BIOMARKERS, VOL. 8, NO. 6 (NOVEMBER–DECEMBER 2003), 461–471

Taylor & Francis
healthsciences

Determinants of serum levels of surfactant proteins A and B and Clara cell protein CC16

CEDRIC HERMANS[1], PING DONG[2], MARJORIE ROBIN[1], MICHEL JADOUL[3], ALFRED BERNARD[1], ANDREW D. BERSTEN[4] and IAN R. DOYLE[2]*

[1] Industrial Toxicology and Occupational Medicine, Medical School, Université Catholique de Louvain, Brussels, Belgium.
[2] Department of Human Physiology, School of Medicine, Flinders University, Flinders Medical Centre, Adelaide, Australia.
[3] Nephrology Unit, Medical School, Université Catholique de Louvain, Brussels, Belgium.
[4] Department of Critical Care Medicine, School of Medicine, Flinders University, Flinders Medical Centre, Adelaide, Australia.

Received 21 May 2003, revised form accepted 11 November 2003

Increased leakage of surfactant proteins A and B (SP-A and SP-B) and Clara cell secretory protein (CC16) from the air spaces into the circulation occurs in a range of respiratory conditions. However, circulating levels depend not only on the rate of entry into the circulation, but also on the rate of clearance. In order to clarify the role of the kidney in the clearance of these proteins, serum levels were related to markers of glomerular filtration in 54 non-smoking patients with varying degrees of renal dysfunction, none of whom had respiratory disease or were receiving dialysis at the time of sampling. Serum SP-A was related to SP-B ($r = 0.53$, $p < 0.001$) and to CC16 ($r = 0.33$, $p < 0.02$). Similarly, SP-B was related to CC16 ($r = 0.39$, $p < 0.004$). Stepwise multiple linear regression analysis suggested that serum SP-A and SP-B are influenced by age (~ 20 and $\sim 25\%$ of variance, respectively), whereas CC16 is determined by renal function and, to a lesser extent, by body weight ($\sim 63\%$ of variance in total). We conclude that CC16 is cleared from blood by the renal route, whereas SP-A and SP-B are not. Serum SP-A and SP-B are influenced by age, which we speculate reflects increased damage to the alveolocapillary barrier.

Keywords: pneumoproteinaemia, alveolocapillary permeability, peripheral markers, plasma protein clearance.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

Introduction

The lung epithelium secretes several specific proteins into the air spaces of the respiratory tract. Among these are the major secretory product of Clara cells, the 16 kDa bronchiolar Clara cell protein (CC16), and the alveolar surfactant-associated proteins A to D. Although it is not known whether surfactant protein (SP) C occurs in blood, small amounts of CC16, SP-A, SP-B and SP-D do. Whereas SP-D is expressed by a number of tissues (Akiyama *et al.* 2002), expression of CC16 and SP-A are largely, and of SP-B is exclusively, confined to the lungs (Hermans and Bernard 1999, Akiyama *et al.* 2002). Consequently, the occurrence of CC16, SP-A and SP-B in the circulation can only be explained by leakage into the vascular compartment. Although the exact mechanisms by which

* Corresponding author: Ian R. Doyle, Department of Human Physiology, Flinders University, Bedford Park, South Australia, Australia 5042. Tel: (+61) 8 8204 5494; Fax: (+61) 8 8204 5768; e-mail: Ian.Doyle@flinders.edu.au

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2003 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/1354750031000164702 1

RIGHTSLINK

these proteins enter the blood remains poorly understood, their concentrations in serum, referred to as 'pneumoproteinaemia', could potentially serve as early and highly sensitive peripheral markers for cellular toxicity and epithelial leakage at different levels of the respiratory tract. Intravascular leakage of the proteins increases in conditions characterized by pulmonary inflammation and/or pulmonary epithelial injury (Kuroki *et al.* 1993, Abe *et al.* 1995, Nomori *et al.* 1995, Lesur *et al.* 1996, Okamoto *et al.* 1997, Doyle *et al.* 1997, Carbonnelle *et al.* 2002, Bernard *et al.* 2003). Moreover, we have recently shown that, whereas tobacco smoking increases serum SP-B (Robin *et al.* 2002), it decreases serum CC16, consistent with increased alveolocapillary leakage and the known toxic effects of tobacco smoke on Clara cells (Bernard *et al.* 1992).

However, circulating levels of pneumoproteins will depend not only on the rate of their entry into the circulation, but also on the rate of their plasma clearance. We have previously shown that in patients with acute lung injury (ALI), levels of CC16, SP-A and SP-B are lower in mixed venous than in arterial plasma, indicating that the proteins are rapidly cleared from the circulation (Doyle *et al.* 1998). The kidney normally accounts for 30–80% of the plasma clearance of low molecular weight proteins (Maack *et al.* 1979). In ALI patients we found that the serum clearance of CC16 was related to that of creatinine, whereas that of SP-A and SP-B was not. Moreover, the arterial–venous gradient for SP-A and SP-B was directly related to their arterial levels, suggesting that temporal increases in their serum levels are due to enhanced alveolocapillary permeability that surpass any increase in plasma clearance. Our findings suggest that, at least in ALI patients, the concentration of CC16 in the vascular compartment is influenced by the glomerular filtration rate (GFR), whereas the levels of SP-A and SP-B reflect flux from the lung that is largely independent of this variable.

