The Soluble Form of the Axl Receptor Tyrosine Kinase

Ekman, Carl

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The soluble form of the Axl receptor tyrosine kinase

Carl Ekman
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Faculty Opponent
Professor Gian Carlo Avanzi
Dipartimento di Medicina Clinica e Sperimentale
Università del Piemonte Arientale A.Avogadro

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Abstract

The present investigation focuses on the receptor Axl, member of the receptor tyrosine kinase family. Axl is activated by the ligand Gas6, which induces cell growth and proliferation, but also regulates inflammation and vascular homeostasis. The Axl receptor can be cleaved outside the membrane, releasing the extracellular, soluble domain, which alone can bind Gas6, removing it from cell-bound receptors.

The first study focuses on soluble Axl (sAxl), which was found to be secreted from several human cell lines and to be present in human circulation. An ELISA was developed against Axl to determine the concentration of sAxl. Through immunoprecipitation and gel filtration, Gas6 was found to be in complex with sAxl, with sAxl in molar excess over Gas6.

In the second study, patients with abdominal aortic aneurysms were investigated with Gas6 and sAxl ELISAs. Gas6 is increased and sAxl is decreased compared to the control group. Aneurysm size correlated to Gas6 concentration and inversely to sAxl concentration. The Gas6/sAxl ratio correlated even better to Aneurysm size, and 40% of all patients with large Aneurysms had higher Gas6/sAxl ratio than any in the control group.

The third study investigated patients with critical limb ischemia. The patients had high Gas6 and sAxl, and both proteins correlated with several inflammatory markers. Patients with high Gas6 and sAxl had worse prognosis with higher mortality, independent of age and gender.

The fourth study assessed patients with Sepsis and related inflammatory conditions. In septic patients, Gas6 was twice the concentration observed in the controls, and sAxl was also increased. Gas6 was especially increased in patients with organ failure, patients demanding intensive care or renal support.

The fifth study focused on patients with systemic lupus erythematosus. Gas6 and sAxl correlated with disease intensity, and was increased in patients with glomerulonephritis and presence of anti-DNA antibodies.

Altogether, the results from this thesis include that Gas6 is bound to sAxl in circulation and that Gas6 and sAxl are increased in various inflammatory conditions.

Key words: Gas6, Axl, sAxl, AAA, CLI, SIRS, SLE, TAM, inflammation

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Carl Ekman
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LUNDS UNIVERSITET
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Jag betraktar människornas liv på jorden som ett ändlöst grupparbete. Uppgiften, formulerad i tidernas begynnelse, lyder som följer: Gör reda i det ni ser omkring er. Kom på hur ni på bästa sätt kan organisera er och ta användning av naturen. Beskriv, med egna ord, hur det är att vara människa. Ta reda på hur allt hänger ihop och varför ni är här. Ta den tid ni behöver.

Erlend Loe

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List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Carl Ekman, Jonas Stenhoff, Björn Dahlbäck. Gas6 is complexed to the soluble tyrosine kinase receptor Axl in human blood. Journal of Thrombosis and Haemostasis, 2010 (8): 838-844

II. Carl Ekman, Despina Flondell Site, Anders Gottsäter, Bengt Lindblad, Björn Dahlbäck. Plasma concentrations of growth arrest specific protein 6 and the soluble form of its tyrosine kinase receptor Axl as marker of large abdominal aortic aneurysms. Clinical Biochemistry 2010 Jan; 43(1-2):110-114

III. Carl Ekman, Anders Gottsäter, Bengt Lindblad, Björn Dahlbäck. Plasma concentrations of Gas6 and soluble Axl correlate with disease and predict mortality in patients with critical limb ischemia. Clinical Biochemistry 2010 Jul;43(10-11):873-6.

IV. Carl Ekman, Adam Linder, Per Åkesson, Björn Dahlbäck. Plasma concentration of Gas6 (growth arrest specific protein 6) and its soluble tyrosine kinase receptor sAxl in sepsis and systemic inflammatory response syndromes. Critical Care 2010 Aug 23;14 (4): R158

V. Carl Ekman, Andreas Jönsen, Gunnar Sturfeldt, Anders A Bengtsson, Björn Dahlbäck. Plasma concentration of Gas6 and sAxl correlate with disease activity in Systemic Lupus Erythematosus. Submitted

Abbreviations

AAA	Abdominal aorta aneurysm
APC	activated protein C
C4BP	C4-binding protein
CLI	Critical limb ischemia
CRP	C-reactive protein
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Gas6	Growth arrest specific 6
Gas6sv	Gas6 splice variant
Gla	gamma carboxylated glutamic acid
Ig	Immunoglobulin
HUVEC	human umbillical vein endothelial cell
LG	Laminin G
Mertk	Mer tyrosine kinase
PI3K	Phosphatidylinositol 3-kinase
PS	Phosphatidylserine
RPE	Retinal pigment epithelial cell
RTK	Receptor tyrosine kinase

sAxl	soluble Axl
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
sMer	soluble Mer
SMC	smooth muscle cell
sTyro3	soluble Tyro3
TAM	Tyro3, Axl and Mer
TNFα	Tumor necrosis factor alpha

Introduction

Cells express multiple receptors to be able to respond to their local microenvironment. These receptors are activated by ligands, which may be proteins, sugars, fats or other molecules. When the ligand binds the receptor, the receptor becomes activated and starts a signalling cascade, which will influence the receptor-bearing cell. One large family of receptors is the enzyme-linked receptors comprising five subfamilies, including receptor tyrosine kinases (RTK). The RTKs have an extracellular domain, one transmembrane domain and one intracellular domain with enzymatic activity. When the ligand binds a RTK, the receptors assemble into dimers, bringing the intracellular domains in close proximity. The intracellular domains will phosphorylate each other at specific tyrosine residues, enabling adaptor proteins to bind to the now activated receptor dimer. The adaptor proteins will transmit the signal into the cell, leading to altered cell behavior.

The human genome encodes 58 receptor tyrosine kinases, which are grouped in 20 families based on homology. The receptors of the TAM family have similar domain organization, with two immunoglobulin domains, two fibronectin domains in the extracellular domain.

The TAM family include the Tyro3, Axl and Mer receptors. My thesis will focus on the soluble form of the Axl receptor, but will also give a introduction to the TAM family at large including the ligands Gas6 and protein S.

Background

The *Gas6* gene and the Gas6 protein

The gene coding for growth arrest specific-6 (*Gas6*) was discovered in 1988 when searching for genes specifically expressed in growth-arrested fibroblasts. 6 genes were found and they were named *gas1* to *gas6*. The expression of these genes was high when cells were treated with media low in nutrients, but when the media was changed to a growth factor rich media, the expression decreased[1]. The full-length human cDNA for Gas6 is 2,461 nucleotides long and encodes a 75 kDa protein consisting of 678 amino acids[2]. Gas6 was found to be similar to the vitamin K-dependent protein S, sharing the domain organization and 44% amino acid identity. However, in contrast to protein S and the other vitamin K-dependent proteins, the expression of *Gas6* is low in liver, but high in lung, intestine, bone marrow and endothelial cells[2].

The vitamin K-dependent proteins share the Gla domain, containing several glutamic acids, which are γ-carboxylated in the endoplasmatic reticulum[3]. The Gla domain is a common feature in several proteins involved in coagulation (Fig 1), and the Gla domain enables them to bind surfaces containing negatively charged phospholipids[4], on which many of the reactions of the coagulation cascade occur[5]. In vivo, negatively charged phospholipids are present on the membranes of endothelial cells and activated platelets[6,7]. The γ-carboxylation of the Gla domain is dependent on vitamin K, and in its absence, the Gla domains have impaired membrane binding[4,5]. The anticoagulant drug

warfarin exerts its function by inhibiting the γ-carboxylation of several of the proteins of the coagulation cascade[8].

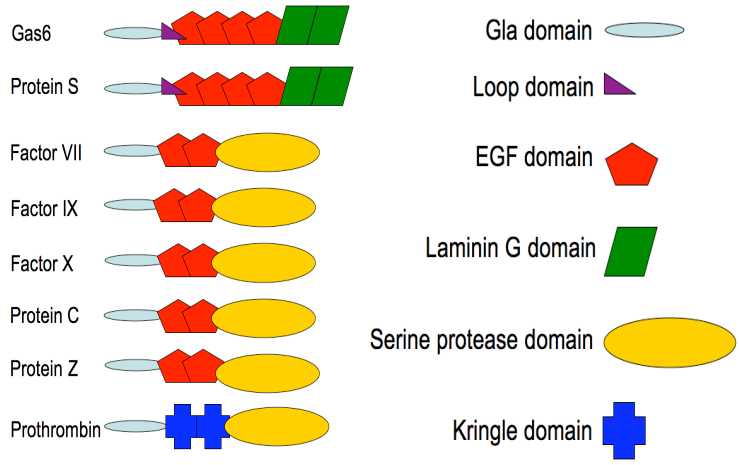

Figure 1

The domain organization of Gas6, protein S, and other Gla-containing proteins.

The first translated amino acids of the Gas6 protein compose the signal peptide, which is cleaved off during secretion. The mature Gas6 protein consists of a N-terminal Gla domain, a loop region, four epidermal growth factor (EGF) domains and two laminin G (LG) domains.

The loop region of Gas6 is the region with lowest homology to protein S[2]. Thrombin cleaves protein S in the loop region, but no such cleavage has been reported for Gas6. EGF domains are found in over 600 proteins[9], including several coagulation factors (Fig 1). Each EGF module has 6 cysteins with a characteristic binding pattern between Cysteins 1-3, 2-4 and 5-6[10].

The LG domains constitute the C-terminal part of Gas6. LG domains are usually present in single modules or pairs, and are found in over 80 proteins[11]. The two LG domains are together sometimes called the SHBG domain. It is called so due to the similarity with sex hormone binding globulin (SHBG), produced by the liver[12]. SHBG binds hormones in circulation and prolongs their half-life[13].

An alternative transcript of *Gas6* has been reported, with an insert of 129 basepairs between the last EGF domain and the SHBG domain. The expression of this splice variant, called *Gas6sv* can be seen in lung, brain, kidney and placenta where it makes up a small fraction of the total *Gas6* expression. However, in the spleen, *Gas6sv* is the dominant form, but the role of this variant is not fully elucidated[14]. The *Gas6* promoter regions are defined[15], and an estrogen response element has been found upstream of the Gas6 gene[16].

Protein S

Gas6 is similar to the more widely known protein S which was discovered in 1977[17] when purifying coagulation factor IX and factor X from plasma. The purification method used included an absorption step using barium citrate, which precipitates proteins containing Gla domains. During the elution of factors IX and X, another protein peak was observed. The protein was purified and amino terminal sequencing revealed a new, Gla-containing protein, which was named protein S[17].

Protein S is mainly produced in the liver, but some expression can also be observed in endothelium, megakaryocytes, smooth muscle cells and osteoblasts[7,18-22].

The mature human protein S consists of 635 amino acids and is composed of a N-terminal Gla domain, a thrombin sensitive loop region, four EGF domains and two LG domains[23]. The Gla domain anchors protein S to negatively charged phospholipids on which it can function as a cofactor to the activated protein C. The loop region is sensitive to thrombin and cleavage at Arg49 and Arg70 removes the cofactor activity to activated protein C[5]. The LG domains in protein S bind with high affinity to the complement regulatory protein C4b binding protein (C4BP).

Protein S is reported to have anticoagulant effects on multiple steps in the coagulation cascade. The most studied effect is the function as a cofactor to activated protein C (APC). Thrombin activates protein C when bound to thrombomodulin, and APC inactivates factor Va and factor VIIIa, limiting further formation of thrombin. With protein S present, the affinity between APC and the membrane is increased tenfold, leading to more efficient inactivation[24]. Protein S is also reported to have direct anticoagulant function on factor X[25], and an anticoagulant function through TFPI[6].

60-70 % of protein S in circulation is normally bound to C4BP[26]. The C4BP-protein S complex has an octopus-like appearance with eight chains linked together by disulfide bonds in the central region[27]. C4BP consists of seven α-chains with complement regulatory activity, and of one β-chain that binds the SHBG domain of protein S[28]. The affinity between the β-chain and protein S is around 0.1 nM[29]. Thus, all β-chains in circulation are bound to protein S. C4BP inhibits complement by acting as a cofactor for factor I in degrading C4b and

C3b[30], and protein S-C4BP inhibits phagocytosis of apoptotic cells[31], as opposed to free protein S[32].

Receptor tyrosine kinases

RTKs comprise a large group of transmembrane proteins with the ability to transfer signals from the outside of the cell to the inside. When a ligand binds a receptor tyrosine kinase, two receptor chains are brought in close contact and will become phosphorylated on specific residues of the RTK[33]. The phosphorylated domain will allow adaptor proteins to bind, which in turn will transfer the signal further into the cell[34]. In the human genome, 58 RTKs have been found, grouped into twenty families due to homology[35].

