Expression of the vitamin K-dependent proteins GAS6 and protein S and the TAM receptor tyrosine kinases in human atherosclerotic carotid plaques

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Summary

The GAS6/ProS-TAM system is composed of two vitamin K-dependent ligands (GAS6 and protein S) and their three protein tyrosine kinase receptors TYRO3, AXL and MERTK, known as the TAM receptors. The system plays a prominent role in conditions of injury, inflammation and repair. In murine models of atherosclerotic plaque formation, mutations in its components affect atherosclerosis severity. Here we used Taqman low-density arrays and immunoblotting to study mRNA and protein expression of GAS6, ProS and the TAM receptors in human carotid arteries with different degrees of atherosclerosis. The results show a clear down-regulation of the expression of AXL in atheroma plaques with respect to normal carotids that is matched by decreased abundance of AXL in protein extracts detected by immunoblotting. A similar decrease was observed in PROS1 mRNA expression in atherosclerotic carotids compared to the normal ones, but in this case protein S (ProS) was clearly increased in protein extracts of carotid arteries with increasing grade of atherosclerosis, suggesting that ProS is carried into the plaque. MERTK was also increased in atherosclerotic carotid arteries with respect to the normal ones, suggesting that the ProS-MERTK axis is functional in advanced human atherosclerotic plaques. MERTK was expressed in macrophages, frequently in association with ProS, while ProS was abundant also in the necrotic core. Our data suggest that the ProS-MERTK ligand-receptor pair was active in advanced stages of atherosclerosis, while AXL signalling is probably down-regulated.

Keywords

PROS1, GAS6, TYRO3, AXL, MERTK, arterioesclerosis, atheroma, carotid plaque, vitamin K-dependent proteins, receptor tyrosine kinases

Introduction

Protein S (ProS) and GAS6 are plasma glycoproteins belonging to the family of vitamin K-dependent proteins (VKDPs). The specific characteristic of VKDPs is the presence of post-transcriptionally modified γ-carboxyglutamic residues (Gla) essential for their interaction with calcium. Plasma VKDPs interact through the Gla-containing (GLA) domain with negatively-charged phospholipids in activated cellular membranes like those in platelets and endothelial cells after a vascular lesion (1). ProS and GAS6 are also able to interact with membrane receptors of the TAM family (Tyro3, Axl and MerTK), through their carboxy-terminal LamG domains, activating tyrosine kinase-initiated signalling pathways (2, 3). ProS is a VKDP with anticoagulant properties due to interactions with components of the coagulation cascade, including activated protein C (PCa) and tissue factor pathway inhibitor (TFPI) (4, 5). GAS6 was identified as the product of a gene turned on by serum deprivation of NIH3T3 fibroblasts. In 1995, GAS6 and ProS were described as ligands of the formerly orphan TAM receptors (6, 7). The specificity of the ligands for each TAM receptor and their relative affinity has been matter of debate (8). It is generally agreed that there is an important level of functional redundancy of ligands and receptors, especially in light of the phenotypes developed by the different knockout mouse models of the genes in the system (3, 9, 10). Interestingly, while even triple

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Tyro3/Axl/MerTK knockout mice survive, Prosl deletion in mice is lethal, emphasizing its specific anticoagulant function (11–13). GAS6 (GeneID: 2621) and PROSI (GeneID: 5627) are paralog genes encoding two proteins with a shared mosaic structure. ProS and GAS6 are found in plasma, but ProS is over 1,000-fold more abundant than GAS6. ProS concentration in plasma is around 30 μg/ml, while GAS6 has a normal range of 20–50 ng/ml in most studies (14). TAM receptor protein tyrosine kinases (RTKs) appear in chordates (3). The extracellular N-terminal domain consists of two modules similar to immunoglobulin, followed by two fibronectin-type III domains in tandem and a transmembrane domain. As the rest of RTKs, TAM receptors are involved in the homeostasis of differentiated cells, tissues and organs. TAM genes are expressed from the later stages of embryo development to adulthood. Studies in cell cultures have shown an effect of GAS6 and TAM activation in processes like vascular smooth muscle survival, division and migration (15, 16) or endothelial cell activation by pro-inflammatory cytokines and survival (17, 18).