Plasma protein clearance is greatly altered in patients with ALI as systemic disease is frequently present (Doyle *et al.* 1998). In order to further elucidate the factors influencing the levels of pneumoproteins in the circulation and to clarify the role of the kidney in the plasma clearance of these proteins, we have related the levels of the pneumoproteins in the vascular compartment to glomerular filtration and to the levels of two other low molecular weight proteins both known to be cleared by a renal route (Donadio *et al.* 2001) – β_2-microglobulin (β_2M), an 11.8 kDa ubiquitous protein (Kin *et al.* 1977, Riska *et al.* 1982), and retinol-binding protein (RBP), a 21 kDa liver protein (Rask *et al.* 1980) – in 55 non-smoking patients with varying degrees of renal dysfunction, none of whom had respiratory disease or were receiving dialysis at the time of sampling.

Material and methods

Study population

The protocol of the study was approved by the Ethical Committee of the Faculty of Medicine, Université Catholique de Louvain, Brussels, Belgium. A single blood sample was drawn from the antecubital vein of 17 non-smoking females with a median age of 53 years (range 28–83 years) and 37 non-smoking males with a median age of 51 years (range 16–87 years) with varying degrees of chronic renal failure, none of whom were yet receiving dialysis. An additional blood sample was taken on a separate occasion approximately 4 weeks later from one of each group of patients with diabetic nephropathy, nephrosclerosis and chronic glomerulonephritis.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Analytical methods

Samples were assayed in a blind, randomized manner.

Glomerular filtration. Serum creatinine was measured using Jaffé's technique (Kabanda *et al.* 1994), and its clearance corrected for age and weight was calculated by the Cockroft formula and used as an index of renal function (Cockcroft and Gault 1976). Creatinine clearance in women was corrected by multiplying by 0.72.

CC16, β_2M and RBP. These proteins were all measured using latex immunoassay (Bernard *et al.* 1991, Kabanda *et al.* 1994). Briefly, to avoid possible interference by complement, rheumatoid factor or chylomicrons, the serum samples were pretreated by heating at 56°C for 30 min and the addition of polyethylene glycol (16%, v/v, 1:1) and trichloroacetic acid (10%, v/v, 1:40). After overnight precipitation at 4°C, the samples were centrifuged (2000 *g* for 10 min). CC16, RBP and β_2M were determined in the supernatants using an automatic agglutination technique with purified standards and antibodies from Dakopatts (Glostrup, Denmark). All samples were analysed in duplicate at two different dilutions. The assays have an average analytical recovery of 95%, with intra- and inter-assay coefficients of variation ranging from 5–10% (Bernard and Lauwerys 1983, Bernard *et al.* 1991).

SP-A and SP-B. SP-A and SP-B were measured using inhibition enzyme-linked immunosorbent assays (ELISAs) (Doyle *et al.* 1997). Briefly, SP-A and SP-B were first freed from any associated components using ethylene diamine tetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS) and Triton X-100. The proteins were then measured with inhibition ELISAs using polyclonal antibodies raised against alveolar proteinosis-derived SP-A and mature SP-B. Absorbance was measured at 405 nm using a Dynatech MR5000 reader (Dynatech Laboratories, Chantilly, Virginia, USA). All samples were assayed in duplicate at four serial dilutions. To compensate for differences in incubation times inherent when manually pipetting across the ELISA plate, each plate included two standard curves, one positioned at the beginning of the plate and one at the end, comprising eight serial dilutions in duplicate (SP-A, 1.95–250.00 ng ml^{-1}; SP-B, 7.8–1000.0 ng ml^{-1}; $r > 0.99$). AssayZap software (Biosoft, Ferguson, Missouri, USA) was used to generate standard curves and to weight the analysis of each sample according to its position within the ELISA plate.

The SP-A assay has intra- and inter-assay coefficients of variation (CVs) of $< 7\%$ and 10%, respectively (Doyle *et al*. 1994). The SP-B assay has intra- and inter-assay CVs of < 9 and 10%, respectively, as determined by repeated measures ($n = 16$) of four plasma samples ranging in concentration from 11.7 to 81.0 μg ml^{-1}. The detection limit of the assay is 4.1 ng ml^{-1} (Ekins 1991) ($n = 30$), with an analytical recovery ($\pm$SD) of $105 \pm 27\%$ as determined by repeated 'spiking' of the same four plasma samples with 0.75, 1.5, 3.0 and 6.0 μg ml^{-1} of SP-B.