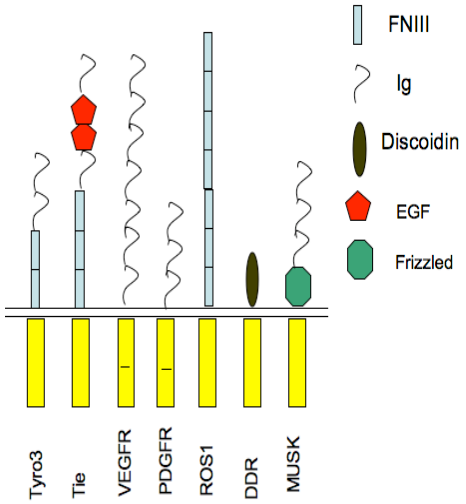

Figure 2

Seven families of receptor tyrosine kinases.

The TAM family of receptor tyrosine kinases

The TAM family consist of three proteins: Tyro3, Axl and Mer. As the proteins have been of interest for many groups, working simultaneously and with different species, a plethora of names are in use. Tyro3 is called Sky, Rse, Brt, Tif, Dtk and Etk-2, Axl is also known as Ufo, Ark and Tyro7 and Mer is referred to as Eyk, Nyk or Tyro12. The whole family of proteins is called the TAM family (**T**yro3, **A**xl and **M**er), the Tyro3 family, or the Axl family. In this thesis, I will use the TAM family and Tyro3, Axl and Mer nomenclature.

The Tyro- names were given after an experiment using primers to conserved parts of RTKs. The primers were used to investigate the nervous system for presence of RTKs, and found several known RTKs and also 13 mRNAs of RTKs not yet described[36]. Three of the new mRNAs, Tyro3, Tyro7 and Tyro12 could not be assigned to any known family of receptors. However, the signalling domains of these three receptors all contained a common recognition motif and were denoted the Tyro3 subfamily.

The *Axl* gene was found as a gene able to induce chronic myeloproliferative leukemia[37], and was later revealed to code for a RTK[38]. The gene was named *Axl* after the Greek anexelekto, meaning the uncontrolled. The same gene was also found by another group as a gene causing chronic myeloproliferative disorder, calling it Ufo, referring to its unknown function[39], and the murine gene was given the name Ark[40]. The *Axl* gene is expressed in brain[36,41], endothelial cells, heart, skeletal muscle, liver, kidney, testis and hematopoietic tissues[42-

[44] and cell lines from epithelial, mesenchymal and hematopoietic origin[38].

Jia et al identified the gene behind the oncogenic properties of the avian virus RPL30, and sequence analysis showed a high homology to *Axl*[45]. After screening a human λgt11 library in *E. Coli* with an antisera against phosphotyrosine a human homologue to the RPL30 gene was identified. High expression was observed in monocytes, epithelium and reproductive tissue, and thus the protein was named Mer[46].

During March and April 1994, four groups independently published findings of a new RTK. They named the receptor tif[47], Sky[48], brt[49] and rse[50], all describing the same protein.
Expression is high in the nervous system[48-51], but can also be observed in ovaries and testis[47], endothelial cells[52] and osteoclasts[53].

Tyro3, Axl and Mer share the same domain organization with two N-terminal immunoglobulin-like domains, two fibronectin type III domains, a transmembrane domain and an intracellular signalling domain. The latter contains a KW(I/L)A(I/L)ES motif which is specific for the TAM family[54,55]. Both Ig and FNIII domains are common in RTKs[35] and are also present in neural cell adhesion molecules[56].

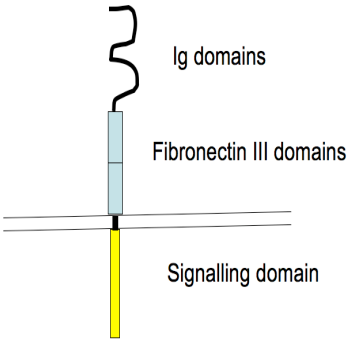

Figure 3

The structure of the receptors of the TAM family

The Tyro3, Axl and Mer proteins contain 890, 894 and 999 amino acids, respectively, which theoretically should encode proteins with a size of 100-110 kDa. Due to extensive glycosylation, the full-length Axl and Tyro3 proteins migrate as 140 kDa[57,58], and Mer as 205 kDa[59].

Gas6 and protein S as ligands of the TAM family

Gas6 was found to function as a ligand to the Axl receptor in 1995. Axl-bearing cells were exposed to media from 70 different cell lines in search for a factor that could activate the receptor, and media from two cell lines were able to stimulate Axl. The cell media was purified on the basis of the Axl stimulatory activity, and after several purification steps, a single protein band was sent to mass spectrometry, which identified the Gas6 protein[60]. Further studies showed that Gas6 bound and activated the Tyro3[61,62], and Mer receptors[63].

The binding of protein S to the TAM receptor family has been debated. Protein S was identified as a ligand to the TAM family of receptors[64], but as the study used human protein S to stimulate murine Tyro3, the relevance of this finding has been questioned[61,63,65,66]. However, Protein S has been reported to activate TAM receptors in several animal models[67-70] and an oxidized, oligomeric form of protein S has been shown to activate Mer and induce phagocytosis[71], suggesting that oligomerization is crucial for signalling.

The domains of Gas6 able to stimulate the TAM receptors have been defined using deletion constructs of Gas6. The C-terminal portion is enough to stimulate the TAM receptors[65,72,73], and Gas6sv can also stimulate receptors[72]. Even so, Gas6 expressed in the presence of warfarin, lacking gamma carboxylated glutamic acids is unable to activate the receptors on cells to give cellular effects[73-77].

The affinity of the human Gas6-TAM receptor interaction has been estimated by several groups. Nagata et al[78] estimated the Gas6-Axl affinity to 1 nM and the Gas6-Tyro3 affinity to 10.8 nM. Chen et al[63] determined the affinity for Gas6 to 1.6 nM for Axl, 3.6 for Tyro3 and 9.7 for Mer. Fisher et al[79] report 0.053, 0.031 and 0.304 nM for the three receptors respectively. Wimmel[80] reported 0.178 nM for Gas6 and Axl, and we have estimated the Gas6-Axl affinity to 0.4 nM (Paper I).

Equilibrium constant of Gas6 (nM)			
Axl	Tyro3	Mer	Reference
1.0	10.8	ND	Nagata 1996
1.6	3.6	9.7	Chen 1997
0.053	0.032	0.304	Fischer 2005

Table 1

Reported strength of the Gas6 interaction with the TAM receptors.

A number of crystals revealing the three-dimensional structure of Gas6 and its receptors have been published. The first published crystal demonstrated the structure of the LG domains of Gas6. The fold was similar to previously described LG-containing proteins and indicated which amino acids that could influence binding to receptors, and when Leu620 was mutated to an alanine, the affinity towards Axl decreased tenfold[81].

Alone, the Ig domains of Tyro3 can bind Gas6, and a crystal of the Tyro3 Ig domains has also been presented[82]. A complex crystal containing the LG domains of Gas6 and the Ig domains of Axl has been presented. In this crystal, two pairs of Ig and LG domains form a 2:2 complex, with one major binding site between Ig1 and LG1, and a minor between Ig2 and LG1[83].

Soluble TAM receptors

Several receptors exist in soluble forms, consisting of the extracellular regions of the receptor. The soluble receptors can remove the ligand from cell bound receptors and thus inhibit signalling[84,85], and several experiments have utilized a extracellular, soluble form of Axl (sAxl) or a dimeric Axl (Axl-Fc) as specific inhibitors for Gas6

signalling[60,86-89]. Soluble receptors can be produced by alternative splicing or by proteolysis of the full-length receptor.

The Axl receptor is cleaved just outside the membrane, and shedding of soluble Axl can be induced by treatment with phorbol esters[58]. Several mouse cells and organs release sAxl constitutively[86,90]. Decreased shedding of sAxl could be observed during treatment of the broad metalloproteinase inhibitor GM6001, the more specific inhibitor GW280264X (targeting TACE and ADAM10) and also siRNA against ADAM10. The cleavage site of Axl was localized with use of deletion mutants, lacking parts of the extracellular domain close to the membrane. In mouse serum, Gas6 could be co-immunoprecipitated with anti-Axl antibodies, which indicated that the proteins circulate in complex[86]. Human dendritic cells shed sAxl upon inflammatory stimuli[91]. sAxl is shedded from human cell lines, and is present in human plasma where it binds Gas6 (see Paper I). Soluble Mer (sMer) is reported to be present in mouse and human serum[59]. Similar to sAxl, the shedding of sMer could be increased with phorbol esters and LPS. The shedding was inhibited by TAPI, an inhibitor of the TACE metalloproteinases. The *Mertk* gene, (encoding the Mer protein) contains a additional polyadenylation site after the extracellular domain, indicating that sMer could be translated without the C-terminal parts of the protein[46]. Expression of this extracellular domain alone has been reported as unpublished observations[55].

Cellular effects of Gas6 signalling

Gas6 binding to the TAM family induces receptor dimerization. The intracellular signalling domains are brought into close proximity and become phosphorylated on specific tyrosine residues. Stimulation of

Tyro3 can stimulate the Src family[92] as well as Erk[93], and has in yeast 2-hybrid systems been shown to interact with PI3K[94] and RanBPM[95]. In Axl, the tyrosine residues in positions 779, 821, and 866 are known to be phosphorylated, which leads to signalling through the Grb2 and PI3K pathways[96,97].
Activation of Mer leads to phosphorylation of the tyrosine residues at 749, 753 and 754[98], and to interaction of PLC, PI3K, Shc, Grb2[99] and Vav[100].

The cellular effects initiated by Gas6 signalling are different in different cell types and can work synergistic with other signals. Gas6 signalling is of importance in many physiological and pathological situations, and below is a brief description of some of the more studied areas.

Antiapoptotis and mitogenesis

Several studies have been presented on the antiapoptotic and mitogenic effects of Gas6 signalling. These effects have been documented in fibroblasts[77,101-103], different endothelial cells[74,76,104-106] vascular smooth muscle cells[75,107,108], oligodendrocytes[109,110], Schwann cells[111], lens epithelial cells[112], neurons[113,114] and liver cells[115-117]. Gas6 decrease apoptosis after serum starvation[74,77,101,104,107,113,115] and TNFα-treatment[106,110,112] in several cell types. Gas6 has a mitogenic effect[75,77,101,111] but the Gas6 effect is lower compared to PDGF and FGF4[101].

The TAM receptors were found as transforming genes, and the role of TAM receptors and Gas6 in cancer has been studied by several

groups. Presence of Gas6 and the TAM receptors have been observed in many malignancies, including leukemia[118], cancer of the thyroid[119,120], lung[80], uterus[121], endometrium[122], ovary[123], prostate[124-127], gastric cancer[87], breast cancer[128], Kaposi's sarcoma[129], malignant gliomas[130,131] and renal cell carcinoma[132].

Gas6 induces proliferation and survival in breast[133] and prostate cancer cells[124,126]. Several animal models have also indicated the role of TAM receptors. In mouse models of glioma[131], breast cancer[134], hepatocellular carcinoma[135] and metastatic ovarian cancer[136], high expression of Axl is deterimental, and blocking Axl expression on transcriptional or protein level increase survival. Gas6 is mitogenic for tumors, and implanted tumors grow faster in wildtype than $Gas6^{-/-}$ mice[137], as infiltrating macrophages released Gas6 to the tumor.

Presence of Gas6 and the TAM receptors have clinical implications for cancer patients. Low expression of Axl mRNA indicate a good prognosis in patients with renal cell carcinoma[132]. High Axl expression in breast cancer[134], pancreatic adenocarcinoma[138], malignant glioma[130] and esophageal adenocarcinoma[139] is a negative prognostic factor. However, in breast cancer, high Gas6 expression is associated with smaller tumor size and decreased metastases[140].

Inhibition of RTKs has been shown to be a viable way of treating several cancers[141-143]. Inhibiting Axl with antibodies and shRNA leads to decreased proliferation and invasiveness in animal models[129,144-146]. Axl-inhibitory small molecules are under development, and have been shown to inhibit breast cancer metastasis in mice[147].

Phagocytosis & Migration

Gas6 can act as a bridging molecule with the Gla domain binding negatively charged phospholipids, coincident with LG domains binding a TAM-bearing phagocytic cell. Phosphatidylserine (PS) normally resides in the inner leaflet of the cell membrane, but as cells become apoptotic, PS accumulates in the outer leaflet[148]. Without proper removal of apoptotic cells, secondary necrosis ensues and leads to inflammation. Gas6 binds PS in microtiter plates, and monocytes bind PS-coated microtiter plates when Gas6 is present, but not in its absence[149]. Liposomes and apoptotic cells, both containing PS are taken up more efficiently by mouse macrophages when Gas6 is present[150].