The implication of the GAS6/ProS-TAM system in atherosclerosis is illustrated by studies using animal models. GAS6 and Axl expression increase after mechanical damage of the vessel wall (19, 20). Inversely, Axl−/− or Gas6−/− mice show decreased neointima formation and less severe restenosis (21). Other studies have shown a role of different components of the system in platelet function, inflammation and efferocytosis of apoptotic cells (3, 22–24), all of them implicated in atheroma development (25, 26). Recently, direct evidence of the effect of this system on mouse atherosclerosis has been available after the description of plaque development in Gas6 and MerTK knockout mice crossed with ApoE−/− and/or LDLr−/− strains. The effects of GAS6 and MerTK deficiencies were strikingly different. Loss of GAS6 increased fibrosis and attenuated inflammation in the plaque, while the number of apoptotic cells was not affected (27). In contrast, loss of MerTK increased the number of apoptotic cells in the plaque, probably due to decreased efferocytosis (28, 29). This effect was due to bone marrow-derived cells, as irradiated LDLr−/− mice transplanted with bone marrows from MerTK−/− mice showed an increase in apoptotic cell content in the plaque, compared to those transplanted with wild type-derived bone marrow cells, with a steeped up progression of atherosclerotic lesions and an exacerbated immune response (29). These results underscore the role of the GAS6/ProS-TAM system in plaque development, but also highlight the specific role of each gene, probably affecting different aspects of the process (26).

Another evidence of the implication of this pathway in human atherosclerosis is found in case-control genetic studies of human populations, which have shown an association of polymorphisms in TYRO3 and MERTK with carotid atherosclerosis, as risk and protective factors, respectively (30). A polymorphism and a haplotype in GAS6 are less frequent in vascular disease (31–33), although they do not seem to be associated with the presence of atherosclerosis (30). The goal of the present study was to determine and analyse quantitatively ProS, GAS6 and their TAM receptors in human carotid arteries with different degrees of atherosclerosis.

**Material and methods**

**Characteristics of the analysed samples**

For gene expression studies, 18 samples of atheroma plaque from non-related patients submitted to programmed endarterectomy were used. Samples were processed for mRNA, protein extraction and histology. Most of the patients had suffered from cerebral-vascular accident previous to endarterectomy. From all samples, stenosis grade (34), presence of bilateral pathology, morphology (35), stability, and angiogenesis inside the plaque were recorded. The type of stroke suffered according to the Oxfordshire classification (36) was confirmed by nuclear magnetic resonance (NMR). Control samples were nine carotid arteries from donors of vascular transplants or post-mortem autopsies, with a similar age of the endarterectomy group and without delay in processing according to a standardised biobank protocol. These arteries had lesions of grades I or II. Samples were obtained with full ethical approval from the local committee.

**RNA extraction, RNA quality assays and reverse transcription**

Cryogenic conditions were used for mRNA extraction, which was performed following the protocol described for the commercial reactive Tripure® (Roche Applied Science). Quantity and integrity/purity of RNA samples was measured by capillary electrophoresis into micorfluidic chips (RNA 6000 Nano Series II kit, Agilent®), using an Agilent 2100 Bioanalyzer (Agilent®) and following the manufacturer’s recommendations. A cut-off of 7 in RNA integrity number (RIN) was used as a measure of RNA quality. RIN uses the entire electrophoretic trace to define RNA quality, as calculated by the 2100 Expert Software (Agilent®). RNA reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), using 500 ng of total RNA in each reaction and following the protocol described in the kit.

**mRNA expression studies**

Relative quantification of mRNA expression for GAS6, PROSI, TYRO3, AXL and MERTK, together with other genes involved in the atherosclerotic process (see ►Suppl. Table 1 available online at www.thrombosis-online.com), was performed by real-time PCR using TaqMan® Low Density Arrays or TLDAs (Applied Biosystems). In the design of the card, special care was taken in selecting assays specific for cDNA. For genes with more than one isoform described, the selected assays were common to all isoforms (see ►Suppl. Table 1 available online at www.thrombosis-online.com).

