■ R E V I E W

Axl-dependent signalling: a clinical update

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A B S T R A C T

Axl is a receptor tyrosine kinase that was originally cloned from cancer cells. Axl belongs to the TAM (Tyro3, Axl and Mertk) family of receptor tyrosine kinases. Gas6 (growth-arrest-specific protein 6) is a ligand for Axl. Activation of Axl protects cells from apoptosis, and increases migration, aggregation and growth through multiple downstream pathways. Up-regulation of the Gas6/Axl pathway is more evident in pathological conditions compared with normal physiology. Recent advances in Axl receptor biology are summarized in the present review. The emphasis is given to translational aspects of Axl-dependent signalling under pathological conditions. In particular, inhibition of Axl reduces tumorigenesis and prevents metastasis as well. Axl-dependent signals are important for the progression of cardiovascular diseases. In contrast, deficiency of Axl in innate immune cells contributes to the pathogenesis of autoimmune disorders. Current challenges in Axl biology are related to the functional interactions of Axl with other members of the TAM family or other tyrosine kinases, mechanisms of ligand-independent activation, inactivation of the receptor and cell–cell interactions (with respect to immune cells) in chronic diseases.

INTRODUCTION

Axl, an RTK (receptor tyrosine kinase), derives its name from the Greek word anexelekto which means uncontrolled. Axl was initially discovered in cancer cells over two decades ago [1]. Axl is a founder of a unique TAM (Tyro3, Axl and Mertk) family of RTKs. Axl is broadly expressed with an onset of expression in late embryogenesis [2]. Overexpression and an increase in Axl activity are evident in a number of chronic pathological conditions. Two ligands that activate TAM receptors are Gas6 (growth-arrest-specific protein 6) and Protein S [3]. Gas6 has the highest affinity for Axl among the TAM receptors and is often called the Gas6/Axl pathway. The Axl receptor regulates various functions, including survival, growth, aggregation, migration and anti-inflammation, in multiple cells. The Gas6/Axl pathway has been predominantly studied in cancer [4]; however, growing evidence supports a pathophysiological role for the Gas6/Axl pathway in chronic immune disorders [5]. Finally, activation of Axl is implicated in the progression of cardiovascular diseases [6]. The major aim of the present review is to summarize recent advances in Axl receptor biology. More attention is given to translational aspects of Axl-dependent signalling in three pathological conditions: cancer, chronic immune disorders and cardiovascular diseases.
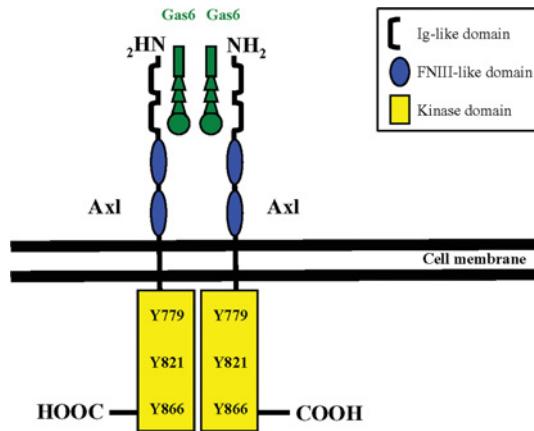
Figure 1   Axl receptor structure

The extracellular domain of Axl has two Ig-like (black bracket) and two FN type III-like (blue) domains. An intracellular kinase domain (yellow) contains autophosphorylation sites (Tyr^{779}, Tyr^{821} and Tyr^{866}). Gas6 (green) can activate Axl. A dimerization of two 1:1 Gas6–Axl complexes is required for signal transduction.