Statistics

Since serum CC16, SP-A and SP-B levels are not normally distributed (Doyle *et al.* 1997, Robin *et al.* 2002), all analyses were made using log-transformed data. The mean and 95% confidence intervals were determined using log-transformed data and then back-transformed. The independent samples *t*-test was used for all comparisons. The associations between the measured variables were tested using the Pearson correlation test.

Multiple linear regression analysis of the serum variables. The levels of serum CC16, SP-A and SP-B must be determined by both their rate of entry into, and clearance from, the circulation. The kidney normally accounts for the majority of plasma clearance of low molecular weight proteins (Maack *et al.* 1979). The GFR is influenced by body weight and is reflected in the serum creatinine clearance. In addition, there is an age- and gender-related influence on the level of many circulating antigens, including CC16, SP-A and SP-B (Robin *et al.* 2002). Therefore, we hypothesized that in subjects without lung injury, the independent variables influencing serum β_2M, RBP, CC16, SP-A and SP-B would include age, body weight, serum creatinine clearance, gender and total protein level. All data was log-transformed for inclusion in the stepwise backwards regression analysis model, where independent variables with p values < 0.05 were sequentially excluded.

Results

Renal function

Patients with diabetic nephropathy and nephrosclerosis were older than patients with chronic glomerulonephritis ($p = 0.005$ and 0.000, respectively), chronic

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

interstitial nephritis ($p = 0.010$ and 0.004) or polycystic kidney disease ($p = 0.060$ and 0.015), although there were no other statistical differences between the groups with regards to indices of renal function (table 1).

Gender

Even though serum creatinine was similar in the male and female subjects, serum creatinine clearance was ~60% higher in males (table 2). Creatinine clearance was ~4-fold lower than normal (120 ml min^{-1}) (Cockcroft and Gault 1976) in the male subjects and ~7-fold lower in the females. CC16 was ~45% less in the male subjects. The males subjects were ~24% heavier than the females. There were no other gender-based differences in any of the other determinants.

Serum variables and indices of renal and lung function

Whereas serum β_2M, RBP and CC16 were all strongly related to serum creatinine and inversely related to its serum clearance ($p = 0.000$), SP-A and SP-B were not (table 3). Serum creatinine clearance was also related to body weight and inversely related to age.

Pneumoprotein determinates

The serum levels of the pneumoproteins were strongly co-related (table 3). However, whereas serum CC16 was also strongly related to β_2M and RBP ($p = 0.000$), SP-A and SP-B were not. β_2M, CC16 and RBP was positively influenced by serum creatinine clearance and, to a lesser extent, by body weight (table 4). RBP was also weakly influenced by age. Notably, SP-A and SP-B were only influenced by age.

Discussion

Although the alveolocapillary barrier is normally extraordinarily effective at partitioning the plasma from the alveolar epithelial lining fluid, increased bidirectional protein flux occurs across the barrier in conditions characterized by pulmonary inflammation and/or pulmonary epithelial injury. The determination of the levels of pneumoproteins in the vascular compartment represents a new and original approach for the non-invasive assessment of the integrity of the lung epithelium. However, circulating levels depend not only on the rate of entry into the circulation, but also the rate of clearance. Of particular significance is the kidney, which is the major organ responsible for the plasma clearance of low molecular weight proteins. Our previous work has suggested that, at least in so far as patients with ALI are concerned, whereas plasma clearance is a determinant of CC16 levels, SP-A and SP-B levels reflect lung function independent of this variable. The present study in patients with varying degrees of renal dysfunction, none of whom had respiratory disease or were receiving dialysis at the time of sampling, confirms that the kidney is an important determinant of intravascular levels of CC16, but is not a determinant of circulating levels of SP-A or SP-B.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

Table 1. Indices of renal function in the study population.

