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Yerkovich, Stephanie; Chang, Anne; Carroll, Melanie; Petsky, Helen; Scrivener, Greta; Upham, John

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Soluble receptor for advanced glycation end products (sRAGE) is present at high concentrations in the lungs of children and varies with age and the pattern of lung inflammation

Stephanie T. Yerkovich[1,2], Anne B. Chang[3,4] Melanie L. Carroll[1], Helen L. Petsky[3], Greta Scrivener[3] & John W. Upham[1]

[1]School of Medicine, The University of Queensland, Brisbane, Australia

[2]Queensland Centre for Pulmonary Transplantation & Vascular Disease, The Prince Charles Hospital, Brisbane, Australia

[3]Queensland Children's Respiratory Centre, and Queensland Children's Medical Research Institute, Royal Children's Hospital, Brisbane, Australia

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[4]Respiratory Program, Child Health Division, Menzies School of Health Research, Charles Darwin University, Darwin, NT

Correspondence: John W. Upham, FRACP PhD, The University of Queensland School of Medicine, Princess Alexandra Hospital, Ipswich Road, Woolloongabba, Brisbane, Qld 4102, Australia. Tel: 61-7-3176-7798 Fax: 61-7-3176-6170 Email: j.upham@uq.edu.au

Summary at a Glance

The soluble receptor for advanced glycation end products (sRAGE) plays an important role in inflammation. This study identified that sRAGE is present at higher concentrations in the lung compared to blood of children, is developmentally regulated and varies with the pattern of lung inflammation.

ABSTRACT

Background and objective: The soluble receptor for advanced glycation end products (sRAGE) plays an important role in inflammation. Few studies have looked at sRAGE levels in human lungs, and there is no information in children. Therefore this study aimed to compare bronchoalveolar lavage fluid (BALF) and plasma sRAGE concentrations in children in relation to age and inflammation.

Methods: BAL was performed in 76 children, and BALF and plasma sRAGE levels were determined by ELISA.

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Results: sRAGE levels were four-fold higher in BALF than in plasma ($p<0.001$). BALF sRAGE was inversely proportional to age ($r=-0.333$, $p=0.008$) and serum IgA ($r=-0.283$, $p=0.028$). Plasma sRAGE showed a positive correlation to the percentage of BAL macrophages and negative correlation to the percentage of neutrophils and lymphocytes ($p<0.05$). Multivariate linear regression analysis identified that the percentage of BAL lymphocytes and neutrophils were significant independent predictors of plasma sRAGE levels, while age and the percentage of BAL macrophages independently predicted BALF sRAGE levels.

Conclusions: In children, sRAGE is present at higher concentrations in the lung compared to blood. It appears that sRAGE varies with age and hence future studies of sRAGE in paediatric lung disease require age-matching. The significant relationship between sRAGE and lung inflammation warrants further research.

Keywords

bronchoalveolar lavage fluid

inflammation

lung

soluble receptor for advanced glycation end products

Short title

sRAGE in BALF and plasma of children

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INTRODUCTION

Early childhood is marked by rapid lung growth and by vulnerability to respiratory infections and inflammatory disorders including pneumonia, bronchiolitis and asthma. This vulnerability is probably due in part to immaturity of innate and adaptive immunity [1]. Current understandings of the mechanisms that regulate inflammatory pathways and immune function in childhood are incomplete.

There has been much recent interest in the receptor for advanced glycation end products (RAGE) which plays an important but complex role in many inflammatory responses. RAGE is expressed in many organs including the lung, and mediates responses to cell injury induced by a variety of stimuli including oxidative stress and hypoxia [2, 3]. The many ligands for RAGE include advanced glycation end-product modified proteins, serum amyloid A, amphoterin and members of the S100 protein family. Binding of these ligands to RAGE on cell membranes initiates a pro-inflammatory cascade. RAGE has several isoforms including a soluble form known as sRAGE that functions as a decoy receptor, mopping up RAGE ligands in the extra-cellular fluid and thereby protecting against tissue injury and inflammation. These protective properties have been shown in a variety of animal models, where treatment with sRAGE can successfully reverse inflammation [4, 5].

Both membrane bound RAGE and sRAGE are expressed constitutively in normal adult lung tissue, even in the absence of inflammation or tissue injury [6-8], perhaps because the lung is constantly exposed to a multitude of environmental stimuli. RAGE expression appears to be crucial for normal type I alveolar epithelial cell function, so RAGE may also play an important homeostatic role in the lung under steady state conditions.