STRUCTURE AND REGULATION OF THE AXL RECEPTOR

Axl is a transmembrane receptor of molecular mass between 100 and 140 kDa that contains an extracellular (N-terminal) domain and an intracellular (C-terminal) tyrosine kinase domain (Figure 1). The extracellular domain of Axl has two Ig and two FN (fibronectin) type III motifs (Figure 1). The presence of Ig-like and FN type III extracellular domains differentiate Axl (along with Tyro3 and Mertk) into a TAM family of RTKs. Axl is a highly conserved gene across species (20 exons), but has two alternative variants due to a splicing site in exon 10 within the transmembrane domain [2,7,8]. RNA *in situ* hybridization analyses have shown the onset of Axl expression in late embryogenesis at day 12.5 post-coitum [2]. Three SNPs (single nucleotide polymorphisms) within introns 6 and 10 of the *Axl* gene were validated recently in humans [9]. Initial genomic studies on Axl showed that its promoter region is GC-rich and contains recognition sites for a variety of transcription factors, including Sp1 (specificity protein 1), AP2 (activating protein 2) and CREB (cAMP-response-element-binding protein) [10]. Studies in cancer cell lines have shown multiple transcriptional mechanisms leading to Axl expression [11–13]. As originally predicted, the expression of Axl is regulated by the Sp1/Sp3 transcription factors and methylation of CpG sites within specific Sp1 motifs which modulate *Axl* gene expression [11]. In addition, MZF1 (myeloid zinc finger 1), a SCAN domain family transcription factor, can bind to the *Axl* promoter and transactivate Axl expression, resulting in progression of colorectal and cervical tumour metastases [12]. Finally, the same group recently showed two specific miRs (microRNAs) that targeted the 3'-UTR
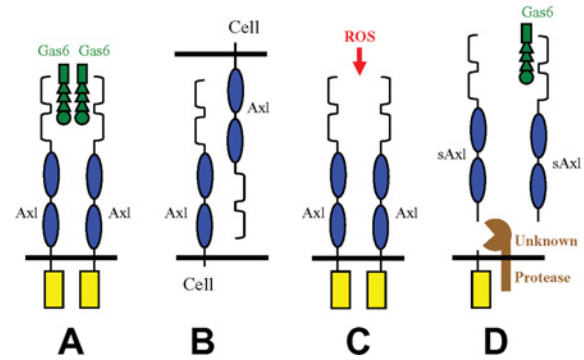

Figure 2   Mechanisms of Axl receptor activation/inactivation

(A) Ligand-induced activation of Axl by Gas6 (green). Initial formation of a high-affinity 1:1 Gas6–Axl complex is followed by dimerization of two 1:1 Gas6–Axl complexes. (B) Homophilic binding of extracellular domains of Axl expressed on neighbouring cells leads to aggregation, especially in regions of excess Axl. (C) Ligand-independent homophilic dimerization of Axl and autophosphorylation in response to ROS (red). (D) Proteolytic cleavage of Axl to form sAxl is by an unknown protease.

(untranslated region) of the *Axl* gene in several cancer lines [13]. Specifically, *miR-34a*, *miR-199a* and *miR-199b* can inhibit the expression and function of Axl in cancer. Taken together, Axl is a very unique RTK that can be induced via multiple molecular mechanisms.

ACTIVATION OF THE AXL RECEPTOR

Gas6 and Protein S are known ligands for the TAM receptor family [3,14]. However, Axl has the highest affinity for Gas6 compared with other members of TAM family, whereas Protein S predominantly binds Mertk and Tyro3 [15]. Both ligands are more than 40% similar in amino acid sequence and require a vitamin K-dependent γ-carboxylation of glutamate to Gla (γ-carboxyglutamate) for biological functions. Gas6 has four EGF (epidermal growth factor)-like repeats and a C-terminal SHBG (sex-hormone-binding globulin)-like domain, which includes two globular LG (laminin G-like) domains, in addition to the Gla domain [16,17]. Ligand-dependent activation of Axl is incompletely understood. Currently, binding of Gas6 to Axl is viewed as a two-step process that involves the initial formation of a high-affinity 1:1 Gas6–Axl complex, followed by dimerization of two 1:1 Gas6–Axl complexes (Figure 2A). A ligand–receptor 2:2 assembly with two Ig-like domains of Axl cross-linked by the LG domain of Gas6 has only been shown by crystal structure analyses of the Gas6–Axl complex [18]. It is likely that both Gas6-binding sites are necessary for Gas6/Axl signalling. In addition, a recombinant protein (Fc–Axl) that mimics the extracellular Ig-binding domain of Axl neutralizes Gas6 and prevents downstream signalling [15].

