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Plasma Concentrations of Growth Arrest Specific Protein 6 and the Soluble Form of its Tyrosine Kinase Receptor Axl in Abdominal Aortic Aneurysms

Carl Ekman[1], Despina Flondell Site[2], Anders Gottsäter[2], Bengt Lindblad[2], and Björn Dahlbäck[1].

[1]University of Lund, Department of Laboratory Medicine, Clinical Chemistry, Wallenberg Laboratory SE-205 02 Malmö,

[2]Vascular Centre Malmö-Lund, Malmö University Hospital UMAS, Malmö, Sweden

Running title: Plasma sAxl and Gas6 in aneurysm patients

Adress correspondence to bjorn.dahlback@med.lu.se

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Abstract

The tyrosine kinase receptor Axl is ubiquitously expressed in the vasculature. The vitamin K-dependent protein growth arrest specific protein 6 (Gas6) is the activating ligand of Axl. Human Axl can be cleaved by an unknown protease thus releasing soluble Axl (sAxl), which can be detected in circulation. The aim of this study was to determine plasma concentrations of Gas6 and sAxl in patients with abdominal aortic aneurysms (AAA) and to evaluate if Gas6 and sAxl can be used as biomarkers for AAA. Immunoassays for sAxl and Gas6 were used to investigate plasma from patients with AAA. Patients with large (>55 mm for men and >50 mm for women, n=123) or small AAAs (n=122) were compared with healthy, age-matched controls (n=141). Gas6 correlated positively with size of AAA, whereas sAxl correlated inversely with AAA size. As a consequence, the calculated Gas6/sAxl ratios correlated even better to AAA size. A Gas6/sAxl ratio above 0.55 was found in 40% in of the subjects in the large AAA group but in no controls. These results suggest that the Gas6/Axl system might be involved in AAA pathogenesis and that the Gas6/sAxl ratio may be useful as a biomarker for AAA.

Abbreviations: AAA, Abdominal aortic aneurysm; Gas6, growth arrest specific protein nr. 6; sAxl, soluble Axl

Abdominal Aortic Aneurysm (AAA) is a disease of the abdominal aorta weakening the vessel wall, resulting in increased vessel diameter, and making the vessel more prone to rupture. Ruptured AAA is currently the 15th leading cause of mortality among men in the United States[1]. The mechanisms governing AAA growth and rupture are not fully elucidated, but heredity, smoking, age, hypertension and high cholesterol have been shown to increase the risk of AAA formation[2]. Inflammation, as reflected by increased plasma concentrations of fibrinogen, IL-6, C-reactive protein[3], and elevation of matrix metalloproteinases[4] has also been suggested to be involved in the pathogenesis of AAA[5]. Biomarkers that could differentiate between stable aneurysms and aneurysms prone to growth and subsequent rupture have been extensively sought for, as they would be of great clinical relevance[1]. However, no marker has yet been identified as "the marker of choice" to use for screening or follow up.

Receptor tyrosine kinases (RTKs) and their ligands are crucially important for the functional integrity of the vasculature[6,7]. Axl is a member of the TAM family of RTKs, consisting of **T**yro3 (Sky), **A**xl and **M**er, and it is expressed in endothelium, vascular smooth muscle cells and fibroblasts of the vessel wall[8,9]. The expression of Axl is upregulated in response to vascular injury being primarily located in the cells of the neointima, suggesting that Axl may be a mediator of vascular smooth muscle migration and proliferation[10]. Axl is stimulated by Gas6 (product of the growth arrest specific gene 6) that was originally found as a protein expressed by growth arrested fibroblasts[9,11,12]. Axl is phosphorylated in response to Gas6 binding and the activation of Axl results in anti-apoptotic and prosurvival effects, mainly due to involvement of the PI3 kinase and Akt pathways[9,12]. Gas6 stimulation can rescue

serum-starved fibroblasts and vascular smooth muscle cells from apoptosis[13-16]. Gas6 also has growth promoting activity, inducing proliferation in normal and malignant cells expressing Axl[9,17,18] The Axl receptor has been shown to be expressed in a wide range of tissues and cell lines[8,9,19]. Overexpression of the Axl receptor is found in many different human cancers [20] and has been demonstrated to be of importance for growth of human gliomas[21] and gastric cell cancer[22].