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The role of RAGE and sRAGE in lung pathology appears complex. Acute lung injury is associated with increased concentrations of RAGE in bronchoalveolar lavage (BAL) fluid and plasma [9-12], while in animal models of pneumonia, RAGE appears to impair host defence [13]. Membrane RAGE and sRAGE are reduced in patients with idiopathic pulmonary fibrosis [7, 14]. Plasma sRAGE is also reduced in chronic obstructive pulmonary disease [15], and there is some evidence that RAGE may have a protective role in animal models of lung disease [7, 14].

Little is known regarding RAGE expression in the lungs of children, though this might be relevant to a number of pulmonary diseases of children. Studies in rats indicate that membrane bound RAGE and sRAGE are expressed to a lesser degree in lungs from neonatal rats than adult lungs [16]. Expression of both RAGE isoforms increases during post-natal life [16], though this has not been examined in humans.

The aims of the current study were (i) to examine BAL fluid and plasma sRAGE in children of various ages and (ii) to determine whether sRAGE is associated with lung inflammatory cells, age and humoral immune function.

METHODS

Subject recruitment

Between March 2008 and September 2009, 76 children who were undergoing bronchoscopy for a clinical indication including evaluation of chronic cough and assessment of the upper airways were enrolled into the study. The latter group of children have no history of current or chronic cough and had stridor. Children were recruited from the respiratory outpatient department, Royal Children's Hospital, Brisbane and were excluded if they were thought to have recurrent aspiration (presence of swallowing disorder or cerebral palsy),

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immunodeficiency, prematurity (<37 weeks gestation), or a specific underlying respiratory disorder (such as cystic fibrosis, interstitial lung disease, bronchiectasis, or more than 2 prior episodes of pneumonia). The study was approved by the Royal Children's Hospital Ethics Committee, Brisbane, Australia and parents consented for all samples to be used.

Bronchoscopy and BAL

Flexible bronchoscopy was performed transnasally under general anaesthesia as previously described [17] and was well tolerated in all subjects. BAL was obtained as per the recommendations of the ERS Task Force on BAL in children [18]. The first aliquot (1ml/kg, max 10mls of normal saline instilled) was used for microbiological examination and detection of bacteria, fungi and viruses using standard methodology. The second and third aliquots (total 2mls/kg, max 20 mls) were pooled for total cell count and differential leukocyte counts. The remaining BAL fluid (from pooled 2nd and 3rd aliquots) was stored at -80°C for subsequent batch analysis of sRAGE levels. Recorded information included yield from BAL, macroscopic appearances and the presence of any anatomical abnormalities.

Blood sampling

At recruitment a peripheral blood sample was taken and immunoglobulins (IgA, IgM, IgE and IgG) were measured by Queensland Pathology using commercial auto-analysers. A second sample was taken at bronchoscopy and plasma was stored at -80°C for later batch analysis of sRAGE levels.

Measurement of sRAGE

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Concentrations of sRAGE in BAL fluid and plasma were determined by commercial ELISA (R&D Systems, Minneapolis, USA) according to manufacturer's directions. Detection limit for the assay was 19.5 pg/ml.

Statistical analysis

Data is presented as the median (interquartile range) unless otherwise stated. SPSS (IBM SPSS Inc, Chicago) was used for data analysis and $p<0.05$ was considered statistically significant. Group differences were assessed by Mann-Whitney U Test, while paired samples were compared by Wilcoxon Signed-Rank Test. Correlations between variables were determined using Spearman's rho. For simple and multiple linear regression analysis, variables were examined for normal distribution and if necessary were natural log-transformed before further analysis. Simple linear regression analysis was initially used to evaluate the relationship between variables and sRAGE. Those variables in which $p<0.1$ were then subjected to multiple linear regression analysis followed by interaction and residual analyses.

RESULTS

Subject Characteristics and BAL differential cell counts

Full characteristics of the study subjects are outlined in Table 1. Briefly, the median age of the children was 20.1 (11.8 – 41) months. Within the lung there was range in the number and pattern of inflammatory cells present, with a median total cell count (TCC) of 150 (94 – 300) cells/ml and 14% (5 - 53) neutrophils and 70% (34 - 87) macrophages in the BALF of the children. Bacterial pathogens (at growth $\geq 10^5$ cfu/ml) were detected in the BALF of 53 (70%)

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children. There was no difference in the age of the children being assessed for chronic cough compared to those having an upper airway assessment (data not shown).