It has been proposed that the Axl homodimer can form heterodimers with Tyro3 or Mertk based on co-expression profiles of the TAM family [5]. No experimental data on heterodimerization across TAM receptors have been reported to date. The homophilic binding of extracellular domains of Axl expressed on neighbouring cells leads to aggregation (Figure 2B). This is a ligand-independent type of receptor activation that occurs with experimental overexpression of Axl [19]. The kinase domain of Axl is not required for cell aggregation, suggesting a distinctive mechanism as compared with the ligand-dependent activation. Finally, the TAM family is capable of ligand-independent homophilic dimerization and autophosphorylation of Axl (Figure 2C). For example, this type of auto-activation may occur after overexpression of Axl [20]. Our group has found that ROS (reactive oxygen species) promote the phosphorylation of Axl in VSMCs (vascular smooth muscle cells), which was independent of Gas6 [21]. Therefore ligand-independent activation of Axl is more typical during pathophysiological conditions with increases in oxidative stress and excess receptor expression.

Release of a soluble form of Axl (sAxl), the extracellular domain of Axl, represents another important feature of Axl receptor biology (Figure 2D). Formation of sAxl–Gas6 complexes limits ligand-dependent signalling, as described previously for cytokine and growth factor receptors. A specific proteinase that is responsible for proteolytic cleavage of sAxl has yet to be identified [22]; however, a metalloproteinase ADAM 17 (a disintegrin and metalloproteinase 17) could be a possible candidate, as it mediates the cleavage of the soluble form of Mertk in macrophages [23]. In addition, the range in the molecular mass of Axl between 100 and 140 kDa might relate to post-translational modifications (glycosylation, phosphorylation and ubiquitination) and these will be discussed below.

We have limited knowledge on the mechanisms of inactivation of Axl. The receptor tyrosine phosphatase C1-TEN (C1 domain-containing protein with homology to tensin) has been shown to bind Axl and affect Axl-dependent downstream signalling pathways in HEK (human embryonic kidney)-293 cells [24]. However, the authors were unable to show direct dephosphorylation of Axl by C1-TEN or increases in C1-TEN enzymatic activity. Endocytosis and lysosomal degradation are probable mechanisms of Axl deactivation upon Gas6 binding to Axl that involves the interaction of Axl with the ubiquitin ligase c-Cbl [25]. Importantly, ROS (H_2O_2) induced Axl tyrosine phosphorylation, but not its ubiquitination, suggesting that oxidative stress may inhibit Axl down-regulation. A proteolytic cleavage of sAxl is another possible mechanism of receptor inactivation (Figure 2D). Thus it is clear that more research should be undertaken to understand Axl receptor biology, especially in pathophysiology.

AXL RECEPTOR SIGNAL TRANSDUCTION

Typically, binding of Gas6 to the extracellular domain of Axl leads to dimerization of Gas6–Axl complexes (Figure 2A). The latter results in autophosphorylation of tyrosine residues on the intracellular tyrosine kinase domain of Axl (Figure 1). Autophosphorylation of the RTK may increase the phosphorylation activity by Axl substrates or the formation of signalling complexes with phosphotyrosine-binding domains. There are three known autophosphorylation sites (Tyr^{779}, Tyr^{821} and Tyr^{866}) on the intracellular domain of Axl (Figure 1). These residues are involved in binding Axl with subunits of PI3K (phosphoinositide 3-kinase), PLC (phospholipase C) and Grb2 (growth-factor-receptor-bound protein 2) [26,27]. In addition, Axl interacts with other signalling molecules, such as C1-TEN, Nck2 (NCK adaptor protein 2), RanBPM (Ran-binding protein in the microtubule-organizing centre) and SOCS1 (suppressor of cytokine signalling 1) [28]. Activation of PI3K and its downstream target, the serine/threonine protein kinase Akt, is a central step in Axl-dependent signal transduction (Figure 3). The Gas6/Axl/PI3K/Akt pathway protects cells from apoptosis via multiple mechanisms. In particular, Akt activates ribosomal protein S6K (S6 kinase) of the mTOR (mammalian target of rapamycin) pathway and phosphorylates Bad (Bcl2-associated agonist of cell death), a pro-apoptotic protein [29]. Akt inhibits the pro-apoptotic caspase 3 and phosphorylates NF-κB (nuclear factor κ-light-chain-enhancer of activated B-cells), which increases the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL [30]. In addition, Akt phosphorylates αIIbβ3 integrins, which are triggered by the Gas6/Axl pathway [31].