Cause of chronic renal failure	*n*	Age (years) Mean	Age (years) 95% CI	Body weight (kg) Mean	Body weight (kg) 95% CI	Creatinine clearance (ml min^{-1}) Mean	Creatinine clearance (ml min^{-1}) 95% CI	Total serum protein (g dl^{-1})[a] Mean	Total serum protein (g dl^{-1})[a] 95% CI	Serum creatinine (mg dl^{-1})[a] Mean	Serum creatinine (mg dl^{-1})[a] 95% CI	Serum RBP (μg ml − 1)[a] Mean	Serum RBP (μg ml − 1)[a] 95% CI	Serum β_2M (μg ml^{-1})[a] Mean	Serum β_2M (μg ml^{-1})[a] 95% CI
Diabetic nephropathy	8	63	52–76	86	76–98	27.3	10.1–74.0	6.7	5.7–7.9	3.0	1.6–5.6	101	74–141	9.1	4.4–18.9
Chronic glomerulonephritis	20	43	30–73	73	49–110	25.6	9.5–69.5	6.7	5.9–7.6	3.5	1.1–11.1	135	66–278	8.3	3.6–18.9
Chronic interstitial nephritis	12	37	16–83	64	46–91	30.5	14.4–67.7	6.8	5.8–7.9	2.6	1.1–6.0	82	38–176	5.3	1.2–22.8
Polycystic kidney disease	6	50	36–70	76	58–99	22.4	7.0–71.3	7.1	6.6–7.6	4.4	2.4–7.8	106	88–127	9.0	4.5–18.2
Nephrosclerosis	7	68	57–80	76	65–89	18.7	4.1–85.3	6.7	6.5–7.0	4.0	2.0–8.0	89	42–189	9.8	3.9–24.5
Multiple myeloma	1	77		70		22.9		6.4		2.7		88		11.9	
Hereditary	1	28		47		19.9		6.5		3.1		92		9.7	
Polyarteritis nodosa	1	79		65		11.9		6.1		4.7		100		15.3	
Wegener's granulomatosis	1	63		82		28.6		6.5		3.1		64		6.3	
Unknown	1	87		73		13.1		5.4		4.1		91		8.9	

95% CI, 95% confidence interval.

The patients with polyarteritis nodosa and Wegener's granulomatosis had no lung involvement of their disease.

[a] The reference intervals used were as follows: total protein, 6.0–8.0 g dl^{-1}; serum creatinine, 0.5–1.2 mg dl^{-1}; serum RBP, 30–60 μg ml^{-1}; serum β_2M, 1.0–3.0 μg ml^{-1} (from Dade Behring Diagnostics Pty Ltd).

RIGHTSLINK

Table 2. Variables measured in the study population.

	Females ($n = 17$)		Males ($n = 37$)		
	Mean	95% CI	Mean	95% CI	p value
Age (years)	52	32–83	49	30–80	0.626
Body weight (kg)	63	41–98	78	62–100	0.001
Creatinine clearance (ml min^{-1})	18.1	6.2–53.2	28.9	11.2–74.5	0.008
Total serum protein (g dl^{-1})	6.7	5.9–7.7	6.7	5.7–7.9	0.971
Serum creatinine (mg dl^{-1})	3.5	1.6–7.6	3.3	1.3–8.3	0.691
Serum RBP (μg ml^{-1})	115	64–206	100	50–198	0.226
Serum β_2M (μg ml^{-1})	10.4	4.8–22.8	7.0	2.4–21.0	0.046
Serum CC16 (ng ml^{-1})	73.8	24.7–220.8	43.2	7.5–247.2	0.020
Serum SP-A (ng ml^{-1})	381	162–897	360	156–832	0.630
Serum SP-B (ng ml^{-1})	3573	1318–9682	3304	1175–9290	0.617

95% CI, 95% confidence interval.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

Study design and validation of methods

Pneumoproteins. The assays employed were validated using independently sourced monoclonal and polyclonal (Bernard and Lauwerys 1983, Bernard *et al*. 1991, Doyle *et al.* 1995, 1997) antibodies. Our CC16 assay includes an initial precipitation step to eliminate serum lipids likely to interfere with its determination (Hermans *et al.* 1998). Similarly, our assay procedure for SP-A and SP-B incorporates EDTA to disrupt the Ca^{2+}–lipid bridges, and SDS and Triton-X 100 to dissociate the proteins and free them from any associated components. We have recently analysed plasma from an infant independently confirmed by two laboratories to have an autosomal recessive frame shift mutation at 121 bp in the SP-B gene that resulted in a premature stop codon and no SP-B mRNA or protein. Analysis of this definitive negative control by ELISA has confirmed the specificity of our assay.

In addition, given that the pneumoproteins share few structural similarities, it is difficult to conceive that the strong correlations we observed with our serum determinations are fortuitous or influenced by non-specific reactivities.

Indices of renal function. Serum creatinine was used as an index of renal function. It is known that serum creatinine is influenced by other factors; indeed, in the present study serum creatinine was slightly lower in females than males and was inversely related to age. We believe that these small differences probably reflect variations in muscle mass rather than renal function. Although the use of single serum creatinine measurements to determine creatinine clearance is limited when renal function changes acutely, all of our patients had stable chronic renal failure in which such changes are unlikely. Indeed, in the three patients where blood was sampled on a second occasion approximately 4 weeks later, creatinine clearance, as estimated by this method, remained similar to that on the previous occasion, and serum CC16, SP-A and SP-B levels were essentially unchanged. As a further precaution, we also measured RBP and β_2M as additional indices of renal function. As with CC16, our assays for RBP and β_2M also included an initial precipitation step to prevent interference by serum lipids. Although we did not correct for multiple comparisons and partial correlation coefficients were not applied, the