Macrophages with a cytoplasmatic truncation of Mer are deficient in phagocytosis of apoptotic thymocytes[151]. Macrophages mainly use the Mer receptor for phagocytosis[152], whereas dendritic cells use Tyro3 and Axl[153], Glial cells use Axl and Mer[154], and the sertoli cells in the testis use all three receptors[155].

The TAM receptors are involved in the phagocytosis of the outer segments of photoreceptors by the retinal pigmental epithelium (RPE) cells. Without trimming of the photoreceptors, they overgrow and eyesight is impaired. The Royal College of Surgeons rat strain has a hereditary retinal degradation, which is caused by a mutation in the *Mertk* gene. The mutation inserts a premature stop codon, truncating the Mer protein, thus hindering effective phagocytosis[156,157]. The phagocytosis of the outer segments in cell cultures can be inhibited by targeting Gas6 or Mer[158]. The Gla domain of Gas6 bind to the outer

segments, and stimulates phagocytosis[159] through Mer, present on the RPE cells[160]. However, Gas6$^{-/-}$ mice do not become blind,[161], but mice lacking *Mertk* do[68], indicating that protein S might play a role. Also in human RPE cells, phagocytosis can be decreased by using anti-Gas6 or anti-Mer antibodies[162]. The genome of several families with hereditary retinal dystrophies have been investigated and revealed several mutations in the *Mertk* gene[163-166].

Gas6 can induce migration in Axl expressing cells, including VSMC[167,168], neurons[169,170], and dendritic cells[91], but is reported to inhibit migration in mouse fibrosarcoma cells[86], renal carcinoma cells[171], and to inhibit chemotaxis of endothelial cells[172], showing that the migration is highly dependent on cell type.

Gas6 and mesangial cell proliferation

Hyperproliferation of mesangial cells in the kidney is a hallmark of glomerular disease. Several studies indicate that mice show lesser symptoms of kidney disease when given warfarin, Axl-Fc, or are deficient in Gas6. In the first published study, mesangial cells were treated with medium from Gas6-producing cells and started to proliferate. However, this proliferation could be inhibited by treating the Gas6-producing cells with warfarin[173]. Gas6 and Axl was later found to be upregulated in the mesangial cells in a mouse model of experimental glomerulonephritis[88]. The STAT3 dependent[89] hyperproliferation of mesangial cells could be inhibited in vivo with Axl-Fc or warfarin[88]. In a related model of nephrotoxic nephritis, Gas6$^{-/-}$ mice survived to a higher degree and showed less albuminuria,

but infusion of Gas6 brought back the symptoms to the level of wildtype animals[174].

Experimentally induced diabetes nephropathy can also be improved with warfarin. Warfarin decreases mesangial cell area and lowers urinary albumin excretion and Gas6$^{-/-}$ mice were also protected[175]. Also in aldosterone induced hypertension, Gas6$^{-/-}$ mice fare better and show less albuminuria and kidney inflammation[176]. Kidney expression of Gas6 is increased during chronic rejection of transplanted kidneys[177], lupus nephritis, glomerulonephritis and IgA nephropathy[178]. These findings indicate that Gas6 signalling can be detrimental in several renal diseases, and the researchers suggest that low dose warfarin could be beneficial for these patients[179].

Gas6 and TAM in the vasculature

Axl and Gas6 are expressed by numerous cell types in the vascular wall, including endothelial cells, smooth muscle cells and fibroblasts[52,102,106,107,180]. Gas6 and Axl are important for vessel integrity and injury response in the vessel wall, and Axl is upregulated after balloon injury[181,182]. Animals deficient in Axl or Gas6 display impaired vessel integrity and have increased vessel leakage compared to their wildtype littermates[18]. Mice deficient in Axl also show decreased intimal proliferation after carotid ligation[183,184] and attenuated intimal growth during hypertension[185].

The role of Gas6 in atherosclerosis has been investigated by comparing mice deficient in ApoE with mice deficient in ApoE and Gas6. The plaques in Gas6 deficient mice had more collagen and smooth muscle cells and less macrophages, indicating higher stability of the plaque in the absence of Gas6[186]. Interestingly, polymorphisms

in the human *Gas6* gene are protective to stroke in two studies[187,188] and polymorphisms in the Mertk and Tyro3 genes are protective against carotid atherosclerosis[189]. In an animal model of blood brain barrier stability, protein S induced phosphorylation of Tyro3 is reported to be protective[70].

Regulation of inflammation

Cell experiments show that Axl stimulation by Gas6 can inhibit release of proinflammatory cytokines from human macrophages[190,191], dendritic cells[192,193] sertoli cells[194], and glial cells[154], thus limiting the immune response. In bone marrow derived dendritic cells Gas6 induces upregulation of SOCS proteins, known for their suppression of cytokine signalling[192].

Interestingly, interferon signalling upregulates Axl mRNA transcription in dendritic cells[91,192], enabling a negative feedback loop.

The role of Gas6 for endothelial activation is complex. Endothelial cells activated by TNFα or phorbol esters showed less granulocyte binding after treatment with high doses of Gas6[195], but animals deficient in Gas6 do not upregulate ICAM-1 and VCAM-1 after TNFα stimulation and exhibit less transmigration of leucocytes[52,196].

Gas6/protein S/TAM-deficient animals

To be able to study the complex role of the Gas6/protein S – TAM system, genetically altered mice lacking one or several of these proteins have been developed. The animals are useful since they can reveal new and unexpected physiological roles of the proteins studied. Mice lacking either Tyro3 or Axl display milder immunological abberations, and animals lacking Mer exhibit a hyperactive response

to LPS and have higher cytokine secretion compared to wildtype littermates[197]. The single knockouts were still fertile, and breeding was directed towards double and triple knockout mice. The double knockouts show increased autoimmune manifestations, and the immune system of the triple knockout is severly deranged[198]. Multiple organ defects were observed, including blindness and infertility[57]. In one-year old mice, the spleen of a triple knockout was ten times the size of the wildtype animal. This growth was due to proliferation of B and T cells, which filled the normal immunological compartments, but also established ectopic colonies in all organs investigated[198]. The animals developed autoimmune disorders with antibody deposition in the glomeruli, and presence of antibodies against DNA, collagen, cardiolipin and α-phosphatidylinositol.

When a mouse deficient in Gas6 was reported, no obvious phenotype could be observed. However, in thrombosis models, the $Gas6^{-/-}$ mice had smaller thrombi and lower mortality[199]. The platelets were found to be less responsive to agonists and platelet aggregates from $Gas6^{-/-}$ mice were less densly packed. Interestingly, addition of anti-Gas6 antibodies could protect mice equally well as total deficiency of Gas6. This study was followed up by a study evaluating hemostasis in animals deficient in all three TAM receptors. These animals were also protected in thrombosis models and had less responsive platelets[200]. Studies on human platelets have also indicated a role for Gas6 during platelet activation[201], but the role is debated[202,203]. Further studies showed that $TAM^{-/-}$ mice have half the number of thrombocytes compared to wildtype littermates. Megakaryocytes were found to

express all TAM receptors and megakaryocytes lacking all the receptors produced less proplatelets[204].

Mice deficient in Gas6 were also found to have lower levels of erythroid cells, and the hematocrit recovered slower from induced anemia[205]. When inducing acute hemolysis with phenylhydrazine, also Axl and Mer knockouts are slower to regain normal hematocrit[206]. Animals deficient in Gas6 show altered endothelial response to inflammatory stimuli. $Gas6^{-/-}$ endothelium activated with TNFα had less upregulation of p-selectin, VCAM-1 and ICAM-1 than wildtype endothelium and adhesion by platelets and leukocytes is decreased[52]. The $Gas6^{-/-}$ animals showed less inflammation following LPS injection and slower rejection of non-matched transplanted hearts. Graft versus host disease is slower in $Gas6^{-/-}$ animals compared to wildtype, presumably due to the slower extravasation of leucocytes, decreasing the rate of organ damage[196]. Gas6 deficient mice also have a higher baseline of many inflammatory cytokines[205].

Mice deficient in protein S do not survive embryogenesis due to severe hemorrhages. Heterozygous $PROS1^{-/+}$ mice however, survive but have decreased levels of protein S in plasma and are more susceptible to induced thrombosis[207].
The vasculature in the $PROS1^{-/-}$ embryos is disorganized and less smooth muscle actin can be found around the vessels at embryonic day 13.5[18]. Furthermore, $PROS1^{-/+}$ mice had defective vasculature, observed by dye leakage from the vessels. This leakage was also found in endothelial $PROS1^{-/-}$ knockouts and $Gas6^{-/-}$ and $Axl^{-/-}$ mice, indicating a role for these proteins for vascular integrity.

Studies of plasma concentrations of Gas6

As Gas6 was indicated to be important for platelet function, early studies focused on investigating human platelets, which do not contain large amounts of Gas6[201,202]. However, plasma was found to contain 18 ng/ml of Gas6, and patients treated with warfarin had decreased Gas6 levels in plasma[202]. Another ELISA was published, which estimated the plasma concentration of Gas6 to 50-63 ng/ml[208]. This ELISA was later used for relating the Gas6 concentration with different coagulation parameters, but found no statistically significant correlation in the investigated patients[203]. However, patients with aspirin pseudoresistance were overrepresented in patients with high Gas6[209], and Gas6 was influenced by oral contraceptives[208]. Plasma from patients with acute coronary syndromes were also investigated, but were not different from a control population[210].

Patients with severe sepsis were evaluated by two groups, and both found increased Gas6 in patients with sepsis, correlating with degree of organ dysfunction, but the concentrations of Gas6 reported were 50 ng/ml[211] and 100 pg/ml[212]. These studies were followed up by a japanese group, measuring Gas6 in patients with acute pancreatitis, another condition characterized by inflammation and organ dysfunction. These patients had triple Gas6 concentration compared to a control group[213].

A Gas6 ELISA made of commercially available reagents was presented[214], and was used to investigate cerebrospinal fluid from patients with neurological diseases, revealing that patients with chronic inflammatory demyelinating polyneuropathy have increased Gas6[215]. The same group investigated patients with acute dyspnea with

the Gas6 ELISA to elucidate if Gas6 could be used for diagnosis, and high Gas6 was observed in patients with heart failure and systemic infections[216].

Patients with acute coronary syndrome were investigate by a chinese group, finding that patients with stable or unstable angina, and also patients with acute myocardial infarction had decreased plasma Gas6 to a control group[217]. Patients with impaired glucose tolerance and type 2 diabetes have also been found to have decreased Gas6[218].

We have recently shown that patients with renal cell carcinoma with higher Gas6 and sAxl have poorer survival compared to patients with low Gas6 and sAxl[132]. A recent study evaluating patients with Systemic Lupus Erythematosus (SLE) indicate that the Gas6 concentrations are similar to controls, but patients with high disease activity have increased Gas6[219].

The present investigation

Overview

The overall aim of my project was to gain structural and functional insight in the TAM family of receptors. Several studies from the lab have been focused on understanding the structural properties of proteins, many times using site directed mutagenesis.
In 2005, when I started in the lab, methods were set up for expressing both protein S and Gas6, and there was a wide collection of antibodies for these proteins, but there were no methods to express the TAM receptors. One could have gone for commercial reagents, but as many experiments demand large quantities of protein, we decided it would make sense to produce the reagents ourselves. Purified TAM receptors could be used for binding studies, signalling experiments and immunizing animals to obtain antibodies. The antibodies could be used for detection of protein, from western blotting, to immunohistochemistry and ELISAs.

The work started with making plasmids with the genes coding for sAxl, sTyro3 and sMer, and transfecting cell lines with these plasmids. sAxl and sTyro3 showed good expression and purification began. Despite several attempts to express sMer, we could not get sufficient expression, and the sMer project was put on hold. Meanwhile, sAxl and sTyro3 were expressed in large scale, purified to single band on silver stained SDS-PAGE, and used for immunization of rabbits. With the anti-Axl antibodies, we soon found out that several cell lines express sAxl, and that sAxl is present in human

circulation, and the work leading to paper I started. With functional ELISAs for Gas6 and sAxl, it was easy to investigate patient materials, and as we got interesting results, we followed that path, leading to papers II-V. The antibodies against Axl have also been useful for immunohistochemistry in several projects.

Paper I – Gas6 in complex with soluble Axl

Background

My work in the lab began with expression of the extracellular domains of the Axl protein. A vector with full-length cDNA for Axl was used as template, which was amplified with specific primers to add restriction sites and a thrombin sensitive His_6-tag. The pcr product was ligated and inserted in a pcDNA3.1 vector, which was transfected into HEK293 cells. sAxl Expression was confirmed by using a commercial affinity purified anti-Axl antibody, and the colony expressing highest amounts of sAxl was chosen for large scale expression.