In some cell types, Axl activates the ERK (extracellular-signal-regulated kinase) pathway and contributes to proliferation (Figure 3). Stimulation of MAPKs (mitogen-activated protein kinases) [ERK, p38 and JNK (c-Jun N-terminal kinase)] by Axl is attributed, in part, to its ability to bind to the adapter protein Grb2 [26]. Stimulation of Axl also regulates Rho family GTPases and actin cytoskeletal re-organization in GRH (gonadotropin-releasing hormone)-stimulated neuronal cell migration [32]. Activation of p38 and phosphorylation of HSP25 (heat-shock protein 25), a regulator of actin remodelling, is downstream of Axl (Figure 3). Interestingly, the Gas6/Axl pathway can inhibit other growth factor signals, as has been shown for VEGFR2 (vascular endothelial growth factor receptor 2) in endothelial cell morphogenesis [33]. Activation of the tyrosine phosphatase SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2) is responsible for Gas6/Axl-mediated VEGFR2 inhibition (Figure 3). The Gas6/Axl pathway is an important inhibitory mechanism for TLR (Toll-like receptor) and cytokine receptor signalling in innate immune cells [34,35].
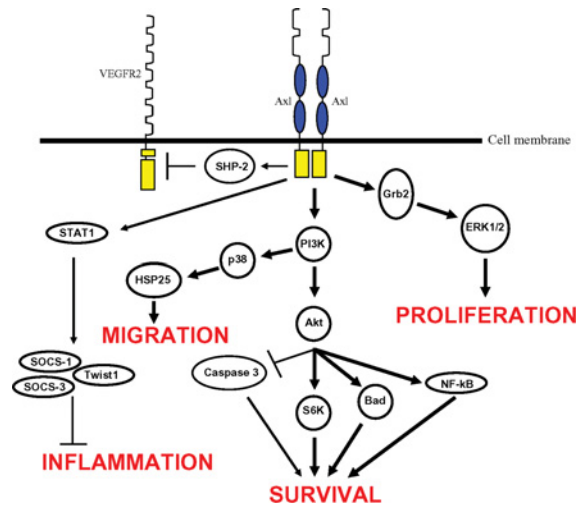
Figure 3   Axl receptor signal transduction

Axl controls cell survival, migration, proliferation and inflammation (red). Major downstream targets of Axl are shown in circles and ellipses. Thick arrows show the direction of signals, and thin lines show the inhibitory effects of Axl. Activation of SHP-2 is responsible for Gas6/Axl-mediated VEGFR2 inhibition.
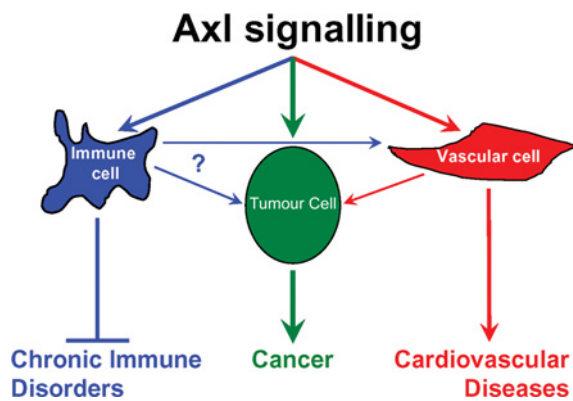

Figure 4   Axl signalling associates with pathological conditions

Axl is involved in immune cell functions that inhibit chronic immune activation (blue), and affects tumour cell function and promotes cancer (green). Activation of Axl is critical for advanced cardiovascular diseases (red). Thin arrows show potential effects of Axl signalling on cell–cell interactions during the three pathological conditions.