BALF sRAGE levels are higher than plasma levels, and are negatively correlated with age

Matched BALF and plasma samples were available from 62 children (82% of cohort). There was no difference in demographic factors between the children with and without matched plasma and BALF. sRAGE levels were approximately 4 fold higher ($p<0.001$) in BALF compared to plasma (Figure 1). Surprisingly, there was no statistically significant association between plasma and BALF sRAGE levels ($r=0.160$ $p=0.213$). We next investigated how these levels varied with the age of the children, and found a significant inverse association between BALF sRAGE levels and age ($p=0.008$, Figure 2A). However, this relationship was not seen between plasma sRAGE and age (Figure 2B).

Relationships between BALF and plasma sRAGE levels and inflammatory cells

There was no relationship between the concentration of sRAGE in plasma or BALF and the BAL TCC (Table 2). However, there was a significant inverse association between plasma sRAGE and the percentage of lymphocytes ($p=0.007$) and neutrophils ($p=0.008$, Table 2) in the BAL differential, such that the lowest plasma sRAGE concentrations were observed in those with greater degrees of BAL lymphocytosis and neutrophilia. In contrast, plasma sRAGE was positively correlated with the percentage of BAL macrophages ($p=0.012$). There was no relationship between any inflammatory cells in BAL and BALF sRAGE levels (Table 2). Similarly, there was no relationship between any inflammatory cell differentials in the blood and BALF or plasma sRAGE levels (Table 2). There was also no difference in the concentration of sRAGE measured in either the BALF or plasma between children who did or

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did not have positive microbial cultures (defined as growth ≥10^5 cfu/ml, Table 2). Finally, BALF cytokine levels were also assessed. IL-6 in BAL fluid was not associated with BALF sRAGE (r=-0.057, p=0.66) and IL-8 was very weakly inversely proportional to BALF sRAGE (r=-0.259, p=0.05, data not shown).

BALF sRAGE levels are negatively correlated with serum IgA levels

There was a weak negative correlation between BALF sRAGE and serum IgA (r = -0.283, p=0.028), but this was not seen with either IgG or IgM (Table 3). Similarly, there was no relationship between plasma sRAGE levels and any serum antibody (Table 3).

Variables associated with plasma and BALF sRAGE

To better understand the relationship between plasma or BALF sRAGE levels and age, sex, serum immunoglobulin levels, TCC and BAL cellularity, regression modelling was performed. Simple univariate linear regression of data from all subjects showed that plasma sRAGE was slightly higher in male children, was inversely predicted by the percentage of BALF lymphocytes and neutrophils and positively predicted by the percentage of macrophages in the BAL (Table 4). When these variables were included in a multivariate linear regression analysis, only the percentage of lymphocytes and neutrophils remained as the strongest independent predictors of plasma sRAGE levels (Table 4). There was no association with immunoglobulin levels, age or the total BAL cell count. Also, there was no association between BALF sRAGE and either the intensity or type of bacterial pathogens isolated from BALF, regardless of whether it was analysed by the total number of organisms grown, or where infection (≥10^5 cfu/ml) was analysed as a dichotomous variable (data not shown).

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Simple univariate linear regression analysis identified that the main predictors of BALF sRAGE levels were male sex, age, serum IgA levels and the percentage of macrophages in the BAL (Table 5). When these variables were included in a multivariate linear regression model, only the percentage of macrophages and age remained as independent predictors (Table 5). There was no association with TCC, or immunoglobulin levels, other than those previously mentioned.

DISCUSSION

In this study we measured sRAGE levels in matched samples from the lungs and circulation of children. The key findings to emerge were that sRAGE levels were considerably higher in BALF than in plasma, and that BALF, but not plasma, sRAGE levels varied in relation to age. Furthermore, multivariate linear regression analysis identified that sRAGE levels within both the circulation and lung were associated with the profile of lung inflammatory cells, highlighting a potentially important role for sRAGE in lung inflammation.

While several recent studies have examined sRAGE in adult lung diseases [7, 9, 14, 15, 19], there are no publications on this important and complex inflammatory axis in children. In addition, few investigators have simultaneously measured sRAGE in both lung and blood compartments [20], and there are no published studies on sRAGE in BAL of children. It is clear from the data shown in Figure 1 that sRAGE is present at much higher concentrations in BALF than in plasma. This is not surprising, given that both membrane bound RAGE and sRAGE are expressed abundantly in normal adult lung tissue [6-8]. Some of the sRAGE in plasma is likely to have originated from the lung. However, it is likely that other tissues also contribute to plasma sRAGE, and the lack of a statistically significant association between

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BALF and plasma concentrations suggests it is too simplistic to attribute plasma sRAGE levels as merely a consequence of leakage of sRAGE from the lung. Proving the 'over spill' of inflammatory mediators from the lung into the circulation is difficult, as discussed recently [21], so we propose that measuring sRAGE in both the lung and blood compartments provides complementary information.