Axl is a transmembrane protein, but the extracellular part of the Axl receptor tyrosine kinase can be shedded from cells, resulting in a soluble receptor. In mice, this process is dependent on the ADAM10 enzyme, but the shedding mechanism for the human sAxl has not been elucidated[23].

The Axl ligand Gas6 is a member of the vitamin K-dependent protein family, Gas6 being homologous to the anticoagulant protein S[11,12,18]. Gas6 is expressed in many cell types, including endothelial cells, vascular smooth muscle cells and fibroblasts[11]. However, expression is low in the liver, explaining the low concentration of Gas6 (approximately 0.2 nM) in plasma[24-26]. The affinity between Gas6 and the Axl receptor is in the subnanomolar range suggesting that Gas6 and sAxl in plasma may form a complex[27,28].

The physiological importance of Gas6 and the TAM receptors have been studied in genetically modified mice models. Gas6$^{-/-}$ mice are found to be resistant to both arterial and venous thrombosis models[29,30]. Similar results were obtained with Axl/Tyro3/Mer$^{-/-}$ mice[31]. Additional studies in mice have suggested that Gas6 is important for endothelial activation, as Gas6$^{-/-}$ animals showed impaired ICAM upregulation, as well as decreased adhesion by immune cells and platelets after exposure to tumor necrosis factor alpha[32].

The aim of this study was to elucidate whether the disease process of AAA are related to circulating concentrations of Gas6 and sAxl and if Gas6 and sAxl are useful as biomarkers for AAA.

Material and Methods

Study Design

The Vascular Center in Malmö serves an area with 750.000 inhabitants. AAA patients visiting the center 2002-2006 for planned AAA operations or for routine ultrasonographic surveillance of AAAs were included in this study. Blood sampling and data collection was done before any AAA treatment. No patients with acute, inflammatory or ruptured AAAs were included. The study included 123 patients with large AAAs considered for operation (>55 mm for men and >50 mm for women), 122 patients with small AAAs undergoing surveillance and 141 healthy, age matched controls without symptomatic cardiovascular disease or peripheral atherosclerosis. Further data is given in table 1. The Ethical Committé of Lund University approved the study and all patients gave written consent to participate in the study.

Assesment of clinical data and medical history

Blood pressure was measured in the right arm after 10 minutes rest. Pharmacological treatment and diseases such as diabetes mellitus, cerebrovascular and ischemic heart disease were recorded. Laboratory analytes such as haemoglobin, mean corpuscular volume, leukocyte and platelet count, creatinine, cholesterol and homocystein were measured in the clinical chemistry laboratory of the hospital with routine methods.

ELISA measurements of Gas6 and sAxl

Gas6 was measured with a sandwich ELISA utilizing a polyclonal goat antibody AF154 (R&D, MN, USA) as a catcher, and a biotinylated rabbit antiserum as a

detector[24]. sAxl was measured with a sandwich ELISA using an Ig fraction of an in-house polyclonal rabbit antiserum (denoted Axl-042), raised against purified recombinant sAxl expressed in a stable HEK293 cell line, as immobilized catcher in Maxisorp microtiter plates (Nunc, Roskilde, Denmark). The biotinylated Ig-fraction of another in-house rabbit antiserum (Axl-041) was used as detector, essentially as described for the Gas6 ELISA[24]. The details of the recombinant sAxl expression and the sAxl ELISA will be described elsewhere (Ekman and Dahlbäck, unpublished data 2009). In brief, all incubation steps were carried out at room temperature and every step was followed by washes with TBS-Tween 20 (50 mM Tris, 150 mM NaCl, pH 7.4 containing 0.1% Tween 20). Samples and reagents were diluted in TBS-Tween containing 3% fish gelatin. A dilution series of a known concentration of the recombinant sAxl was used as a standard and wells with only Fish-TBS-Tween were used as negative controls. Samples were incubated overnight. The detecting antibody was incubated 1 hour, using 3 μg/ml of biotinylated 041. The ELISA incubated for 30 minutes with StreptABC/HRP (Dako, Glostrup, Denmark) before development with OPD tablets (Dako).

Statistical analysis

Statistical analysis was made with Graphpad 4.0 (Graphpad software, CA, USA), using the Mann-Whitney statistical test for difference between groups, and Spearman's rank correlation test was tested to evaluate correlations. Statview 5.0.1 (SAS institute, NC, USA) was used for multiple regression analysis. A $p<0,05$ was considered significant for all analyses.