Specifically, Gas6/Axl co-activates the INFAR (IFNα/β-receptor)/STAT1 (signal transducer and activator of transcription 1) pathway, which increases the expression of suppressors of pro-inflammatory signals, such as Twist1, SOCS1 and SOCS3 (Figure 3). Taken together, Axl-dependent signalling is responsible for cell survival, aggregation, migration and growth. However, the specific pathophysiological response varies depending on the stimuli, cell type and binding partner.

Examples implicating Axl-dependent signalling in three pathological conditions are discussed in the subsequent sections (see Figure 4).

AXL-DEPENDENT SIGNALLING IN CANCER

It is logical to start the discussion of the role of Axl in pathological conditions by looking at oncology, as the receptor was cloned from human cancer cells [1]. A large body of data suggest the TAM family play an important role in cancer. Overexpression and activation of Axl protein, but not *Axl* gene mutations, are responsible for tumour growth in mesothelioma [36]. Tumour cell survival and growth, increased migration and angiogenesis are probable mechanisms by which Axl signalling regulates tumorigenesis [37]. Up-regulation of Axl has been documented in the vast majority of tumours and has been summarized recently [4]. For example, Axl is one of the most common RTKs detected in human breast cancer [38]. In fact, Axl expression correlates with metastasis and poor prognosis in breast cancer [39]. It was shown that oestrogen-induced Axl expression increased the survival of cells via the PI3K/Akt pathway in breast cancer [40]. A recent study has suggested that intermediate filament protein vimentin-dependent cell migration requires Axl in breast tumour formation [41]. In addition to a significant role in tumour growth, anti-apoptosis, migration and metastasis, Axl has also been implicated in angiogenesis [42]. Finally, Gas6/Axl signalling may also affect tumour–stromal cell interactions via changes in the immune response during tumorigenesis. Previous experiments have suggested that communications between multiple cell types, including vascular and immune cells, are required for Gas6/Axl-dependent immune responses [43]. Inhibition of Axl significantly reduced the expression of pro-inflammatory cytokines, which are important mediators of metastasis [44]. Thus the Gas6/Axl pathway increases cell survival, promotes proliferation, aggregation and migration, and is necessary for angiogenesis and immune cell activation in cancer.

AXL-DEPENDENT SIGNALLING IN CHRONIC IMMUNE DISORDERS

The Gas6/Axl pathway plays a crucial role in immune biology [5]. Specifically, TAM receptors protect innate immune cells [macrophages, dendritic cells and NK (natural killer) cells] from apoptosis and are involved in the phagocytosis of apoptotic bodies. Insights from triple TAM-knockout mice revealed the importance of the TAM family in the immune system [45]. Autoimmunity phenotypes in TAM-knockout mice suggest the protective role for the Axl receptor in chronic immune disorders, such as rheumatoid arthritis and systemic lupus erythematosus. Negative regulation of pro-inflammatory signals by TAM and phagocytosis in innate immune cells is the proposed mechanism in autoimmune disorders. In particular, activation of INFAR triggers the Gas6/Axl pathway that inhibits

TLR and cytokine receptor signalling via suppressors of pro-inflammatory signals, such as Twist1, SOCS-1 and SOCS-3, in innate immune cells [34,35]. It has been suggested that the autoimmune phenotypes are linked to the function of TAM in innate immune cells, e.g. macrophages, dendritic cells and NK cells. Previous findings have shown that Axl is undetectable in lymphocytes and granulocytes [7]. Recent experiments in Axl/Mertk-double-knockout mice showed a greater ability of antigen-presenting cells to drive Th1 responses via increases in pro-inflammatory cytokine levels [46]. However, recent findings in chronic lymphocytic leukaemia suggests that a phosphorylated form of Axl is derived from B-cells [47,48]. In addition, inhibition of Axl decreased the expression of pro-inflammatory cytokines in breast cancer [44]. These findings show the complexity of Axl-dependent signalling in homoeostasis of the immune system compared with pathological stimulation. Alternatively, up-regulation of other members of the TAM family (e.g. Mertk) and/or ligands (Protein S compared with Gas6) determine the functions of immune cell populations, as has been shown for Mertk-dependent phagocytosis [49]. A complex mechanism of interactions among multiple cell types was recently shown in the 26T model of colon cancer [50]. In particular, authors found a paracrine mechanism of tumour growth, which was driven by infiltrating leucocytes producing Gas6. Taken together, Axl-dependent signalling is involved in immunosuppressive pathways in innate immune cells; however, more studies are required to determine the effects of Axl on cell populations in chronic immune diseases (Figure 4).