Two hundred fifty nanograms of cDNA from eight samples were analysed in each card and all samples were analysed by duplicate using the cDNA from two independent reverse transcriptions.
Amplification was performed according to the manufacturer’s instructions in an ABI 7900HT (Applied Biosystems). Ct values were determined automatically by the SDS v2.2.2 software (Applied Biosystems) and analysed using the qBASE® software (37). The geometric mean of GAPDH and PPIA endogenous expression was used for the normalisation of the expression.

**Western blot analysis**

Specimens from human carotids with different plaque stages were analysed. When possible, but not always, the same protein extract was used for all antigens. A tissue sample of 1 to 5 mg from carotid arteries was used for protein extraction. The samples were homogenised in a mortar pestle under cryogenic conditions and proteins extracted using a lysis buffer (50 mM Tris-HCl pH 8.0; 0.5% SDS, 1 mM DTT) supplemented with a mixture of protease and phosphatase inhibitors (PhosSTOP and Complete Protease Inhibitor Cocktail, Roche Applied Science). The homogenate was incubated in ice for 30 minutes (min) and centrifuged at 13,000 g 15 min at 4°C, collecting the supernatant. Total protein was quantified using a selective colorimetric detection by bicinchoninic acid commercial kit (BCA Protein Assay Reagent Kit, Pierce) and samples were boiled in Laemmlı Buffer (70% (v/v) 0.5M Tris-HCl-0.4% (w/v) sodium dodecyl sulphate pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 9.3% (w/v) dithiothreitol, 0.012% (w/v) bromophenol blue and 10% (v/v) β-mercaptoethanol) at 98°C for 10 min.

Western blot analyses were performed following a standard protocol. Briefly, 30 μg of total protein extract were analysed by 10% SDS-PAGE under reducing conditions before transfer to a nitrocellulose membrane. For ProS, GAS6 and AXL, control protein samples were also loaded in each electrophoresis. These consisted of 25 ng of commercially purified human plasma ProS (Enzyme Research Laboratories), 30 μg of total protein extract from human umbilical vein endothelial cells (HUVECs) that express AXL and 25 μl of conditioned media from cells expressing recombinant human GAS6. Membranes were blocked for 1 hour (h) at room temperature with 5% (w/v) of skimmed milk in TBS-T (50 mM Tris-Cl, 100 mM NaCl, 0.1% Tween-20, pH 7.4). They were incubated overnight at 4°C with the following antibodies: rabbit polyclonal anti-ProS HRP conjugated (P0419, Dako); rabbit polyclonal anti-GAS6 (R&D systems); goat polyclonal anti-AXL (Sc-1096, Santa Cruz); mouse monoclonal anti-MERTK (MAB8912, R&D Systems).

![Figure 1: Relative mRNA expression of the genes of the GAS6/ProS-TAM system. A) Relative expression of PROS1 and GAS6 both in carotids without plaque (grade I-II; n=9) and with atherosclerotic plaque (grades IV-VI; n=18). B) Relative expression of TAM genes both in carotids without (grade I-II) and with plaque (grade IV-VI). C) Relative expression of PROS1 and GAS6 in samples with plaque grade IV (n=7); grade V (n=6) and grade VI (n=5). D) Relative expression of TAM genes according to carotid plaque grade. Asterisks *, ** and *** indicate a Mann-Whitney test result with p-values<0.05, p <0.005 and p <0.001, respectively.](image-url)
systems) and mouse monoclonal anti-tubulin (ab56676, Abcam). All membranes were rinsed three times with TBS-T and incubated with proper secondary antibodies conjugated with HPR. Protein detection was done using the ECL Advance Western Blotting Detection System (Amersham Biosciences), following the recommendations of the manufacturer. Membranes were exposed to a film at different time intervals and quantified in a densitometer GS800 (Bio-Rad) using the Quantity One software (Bio-Rad).