Results

Gas6 and sAxl in plasma of patients with AAA

Gas6 and sAxl were measured in plasma from patients with AAA and healthy controls (Fig 1). Patients with large AAAs were found to have higher Gas6 concentrations than those with smaller AAAs and healthy controls (Fig 1A). In contrast, the concentrations of sAxl were inversely correlated to AAA size, the patients with large AAAs having lower sAxl than those with small AAA and healthy controls (Fig 1B). The calculated Gas6/sAxl ratio stratified even better between the groups (Fig 1C). Many patients were found to have a Gas6/sAxl ratio above 0.55, which was the upper limit of the healthy controls. In the group of patients with large AAA many individuals (around 40%) had high Gas6/sAxl ratios (Fig 1C). Thus, a high Gas6/sAxl ratio showed a high positive predictive value for large AAA, whereas a low Gas6/sAxl ratio did not exclude the presence of an AAA.
When the measured size of the AAA was compared to the Gas6 concentration, a positive correlation was observed (Fig 2A), whereas for sAxl, a negative correlation was shown (Fig 2B). The correlation between the Gas6/sAxl ratio and AAA size was stronger than between Gas6 or sAxl and AAA size (Fig 2C).

Correlation analysis was performed to evaluate whether other commonly used biochemical markers correlated with Gas6, sAxl, and the Gas6/sAxl ratio. Gas6 correlated significantly to TIMP-1 and IL-6 concentrations, whereas it had an inverse correlation to diastolic blood pressure and cholesterol. sAxl correlated to TIMP-1, MMP2, MMP9, antitrypsin and ceruloplasmin. The Gas6/sAxl ratio correlated positively to IL-6 and inversely to MMP2, MMP9 and leucocyte concentrations (Table 2).

Logistic regression was performed to evaluate if Gas6, sAxl and the Gas6/sAxl ratio were independent determinants of AAA larger than 55 mm in size. We found that all three variables were independent determinants from the other main known risk factors (Table 3). Independent odds ratios per standard deviation were 1.119 (CI 1.013-1.419, p=0.0354) for Gas6 and 0.778 (CI 0.651-0.933, p=0.0066) for sAxl, and 1.228 (CI 1.045-1.442, p=0.0125, (Table 3)) for the Gas6/sAxl ratio.

Discussion

In this study, we report data regarding the plasma concentrations of Gas6 and sAxl proteins in a cohort of patients with AAA. Gas6 concentration correlated positively, and sAxl correlated negatively with AAA size. The correlation between Gas6/sAxl ratio and AAA size was also positive and stronger than the separate correlations for Gas6 or sAxl. Furthermore, concentrations of Gas6, sAxl and Gas6/sAxl ratio were independent determinants for having an AAA above 55 mm. About 40% of all patients with large AAAs had Gas6/sAxl ratios above the highest values found among healthy controls. The results suggest that the Gas6/sAxl ratio cannot be used to exclude the presence of AAA, but may be useful for screening purposes to find individuals with risk of having large AAA. However, additional studies are needed to elucidate the validity of using Gas6/sAxl ratios for AAA screening purposes.

The Gas6 plasma concentrations have been reported to be decreased by Warfarin[24] and oral contraceptives[26] whereas increased levels have been found in patients suffering from sepsis[33,34]. To our knowledge, the plasma concentrations of sAxl have not previously been measured in patients with different diseases. Gas6 and Axl are expressed in endothelial cells, where they are of importance for endothelial activation[32]. Gas6 is also involved in phagocytosis of apoptotic cells[35-37], and increased circulating Gas6 might be a sign of upregulated expression of Gas6 because of increased apoptosis. sAxl has been shown to bind and inhibit Gas6 in a variety of experimental situations[17,38,39], and increased Gas6/sAxl ratios observed in plasma could be associated with less inhibition by sAxl and stronger Gas6-mediated signalling.

The mechanism behind the increased Gas6/sAxl ratios in patients having large AAAs is unknown. Possibly, the increased levels of Gas6 suggest activation of Gas6 gene expression, either locally in the aneurysm or systemically in the vasculature. Whether the decreased sAxl is a reflection of decreased Axl gene expression, increased internalization of Axl due to the increased Gas6 concentration and subsequent Axl activation, or decreased cleavage of Axl by proteinases, remains to be elucidated. Moreover, it is not yet known whether patients having increased Gas6/sAxl ratios constitute a subgroup with respect to progression and prognosis of the disease.