Our finding that BALF sRAGE varied in relation to age (Figure 2 and Table 5) suggests that it may be developmentally regulated. To our knowledge there has only been one other study looking at sRAGE in relation to age, a murine study comparing neonatal and adult sRAGE expression [16]. While these investigators showed that sRAGE expression was lower in neonatal than in adult mice, sRAGE was measured by western blotting which can be difficult to accurately quantitate. Interestingly, the investigators reported that sRAGE glycosylation patterns vary with age which may account for their observations. It is unknown if age-related differences in glycosylation exist in humans, but as we measured sRAGE using a commercial ELISA which detects the same sRAGE antigen over the entire cohort we believe that BALF sRAGE is developmentally regulated. It is intriguing that BALF sRAGE is inversely proportional to serum IgA, which may suggest that sRAGE is produced at high levels in the lung at a time in early childhood when the main mucosal antibody, IgA, is relatively deficient. Hence, future studies of sRAGE in children need to consider age as an important variable.

It is notable that BALF sRAGE levels showed an approximately 500 fold range in concentration compared to the 20 fold range in blood sRAGE. It is unknown why BALF sRAGE shows such a high degree of variability across the cohort, and while age would account for some of the spread, further investigations will be required to more accurately address this issue. However a high degree of variability was also found in a study that

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evaluated the effect of mechanical ventilation on BALF sRAGE in adults (range from 0-2500 pg/ml) [20]. This study has highlighted the strong statistical association between lung inflammation and both BALF and plasma sRAGE (Tables 4 & 5). Inflammatory cell populations in the lung are dependent on differentiation within bone marrow, trafficking through the circulation and recruitment to the lung, and plasma sRAGE might be involved in some or all of these processes. The strong association between lung inflammation and BALF and plasma sRAGE may also suggest that either sRAGE is being produced or utilised in response to current inflammation. Alternatively, it may be expressed in response to current infection as previously suggested [12], although our data in children does not appear to support this (Table 2). The extent to which variations in lung and blood sRAGE contribute to the pathogenesis of lung inflammation or are instead a downstream consequence of lung inflammation is an unresolved issue that warrants further research. It is noteworthy that variations in the *AGER* gene that regulates RAGE expression appear to be risk factors for some diseases [22-26]. Longitudinal studies will be required to more accurately define if variations in both plasma and BALF sRAGE levels are indeed risk factors for respiratory infections, inflammation and disease in children.

There are a number of limitations of the current study that should be acknowledged. While it would have been ideal to have recruited a group of truly healthy children for the study, there are considerable ethical issues around performing bronchoscopy on asymptomatic children. Therefore, although 15 children did not have any airway inflammation (ie bronchoscopy was to performed to evaluate upper airway lesions), we acknowledge that our cohort may not be reflective of the normal healthy situation. Nonetheless, our study provides an important foundation for further investigation of the role of sRAGE in airway inflammatory conditions in children.

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In conclusion, BALF sRAGE is present at higher concentrations than circulating plasma sRAGE and appears to vary with age. Hence comparative studies of sRAGE in children with lung disease should include age-matching of cases and controls. The extent to which variations in lung and blood sRAGE contribute to the pathogenesis of lung inflammation or are instead a downstream consequence of lung inflammation is an unresolved issue that warrants further research.

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Table 1: Subject Characteristics

	Study Subject Characteristics (n=76)
Age, median (IQR) months	20.1 (11.8 – 41.0)
Male, n (%)	53 (70)
Reasons for bronchoscopy, n (%)	
Assessment of upper airways	15 (20)
Presence of lower respiratory symptoms (cough or wheeze)	61 (80)
BALF characteristics	
TCC, median (IQR)	150 (94 – 330)
%macrophages, median (IQR)	70 (34 – 87)
%lymphocytes, median (IQR)	9 (5 – 12)
%neutrophils, median (IQR)	14 (5 – 53)
Number of bacterial pathogens grown at $\geq 10^5$ cfu/ml, median (IQR)	2 (0 - 3)
Serum characteristics	
IgA (g/l), median (IQR)	0.5 (0.2 – 0.7)
IgG (g/l), median (IQR)	6.4 (4.9 – 8.2)
IgM (g/l), median (IQR)	0.8 (0.6 – 1.1)

IQR= interquartile range,

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Table 2: Non-parametric correlations between sRAGE levels and inflammatory cells