The first purification step was precipitation with saturated ammonium sulphate. The pellet was resuspended and dialyzed in a low salt buffer to allow for anion exchange chromatography. The fractions containing sAxl from the anion exchange were pooled and applied to a nickel affinity column. After elution, the sAxl-containing fractions from this column was concentrated and futher purified using gel filtration. The resulting protein could be seen as a single band on a silver stained gel. After removal of the His_6-tag, the purified sAxl protein was used to immunize two rabbits to obtain antibodies against Axl. After several immunizations, the two antisera (041 and 042) contained high titers of anti-Axl antibodies and the antibodies were collected on Protein A and Protein G columns. The antibodies bound sAxl in Western blotting and in microtiter plates with good specificity and low background. Parts of each antisera were biotinylated to allow visualization with streptavidin-coupled horseradish peroxidase. Different concentrations were tested to identify the optimal concentrations of antibodies for an Axl ELISA. The recombinant purified sAxl was quantified using total

amino acid analysis after acidic hydrolysis, and a serial dilution of the sAxl was used as standard in the ELISA.

Results and Discussion

Human serum and plasma was investigated with the Axl ELISA. A positive signal could be detected in all the samples tested, corresponding to a concentration around 0.6 nM.

To evaluate that the ELISA was measuring the correct protein, recombinant sAxl was added to serum samples, and the signal increased accordingly. To determine the specificity of the ELISA, samples were immunoprecipitated with a commercially available anti-Axl antibody, which effectively removed the sAxl signal in these samples. To further show specificity of the antibodies, the 042 and 041 antibodies were coupled to NHS-activated columns. Large amounts of plasma were added to the 042 column, followed by extensive washing and elution. The eluate was added to the 041 column which was washed and eluted similarly. The collected sample was evaluated by SDS-PAGE stained with collodial coomassie. A 65 kDa band was excised and sent to mass spectrometry, which could confirm the identity of the band as Axl. Four peptides, all from the extracellular domain could be identified. Immunoprecipitation of human serum with the antibodies revealed a 65 kDa band, and several cell lines also seemed to express sAxl, although the glycosylation pattern differed slighlty.

Due to the high affinity between Axl and Gas6, we used gel filtration to determine if Gas6 and sAxl are complexed in human circulation. The elution patterns of recombinant Gas6 and serum Gas6 was compared, and recombinant Gas6 eluted later than serum Gas6,

indicating that serum Gas6 was a part of a larger complex. The recombinant sAxl eluted as serum sAxl, except a small part of the serum sAxl that eluted in the Gas6 fractions. Antibodies to Gas6 displaced sAxl to the void volume, and antibodies to Axl displaced Gas6, indicating that Gas6 and sAxl were in complex. In an ELISA, with catching anti-Axl antibodies, and detecting anti-Gas6 antibodies, a signal was detected in the Gas6 peak of serum and plasma. Immunoprecipitation of serum was performed to precipitate the complex. After immunoprecipitation with an anti-Gas6 and an anti-Axl antibody, similar amounts of Gas6 could be immunoblotted, indicating that practically all Gas6 is in complex with Axl. Immunoblotting with anti-Axl antibodies revealed that much more sAxl is precipitated with anti-Axl compared to anti-Gas6 antibodies, indicating excess of sAxl in human serum. When testing serum samples with the complex ELISA, an increased signal could be seen after adding Gas6 to the serum, but not after adding sAxl, indicating that Gas6 is the limiting factor for complex formation.

To further show presence of the complexes, native gels were used to investigate the migration of Gas6 alone and together with sAxl and Axl-Fc. Gas6 travelled shorter distances when the sAxl and Axl-Fc were present, indicating that a larger complex was formed.

To rule out that sTyro3 or sMer took a part in complex formation, gel filtration of serum was performed with and without anti-Gas6 antibodies. No change in the elution of sTyro3 or sMer could be observed, indicating that sAxl is the main binder of Gas6 in human serum.

These findings indicate that the Gas6 present in human serum is bound to sAxl and suggests that serum Gas6 is incapable of

stimulating cell-bound receptors. The signalling of Gas6 ought be local to its nature, as Gas6 leaked to the circulation would be bound by the excess sAxl. Earlier research in mice models have indicated that Gas6 is an important mediator for platelet activation, and that soluble TAM receptors or antibodies against Gas6 could be useful against thrombosis. This study indicates that Gas6 in circulation isn't available for binding in humans. Further studies on TAM receptor content of human platelets or Gas6 release by endothelium could be very useful to better understand platelet activation, as some studies suggest that Gas6 has a role in human platelet activation[201].

The developed ELISA to quantify sAxl in human sera is a useful tool to investigate the role of sAxl in a number of human conditions. As the lab have setup an ELISA for Gas6, we found it natural to investigate these two proteins in a number of patient materials.

Paper II - Gas6 and sAxl in patients with Abdominal Aorta Aneurysms

Background

Abdominal Aorta Aneurysm (AAA) is a disease characterized by weakening of the abdominal aorta, causing the vascular wall to bulge outwards, resulting in increased vessel diameter. Inflammation, mechanical stress and proteolytic degradation of the aortic wall are all proposed to be of importance for the development of AAAs, but the mechanisms are not fully elucidated[220,221]. High age, smoking, heredity, coronary heart disease, high cholesterol and blood pressure are all risk factors for development of AAA[222]. Increased diameter of the AAA increases the likelihood of rupture, causing massive bleeding, which often is lethal[223]. AAAs are present in approximately 5% of elderly men and ruptured aorta is estimated to be responsible for 2% of all deaths in the Western world[222].

Surgery for all aneurysms larger than 5.5 cm is the recommended treatment for all patients suitable for operation[224]. Due to the suggested role for Gas6 and Axl in the vasculature, we decided to evaluate the concentrations of these proteins in patients with large and small aortic aneurysms and compare them to a control population. The study included 123 patients with large AAAs, 122 with small AAAs, and 141 healthy controls.

Results and discussion

Compared to the control population, Gas6 was increased and sAxl decreased in patients with large aneurysms, with the small aneurysms having intermediate Gas6 and sAxl concentrations. The diameter of

the AAA correlated positively to Gas6 and negatively to sAxl concentration. Due to Gas6 being bound to sAxl, we calculated the Gas6/sAxl-ratio and this ratio correlated better than Gas6 or sAxl to the diameter of the AAAs. Thus, the Gas6/sAxl ratio was high in the patients with large AAAs, and 40% of the patients with large AAA had higher Gas6/sAxl-ratios compared to the highest ratio in the control group. The Gas6/sAxl-ratio is not specific enough to allow screening of AAAs, as 60% of all patients with large AAAs have Gas6/sAxl ratios in the normal range. The causes of this altered ratio remains to be elucidated, but increased Gas6 is observed during severe inflammatory conditions[211-213]. Gas6 and Axl are important for vascular integrity[18] and involved in vascular remodelling[181,183], likely to take place during the development of AAA. The patients with high Gas6/sAxl-ratio might constitute a subgroup with different AAA properties, with higher involvement of the Gas6/Axl-system.
The study demonstrated that Gas6 and sAxl were altered in the patient group and encouraged us to further study the role of Gas6 and sAxl in patient materials.

Paper III - Gas6 and sAxl in Critical Limb Ischemia

Background

Critical limb ischemia (CLI) is a disease caused by atherosclerosis. Atherosclerosis is characterized by fat accumulation under the intima of the larger vessels, causing inflammation[225]. Atherosclerosis causes several diseases, including coronary heart disease and cerebrovascular disease, and patients with critical limb ischemia have high co-morbidities with these diseases[226]. CLI is characterized by low blood supply to the tissues, which leads to rest pain, ulcers or gangrene in the affected limbs[227]. The diagnosis of CLI is made after measuring blood pressure in ankles and toes or by transcutaneous measurements of the oxygen pressure[228]. CLI is present of 0.26% in the population between 40 and 69 years of age[229], and patients have a high mortality, often due to cardiovascular disease and stroke[230]. The treatment of CLI includes revascularization when possible, as well as controlling atherosclerotic risk factors as smoking, hypertension, hyperlipidemia and diabetes mellitus. In our study, 189 patients with CLI and 204 controls were included.

Results and discussion

Patients with CLI have increased Gas6 and sAxl compared to healthy controls, and both Gas6 and sAxl correlated with inflammatory markers as C-reactive protein, interleukin-6, TNFα and neopterin. Gas6 and sAxl were increased in patients who have had ulcers, were amputated, had gangrene or angina. A small number of patients were investigated with echocardiography and in these patients, Gas6 correlated strongly to left heart strain. Patients who died within three

years of sampling had increased Gas6 and sAxl compared to the survivors. Gas6 and sAxl predicted mortality independent of age and gender, and Gas6 was also independent to many known risk factors of CLI, indicating a role in the disease. This study shows that Gas6 and sAxl correlate to several inflammatory markers and many aspects of CLI.

Gas6 has earlier been shown to be important for cortisone induced heart hypertrophy[176], and the strong correlation to left heart strain indicates that Gas6 might be of importance for human heart remodelling. Only 35 patients were investigated with echocardiagraphy, so a larger study on patients with heart insufficiency would be enlightening for the role of Gas6 in heart remodelling. sAxl correlated strongly with neopterin, which indicates that sAxl and neopterin release are connected in CLI, which can be a useful starting point when elucidating the mechanisms of sAxl shedding.

Paper IV - Gas6 and sAxl in Sepsis and SIRS

Background

Sepsis is a complex disease, defined as presence of infection and systemic inflammatory response syndrome (SIRS). A patient has SIRS when two or more of the following criteria are fulfilled: (1) Temperature above 38 °C or under 36 °C; (2) Heart rate above 90 beats per minute; (3) Breathing frequency above 20 breaths per minute, or PaO_2 less than 32 mm Hg or (4) White blood cell count above $12\text{^}10^9$ or below $4\text{^}10^9$ per liter, or the presence of more than 10% of immature neutrophils[231]. The patients are treated with antibiotics to eradicate the infection, and fluid replacement to avoid shock, and otherwise given supportive treatment in case of organ failure. Controlling glucose and countering anemia does also increase survival[232]. Increased plasma concentrations of Gas6 have earlier been reported in two studies of sepsis[211,212], but sAxl has never been measured. Due to the complex between these two molecules, we decided to evaluate the concentrations of both molecules in patients with sepsis and related conditions. The Division of Infection Medicine at Lund University has a well-characterized sepsis material consisting of blood samples from 232 patients with severe sepsis, sepsis, infections and SIRS and 100 blood donors as controls[233].

Results and discussion

All patient groups showed approximately doubled Gas6 concentrations compared to healthy controls, whereas sAxl is increased by around 20%. Our results confirm that Gas6 is increased in patients with sepsis, but also show that patients with milder

infections have Gas6 levels similar to the sepsis patients. Gas6 is increased in patients developing organ failure and patients in need of intensive care, indicating that increased Gas6 correlates with severe disease. Plasma sAxl is altered in the patients, but the increase is lower compared to Gas6, indicating that Gas6 can travel longer before complexed to sAxl, inducing more signalling during sepsis. Again, Gas6 and sAxl correlate to markers of inflammation, and especially Gas6 seems to behave as an acute phase protein.

Paper V - Gas6 and sAxl in systemic lupus erythematosus

Background

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with symptoms including rash, arthritis, anemia, nephritis and serositis[234]. The disease is associated with impaired clearance of apoptotic cells[235,236] and antibodies directed to nuclear antigens[237], but the etiology of the disease is not fully elucidated. SLE patients are predominantly female and African or Asian descent is overrepresented amongst the patients[238]. SLE is usually treated by immunosuppression with corticosteroids, cyclosporine or antimalarials, but a new generation of drugs as TNFα blockers and other specific therapies based on monoclonal antibodies are coming into the clinic[239].
Mice deficient in the TAM receptors develop several symptoms reminiscent of SLE, including deficient uptake of apoptotic cells[151], glomerulonephritis and antibodies to DNA and phospholipids[198]. As Gas6 and the TAM receptors are of importance for immune regulation, we measured Gas6 and sAxl in a 96 SLE patients with a wide range of symptoms to evaluate the role for Gas6 and sAxl in the disease.

Results and discussion

In the 96 patients, the plasma concentrations of Gas6 and sAxl correlated with the disease intensity estimated by the SLEDAI-2K index. For 45 of the patients, two samples were available, one with high and one with low SLEDAI. Gas6 and sAxl were significantly increased in the high SLEDAI sample. Gas6 and sAxl correlated to

sedimentation rate, C-reactive protein and negatively to hemoglobin. Especially patients with glomerulonephritis or anti-DNA antibodies had increased levels of Gas6 and sAxl. The study shows that Gas6 and sAxl correlate with disease intensity in SLE, indicating a role in the disease, probably linked to inflammation.