Immunohistochemical staining

Carotid plaque specimens were formalin-fixed and briefly decalcified to remove excess calcium with 1% nitric acid in distilled water during 2 h, followed by acid neutralisation of the acid by sodium sulfate for 12 h before embedding in paraffin. For histological analysis of plaque phenotype, cross-sections (4 μm thick) were cut and mounted on polylysine-coated glass slides. To analyse plaque morphology, the carotid sections were stained with hematoxylin and eosin. Each section was classified according to the degree of atherosclerosis using the American Heart Association (AHA) criteria.

Paraffin sections were deparaaffinised in xylene and rehydrated through a series of graded alcohols. For antigen retrieval, rehydrated paraffin sections were boiled for 5 min in 10 mM citrate buffer at pH 6.0 and incubated with 0.1% of sodium borohydure in 100 mM phosphate-buffered saline (PBS) for 30 min at room temperature under soft shaking, to reduce endogenous fluorescence. Sections were incubated 1 h at room temperature with normal goat serum blocking solution (2% goat serum, 1% BSA, 0.1% cold fish skin gelatine, 0.1% Triton X-100, 0.05% Tween-20 and 0.05% sodium azide in PBS at pH 7.2). Sections were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-human MERTK (MAB8912, R&D Systems, final concentration 10 μg/ml), rabbit polyclonal anti-human ProS (HPA007724, Atlas Antibodies, final concentration 30 μg/ml), goat polyclonal anti-human CD68 (sc-7083, Santa Cruz, final concentration 2 μg/ml), polyclonal antibody against cleaved caspase-3 (#9661, Cell Signalling Technologies, 1:200). Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was carried out following the manufacturer’s recommendations (Roche Applied Science). The 3,3’-diaminobenzidine (DAB) immunostaining of ProS and MERTK was performed following the protocol described for Luque et al., using the antibodies indicated above (39). For immunofluorescence, primary antibodies were detected by incubating the sections with Alexa-conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature. The slides were washed three times for 3 min in 0.05% of Tween-20 in

Figure 2: Immunoblot analysis of ProS, GAS6, AXL and MERTK in total protein extracts from carotid arteries without and with atheroma plaque. Representative Western blot analysis of each protein is shown with the α-tubulin control of each immunoblot. A) ProS Western blot analysis of 30 μg of total protein extract of each sample were compared to 25 ng of purified human plasma ProS as positive control (C+). B) GAS6 Western blot using as positive control 25 μl of conditioned media from recombinant human GAS6-expressing HEK cells. C) AXL Western blot of samples compared to 30 μg of total protein extract from HUVECs. D) Western blot analysis of MERTK. The proteolyzed soluble isoform is indicated by an arrow.
PBS, and cell nuclei were counterstained with DAPI (Roche Applied Science). The slides were mounted using fluorescent mounting medium (DAKO), examined under a Leica CRT 4000 fluorescent microscope equipped with appropriate filters, and photographed with a CCD camera (DFC 300FXR2, Leica).

To check the specificity of the antibodies used in the immunofluorescent assays, several negative control were performed. First, the tissue was incubated with antibody diluent, without the primary antibody included and followed by incubation with secondary antibodies. Second, a preparation was incubated with antibody diluent, supplemented with a non-immune immunoglobulin of the same isotype and concentration as the primary antibody (30 μg/ml) and then was incubated with the secondary antibody. Third, the anti-human ProS antibody was first pre-incubated overnight at 4°C in a soft orbital shaker with the immunogen in a molar ratio of 1:10 antibody-immunogen, respectively. The pre-absorbed antibody was then incubated with the sample in place of the primary antibody alone. All negative controls, together with a positive control using the specific antibody, were performed in serial sections of tissue placed in the same slide, for triplicate. All pictures were acquired the same day and their settings (exposure time, contrast and colour balance) were exactly the same for all pictures (see Suppl./L50480 Fig. 1 available online at www.thrombosis-online.com).