AXL-DEPENDENT SIGNALLING IN CARDIOVASCULAR DISEASES

The Gas6/Axl pathway is equally important for the cardiovascular system, especially under pathological conditions (Figure 4). Late-onset up-regulation of Gas6 and Axl has been shown in rat carotid arteries after balloon injury [51]. Our findings in Axl-knockout mice further support a role of Axl in vascular remodelling [21,52]. The smooth muscle cell response to injury is complex and regulated through multiple autocrine growth mechanisms [6]. Induction of growth factors [including PDGF (platelet-derived growth factor), ET-1 (endothein-1), IL-6 (interleukin-6) and Gas6] and growth factor receptors [PDGFR (PDGF receptor) and Axl] mediates long-term pathophysiological adaptations (e.g. restenosis) in arteries. In fact, G-protein-coupled receptor agonists [AngII (angiotensin II) and thrombin] increased Axl expression in VSMCs *in vitro* [51]. Mechanistic studies showed that Gas6/Axl activates PI3K and Akt, which protect VSMCs from apoptosis [53]. Similar anti-apoptotic mechanisms have

been described for Axl in endothelial cells [30,54]. It is important to note that Axl signalling may direct not only VSMC survival, but also migration depending upon the cellular microenvironment. Specifically, the lower-molecular-mass isoform of Axl (114 kDa) activates the PI3K/Akt/mTOR pathway and survival in low glucose, whereas the higher-molecular-mass isoform of Axl (140 kDa) leads to increased ERK1/2-mediated migration in high-glucose conditions [55]. The Gas6/Axl pathways not only increase survival, but also protect VSMCs from calcium deposition *in vitro* [56]. However, we observed no evidence of calcification in arteries with increased apoptosis in Axl$^{-/-}$ mice (J. Gerloff and V.A. Korshunov, unpublished work). Activation of Axl is regulated by oxidative stress in VSMCs [21], which could be a common pathophysiological mechanism in all of the pathologies discussed. We recently found a novel post-translational redox modification that leads to Axl-dependent migration of VSMCs [57]. Glutathiolation (a reaction of glutathione with cysteine residues) of non-muscle MHC (myosin heavy chain)-IIB interacts with Axl in response to ROS and increases migration during vascular remodelling. In fact, vascular oxidative stress and vasoreactivity were improved in Axl$^{-/-}$ mice compared with Axl wild-type littermates in a mouse model of hypertension [58]. It is also possible that Axl controls phagocytosis which, in combination with pro-survival effects, determines vascular remodelling [52]. Finally, *Gas6* gene polymorphisms are associated with stroke and acute coronary syndrome in humans [59–61]. The Gas6/Axl pathway is shown to regulate thrombosis, which is crucial for cardiovascular events. In particular, Gas6/TAM transgenic mice were protected from experimental thrombosis, and Fc–Axl treatment protected wild-type mice against fatal thromboembolism [31]. Plasma concentrations of Gas6 and sAxl proteins correlated with large abdominal aortic aneurysms in humans [62]. Not surprisingly (as noted in cancer), a weak causal relationship has recently been reported between *Gas6* or *Axl* gene mutations and human atherosclerosis [9]. Further exploration of the Gas6/Axl pathway with respect to cell origin (vascular, blood and immune) will be important for clinical studies in patients with cardiovascular diseases.

In summary, the Gas6/Axl pathway is critical for the progression of cardiovascular diseases, via the regulation of survival, proliferation and migration of vascular cells, and various functions of circulating blood cells.