Future perspectives

Gas6 and the TAM receptors are important for many physiological and pathological processes. The main findings of my PhD project are that Gas6 is bound to sAxl in human circulation, that Gas6 and sAxl levels are increased during various inflammatory conditions. Released Gas6 can influence the adjacent cells, before sAxl can inactivate it, but the site of Gas6 release is still poorly defined. We have preliminary data indicating that TNFα-activated endothelial cells don't upregulate their Gas6 expression on the mRNA level, arguing for that the main Gas6 expression in inflammation come from other cell types. Systematic studies of cells beeing exposed to proinflammatory stimuli could reveal cell types involved in the Gas6 production.

The release of sAxl also remains poorly defined. In the CLI material, neopterin correlated strongly to sAxl, which indicates that the release of sAxl could be linked to activated macrophages. Dendritic cells have been reported to shed sAxl upon inflammatory stimulation, but macrophages might induce considerable sAxl shedding, and should be further investigated to reveal if this is a direct or an indirect effect.

Warfarin seems to inhibit Gas6 function in some animal models, and further characterization of mouse and human Gas6 produced during treatment with low dose warfarin would be interesting. As low dose warfarin interferes with formation of functional Gas6, it might be an interesting experimental drug in animal models, as shown before with several kidney manifestations. As animal models for AAA, CLI, Sepsis and SLE exist, it is tempting to evaluate warfarin treated

animals to determine if lack of functional Gas6 is making a difference in those animal models.

Due to the findings in the patient materials, we believe that further studies of Gas6 and sAxl in other diseases are motivated. Vasculitis, multiple sclerosis and gout would be interesting to study to widen our knowledge of Gas6 and sAxl in inflammatory conditions. Soluble Mer and Tyro3 seems to be present in circulation, and systematic studies of these proteins might also give us additional clues of the role of the TAM system in human physiology.

Populärvetenskaplig sammanfattning

Cellerna i vår kropp kommunicerar med varandra genom att skicka ut substanser, som binder till receptorer på andra celler. Min forskning har handlat om en sådan substans som heter Gas6, och dess receptorer Tyro3, Axl och Mer, som gemensamt kallas TAM-receptorerna. Receptorerna sitter genom cellens vägg och när Gas6 binder en av sina receptorer, så förändras de inre delarna av receptorn, vilket drar igång processer inne i cellen. Gas6 och TAM-receptorerna är viktiga för cellöverlevnad, reglering av immunförsvaret och för blodkärlens väggar.

Celler som inte regelbundet får överlevnadssignaler där de befinner sig brukar genomgå en kontrollerad dödsprocess. Detta gör att onödiga cellansamlingar undviks och försvårar okontrollerad celltillväxt, som kan ge upphov till cancer. När svältande celler behandlas med Gas6 dör de inte, och kan till och med börja dela sig. Flera olika celler från immunförsvaret dämpas av Gas6. Om man behandlar celler med bakteriella ämnen drar de igång ett starkt immunsvar, men om man samtidigt stimulerar med Gas6, så blir produktionen av inflammatoriska ämnen mindre. Eftersom en överdriven reaktion av immunförsvaret kan ställa till med mycket skada, är det viktigt att reglera det så det inte skadar kroppen.

Gas6 och Axl finns i cellerna som klär blodkärlens insida. Möss som på genetisk väg saknar Axl eller Gas6 har läckande kärl och minskad reparationstakt av skadad kärlvävnad.

Mitt projekt inleddes med att tillverka den del av Axl som finns utanför cellen (sAxl) genom att sätta in genen för detta i celler som sedan producerade sAxl-proteinet. sAxl-proteinet renades och användes för att vaccinera kaniner, för att få antikroppar mot Axl. Med antikropparna kan koncentrationen av Axl mätas i en metod som heter ELISA. I denna metod används en plastbricka med 96 små brunnar. Antikroppar mot sAxl fästs i botten av varje brunn, varpå en standard med kända mängder sAxl tillsätts, samt prov med okända mängder sAxl i olika brunnar. Sedan tvättas brickan och andra anti-Axl antikroppar tillsätts. Dessa är märkta med en enzym som producerar ett färgat ämne. Mer Axl innebär mer färg, och genom att jämföra med standarden kan mängden Axl i varje brunn räknas ut. Metoden är känslig och kan mäta prov med Axl från 0.4 nanogram per milliliter. Löslig sAxl uppmättes i blodprov från friska frivilliga och medelkoncentrationen var 25 nanogram per milliliter. Eftersom Gas6 också finns i blod, och Gas6 och Axl binder starkt till varandra, undersökte vi om de var bunda till varandra i blodet. Till detta användes antikroppar mot Gas6 och Axl som var fästa på mikrometerstora kulor av socker som kan centrifugeras till en liten prick på botten av ett provrör. Vi tillsatte antikropp/kulblandningen till blodprov, lät det dra sig en stund och centrifugerade sedan ner kulorna. Med anti-Axl antikropparna drog vi ner Axl, men också Gas6, vilket visar att de sitter ihop i blod. Gas6 kan inte binda till Axl på celler då det är bundet till lösligt Axl. Det Gas6 som finns i blodet är således inaktivt.

Eftersom vi vet ganska lite om Gas6 och sAxl i olika sjukdomar, beslöt vi oss för att mäta sAxl och Gas6 i patientmaterial för att se om

dessa proteiner kan vara av betydelse. Det finns data på många andra prover och tester på patienterna, som gör att man kan hitta något oväntat som har ett samband Gas6 eller sAxl.

Första materialet som undersöktes var patienter med bråck på stora kroppspulsådern. Patienter med stora bråck hade mer Gas6 och mindre sAxl än friska frivilliga. Skillnaden är intressant, men räcker inte för att diagnosticera, men visar att Gas6 och sAxl är förändrade i en kärlsjukdom, vilket uppmuntrade oss till nya studier.

Andra materialet som undersöktes var patienter med en annan kärlsjukdom, kritisk ischemi. Det är en sjukdom när blodcirkulationen i fötterna är otillräcklig på grund av förträngningar i blodkärlen. Smärta, långvariga sår och kallbrand kan bli följden. Gas6 och sAxl var förhöjda i patienterna, och de som dog inom tre års uppföljning hade förhöjda koncentrationer av både Gas6 och sAxl när provet togs, detta oberoende av andra kända riskfaktorer.

Det tredje gruppen av patienter som undersöktes hade blodförgiftning. Man har tidigare vetat att det ger förhöjda värden av Gas6, men ingen har mätt sAxl i dessa patienter. Eftersom sAxl binder till och inaktiverar Gas6 är det viktigt att veta om sAxl också ökar. Gas6 var dubblerat, och sAxl ökade med tjugo procent, så det fanns mer fritt Gas6. Gas6 var särskilt högt i de patienter som hade organsvikt och de som behövde intensivvård.

Den fjärde gruppen var patienter med SLE, som är en sjukdom där immunförsvaret skadar den egna kroppen av okända orsaker. sAxl och

Gas6 var förhöjda i patienter med aktiv sjukdom, och Gas6 och sAxl-koncentrationerna korrelerade med sjukdomsaktivitet, så att patienter med många symptom hade mer Gas6 och sAxl än patienter med få symptom.

Sammantaget har vi visat att Gas6 är bundet till sAxl i blodet, att Gas6 och sAxl är förhöjda i flera sjukdomar.

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References

1. Schneider C, King RM, Philipson L. Genes specifically expressed at growth arrest of mammalian cells. Cell. 1988;54:787-793.
2. Manfioletti G, Brancolini C, Avanzi G, Schneider C. The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol Cell Biol. 1993;13:4976-4985.
3. Wu SM, Cheung WF, Frazier D, Stafford DW. Cloning and expression of the cDNA for human gamma-glutamyl carboxylase. Science. 1991;254:1634-1636.
4. Stenflo J. Contributions of Gla and EGF-like domains to the function of vitamin K-dependent coagulation factors. Crit Rev Eukaryot Gene Expr. 1999;9:59-88.
5. Dahlback B. Blood coagulation. Lancet. 2000;355:1627-1632.
6. Hackeng TM, Sere KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. Proc Natl Acad Sci U S A. 2006;103:3106-3111.
7. Stern D, Brett J, Harris K, Nawroth P. Participation of endothelial cells in the protein C-protein S anticoagulant pathway: the synthesis and release of protein S. J Cell Biol. 1986;102:1971-1978.
8. Whitlon DS, Sadowski JA, Suttie JW. Mechanism of coumarin action: significance of vitamin K epoxide reductase inhibition. Biochemistry. 1978;17:1371-1377.
9. Campbell I, Bork P. Epidermal growth factor-like modules Current Opinions in Structural Biology 1993:385-392
.
10. Stenflo J, Stenberg Y, Muranyi A. Calcium-binding EGF-like modules in coagulation proteinases: function of the calcium ion in module interactions. Biochim Biophys Acta. 2000;1477:51-63.

11. Timpl R, Tisi D, Talts JF, Andac Z, Sasaki T, Hohenester E. Structure and function of laminin LG modules. Matrix Biol. 2000;19:309-317.
12. Gershagen S, Lundwall A, Fernlund P. Characterization of the human sex hormone binding globulin (SHBG) gene and demonstration of two transcripts in both liver and testis. Nucleic Acids Res. 1989;17:9245-9258.
13. Kahn SM, Hryb DJ, Nakhla AM, Romas NA, Rosner W. Sex hormone-binding globulin is synthesized in target cells. J Endocrinol. 2002;175:113-120.
14. Marcandalli P, Gostissa M, Varnum B, Goruppi S, Schneider C. Identification and tissue expression of a splice variant for the growth arrest-specific gene gas6. FEBS Lett. 1997;415:56-58.
15. Wang J, Qiao Y, Sun F, et al. Identification and characterization of mouse Gas6 promoter. Biochem Biophys Res Commun. 2008;371:567-572.
16. Mo R, Tony Zhu Y, Zhang Z, Rao SM, Zhu YJ. GAS6 is an estrogen-inducible gene in mammary epithelial cells. Biochem Biophys Res Commun. 2007;353:189-194.
17. Di Scipio RG, Hermodson MA, Yates SG, Davie EW. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. Biochemistry. 1977;16:698-706.
18. Burstyn-Cohen T, Heeb MJ, Lemke G. Lack of protein S in mice causes embryonic lethal coagulopathy and vascular dysgenesis. J Clin Invest. 2009;119:2942-2953.
19. Dahlback B. Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. Thromb Haemost. 1991;66:49-61.
20. Fair DS, Marlar RA. Biosynthesis and secretion of factor VII, protein C, protein S, and the Protein C inhibitor from a human hepatoma cell line. Blood. 1986;67:64-70.
21. Fair DS, Marlar RA, Levin EG. Human endothelial cells synthesize protein S. Blood. 1986;67:1168-1171.
22. Malm J, He XH, Bjartell A, Shen L, Abrahamsson PA, Dahlback B. Vitamin K-dependent protein S in Leydig cells of human testis. Biochem J. 1994;302 (Pt 3):845-850.

23. Lundwall A, Dackowski W, Cohen E, et al. Isolation and sequence of the cDNA for human protein S, a regulator of blood coagulation. Proc Natl Acad Sci U S A. 1986;83:6716-6720.
24. Esmon CT. The protein C pathway. Chest. 2003;124:26S-32S.
25. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732.
26. Dahlback B, Stenflo J. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci U S A. 1981;78:2512-2516.
27. Dahlback B, Smith CA, Muller-Eberhard HJ. Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b. Proc Natl Acad Sci U S A. 1983;80:3461-3465.
28. Hillarp A, Dahlback B. Novel subunit in C4b-binding protein required for protein S binding. J Biol Chem. 1988;263:12759-12764.
29. Linse S, Hardig Y, Schultz DA, Dahlback B. A region of vitamin K-dependent protein S that binds to C4b binding protein (C4BP) identified using bacteriophage peptide display libraries. J Biol Chem. 1997;272:14658-14665.
30. Blom AM, Villoutreix BO, Dahlback B. Functions of human complement inhibitor C4b-binding protein in relation to its structure. Arch Immunol Ther Exp (Warsz). 2004;52:83-95.
31. Kask L, Trouw LA, Dahlback B, Blom AM. The C4b-binding protein-protein S complex inhibits the phagocytosis of apoptotic cells. J Biol Chem. 2004;279:23869-23873.
32. Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol. 2003;4:87-91.
33. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2000;103:211-225.
34. Pawson T. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell. 2004;116:191-203.

35. Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. Oncogene. 2000;19:5548-5557.
36. Lai C, Lemke G. An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron. 1991;6:691-704.
37. Liu E, Hjelle B, Bishop JM. Transforming genes in chronic myelogenous leukemia. Proc Natl Acad Sci U S A. 1988;85:1952-1956.
38. O'Bryan JP, Frye RA, Cogswell PC, et al. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol. 1991;11:5016-5031.
39. Janssen JW, Schulz AS, Steenvoorden AC, et al. A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene. 1991;6:2113-2120.
40. Rescigno J, Mansukhani A, Basilico C. A putative receptor tyrosine kinase with unique structural topology. Oncogene. 1991;6:1909-1913.
41. Bellosta P, Costa M, Lin DA, Basilico C. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. Mol Cell Biol. 1995;15:614-625.
42. Avanzi GC, Gallicchio M, Cavalloni G, et al. GAS6, the ligand of Axl and Rse receptors, is expressed in hematopoietic tissue but lacks mitogenic activity. Exp Hematol. 1997;25:1219-1226.
43. Graham DK, Bowman GW, Dawson TL, Stanford WL, Earp HS, Snodgrass HR. Cloning and developmental expression analysis of the murine c-mer tyrosine kinase. Oncogene. 1995;10:2349-2359.
44. Neubauer A, Fiebeler A, Graham DK, et al. Expression of axl, a transforming receptor tyrosine kinase, in normal and malignant hematopoiesis. Blood. 1994;84:1931-1941.
45. Jia R, Mayer BJ, Hanafusa T, Hanafusa H. A novel oncogene, v-ryk, encoding a truncated receptor tyrosine kinase is transduced into the RPL30 virus without loss of viral sequences. J Virol. 1992;66:5975-5987.
46. Graham DK, Dawson TL, Mullaney DL, Snodgrass HR, Earp HS. Cloning and mRNA expression analysis of a novel

human protooncogene, c-mer. Cell Growth Differ. 1994;5:647-657.
47. Dai W, Pan H, Hassanain H, Gupta SL, Murphy MJ, Jr. Molecular cloning of a novel receptor tyrosine kinase, tif, highly expressed in human ovary and testis. Oncogene. 1994;9:975-979.
48. Ohashi K, Mizuno K, Kuma K, Miyata T, Nakamura T. Cloning of the cDNA for a novel receptor tyrosine kinase, Sky, predominantly expressed in brain. Oncogene. 1994;9:699-705.
49. Fujimoto J, Yamamoto T. brt, a mouse gene encoding a novel receptor-type protein-tyrosine kinase, is preferentially expressed in the brain. Oncogene. 1994;9:693-698.
50. Mark MR, Scadden DT, Wang Z, Gu Q, Goddard A, Godowski PJ. rse, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain. J Biol Chem. 1994;269:10720-10728.
51. Lai C, Gore M, Lemke G. Structure, expression, and activity of Tyro 3, a neural adhesion-related receptor tyrosine kinase. Oncogene. 1994;9:2567-2578.
52. Tjwa M, Bellido-Martin L, Lin Y, et al. Gas6 promotes inflammation by enhancing interactions between endothelial cells, platelets, and leukocytes. Blood. 2008;111:4096-4105.
53. Katagiri M, Hakeda Y, Chikazu D, et al. Mechanism of stimulation of osteoclastic bone resorption through Gas6/Tyro 3, a receptor tyrosine kinase signaling, in mouse osteoclasts. J Biol Chem. 2001;276:7376-7382.
54. Hafizi S, Dahlback B. Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases. Cytokine Growth Factor Rev. 2006;17:295-304.
55. Linger RM, Keating AK, Earp HS, Graham DK. TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. Adv Cancer Res. 2008;100:35-83.
56. Yamagata M, Sanes JR, Weiner JA. Synaptic adhesion molecules. Curr Opin Cell Biol. 2003;15:621-632.
57. Lu Q, Gore M, Zhang Q, et al. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature. 1999;398:723-728.

58. O'Bryan JP, Fridell YW, Koski R, Varnum B, Liu ET. The transforming receptor tyrosine kinase, Axl, is post-translationally regulated by proteolytic cleavage. J Biol Chem. 1995;270:551-557.
59. Sather S, Kenyon KD, Lefkowitz JB, et al. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood. 2007;109:1026-1033.
60. Varnum BC, Young C, Elliott G, et al. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature. 1995;373:623-626.
61. Godowski PJ, Mark MR, Chen J, Sadick MD, Raab H, Hammonds RG. Reevaluation of the roles of protein S and Gas6 as ligands for the receptor tyrosine kinase Rse/Tyro 3. Cell. 1995;82:355-358.
62. Ohashi K, Nagata K, Toshima J, et al. Stimulation of sky receptor tyrosine kinase by the product of growth arrest-specific gene 6. J Biol Chem. 1995;270:22681-22684.
63. Chen J, Carey K, Godowski PJ. Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. Oncogene. 1997;14:2033-2039.
64. Stitt TN, Conn G, Gore M, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell. 1995;80:661-670.
65. Mark MR, Chen J, Hammonds RG, Sadick M, Godowsk PJ. Characterization of Gas6, a member of the superfamily of G domain-containing proteins, as a ligand for Rse and Axl. J Biol Chem. 1996;271:9785-9789.
66. Nyberg P, He X, Hardig Y, Dahlback B, Garcia de Frutos P. Stimulation of Sky tyrosine phosphorylation by bovine protein S--domains involved in the receptor-ligand interaction. Eur J Biochem. 1997;246:147-154.
67. McColl A, Bournazos S, Franz S, et al. Glucocorticoids induce protein S-dependent phagocytosis of apoptotic neutrophils by human macrophages. J Immunol. 2009;183:2167-2175.

68. Prasad D, Rothlin CV, Burrola P, et al. TAM receptor function in the retinal pigment epithelium. Mol Cell Neurosci. 2006;33:96-108.
69. Takagi T, Taguchi O, Aoki S, et al. Direct effects of protein S in ameliorating acute lung injury. J Thromb Haemost. 2009;7:2053-2063.
70. Zhu D, Wang Y, Singh I, et al. Protein S controls hypoxic/ischemic blood-brain barrier disruption through the TAM receptor Tyro3 and sphingosine 1-phosphate receptor. Blood. 2010;115:4963-4972.
71. Uehara H, Shacter E. Auto-oxidation and oligomerization of protein S on the apoptotic cell surface is required for Mer tyrosine kinase-mediated phagocytosis of apoptotic cells. J Immunol. 2008;180:2522-2530.
72. Goruppi S, Yamane H, Marcandalli P, et al. The product of a gas6 splice variant allows the release of the domain responsible for Axl tyrosine kinase receptor activation. FEBS Lett. 1997;415:59-63.
73. Tanabe K, Nagata K, Ohashi K, Nakano T, Arita H, Mizuno K. Roles of gamma-carboxylation and a sex hormone-binding globulin-like domain in receptor-binding and in biological activities of Gas6. FEBS Lett. 1997;408:306-310.
74. Hasanbasic I, Rajotte I, Blostein M. The role of gamma-carboxylation in the anti-apoptotic function of gas6. J Thromb Haemost. 2005;3:2790-2797.
75. Nakano T, Kawamoto K, Kishino J, Nomura K, Higashino K, Arita H. Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. Biochem J. 1997;323 (Pt 2):387-392.
76. Rajotte I, Hasanbasic I, Blostein M. Gas6-mediated signaling is dependent on the engagement of its gamma-carboxyglutamic acid domain with phosphatidylserine. Biochem Biophys Res Commun. 2008;376:70-73.
77. Stenhoff J, Dahlback B, Hafizi S. Vitamin K-dependent Gas6 activates ERK kinase and stimulates growth of cardiac fibroblasts. Biochem Biophys Res Commun. 2004;319:871-878.
78. Nagata K, Ohashi K, Nakano T, et al. Identification of the product of growth arrest-specific gene 6 as a common ligand

for Axl, Sky, and Mer receptor tyrosine kinases. J Biol Chem. 1996;271:30022-30027.
79. Fisher PW, Brigham-Burke M, Wu SJ, et al. A novel site contributing to growth-arrest-specific gene 6 binding to its receptors as revealed by a human monoclonal antibody. Biochem J. 2005;387:727-735.
80. Wimmel A, Glitz D, Kraus A, Roeder J, Schuermann M. Axl receptor tyrosine kinase expression in human lung cancer cell lines correlates with cellular adhesion. Eur J Cancer. 2001;37:2264-2274.
81. Sasaki T, Knyazev PG, Cheburkin Y, et al. Crystal structure of a C-terminal fragment of growth arrest-specific protein Gas6. Receptor tyrosine kinase activation by laminin G-like domains. J Biol Chem. 2002;277:44164-44170.
82. Heiring C, Dahlback B, Muller YA. Ligand recognition and homophilic interactions in Tyro3: structural insights into the Axl/Tyro3 receptor tyrosine kinase family. J Biol Chem. 2004;279:6952-6958.
83. Sasaki T, Knyazev PG, Clout NJ, et al. Structural basis for Gas6-Axl signalling. Embo J. 2006;25:80-87.
84. Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. Mol Aspects Med. 2008;29:258-289.
85. Murphy G. The ADAMs: signalling scissors in the tumour microenvironment. Nat Rev Cancer. 2008;8:929-941.
86. Budagian V, Bulanova E, Orinska Z, et al. Soluble Axl is generated by ADAM10-dependent cleavage and associates with Gas6 in mouse serum. Mol Cell Biol. 2005;25:9324-9339.
87. Sawabu T, Seno H, Kawashima T, et al. Growth arrest-specific gene 6 and Axl signaling enhances gastric cancer cell survival via Akt pathway. Mol Carcinog. 2007;46:155-164.
88. Yanagita M, Arai H, Ishii K, et al. Gas6 regulates mesangial cell proliferation through Axl in experimental glomerulonephritis. Am J Pathol. 2001;158:1423-1432.
89. Yanagita M, Arai H, Nakano T, et al. Gas6 induces mesangial cell proliferation via latent transcription factor STAT3. J Biol Chem. 2001;276:42364-42369.

90. Costa M, Bellosta P, Basilico C. Cleavage and release of a soluble form of the receptor tyrosine kinase ARK in vitro and in vivo. J Cell Physiol. 1996;168:737-744.
91. Scutera S, Fraone T, Musso T, et al. Survival and Migration of Human Dendritic Cells Are Regulated by an IFN-{alpha}-Inducible Axl/Gas6 Pathway. J Immunol. 2009.
92. Toshima J, Ohashi K, Iwashita S, Mizuno K. Autophosphorylation activity and association with Src family kinase of Sky receptor tyrosine kinase. Biochem Biophys Res Commun. 1995;209:656-663.
93. Prieto AL, O'Dell S, Varnum B, Lai C. Localization and signaling of the receptor protein tyrosine kinase Tyro3 in cortical and hippocampal neurons. Neuroscience. 2007;150:319-334.
94. Lan Z, Wu H, Li W, et al. Transforming activity of receptor tyrosine kinase tyro3 is mediated, at least in part, by the PI3 kinase-signaling pathway. Blood. 2000;95:633-638.
95. Hafizi S, Gustafsson A, Stenhoff J, Dahlback B. The Ran binding protein RanBPM interacts with Axl and Sky receptor tyrosine kinases. Int J Biochem Cell Biol. 2005;37:2344-2356.
96. Braunger J, Schleithoff L, Schulz AS, et al. Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. Oncogene. 1997;14:2619-2631.
97. Weinger JG, Gohari P, Yan Y, Backer JM, Varnum B, Shafit-Zagardo B. In brain, Axl recruits Grb2 and the p85 regulatory subunit of PI3 kinase; in vitro mutagenesis defines the requisite binding sites for downstream Akt activation. J Neurochem. 2008;106:134-146.
98. Ling L, Templeton D, Kung HJ. Identification of the major autophosphorylation sites of Nyk/Mer, an NCAM-related receptor tyrosine kinase. J Biol Chem. 1996;271:18355-18362.
99. Ling L, Kung HJ. Mitogenic signals and transforming potential of Nyk, a newly identified neural cell adhesion molecule-related receptor tyrosine kinase. Mol Cell Biol. 1995;15:6582-6592.
100. Mahajan NP, Earp HS. An SH2 domain-dependent, phosphotyrosine-independent interaction between Vav1 and the Mer receptor tyrosine kinase: a mechanism for localizing

guanine nucleotide-exchange factor action. J Biol Chem. 2003;278:42596-42603.
101. Bellosta P, Zhang Q, Goff SP, Basilico C. Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. Oncogene. 1997;15:2387-2397.
102. Goruppi S, Ruaro E, Schneider C. Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. Oncogene. 1996;12:471-480.
103. Goruppi S, Ruaro E, Varnum B, Schneider C. Gas6-mediated survival in NIH3T3 cells activates stress signalling cascade and is independent of Ras. Oncogene. 1999;18:4224-4236.
104. Hasanbasic I, Cuerquis J, Varnum B, Blostein MD. Intracellular signaling pathways involved in Gas6-Axl-mediated survival of endothelial cells. Am J Physiol Heart Circ Physiol. 2004;287:H1207-1213.
105. Healy AM, Schwartz JJ, Zhu X, Herrick BE, Varnum B, Farber HW. Gas 6 promotes Axl-mediated survival in pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2001;280:L1273-1281.
106. O'Donnell K, Harkes IC, Dougherty L, Wicks IP. Expression of receptor tyrosine kinase Axl and its ligand Gas6 in rheumatoid arthritis: evidence for a novel endothelial cell survival pathway. Am J Pathol. 1999;154:1171-1180.
107. Melaragno MG, Cavet ME, Yan C, et al. Gas6 inhibits apoptosis in vascular smooth muscle: role of Axl kinase and Akt. J Mol Cell Cardiol. 2004;37:881-887.
108. Nakano T, Kawamoto K, Higashino K, Arita H. Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, gas6. FEBS Lett. 1996;387:78-80.
109. Shankar SL, O'Guin K, Cammer M, et al. The growth arrest-specific gene product Gas6 promotes the survival of human oligodendrocytes via a phosphatidylinositol 3-kinase-dependent pathway. J Neurosci. 2003;23:4208-4218.
110. Shankar SL, O'Guin K, Kim M, et al. Gas6/Axl signaling activates the phosphatidylinositol 3-kinase/Akt1 survival