Figure 3: Densitometric analysis of GAS6, ProS, AXL and MERTK immunoblots. Densitometric values of each protein detected in Western blot analysis (Fig. 2) were normalised with the corresponding values of the loading control (Tubulin). At least two Western blots were performed for each protein, assaying 8–11 samples from carotids without atherosclerosis (grade I and II) and 12–18 carotids with advanced atherosclerosis. The densitometric values were referred to the median of the relative values obtained for all samples without atheroma plaque in each protein (grade I-II). *, ** and ***, Mann-Whitney test p-values<0.05, p<0.005 and p<0.001, respectively.
Statistical analysis

All statistical analyses were performed using the STATA and SPSS packages. To determine the differences in relative gene expression between groups of samples we performed non-parametric tests (Mann-Whitney-Wilcoxon or Kruskal-Wallis, depending on the number of samples to analyse). Pearson’s correlation test ($\rho$) was used to determine the degree of linear relationship of the mRNA relative expression between pairs of genes analysed. Mann-Whitney-Wilcoxon and Kruskal-Wallis, were also used in the analysis of the densitometric data of the levels of proteins detected in the western blot analysis.

Results

mRNA expression studies

Analyses were performed using 18 samples from endarterectomised carotid arteries with different degrees of atherosclerotic plaque, diagnosed by their histological features (grades IV to VI). These samples were compared to nine control samples of post-mortem or vascular transplants with initial or no atherosclerotic lesions (grades I or II according to the AHA classification). $\text{PROS1}$ mRNA decreased significantly in samples with atheroma plaque to around 50% of the control samples, and the decrease followed the severity of the lesion, being maximal in grade VI lesions (Fig. 1A and C). We did not observe differences in the expression of $\text{GAS6}$ mRNA (Fig. 1A and C).

$\text{AXL}$ and $\text{TYRO3}$ also showed less expression in atheroma plaque compared to normal carotids (Fig. 1B and D). Although the down-regulation in both genes was statistically significant, it was more marked for $\text{AXL}$ (approximately a 60% decrease of the control samples). Reduced expression was maintained in all plaque phenotypes without differences among types of plaque, although in $\text{AXL}$ there was a significant trend towards lower level with plaque grade (Fig. 1D). In contrast, $\text{MERTK}$ was significantly overexpressed in grade IV plaques, returning to normal values in grades V and VI. Interestingly, $\text{AXL}$ mRNA showed a strong linear correlation with the expression of both ligands, $\text{GAS6}$ and $\text{PROS1}$ ($\text{AXL}$ vs. $\text{GAS6}$ $r=0.577$; p-value<0.01 and $\text{AXL}$ vs. $\text{PROS1}$ $r=0.837$; p-value<0.001) in samples with atherosclerosis, despite the small difference in $\text{GAS6}$ expression among groups (see Suppl. Table 2 available online at www.thrombosis-online.com), while $\text{MERTK}$ did not correlate with the ligands.

In general, the rest of the genes studied in the array gave consistent results with those reported the literature (see Suppl. Table 2 available online at www.thrombosis-online.com). Thus, mRNA levels of the pro-inflammatory cytokines interleukin ($\text{IL}$)-1$\beta$ and monocyte chemoattractant protein 1 ($\text{CCL2}$) increased considerably (40), although we did not observe a significant increase in IL-6 expression in the plaque (41). The intracellular transcription factors hypoxia-inducible factor ($\text{HIF}$)-1$\alpha$ showed a significant increase (39, 42), although it was the opposite in the case of $\text{HIF}$-2$\alpha$ ($\text{EPAS1}$), which decreased especially in grade VI plaques (40% of the expression in control arteries). The gene coding for the metalloprotease involved in $\text{AXL}$ and $\text{MERTK}$ shedding, $\text{ADAM10}$ (43), showed a minor increase, lower than the observed for other metalloproteases (44, 45). In agreement with the common finding of in-
increased cell death and caspase-3 overexpression in atherosclerosis (46), caspase-3 mRNA expression was double in plaque-containing arteries than in controls.