	Plasma sRAGE	BALF sRAGE
TCC	r = -0.096, p=0.416	r = 0.007, p=0.955
%macrophages in BALF	**r = 0.293, p=0.012**	r = 0.142, p=0.280
%lymphocytes in BALF	**r = -0.311, p=0.007**	r = -0.007, p=0.954
%neutrophils in BALF	**r = -0.306, p=0.008**	r = -0.091 p=0.492
Number of bacterial pathogens with growth ≥10^5 cfu/ml	r = 0.0320, p = 0.784	r = -0.0694, p= 0.591
%neutrophils in blood	r = -0.198, p=0.092	r = -0.242, p=0.064
%monocytes in blood	r = -0.209, p=0.076	r = -0.022, p=0.867
%lymphocytes in blood	r = -0.056, p=0.637	r = 0.145, p=0.274
White blood cell count	r = -0.176, p=0.139	r = 0.050, p=0.711

Table 3: Correlations between sRAGE levels and serum immunoglobulin levels

	Plasma sRAGE	BALF sRAGE
IgA	r = -0.203, p=0.084	**r = -0.283, p=0.028**
IgM	r = -0.102, p=0.391	r = -0.157, p=0.230
IgG	r = -0.161, p=0.165	r = -0.172, p=0.180

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Table 4: Linear regression analysis of plasma sRAGE levels as the dependant variable

Univariate analysis	***β***	***p***	***95% CI for β***
Male sex	-0.265	0.093	-0.577 – 0.045
* % lymphocytes in BALF	-0.265	0.004	-0.445 - -0.085
* % neutrophils in BALF	-0.146	0.008	-0.254 - -0.039
* % macrophages in BALF	0.172	0.063	-0.009 – 0.353
Multivariate analysis	***β***	***p***	***95% CI for β***
* % lymphocytes in BALF	-0.222	0.018	-0.405 - -0.039
* % neutrophils in BALF	-0.119	0.028	-0.226 - -0.013

Variables marked with (*) and sRAGE were natural log-transformed. Only variables with $p<0.01$ are shown in the table. r^2 of the multivariate linear regression = 0.166.

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Table 5 : Linear regression analysis of BALF sRAGE levels as the dependant variable

Univariate analysis	***β***	***p***	***95% CI for β***
Male sex	-0.614	0.045	-1.213 - -0.015
* Age (months)	-0.322	0.030	-0.611 - -0.033
* plasma IgA	-0.409	0.013	-0.729 - -0.088
* % macrophages in BALF	0.591	<0.001	0.295 – 0.887
Multivariate analysis	***β***	***p***	***95% CI for β***
* Age (months)	-0.272	0.042	-0.535 - -0.010
* % macrophages in BALF	0.561	<0.001	0.271 – 0.850

Variables marked with (*) and sRAGE were natural log-transformed. Only variables with $p<0.01$ are shown in the table. r^2 of the multivariate linear regression = 0.279.

FIGURE LEGENDS

Figure 1: BALF and plasma sRAGE levels

sRAGE levels were measured in matched (n=62) BALF and plasma samples, and are presented as box and whisker plots with significant differences indicated.

Figure 2: BALF and plasma sRAGE levels plotted against age

BALF (A) and plasma (B) sRAGE levels are shown against the age of the children at sampling. Data are presented a scatter plots with significant correlations indicated.

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Figure 1

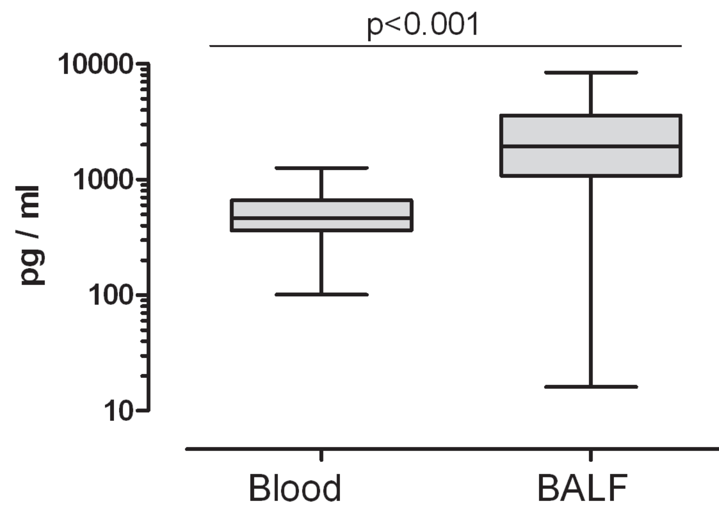

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Figure 2