Disclosure of interest: The authors have no conflicts of interest to disclose.

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Table 1.

	Large AAA	range	Small AAA	range	Healthy individuals	range
Number	123		122		141	
% Male	87		75		51	
% Smokers	28		31		10	
% Hypertensive	70		74		52	
Age in years	75	55-87	73	55-87	67	67-78
BMI (Kg/m2)	25	14-39	25	15-39	27	17-39
SBP (mmHg)	140	85-200	140	100-200	140	100-190
Gas6 (ng/ml)	13.5	5.4-32.2	11.6	5.1-28.9	11.9	4.6-23.8
sAxl (ng/ml)	26.0	13.4-64.2	29.0	17.0-64.4	32.1	21.2-59.8
Gas6/sAxl	0.49	0.25-1.54	0.39	0.21-1.02	0.36	0.08-0.55

Table 1. Comparison of patients with and without AAA investigated for Gas6 and sAxl concentration. The median value and the range are given for all variables. BMI = body mass index, SBP = systolic blood pressure. % Complications is defined as former myocardial infarction, cerebrovascular infarction or claudocatio intermittens.

Table 2.

Gas6 correlations	r	p-value
TIMP-1	0.1925	0.0003
Diastole BP	-0.1741	0.0011
Cholesterol	-0.1711	0.0015
IL-6	0.119	0.026

sAxl correlations	r	p-value
TIMP-1	0.2784	<0.0001
MMP2	0.3381	<0.0001
MMP9	0.2343	<0.0001
Antitrypsin	0.1953	0.0018
Ceruloplasmin	0.1724	0.0059

Gas6/sAxl correlations	r	p-value
MMP2	-0.3747	<0.0001
MMP9	-0.1648	0.0092
Leucocytes	-0.1875	0.0068
IL-6	0.1664	0.0052

Correlations of Gas6, sAxl and Gas6/sAxl ratio to the biochemical analytes in the patient material. The correlations were evaluated using the Spearman rank correlation test. TIMP-1 = tissue inhibitor of metalloproteinase 1, IL-6 = interleukin 6, BP = blood pressure, MMP = matrix metalloproteinase.

Table 3.

Independent determinants of AAA >55 mm in the AAA cohort

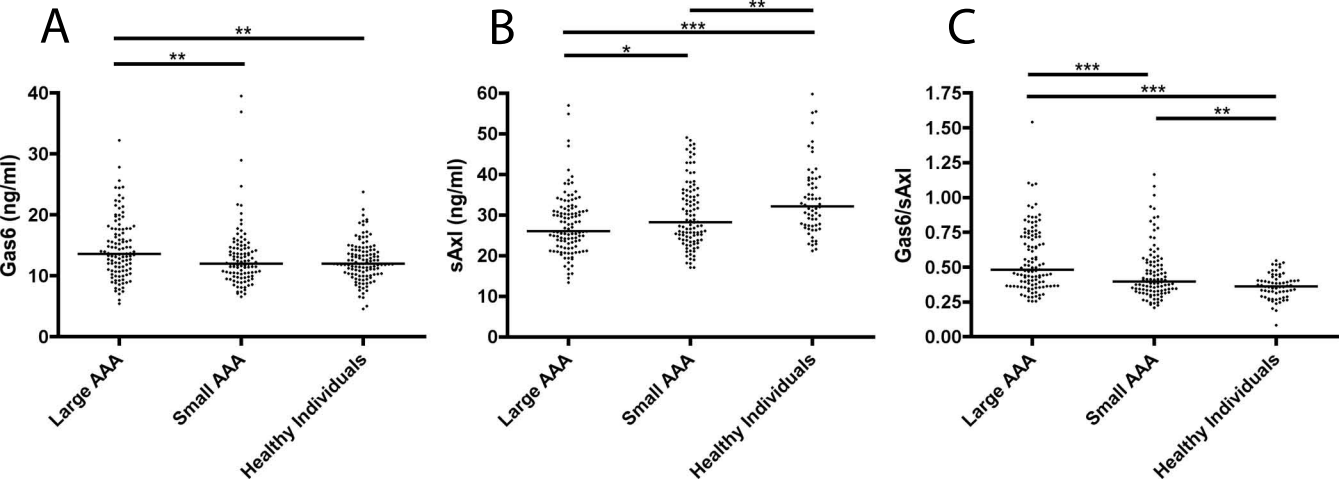
Characteristic	P-value	Odds ratio	CI lower	CI higher
Age in years	0.3072	1.099	0.916	1.314
Systolic BP	0.1496	0.881	0.735	1.052
Diastolic BP	0.4119	0.915	0.739	1.132
Male gender	0.0332	3.762	1.111	12.738
Current smoker	0.3923	1.463	0.612	3.498
Diabetes Mellitus	0.1603	2.474	0.699	8.763
Statin treatment	0.3679	1.507	0.617	3.677
C-Reactive Protein	0.078	1.149	0.982	1.348
S-Creatinine	0.1063	0.882	0.761	1.021
COPD	0.8461	1.039	0.704	1.534
Hemoglobin	0.0278	0.804	0.664	0.974
Leucocyte count	0.0077	1.316	1.075	1.610
Thrombocyte count	0.0139	0.746	0.612	0.937
BMI (Kg/m2)	0.9849	0.998	0.819	1.217
Myocardial infarction	0.0672	2.460	0.938	6.450
ACE inhibitor treatment	0.6154	0.813	0.362	1.824
Antihypertensive treatment	0.9694	0.985	0.457	2.122
Gas6/sAxl ratio	0.0125	1.228	1.045	1.442