pathway to protect oligodendrocytes from tumor necrosis factor alpha-induced apoptosis. J Neurosci. 2006;26:5638-5648.
111. Li R, Chen J, Hammonds G, et al. Identification of Gas6 as a growth factor for human Schwann cells. J Neurosci. 1996;16:2012-2019.
112. Valverde P, Obin MS, Taylor A. Role of Gas6/Axl signaling in lens epithelial cell proliferation and survival. Exp Eye Res. 2004;78:27-37.
113. Funakoshi H, Yonemasu T, Nakano T, Matumoto K, Nakamura T. Identification of Gas6, a putative ligand for Sky and Axl receptor tyrosine kinases, as a novel neurotrophic factor for hippocampal neurons. J Neurosci Res. 2002;68:150-160.
114. Yagami T, Ueda K, Asakura K, et al. Gas6 rescues cortical neurons from amyloid beta protein-induced apoptosis. Neuropharmacology. 2002;43:1289-1296.
115. Couchie D, Lafdil F, Martin-Garcia N, Laperche Y, Zafrani ES, Mavier P. Expression and role of Gas6 protein and of its receptor Axl in hepatic regeneration from oval cells in the rat. Gastroenterology. 2005;129:1633-1642.
116. Lafdil F, Chobert MN, Couchie D, et al. Induction of Gas6 protein in CCl4-induced rat liver injury and anti-apoptotic effect on hepatic stellate cells. Hepatology. 2006;44:228-239.
117. Lafdil F, Chobert MN, Deveaux V, et al. Growth arrest-specific protein 6 deficiency impairs liver tissue repair after acute toxic hepatitis in mice. J Hepatol. 2009;51:55-66.
118. Dirks W, Rome D, Ringel F, Jager K, MacLeod RA, Drexler HG. Expression of the growth arrest-specific gene 6 (GAS6) in leukemia and lymphoma cell lines. Leuk Res. 1999;23:643-651.
119. Ito M, Nakashima M, Nakayama T, et al. Expression of receptor-type tyrosine kinase, Axl, and its ligand, Gas6, in pediatric thyroid carcinomas around chernobyl. Thyroid. 2002;12:971-975.
120. Ito T, Ito M, Naito S, et al. Expression of the Axl receptor tyrosine kinase in human thyroid carcinoma. Thyroid. 1999;9:563-567.
121. Sun WS, Fujimoto J, Tamaya T. Clinical implications of coexpression of growth arrest-specific gene 6 and receptor

tyrosine kinases Axl and Sky in human uterine leiomyoma. Mol Hum Reprod. 2003;9:701-707.
122. Sun WS, Fujimoto J, Tamaya T. Coexpression of growth arrest-specific gene 6 and receptor tyrosine kinases Axl and Sky in human uterine endometrial cancers. Ann Oncol. 2003;14:898-906.
123. Sun W, Fujimoto J, Tamaya T. Coexpression of Gas6/Axl in human ovarian cancers. Oncology. 2004;66:450-457.
124. Sainaghi PP, Castello L, Bergamasco L, Galletti M, Bellosta P, Avanzi GC. Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. J Cell Physiol. 2005;204:36-44.
125. Shain SA. Exogenous fibroblast growth factors maintain viability, promote proliferation, and suppress GADD45alpha and GAS6 transcript content of prostate cancer cells genetically modified to lack endogenous FGF-2. Mol Cancer Res. 2004;2:653-661.
126. Shiozawa Y, Pedersen EA, Patel LR, et al. GAS6/AXL axis regulates prostate cancer invasion, proliferation, and survival in the bone marrow niche. Neoplasia. 2010;12:116-127.
127. Wu YM, Robinson DR, Kung HJ. Signal pathways in up-regulation of chemokines by tyrosine kinase MER/NYK in prostate cancer cells. Cancer Res. 2004;64:7311-7320.
128. Abba MC, Fabris VT, Hu Y, et al. Identification of novel amplification gene targets in mouse and human breast cancer at a syntenic cluster mapping to mouse ch8A1 and human ch13q34. Cancer Res. 2007;67:4104-4112.
129. Liu R, Gong M, Li X, et al. Induction, regulation, and biologic function of Axl receptor tyrosine kinase in Kaposi sarcoma. Blood. 2010;116:297-305.
130. Hutterer M, Knyazev P, Abate A, et al. Axl and growth arrest-specific gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with glioblastoma multiforme. Clin Cancer Res. 2008;14:130-138.
131. Vajkoczy P, Knyazev P, Kunkel A, et al. Dominant-negative inhibition of the Axl receptor tyrosine kinase suppresses brain tumor cell growth and invasion and prolongs survival. Proc Natl Acad Sci U S A. 2006;103:5799-5804.

132. Gustafsson A, Martuszewska D, Johansson M, et al. Differential expression of Axl and Gas6 in renal cell carcinoma reflecting tumor advancement and survival. Clin Cancer Res. 2009;15:4742-4749.
133. Goruppi S, Chiaruttini C, Ruaro ME, Varnum B, Schneider C. Gas6 induces growth, beta-catenin stabilization, and T-cell factor transcriptional activation in contact-inhibited C57 mammary cells. Mol Cell Biol. 2001;21:902-915.
134. Gjerdrum C, Tiron C, Hoiby T, et al. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. Proc Natl Acad Sci U S A. 2010;107:1124-1129.
135. He L, Zhang J, Jiang L, et al. Differential expression of Axl in hepatocellular carcinoma and correlation with tumor lymphatic metastasis. Mol Carcinog. 2010;49:882-891.
136. Rankin EB, Fuh KC, Taylor TE, et al. AXL is an essential factor and therapeutic target for metastatic ovarian cancer. Cancer Res. 2010;70:7570-7579.
137. Loges S, Schmidt T, Tjwa M, et al. Malignant cells fuel tumor growth by educating infiltrating leukocytes to produce the mitogen Gas6. Blood. 2010;115:2264-2273.
138. Song X, Wang H, Logsdon CD, et al. Overexpression of receptor tyrosine kinase Axl promotes tumor cell invasion and survival in pancreatic ductal adenocarcinoma. Cancer. 2010.
139. Alvarez H, Montgomery EA, Karikari C, et al. The Axl receptor tyrosine kinase is an adverse prognostic factor and a therapeutic target in esophageal adenocarcinoma. Cancer Biol Ther. 2010;10.
140. Mc Cormack O, Chung WY, Fitzpatrick P, et al. Growth arrest-specific gene 6 expression in human breast cancer. Br J Cancer. 2008;98:1141-1146.
141. Agrawal M, Garg RJ, Cortes J, Quintas-Cardama A. Tyrosine kinase inhibitors: the first decade. Curr Hematol Malig Rep. 2010;5:70-80.
142. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med. 2006;355:2408-2417.
143. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast

crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038-1042.
144. Keating AK, Kim GK, Jones AE, et al. Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. Mol Cancer Ther. 2010;9:1298-1307.
145. Ye X, Li Y, Stawicki S, et al. An anti-Axl monoclonal antibody attenuates xenograft tumor growth and enhances the effect of multiple anticancer therapies. Oncogene. 2010;29:5254-5264.
146. Zhang YX, Knyazev PG, Cheburkin YV, et al. AXL is a potential target for therapeutic intervention in breast cancer progression. Cancer Res. 2008;68:1905-1915.
147. Holland SJ, Pan A, Franci C, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. Cancer Res. 2010;70:1544-1554.
148. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends Cell Biol. 2006;16:189-197.
149. Nakano T, Ishimoto Y, Kishino J, et al. Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. J Biol Chem. 1997;272:29411-29414.
150. Ishimoto Y, Ohashi K, Mizuno K, Nakano T. Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. J Biochem. 2000;127:411-417.
151. Scott RS, McMahon EJ, Pop SM, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001;411:207-211.
152. Shao WH, Zhen Y, Eisenberg RA, Cohen PL. The Mer receptor tyrosine kinase is expressed on discrete macrophage subpopulations and mainly uses Gas6 as its ligand for uptake of apoptotic cells. Clin Immunol. 2009;133:138-144.
153. Seitz HM, Camenisch TD, Lemke G, Earp HS, Matsushima GK. Macrophages and dendritic cells use different Axl/Mertk/Tyro3 receptors in clearance of apoptotic cells. J Immunol. 2007;178:5635-5642.

154. Grommes C, Lee CY, Wilkinson BL, et al. Regulation of microglial phagocytosis and inflammatory gene expression by Gas6 acting on the Axl/Mer family of tyrosine kinases. J Neuroimmune Pharmacol. 2008;3:130-140.
155. Xiong W, Chen Y, Wang H, et al. Gas6 and the Tyro 3 receptor tyrosine kinase subfamily regulate the phagocytic function of Sertoli cells. Reproduction. 2008;135:77-87.
156. D'Cruz PM, Yasumura D, Weir J, et al. Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. Hum Mol Genet. 2000;9:645-651.
157. Nandrot E, Dufour EM, Provost AC, et al. Homozygous deletion in the coding sequence of the c-mer gene in RCS rats unravels general mechanisms of physiological cell adhesion and apoptosis. Neurobiol Dis. 2000;7:586-599.
158. Hall MO, Prieto AL, Obin MS, et al. Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. Exp Eye Res. 2001;73:509-520.
159. Hall MO, Obin MS, Prieto AL, Burgess BL, Abrams TA. Gas6 binding to photoreceptor outer segments requires gamma-carboxyglutamic acid (Gla) and Ca(2+) and is required for OS phagocytosis by RPE cells in vitro. Exp Eye Res. 2002;75:391-400.
160. Hall MO, Agnew BJ, Abrams TA, Burgess BL. The phagocytosis of os is mediated by the PI3-kinase linked tyrosine kinase receptor, mer, and is stimulated by GAS6. Adv Exp Med Biol. 2003;533:331-336.
161. Hall MO, Obin MS, Heeb MJ, Burgess BL, Abrams TA. Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. Exp Eye Res. 2005;81:581-591.
162. Karl MO, Kroeger W, Wimmers S, et al. Endogenous Gas6 and Ca(2+)-channel activation modulate phagocytosis by retinal pigment epithelium. Cell Signal. 2008;20:1159-1168.
163. Gal A, Li Y, Thompson DA, et al. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat Genet. 2000;26:270-271.
164. McHenry CL, Liu Y, Feng W, et al. MERTK arginine-844-cysteine in a patient with severe rod-cone dystrophy: loss of

mutant protein function in transfected cells. Invest Ophthalmol Vis Sci. 2004;45:1456-1463.
165. Tada A, Wada Y, Sato H, et al. Screening of the MERTK gene for mutations in Japanese patients with autosomal recessive retinitis pigmentosa. Mol Vis. 2006;12:441-444.
166. Tschernutter M, Jenkins SA, Waseem NH, et al. Clinical characterisation of a family with retinal dystrophy caused by mutation in the Mertk gene. Br J Ophthalmol. 2006;90:718-723.
167. Cavet ME, Smolock EM, Ozturk OH, et al. Gas6-axl receptor signaling is regulated by glucose in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2008;28:886-891.
168. Fridell YW, Villa J, Jr., Attar EC, Liu ET. GAS6 induces Axl-mediated chemotaxis of vascular smooth muscle cells. J Biol Chem. 1998;273:7123-7126.
169. Allen MP, Linseman DA, Udo H, et al. Novel mechanism for gonadotropin-releasing hormone neuronal migration involving Gas6/Ark signaling to p38 mitogen-activated protein kinase. Mol Cell Biol. 2002;22:599-613.
170. Pierce A, Bliesner B, Xu M, et al. Axl and Tyro3 modulate female reproduction by influencing gonadotropin-releasing hormone neuron survival and migration. Mol Endocrinol. 2008;22:2481-2495.
171. Gustafsson A, Bostrom AK, Ljungberg B, Axelson H, Dahlback B. Gas6 and the receptor tyrosine kinase Axl in clear cell renal cell carcinoma. PLoS One. 2009;4:e7575.
172. Gallicchio M, Mitola S, Valdembri D, et al. Inhibition of vascular endothelial growth factor receptor 2-mediated endothelial cell activation by Axl tyrosine kinase receptor. Blood. 2005;105:1970-1976.
173. Yanagita M, Ishii K, Ozaki H, et al. Mechanism of inhibitory effect of warfarin on mesangial cell proliferation. J Am Soc Nephrol. 1999;10:2503-2509.
174. Yanagita M, Ishimoto Y, Arai H, et al. Essential role of Gas6 for glomerular injury in nephrotoxic nephritis. J Clin Invest. 2002;110:239-246.
175. Nagai K, Arai H, Yanagita M, et al. Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. J Biol Chem. 2003;278:18229-18234.