RIGHTSLINK

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

Table 3. CROSS CORRELATION BETWEEN THE MEASURED VARIABLES

	β2M	CC16	Creat Clear	Creat	RBP	SP-A	SP-B	Protein	Body Weight
Age	0.31	0.31	−0.33	0.15	0.10	0.45	0.50	−0.26	0.30
	0.017	0.019	0.013	0.266	0.438	*0.001*	*0.000*	0.050	0.024
β2M		0.77	−0.86	0.82	0.58	0.26	0.34	−0.09	−0.20
		0.000	*0.000*	*0.000*	*0.000*	0.058	0.010	0.496	0.144
CC16			−0.78	0.73	0.47	0.33	0.39	0.00	−0.18
			0.000	*0.000*	*0.000*	0.014	*0.003*	0.970	0.173
Creat				−0.88	−0.49	−0.09	−0.35	0.04	0.42
Clear				*0.000*	*0.000*	0.518	0.010	0.758	*0.001*
Creat					0.58	0.08	0.19	0.06	−0.09
					0.000	0.575	0.168	0.644	0.499
RBP						0.21	0.13	0.10	−0.00
						0.119	0.331	0.455	0.992
SP-A							0.53	−0.07	0.31
							0.000	0.615	0.023
SP-B								−0.24	−0.01
								0.075	0.967
Protein									−0.13
									0.329

Cross correlation between the measured variables where the "r" value is given in the top and the "p-value" in the bottom line. Relations where $p < 0.01$ are underlined. Legend as in Table 1.

RIGHTSLINK

Table 4. Determinants of serum proteins.

β_2M	F = 91.0, $p = 0.0000$, $r^2 = 0.77$
	Creatinine clearance: $p = 0.0000$, $\beta = -0.94$
	Body weight: $p = 0.0070$, $\beta = 0.20$
RBP	F = 8.8, $p = 0.0001$, $r^2 = 0.33$
	Creatinine clearance: $p = 0.0000$, $\beta = -0.73$
	Age: $p = 0.0757$, $\beta = -0.25$
	Body weight: $p = 0.0104$, $\beta = 0.38$
CC16	F = 45.9, $p = 0.0000$, $r^2 = 0.63$
	Creatinine clearance: $p = 0.0000$, $\beta = -0.85$
	Body weight: $p = 0.0602$, $\beta = 0.18$
SP-A	Age: $r^2 = 0.20$
	F = 13.1, $p = 0.0007$, $\beta = 0.45$
SP-B	Age: $r^2 = 0.25$
	F = 17.1, $p = 0.0001$, $\beta = 0.50$

We hypothesized that independent variables influencing serum β_2M, CC16, RBP, SP-A and SP-B would include age, body weight, serum creatinine clearance, gender and total protein.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

cross correlations between our indices of renal function (serum creatinine, serum creatinine clearance, RBP and β_2M) were very strong ($p = 0.000$), further strengthening our findings.

β_2M has a diagnostic accuracy very similar to that of creatinine, whereas RBP has been purported to be a less adequate marker of glomerular filtration (Donadio *et al.* 2001), possibly because its serum level varies with hormonal fluctuations and nutritional status. Consistent with this, β_2M and RBP were both predicted by serum creatinine clearance, our index of GFR, but to different extents. Whereas multiple linear regression analysis suggests that GFR, as reflected by serum creatinine clearance, and body weight together accounted for $\sim$77% of the serum β_2M, RBP was also influenced by age, which together accounted for only $\sim$33% of its serum level. Indeed, whereas CC16 (Robin *et al.* 2002) and β_2M (Donadio *et al.* 2001) were elevated approximately in proportion to the decrease in GFR ($\sim$5-fold), RBP (Donadio *et al.* 2001) was increased by a much lesser amount ($\sim$2-fold), consistent with our own (Doyle *et al.* 1998) and other observations (Maack *et al.* 1979, Kabanda *et al.* 1994) that, when the GFR is reduced, some proteins that would otherwise cleared by the kidney are removed by unknown pathways that may partially, or even totally, compensate for the reduced renal protein clearance.

The kidney as an organ of serum protein clearance

The filtration of proteins across the renal glomerular capillary membrane occurs with an approximate log-log relationship to the molecular weight (Maack *et al.* 1979, Kabanda *et al.* 1995, Hermans *et al.* 1998). Although steric considerations also play a major role, most proteins smaller than 50 kDa normally pass relatively freely through the glomerulus, and virtually all (95–99%) are taken up and catabolized by fusion with lysosomes in the heterolysosomes of proximal tubular cells (Guder 1988, Kabanda *et al.* 1995).

