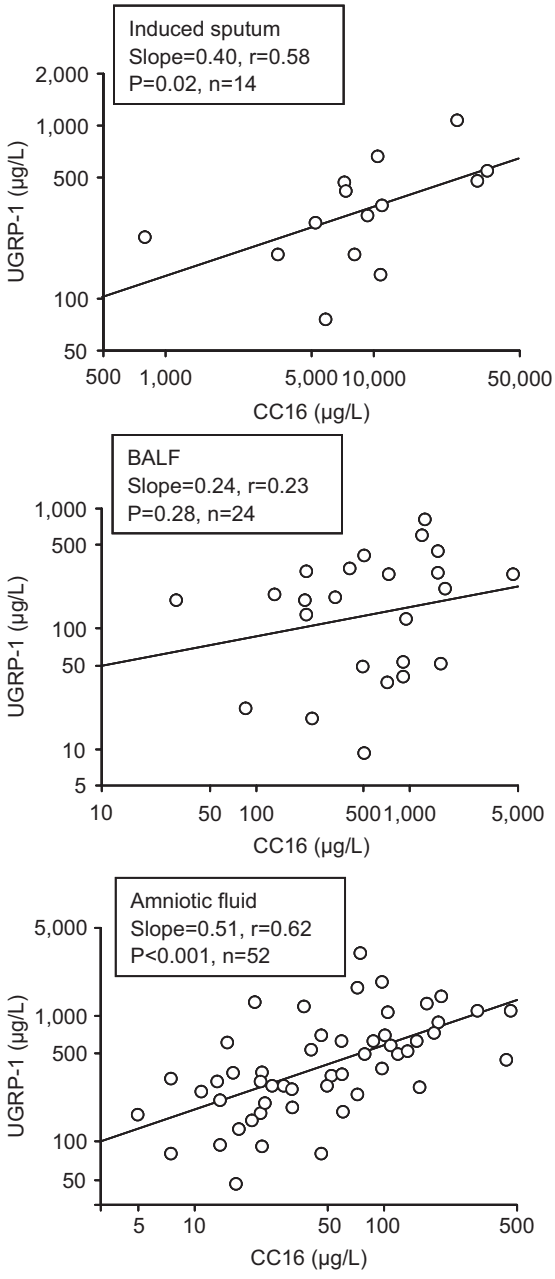

Figure 3. Comparison between the concentrations of uteroglobin-related protein-1 (UGRP-1) and of Clara cell protein (CC16) in induced sputum, bronchoalveolar lavage fluid (BALF) and amniotic fluid.

monoclonal antibody is indeed likely to be more specific than an immunoassay based on two inhouse polyclonal antibodies. In addition, a latex agglutination technique might also be more sensitive than an ELISA to steric hindrance and thus yield different results depending on whether UGRP-1 occurs free as a homodimer, monomer or possibly bound to other molecules (Lu et al.1996).

To our knowledge, our study is the first to have quantified UGRP-1 in human serum using a carefully validated immunoassay. It is also the first study to report concentrations of UGRP-1 in amniotic fluid. Although having a detection limit similar to the present ELISA, the latex immunoassay could not detect the protein in serum because of the large dilution (20 and 40 times) required to eliminate matrix interferences in this test (Bernard et al. 1992). Previously, a Japanese team has reported concentrations of UGRP-1 in human plasma measured by ELISA (Inoue et al. 2008). These authors, however, did not report any data regarding the analytical validity of their ELISA, nor did they attempt to validate their assay by measuring UGRP-1 in pulmonary fluids or comparing their results with another method. UGRP-1 concentrations in plasma reported in this earlier study should thus be interpreted with caution especially as they were abnormally low (on average 0.11 $\mu g\, l^{-1}$ in normal plasma) compared with our values and those reported for other lung secretory proteins. If one refers to values of UGRP-1 in BALF measured by ELISA or latex immunoassay, the BALF/serum ratio in the Japanese study amounted to 2000, a value clearly outside the range of ratios reported for lung-specific proteins of about the same size as UGRP-1 (CC16 ~80, SP-D ~10) or even larger much larger (SP-A ~50) (Hermans& Bernard 1999).

The pattern of UGRP-1 concentrations in biological fluids confirms that UGRP-1 leaks across the broncho-alveolar–blood barrier into plasma in a similar way to CC16 and other lung-specific proteins. The BALF/serum ratio observed in our study (~40) falls perfectly well in the range of ratios reported so far for other lung secretory proteins. Our data suggest that serum UGRP-1 might be a lung epithelium biomarker even more specific than serum CC16 for which a contribution from some other organs (upper airways, prostate and kidney) cannot be formally excluded (Hermans& Bernard 1999). Interestingly, the concentration of serum UGRP-1 in women was almost twice higher as in men, a sex-related difference which is not found with serum CC16. This sex difference certainly deserves further investigation given the anti-inflammatory properties of UGRP-1 and the suspected link of this protein with allergic diseases, which indeed are less common in women. Unlike CC16, UGRP-1 could not be detected in NALF, meaning that the protein is not secreted by the upper airways or at least in minute amounts undetectable by our assays. Concentrations of UGRP-1 in the urine of women and men were rather similar, which contrasts with the huge sex difference in the urinary excretion of CC16 due to the secretion of the protein by the prostate (Bernard et al. 1991). This lack of post-renal secretion of UGRP-1 suggests that compared with CC16, urinary UGRP-1 might be a more specific marker of the proximal tubular function (Martin-Granadoet al. 2009) and perhaps also a better surrogate marker of the airways epithelium (Anderssonet al. 2007). Another feature that differentiates UGRP-1 from CC16 is the concentration in amniotic fluid, where levels of UGRP-1 were about one order of magnitude greater than those of CC16 while they

were systematically lower in the other biological fluids. This is in agreement with the experimental studies suggesting that UGRP-1 plays a role in the airways development (Porter et al. 2002, Kurotaniet al. 2008).

In conclusion, the lung-specific protein UGRP-1 can be quantified in pulmonary fluids but also in serum where by analogy with CC16 it might serve as a peripheral marker of the airways epithelium integrity. Because of its potential anti-inflammatory function, the UGRP-1 assay might be useful to explore mechanisms or risk factors in the development of asthma and respiratory allergies.

Acknowledgements

The authors thank Michael Eppe for his technical assistance.

Declaration of interests

This study was supported by the Fund for Research in Industry and Agriculture (FRIA), Belgium and by the Belgian Science Policy (ANIMO project). A.B. is research director of the National Fund for Scientific Research, Belgium. None of the authors has a conflict of interest to disclose.

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