176. Park JK, Theuer S, Kirsch T, et al. Growth arrest specific protein 6 participates in DOCA-induced target-organ damage. Hypertension. 2009;54:359-364.
177. Yin JL, Hambly BD, Bao SS, Painter D, Bishop GA, Eris JM. Expression of growth arrest-specific gene 6 and its receptors in dysfunctional human renal allografts. Transpl Int. 2003;16:681-688.
178. Fiebeler A, Park JK, Muller DN, et al. Growth arrest specific protein 6/Axl signaling in human inflammatory renal diseases. Am J Kidney Dis. 2004;43:286-295.
179. Arai H, Nagai K, Doi T. Role of growth arrest-specific gene 6 in diabetic nephropathy. Vitam Horm. 2008;78:375-392.
180. Nakano T, Higashino K, Kikuchi N, et al. Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. J Biol Chem. 1995;270:5702-5705.
181. Melaragno MG, Wuthrich DA, Poppa V, et al. Increased expression of Axl tyrosine kinase after vascular injury and regulation by G protein-coupled receptor agonists in rats. Circ Res. 1998;83:697-704.
182. Yin J, McLachlan C, Chaufour X, et al. Growth arrest-specific gene 6 expression in proliferating rabbit vascular smooth muscle cells in vitro and in vivo. Electrophoresis. 2000;21:3851-3856.
183. Konishi A, Aizawa T, Mohan A, Korshunov VA, Berk BC. Hydrogen peroxide activates the Gas6-Axl pathway in vascular smooth muscle cells. J Biol Chem. 2004;279:28766-28770.
184. Korshunov VA, Mohan AM, Georger MA, Berk BC. Axl, a receptor tyrosine kinase, mediates flow-induced vascular remodeling. Circ Res. 2006;98:1446-1452.
185. Korshunov VA, Daul M, Massett MP, Berk BC. Axl mediates vascular remodeling induced by deoxycorticosterone acetate-salt hypertension. Hypertension. 2007;50:1057-1062.
186. Lutgens E, Tjwa M, Garcia de Frutos P, et al. Genetic loss of Gas6 induces plaque stability in experimental atherosclerosis. J Pathol. 2008;216:55-63.

187. Munoz X, Obach V, Hurtado B, de Frutos PG, Chamorro A, Sala N. Association of specific haplotypes of GAS6 gene with stroke. Thromb Haemost. 2007;98:406-412.
188. Munoz X, Sumoy L, Ramirez-Lorca R, Villar J, de Frutos PG, Sala N. Human vitamin K-dependent GAS6: gene structure, allelic variation, and association with stroke. Hum Mutat. 2004;23:506-512.
189. Hurtado B, Abasolo N, Munoz X, et al. Association study between polymorphims in GAS6-TAM genes and carotid atherosclerosis. Thromb Haemost. 2010;104:592-598.
190. Alciato F, Sainaghi PP, Sola D, Castello L, Avanzi GC. TNF-alpha, IL-6, and IL-1 expression is inhibited by GAS6 in monocytes/macrophages. J Leukoc Biol. 2010;87:869-875.
191. Sharif MN, Sosic D, Rothlin CV, et al. Twist mediates suppression of inflammation by type I IFNs and Axl. J Exp Med. 2006;203:1891-1901.
192. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell. 2007;131:1124-1136.
193. Wallet MA, Sen P, Flores RR, et al. MerTK is required for apoptotic cell-induced T cell tolerance. J Exp Med. 2008;205:219-232.
194. Sun B, Qi N, Shang T, Wu H, Deng T, Han D. Sertoli cell-initiated testicular innate immune response through toll-like receptor-3 activation is negatively regulated by Tyro3, Axl, and mer receptors. Endocrinology. 2010;151:2886-2897.
195. Avanzi GC, Gallicchio M, Bottarel F, et al. GAS6 inhibits granulocyte adhesion to endothelial cells. Blood. 1998;91:2334-2340.
196. Burnier L, Saller F, Kadi L, et al. Gas6 deficiency in recipient mice of allogeneic transplantation alleviates hepatic graft-versus-host disease. Blood. 2010;115:3390-3397.
197. Camenisch TD, Koller BH, Earp HS, Matsushima GK. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol. 1999;162:3498-3503.
198. Lu Q, Lemke G. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science. 2001;293:306-311.

199. Angelillo-Scherrer A, de Frutos P, Aparicio C, et al. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med. 2001;7:215-221.
200. Angelillo-Scherrer A, Burnier L, Flores N, et al. Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest. 2005;115:237-246.
201. Cosemans JM, van Kruchten R, Olieslagers S, et al. Potentiating role of Gas6 and TAM receptors in human and murine platelet activation and thrombus stabilization. J Thromb Haemost. 2010.
202. Balogh I, Hafizi S, Stenhoff J, Hansson K, Dahlback B. Analysis of Gas6 in human platelets and plasma. Arterioscler Thromb Vasc Biol. 2005;25:1280-1286.
203. Clauser S, Bachelot-Lozat C, Fontana P, et al. Physiological plasma Gas6 levels do not influence platelet aggregation. Arterioscler Thromb Vasc Biol. 2006;26:e22.
204. Wang H, Chen C, Chen Y, et al. The role of Tyro 3 subfamily receptors in the regulation of hemostasis and megakaryocytopoesis. Haematologica. 2007;92:643-650.
205. Angelillo-Scherrer A, Burnier L, Lambrechts D, et al. Role of Gas6 in erythropoiesis and anemia in mice. J Clin Invest. 2008;118:583-596.
206. Tang H, Chen S, Wang H, Wu H, Lu Q, Han D. TAM receptors and the regulation of erythropoiesis in mice. Haematologica. 2009;94:326-334.
207. Saller F, Brisset AC, Tchaikovski SN, et al. Generation and phenotypic analysis of protein S-deficient mice. Blood. 2009;114:2307-2314.
208. Clauser S, Peyrard S, Gaussem P, et al. Development of a novel immunoassay for the assessment of plasma Gas6 concentrations and their variation with hormonal status. Clin Chem. 2007;53:1808-1813.
209. Burnier L, Borgel D, Angelillo-Scherrer A, Fontana P. Plasma levels of the growth arrest-specific gene 6 product (Gas6) and antiplatelet drug responsiveness in healthy subjects. J Thromb Haemost. 2006;4:2283-2284.

210. Borgel D, Durand E, Clauser S, et al. Plasma Gas6 levels and coronary artery disease. Thromb Haemost. 2009;101:215-216.
211. Borgel D, Clauser S, Bornstain C, et al. Elevated growth-arrest-specific protein 6 plasma levels in patients with severe sepsis. Crit Care Med. 2006;34:219-222.
212. Gibot S, Massin F, Cravoisy A, et al. Growth arrest-specific protein 6 plasma concentrations during septic shock. Crit Care. 2007;11:R8.
213. Uehara S, Handa H, Gotoh K, Tomita H, Sennshuu M. Plasma concentrations of growth arrest-specific protein 6 and protein S in patients with acute pancreatitis. J Gastroenterol Hepatol. 2009;24:1567-1573.
214. Alciato F, Sainaghi PP, Castello L, Bergamasco L, Carnieletto S, Avanzi GC. Development and validation of an ELISA method for detection of growth arrest specific 6 (GAS6) protein in human plasma. J Immunoassay Immunochem. 2008;29:167-180.
215. Sainaghi PP, Collimedaglia L, Alciato F, et al. Elevation of Gas6 protein concentration in cerebrospinal fluid of patients with chronic inflammatory demyelinating polyneuropathy (CIDP). J Neurol Sci. 2008;269:138-142.
216. Sainaghi PP, Alciato F, Carnieletto S, et al. Gas6 evaluation in patients with acute dyspnea due to suspected pulmonary embolism. Respir Med. 2009;103:589-594.
217. Jiang L, Liu CY, Yang QF, Wang P, Zhang W. Plasma level of growth arrest-specific 6 (GAS6) protein and genetic variations in the GAS6 gene in patients with acute coronary syndrome. Am J Clin Pathol. 2009;131:738-743.
218. Hung YJ, Lee CH, Chu NF, Shieh YS. Plasma protein growth arrest-specific 6 levels are associated with altered glucose tolerance, inflammation, and endothelial dysfunction. Diabetes Care. 2010;33:1840-1844.
219. Suh CH, Hilliard B, Li S, Merrill JT, Cohen PL. TAM receptor ligands in lupus: Protein S but not Gas6 levels reflect disease activity in systemic lupus erythematosus. Arthritis Res Ther. 2010;12:R146.
220. Middleton RK, Lloyd GM, Bown MJ, Cooper NJ, London NJ, Sayers RD. The pro-inflammatory and chemotactic

cytokine microenvironment of the abdominal aortic aneurysm wall: a protein array study. J Vasc Surg. 2007;45:574-580.
221. Wassef M, Baxter BT, Chisholm RL, et al. Pathogenesis of abdominal aortic aneurysms: a multidisciplinary research program supported by the National Heart, Lung, and Blood Institute. J Vasc Surg. 2001;34:730-738.
222. Golledge J, Muller J, Daugherty A, Norman P. Abdominal aortic aneurysm: pathogenesis and implications for management. Arterioscler Thromb Vasc Biol. 2006;26:2605-2613.
223. Choke E, Cockerill G, Wilson WR, et al. A review of biological factors implicated in abdominal aortic aneurysm rupture. Eur J Vasc Endovasc Surg. 2005;30:227-244.
224. Lederle FA, Wilson SE, Johnson GR, et al. Immediate repair compared with surveillance of small abdominal aortic aneurysms. N Engl J Med. 2002;346:1437-1444.
225. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-126.
226. Novo S, Coppola G, Milio G. Critical limb ischemia: definition and natural history. Curr Drug Targets Cardiovasc Haematol Disord. 2004;4:219-225.
227. Minar E. Critical limb ischaemia. Hamostaseologie. 2009;29:102-109.
228. Norgren L, Hiatt WR, Dormandy JA, et al. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg. 2007;33 Suppl 1:S1-75.
229. Jensen SA, Vatten LJ, Myhre HO. The prevalence of chronic critical lower limb ischaemia in a population of 20,000 subjects 40-69 years of age. Eur J Vasc Endovasc Surg. 2006;32:60-65.
230. Dormandy J, Heeck L, Vig S. The fate of patients with critical leg ischemia. Semin Vasc Surg. 1999;12:142-147.
231. Bone RC, Balk RA, Cerra FB, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992;101:1644-1655.
232. Dellinger RP, Levy MM, Carlet JM, et al. Surviving Sepsis Campaign: international guidelines for management of

severe sepsis and septic shock: 2008. Crit Care Med. 2008;36:296-327.
233. Linder A, Christensson B, Herwald H, Bjorck L, Akesson P. Heparin-binding protein: an early marker of circulatory failure in sepsis. Clin Infect Dis. 2009;49:1044-1050.
234. Rahman A, Isenberg DA. Systemic lupus erythematosus. N Engl J Med. 2008;358:929-939.
235. Gaipl US, Voll RE, Sheriff A, Franz S, Kalden JR, Herrmann M. Impaired clearance of dying cells in systemic lupus erythematosus. Autoimmun Rev. 2005;4:189-194.
236. Munoz LE, Gaipl US, Franz S, et al. SLE--a disease of clearance deficiency? Rheumatology (Oxford). 2005;44:1101-1107.
237. Isenberg DA, Manson JJ, Ehrenstein MR, Rahman A. Fifty years of anti-ds DNA antibodies: are we approaching journey's end? Rheumatology (Oxford). 2007;46:1052-1056.
238. Johnson AE, Gordon C, Palmer RG, Bacon PA. The prevalence and incidence of systemic lupus erythematosus in Birmingham, England. Relationship to ethnicity and country of birth. Arthritis Rheum. 1995;38:551-558.
239. Lateef A, Petri M. Biologics in the treatment of systemic lupus erythematosus. Curr Opin Rheumatol. 2010;22:504-509.
240. Orth-Gomer K, Johnson JV. Social network interaction and mortality. A six year follow-up study of a random sample of the Swedish population. J Chronic Dis. 1987;40:949-957.