BP = Blood pressure, BMI = Body mass index, ACE = Angiotensin converting enzyme. The odds ratio for the continous variables are given in odds ratio per standard deviation.

Titles and legends to figures

Figure 1. Gas6 and sAxl concentrations, and Gas6/sAxl ratios in AAA patients. (A), Gas6 plasma concentrations in patients with large AAAs, small AAAs and healty controls. (B), sAxl plasma concentrations and (C), Gas6/sAxl ratios in the same groups. The p-values in the graph are defined * $p<0.05$, ** $p<0.01$ *** $p<0.001$ using the Mann-Whitney test.

Figure 2. Correlations between AAA size and plasma Gas6 and sAxl concentrations, between AAA size and Gas6/sAxl ratios. The Spearman's correlations were significant between AAA size and (A) Gas6 ($p=0.0145$), (B) sAxl ($p=0.0027$) and (C) the Gas6/sAxl ratio ($p<0.0001$).


A
B
C
Gas6 (ng/ml)
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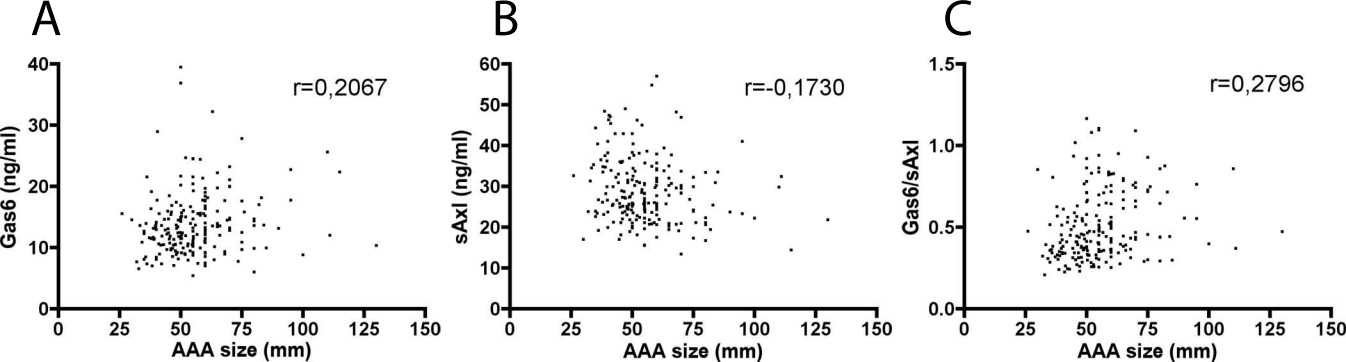
Large AAA
Small AAA
Healthy Individuals


A
Gas6 (ng/ml)
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AAA size (mm)
B
sAxl (ng/ml)
r=-0,1730
AAA size (mm)
C
Gas6/sAxl
r=0,2796
AAA size (mm)