Consistent with this, CC16 was strongly predicted by serum creatinine clearance, our index of glomerular filtration. In contrast, and consistent with our findings in ALI patients (Doyle *et al.* 1998), SP-A and SP-B levels were not predicted by serum creatinine clearance. In blood, native SP-A appears to be complexed with immunoglobulin G (IgG) and immunoglobulin M (IgM) (Kuroki

RIGHTSLINK

et al. 1993, Doyle *et al.* 1995). It is possible that such complexes are too large to be filtered in the glomerulus. It is not apparent why SP-B is not cleared by the renal route, since the immunoreactive forms detected in plasma, at least under denaturing electrophoretic conditions, are sufficiently small ($\sim$18–45kDa) (Doyle *et al.* 1997). One possibility is that the hydrophobicity of SP-B is such that it complexes with serum lipids, and the derived complexes are too large to be filtered through the glomerulus.

Systemic clearance of SP-A and SP-B

The pathway by which SP-A and SP-B are cleared from the circulation remains controversial. SP-A may be phagocytosed though binding directly to specific receptor(s) found on macrophages (Chroneos *et al.* 1996), monocytes (Geertsma *et al.* 1994) and neutrophils (Hartshorn *et al.* 1998), or through Fc-receptor-mediated phagocytosis in association with complexed IgG and IgM. Although it is not known whether SP-B specifically binds to phagocytes, SP-B is phagocytosed by alveolar macrophages (Phelps and Floros 1991). Moreover, infants deficient in SP-B die of alveolar proteinosis (deMello and Lin 2001), probably caused by impaired alveolar macrophage clearance of surfactant lipids, while airway administration of SP-B-containing vesicles can enhance the induction of IgM-mediated systemic immunity (van Iwaarden *et al.* 2001). Systemic phagocytes may also clear SP-B.

The effect of age

The present study confirms our recent findings that CC16, SP-A and SP-B increase with age. The levels of CC16, SP-A and SP-B in our patients are similar to those recently reported by us in healthy, aged-matched subjects (Robin *et al.* 2002) and were $\sim$50% higher than we previously reported in subjects aged on average 25 years younger (Bernard *et al.* 1994, Hermans and Bernard 1999). Age-related structural and functional changes in the lung have been previously described. Gas trapping increases with age (Lee *et al.* 2000), as do air space abnormalities (Soejima *et al.* 2000) and diameter (Zeman and Bennett 1995), whereas lung compliance and shear modulus decrease (Lai-Fook and Hyatt 2000) in association with increased lung elastin content (Foster and Curtiss 1990). Moreover, whereas the internal surface area of the lung increases to a maximum of $\sim$80 m^2 at the age of $\sim$20 years, it then decreases $\sim$2.7 m^2 per decade thereafter (Thurlbeck 1967). Finally, increased albumin levels have been reported in bronchoalveolar lavage fluid from older subjects (Roberts *et al.* 1993). We have no information concerning the effect of age on the alveolar levels of SP-A or SP-B. However, since CC16 mRNA expression is not related to age (Jensen *et al.* 1994), we suggest that the age-related pneumoproteinaemia in non-smokers reflects increased alveolocapillary leakage and the effects of non-specific deterioration of the lung.

Conclusion

Our findings suggest that, whereas serum CC16 is influenced by GFR, serum SP-A and SP-B levels are largely independent of this variable. Serum SP-A and SP-B are influenced by age, which we speculate reflects increased damage to the alveolocapillary barrier with ageing.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Acknowledgements

This research was supported by grant no. 102175 from the National Health and Medical Research Council of Australia, the Australian ARDS Association, and by the European Union (EV4-CT96-0171).

References

Abe, S., Honda, Y., Ando, M., Saita, N., Kida, K., Jinno, S., Kondo, A., Kuroki, Y. and Akino, T. 1995, Clinical significance of levels of lung surfactant protein A in serum, in various lung diseases. *Nippon Kyobu Shikkan Gakkai Zasshi*, **33**, 1219–1225.

Akiyama, J., Hoffman, A., Brown, C., Allen, L., Edmondson, J., Poulain, F. and Hawgood, S. 2002, Tissue distribution of surfactant proteins A and D in the mouse. *Journal of Histochemistry and Cytochemistry*, **50**, 993–996.

Bernard, A. and Lauwerys, R. 1983, Continuous flow system for the automation of latex immunoassay by particle counting. *Clinical Chemistry*, **29**, 1007–1011.

Bernard, A., Lauwerys, R., Noël, A., Vandeleene, B. and Lambert, A. 1991, Determination by latex immunoassay of protein 1 in normal and pathological urine. *Clinica Chimica Acta*, **201**, 213–216.

Bernard, A., Marchandisem, F. X., Depelchin, S., Lauwerys, R. and Sibille, Y. 1992, Clara cell protein in serum and bronchoalveolar lavage. *European Respiratory Journal*, **5**, 1231–1238.

Bernard, A. M., Roels, H. A., Buchet, J. P. and Lauwerys, R. R. 1994, Serum Clara cell protein: an indicator of bronchial cell dysfunction caused by tobacco smoking. *Environmental Research*, **66**, 96–104.