**Analysis of protein expression by Western blot**

In order to compare the data on mRNA expression with protein products, we studied protein expression by western blots on total protein extracts of a subset of samples. All proteins analysed, either from normal carotids or from carotid plaque extracts, were revealed at the expected molecular weight. ProS analysis showed a clear increase in protein content in the plaque (Fig. 2A), contrary to the reduction observed in PROSI mRNA (Fig. 1). It is interesting to note that ProS increased with atherosclerosis grade, being more than 10 times higher in grade VI carotid plaques than in control samples (Fig. 3). The electrophoretic pattern observed was the same as the plasma purified ProS, which consist of two bands around 75 kDa. In contrast, GAS6 protein followed the mRNA expression, showing similar values in control and in plaque samples with an electrophoretic pattern similar to the previously described, which consist of two bands around 70 kDa (Fig. 2B).

Next, we studied protein expression of TAM receptors by Western blot in the same group of samples. AXL was detected as a double band around 140 kDa, slightly below the one observed for control AXL in cultured HUVECs (Fig. 2C). In agreement with the results obtained for AXL mRNA, the protein was also significantly and increasingly reduced in the plaques, according to their grade (Fig. 3). MERTK was revealed as a double band of molecular weights 160 kDa and 100 kDa, approximately (Fig. 2D). These correspond with the full-length and soluble processed forms, as has been previously described (45). The levels of the full-length protein expression in carotid plaque increased three times (1 ± 0.19 in controls vs. 3.08 ± 0.7 in the plaque group, p<0.005; Fig. 3D). We were not able to detect TYRO3 in protein extracts from carotid arteries, while it was easily detectable in control samples from brain-derived cell lines.

**Immunohistochemical localisation of ProS and MERTK**

Given the increased expression of ProS and MERTK in the plaques, we studied their histological localisation in relation to macrophage and cell death markers. As it was previously reported in human atherosclerotic coronary lesions (38), ProS was localised in both the endothelium of carotid arteries (not shown) and vessels of the intima, and the extracellular sub-endothelial intima (Fig. 4), while it was not observed in carotids without advanced atherosclerosis (not shown). In plaques with high grade of lipid infiltration, we also observed a strong staining of ProS into the lipid core (Figs. 4A, 5A), surrounded by a strong staining for cellular death, as assayed by TUNEL (Fig. 5B). Double staining of ProS with the macrophage specific marker CD68 was observed in some cells of the intima and close to the necrotic core of the plaques (Fig. 5C).

In contrast to ProS, MERTK immunostaining was not positive in the lipid core, but it was found in perivascular cells of the intima and surrounding the necrotic core (Fig. 4B). In a majority of cases, MERTK and CD68 co-localised (Fig. 5D), suggesting that most MERTK-expressing cells are also CD68 positive. To test if these macrophages could be phagocytosing apoptotic cells in the core of the plaque, we performed staining using MERTK and both TUNEL and cleaved caspase-3, which are markers of cellular death and apoptosis, respectively. Some cells were co-stained with MERTK and TUNEL (not shown) and most cells were double stained with MERTK and cleaved caspase-3 (Fig. 5E). When we stained for MERTK and ProS, some of the MERTK positive cells were also positive for ProS immunostaining (Fig. 5F).

**Discussion**

As summarised in the introduction, different lines of research have demonstrated an implication of the GAS6/ProS-TAM system in atherosclerotic plaque progression (26). In the present study, we did not observe differences in mRNA of GAS6 nor in GAS6 protein associated with atherosclerosis. At the same time AXL and TYRO3, which could be the receptor of GAS6 in the context of the vessel wall (20, 47, 48), are down-regulated in advanced human carotid plaques. In animal models, GAS6 expression is increased after mechanical vascular damage (19, 21, 27) and the absence of Gas6 in mice promotes the development of more stable atheroma plaques in ApoE-/- mice, increasing fibrosis and attenuating inflammation (15, 49).