DIAGNOSTIC AND THERAPEUTIC POTENTIAL OF INHIBITION OF THE AXL RECEPTOR

The Gas6/Axl pathway is a plausible target for diagnostic test development given its role in an array of chronic disorders. Detection of Gas6, the Axl ligand,

is challenging due to low concentrations of Gas6 in human plasma [63]. However, measurements of sAxl could be an alternative diagnostic strategy for evaluation of the Gas6/Axl pathway (Figure 2D). In fact, Gas6 circulates in a complex with sAxl in human plasma [64]. It was proposed that sAxl acts as a decoy for Gas6 systemically and the Gas6/sAxl balance shifted towards Gas6 in chronic inflammatory and vascular diseases [62,65]. Although these studies are encouraging, systemic compared with local activity of the Gas6/Axl pathway remains an open question, especially in a cell- and disease-specific manner.

Despite the limitations of detection of abnormal Axl signalling in humans, we have a number of established pre-clinical approaches to inhibit the Axl receptor (Figure 2). First, a decrease in Gas6/Axl binding can be achieved in two ways. Decreased activity of Gas6 can occur by the well-known anticoagulant warfarin, which inhibits vitamin K-dependent γ-carboxylation of Gas6 [66], although the narrow therapeutic index and low specificity of warfarin clearly limits such an approach. Alternatively, administration of the recombinant protein Fc–Axl blocks the interaction of Gas6 with Axl [15]. One report has shown that Fc–Axl significantly protected mice against pulmonary thrombosis [31]. Secondly, targeting the Axl receptor with inhibitory antibodies can prevent downstream signalling. Two reports have shown that anti-Axl antibodies affected not only tumour cells, but also modulated tumour-associated vasculature and immune cell functions [67,68]. The anti-Axl antibodies are highly specific, but have limitations due to poor pharmacokinetics in humans. Thirdly, utilization of Axl-specific SMIs (small-molecule inhibitors) is currently the best therapeutic strategy. Again, the majority of reports on Axl inhibition by SMIs have been shown in cancer [4]. Among several inhibitors, R428 (Rigel Pharmaceuticals) was effective in multiple animal models of tumorigenesis, and also has favourable pharmacokinetic profiles and specificity to Axl [44]. We recently found that R428 strongly inhibited Axl signalling in VSMCs [69]. In addition, we have observed that R428 was more effective than Fc–Axl in ligand-independent activation of Axl in response to oxidative stress in VSMCs. One possibility could be a post-translational redox modification that leads to Axl signal transduction in VSMCs [57]. Chronic administration of R428 also promoted adipocyte hypotrophy, and enhanced macrophage infiltration and apoptosis in a murine obesity model [70]. These findings suggest that R428 is an effective and selective compound that inhibits Axl signalling in multiple cell types. All possible Axl inhibition approaches need to be carefully evaluated in humans. For example, the specificity of Axl inhibitors to other members of the TAM receptor family may lead to harmful effects on immunity, as has been shown in TAM-knockout mice [45]. Thus recent progress in translational aspects of Axl signalling should be evaluated further in a wider spectrum of chronic diseases.

CONCLUSIONS

The Gas6/Axl pathway is highly regulated in chronic pathological conditions. Axl is a very unique RTK that can be induced via several molecular mechanisms. Axl-dependent signalling is responsible for cell survival, aggregation, migration and growth through multiple downstream pathways. Axl signalling has mostly been implicated in cancer and, naturally, translational advances of Gas6/Axl (diagnostics and drug development) are seen in cancer. However, a rapid increase in publications supports the significance of Axl in other chronic pathological conditions (immune and cardiovascular diseases). It appears that overexpression of the Axl receptor in cardiovascular diseases dramatically affects signalling as compared with normal physiology. Axl-dependent signals are important for physiological homoeostasis of the immune system (Figure 4). Despite these discrepancies, targeting of Axl is beneficial in advanced stages of cancer and more effective in overcoming chemoresistance [71]. Current challenges in Axl biology are related to the functional interaction of the receptor with other members of the TAM family or other tyrosine kinases, mechanisms of ligand-independent activation, inactivation of the Axl receptor and cell–cell interactions (with respect to immune cells) in chronic diseases.

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