Bernard, A., Carbonnelle, S., Michel, O., Higuet, S., de Burbure, C., Buchet, J-P., Hermans, C., Dumont, X. and Doyle, I. R. 2003, Lung hyperpermeability and asthma prevalence in schoolchildren: unexpected associations with the attendance of indoor chlorinated pools. *Occupational Environmental Medicine*, **60**, 385–394.

Carbonnelle, S., Francau, X. M., Doyle, I. R., Dumont, X., de Burbure, C., Morel, G., Michel, O. and Bernard, A. 2002, Changes of serum pneumoproteins caused by short-term exposures to nitrogen trichloride in indoor chlorinated pools. *Biomarkers*, **7**, 464–478.

Chroneos, Z., Abdolrasulnia, R., Whitsett, J., Rice, W. and Shepherd, V. 1996, Purification of a cell-surface receptor for surfactant protein A. *Journal of Biological Chemistry*, **271**, 16375–16383.

Cockcroft, D. W. and Gault, M. H. 1976, Prediction of creatinine clearance from serum creatinine. *Nephron*, **16**, 31–41.

deMello, D. E. and Lin, Z. 2001, Pulmonary alveolar proteinosis: a review. *Pediatric Pathology and Molecular Medicine*, **20**, 413–432.

Donadio, C., Lucchesi, A., Ardini, M. and Giordani, R. 2001, Cystatin C, beta 2-microglobulin, and retinol-binding protein as indicators of glomerular filtration rate: comparison with plasma creatinine. *Journal of Pharmaceutical and Biomedical Analysis*, **24**, 835–842.

Doyle, I. R., Jones, M. E., Barr, H. A., Orgeig, S., Crockett, A. J., McDonald, C. F. and Nicholas, T. E. 1994, Composition of human pulmonary surfactant varies with exercise and level of fitness. *American Journal of Respiratory and Critical Care Medicine*, **149**, 1619–1627.

Doyle, I. R., Nicholas, T. E. and Bersten, A. D. 1995, Serum surfactant protein A levels in patients with acute cardiogenic pulmonary edema and adult respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine*, **152**, 307–317.

Doyle, I. R., Nicholas, T. E. and Bersten, A. D. 1997, Surfactant proteins A and B are elevated in plasma of patients with acute respiratory failure. *American Journal of Respiratory and Critical Care Medicine*, **156**, 1217–1229.

Doyle, I. R., Hermans, C., Bernard, A., Nicholas, T. E. and Bersten, A. D. 1998, Clearance of Clara cell secretory protein and surfactant proteins A and B from blood in acute respiratory failure. *American Journal of Respiratory and Critical Care Medicine*, **158**, 1528–1535.

Ekins, R. P. 1991, Immunoassay design and optimization. In *Principles and Practice of Immunoassays*, edited by C. P. Price and D. J. Newmans (New York: Stockton Press), pp. 93–153.

Foster, J. A. and Curtiss, S. W. 1990, The regulation of lung elastin synthesis. *American Journal of Physiology*, **259**, L13–L23.

Geertsma M, F., Nibbering, P. H., Haagsman, H. P., Daha, M. R. and van Furth, R. 1994, Binding of surfactant protein A to C1q receptors mediates phagocytosis of *Staphylococcus aureus* by monocytes. *American Journal of Physiology*, **267**, L578–L584.

Guder, W. G. 1988, Proteinuria: causes, forms and methods of determination. *Clinical and Diagnostic Laboratory Medicine*, **1**, 49–55.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK

Hartshorn, K. L., Crouch, E., White, M. R., Colamussi, M. L., Kakkanatt, A., Tauber, B., Shepherd, V. and Sastry, K. N. 1998, Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria. *American Journal of Physiology*, **274**, L958–L969.

Hermans, C. and Bernard, A. 1999, Lung epithelium specific proteins: characteristics and potential applications as markers. *American Journal of Respiratory and Critical Care Medicine*, **159**, 646–678.

Hermans, C., Aly, O., Nyberg, B. I., Peterson, C. and Bernard, A. 1998, Determinants of Clara cell protein (CC16) concentration in serum: a reassessment with two different immunoassays. *Clinica Chimica Acta*, **272**, 101–110.

Jensen, S. M., Jones, J. E., Pass, H., Steinberg, S. M. and Linnoila, R. I. 1994, Clara cell 10 kDa protein mRNA in normal and atypical regions of human respiratory epithelium. *International Journal of Cancer*, **58**, 629–637.

Kabanda, K., Jadoul, M., Pochet, J. M., Lauwerys, R., van Ypersele de Strihou, C. and Bernard, A. 1994, Determinants of the serum concentrations of low molecular weight proteins in patients on maintenance hemodialysis. *Kidney International*, **45**, 1689–1696.

Kabanda, K., Goffin, E., Bernard, A., Lauwerys, R. and van Ypersele de Strihou, C. 1995, Factors influencing serum levels and peritoneal clearances of low molecular weight proteins in continuous ambulatory peritoneal dialysis. *Kidney International*, **48**, 1946–1952.