In our study, the only TAM receptor whose expression was increased in human carotid plaques was MERTK. MERTK function has been implicated in efficient apoptotic cell removal (efferocytosis), as illustrated by the effect of genetic deficiencies in MERTK, which are associated with retinitis pigmentosa (2). Merk-deficient mice show a severe lymphoproliferative disorder accompanied by broad-spectrum autoimmunity, associated with a decreased efficiency in apoptotic cell clearance (9, 10). Mouse models of atherosclerosis development have shown that Mertk is implicated in efferocytosis, which in turns diminishes the inflammatory response in the atheroma plaque (28, 29). In this context, the increase in MERTK in the initial stages of atheroma formation could be involved in the efficient clearance of apoptotic cells. In animal models, the VKDP ligand of MERTK in the plaque was not determined. With the present data, it is tempting to consider that ProS could be the main candidate. ProS has been suggested to be a crucial MERTK ligand in retinal (50) and macrophages (51). Interestingly, ProS accumulated significantly in the atheroma carotid plaques analysed, according to previous observations (38, 52), but PROSI mRNA decreased, indicating that this is not due to local production of ProS. Since ProS is an abundant plasmatic protein, it is probable that ProS detected in the plaque is derived from plasma. Localisation of ProS in the plaque is mainly extracellular, with an
Figure 5: Fluorescence immunostaining of ProS, MERTK, CD68, cleaved caspase-3 and TUNEL in advanced carotid plaques. A) Nuclear DAPI staining of the atheroma carotid plaques at low magnification. Yellow circles indicate the areas magnified in B to F. M: media; LC: Lipid core; FC: fibrous cap; L: lumen. B) Double immunostaining for ProS and TUNEL. ProS was localised in lipid core surrounding, but not co-localising, with a positive cell staining for cellular death (TUNEL). C) Double immunostaining for ProS and CD68, as a marker of macrophages. A double positive staining in some cells in areas close to the necrotic core was observed and it is indicated by a white arrow; empty arrows indicate some cells only stained with CD68. D) Double immunostaining for MERTK and CD68. As in ProS-CD68 immunostaining, a double positive pattern for several cells (white arrows) was observed both in necrotic core and in areas next to it. E) Immunostaining for MERTK and cleaved caspase-3. A large proportion of the cells in the inflammatory infiltrate were positive for MERTK, with co-localisation with cleaved caspase-3 in most of them (white arrows). F) Double immunostaining for ProS and MERTK. A double-positive staining in some cells was observed next to the necrotic core and is indicated by white arrows while the cells with a simple MERTK staining are indicated by empty arrows.
What is known about this topic?

- The TAM system of VKDP ligands/receptor tyrosine kinases is implicated in atherosclerosis.
- Mertk<sup>a</sup> and Gas6<sup>c</sup> mice show altered plaque development in high cholesterol diets.
- Genetic association studies have suggested an implication of GAS6 and TAM receptors in human vascular disease.

What does this paper add?

- This report describes the expression of components of the GAS6/ProS-TAM system in human carotid plaques.
- AXL and Tyro3 expression are downregulated in atherosclerotic carotids.
- MERTK is overexpressed in the plaque and ProS is abundant in the plaque but it is not produced locally.
- The results support the functionality of the ProS-MERTK interaction in advanced atherosclerosis.

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important staining in the necrotic core of developed plaques. This could be caused by interaction of ProS with negatively charged phospholipid membranes of dying cells or by interactions with lipoproteins. Interestingly, ProS is able to inhibit acetylated LDL uptake by macrophages through its interaction with MERTK and down-regulation of scavenger receptor A, while GAS6 is a much less potent inhibitor (52). Therefore, in advanced plaques, ProS could have a dual role of inhibiting LDL uptake by macrophages, while favouring the uptake of death cells. In any case, we cannot rule out a possible additional role of GAS6, as we could detect its presence in all samples analysed and its mRNA expression did not decrease, as is the case of AXL.

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References


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