Kin, K., Sakurabayashi, I. and Kawai, T. 1977, Beta 2-microglobulin levels of serum and ascites in malignant diseases. *Japanese Journal of Cancer Research*, **68**, 427–434.

Kuroki, Y., Tsutahara, S., Shijubo, N., Takahashi, H., Shiratori, M., Hattori, A., Honda, Y., Abe, S. and Akino, T. 1993, Elevated levels of lung surfactant protein A in sera from patients with idiopathic pulmonary fibrosis and pulmonary alveolar proteinosis. *American Review of Respiratory Disease*, **147**, 723–729.

Lai-Fook, S. J. and Hyatt, R. E. 2000, Effects of age on elastic moduli of human lungs. *Journal of Applied Physiology*, **89**, 163–168.

Lee, K. W., Chung, S. Y., Yang, I., Lee, Y., Ko, E. Y. and Park, M. J. 2000, Correlation of aging and smoking with air trapping at thin-section CT of the lung in asymptomatic subjects. *Radiology*, **214**, 831–836.

Lesur, O., Bernard, A. and Begin, R. O. 1996, Clara cell protein (CC16) and surfactant associated protein A (SP-A) in asbestos-exposed workers. *Chest*, **109**, 467–474.

Maack, T., Johnson, V., Kau, S. T., Figueiredo, J. and Sigulem, D. 1979, Renal filtration, transport, and metabolism of low-molecular-weight proteins: a review. *Kidney International*, **16**, 251–270.

Nomori, H., Horio, H., Fuyuno, G., Kobayashi, R., Morinaga, S. and Hirabayashi, Y. 1995, Protein 1 (Clara cell protein) serum levels in healthy subjects and patients with bacterial pneumonia. *American Journal of Respiratory and Critical Care Medicine*, **152**, 746–750.

Okamoto, Y., Yoneda, T., Tokuyama, T., Hamada, K., Tsukaguchi, K., Yoshikawa, M., Fu, A., Yamamoto, C., Takenaka, H., Nakaya, M., Kobayashi, A., Narita, N., Tamura, M. and Miyazaki, R. 1997, Serum pulmonary surfactant protein A in asbestosis and asbestos-exposed workers. *American Journal of Respiratory and Critical Care Medicine*, **155**, A808.

Phelps, D. S. and Floros, J. 1991, Localization of pulmonary surfactant proteins using immunohistochemistry and tissue in situ hybridization. *Experimental Lung Research*, **17**, 985–995.

Rask, L., Anundi, H., Böhme, J., Eriksson, U., Fredriksson, A., Nilsson, S. F., Ronne, H., Vahlquist, A. and Peterson, P. A. 1980, The retinol-binding protein. *Scandinavian Journal of Laboratory Investigation*, **40**, 45–59.

Riska, H., Petterson, T., Fröseth, B. and Klockars, M. 1982, Beta 2-microglobulin in pleural effusions. *Acta Medica Scandinavica*, **211**, 45–50.

Roberts, C. M., Cairns, D., Bryant, D. H., Burke, W. M., Yeates, M., Blake, H., Penny, R., Shelley, L., Zaunders, J. J. and Breit, S. N. 1993, Changes in epithelial lining fluid albumin associated with smoking and interstitial lung disease. *European Respiratory Journal*, **6**, 110–115.

Robin, M., Dong, P., Hermans, C., Bernard, A., Broeckaert, F., Clippe, A., Mc Cormack, F., Bersten, A. D. and Doyle, I. R. 2002, Serum CC16, SP-A, and SP-B levels reflect lung epithelial damage in tobacco smokers. *European Respiratory Journal*, **20**, 1152–1161.

Soejima, K., Yamaguchi, K., Kohda, E., Takeshita, K., Ito, Y., Mastubara, H., Oguma, T., Inoue, T., Okubo, Y., Amakawa, K., Tateno., H. and Shiomi, T. 2000, Longitudinal follow-up study of smoking-induced lung density changes by high-resolution computed tomography. *American Journal of Respiratory and Critical Care Medicine*, **161**, 1264–1273.

Thurlbeck, W. M. 1967, Internal surface area and other measurements in emphysema. *Thorax*, **22**, 483–496.

van Iwaarden, J. F., Claassen, E., Jeurissen, S. H., Haagsman, H. P. and Kraal, G. 2001, Alveolar macrophages, surfactant lipids, and surfactant protein B regulate the induction of immune responses via the airways. *American Journal of Respiratory Cell and Molecular Biology*, **24**, 452–458.

Zeman, K. L. and Bennett, W. D. 1995, Measuring alveolar dimensions at total lung capacity by aerosol-derived airway morphometry. *Journal of Aerosol Medicine*, **8**, 135–147.

Biomarkers Downloaded from informahealthcare.com by Hacettepe Univ. on 11/18/12
For personal use only.

RIGHTSLINK