Axl/Phosphatidylinositol 3-Kinase Signaling Inhibits Mineral Deposition by Vascular Smooth Muscle Cells

Georgina D.M. Collett,* Andrew P. Sage,* John Paul Kirton, M. Yvonne Alexander, Andrew P. Gilmore, Ann E. Canfield

Abstract—The calcification of blood vessels correlates with increased morbidity and mortality in patients with atherosclerosis, diabetes, and end-stage kidney disease. The receptor tyrosine kinase Axl is emerging as an important regulator of adult mammalian physiology and pathology. This study tests the hypothesis that Axl prevents the deposition of a calcified matrix by vascular smooth muscle cells (VSMCs) and that this occurs via the phosphatidylinositol 3-kinase (PI3K) signaling pathway. First, we demonstrate that Axl is expressed and phosphorylated in confluent VSMCs and that its expression is markedly downregulated as these cells calcify their matrix. Second, we demonstrate that overexpression of wild-type Axl, using recombinant adenoviruses, enhances Axl phosphorylation and downstream signaling via PI3K and Akt. Furthermore, overexpression of Axl significantly inhibits mineral deposition by VSMCs, as assessed by alizarin red staining and 45Ca accumulation. Third, the addition of a PI3K inhibitor, wortmannin, negates the inhibition of mineralization by overexpression of wild-type Axl, suggesting that activation of downstream signaling via PI3K is crucial for its inhibitory activity. In contrast, Axl-mediated signaling is not enhanced by overexpression of kinase-dead Axl and mineralization is accelerated, although β-glycerophosphate is still required for this effect. Finally, the caspase inhibitor zVAD.fmk attenuates the increased mineralization induced by kinase-dead Axl, suggesting that kinase-dead Axl stimulates mineralization by inhibiting the antiapoptotic effect of endogenous Axl. Together, these results demonstrate that signaling through Axl inhibits vascular calcification in vitro and suggest that therapeutics targeting this receptor may open up new avenues for the prevention of vascular calcification in vivo. (Circ Res. 2007;100:502-509.)

Key Words: Axl receptor tyrosine kinase ■ calcification ■ differentiation ■ signaling pathways ■ vascular smooth muscle cells

Vascular calcification has severe clinical consequences in a number of diseases including atherosclerosis, diabetes, and end-stage renal disease, and it is now considered a prognostic indicator of future adverse cardiovascular events.1–3 Mineralized bone-like tissue containing marrow, tissue resembling cartilage, and dispersed hydroxyapatite crystals have all been demonstrated within vascular lesions.4–6 The cellular mediators of vascular calcification include vascular smooth muscle cells (VSMCs),7 calcifying vascular cells (CVCs),8 pericytes,9 and adventitial myofibroblasts,10 but the underlying mechanism by which vascular calcification is regulated is still not fully understood.1,11

The Axl receptor tyrosine kinase signaling system is now emerging as an important regulator of mammalian physiology and pathology with distinct and diverse mechanisms of action, depending on the cell type or organ system involved. Axl autophosphorylation can be induced by receptor dimerization in response to binding of its ligand, Gas6,12 the activity of which is dependent on the presence of its Gla domain.13 In addition, recent studies have shown that Axl can also be activated in a Gas6-independent way.14–16 Axl phosphorylation can result in activation of a number of downstream signaling pathways. The “canonical” pathway is considered to be the phosphatidylinositol 3-kinase (PI3K) pathway, resulting in cell survival17,18; however, in some cell types, other pathways are activated.19,20 It is also becoming apparent that Axl can further influence cell function by modulating Wnt, vascular endothelial growth factor, and integrin-mediated signaling21–23; signaling-independent mechanisms have even been suggested.24 Axl signaling plays important roles in spermatogenesis, platelet function, vascular remodeling, and cancer,25–29 and this study now reports its role as a critical regulator of vascular cell mineralization.

Axl and Gas6 expression is prominent in the vasculature; however, the potential importance of this signaling pathway in vascular disease is only just emerging. Gas6 inhibits apoptosis of endothelial cells17 and VSMCs,18,30 Axl/Gas6 signaling also induces VSMC migration in vitro and in

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502
vivo. The possibility that the Axl/Gas6 pathway regulates vascular calcification has also been suggested, although the mechanism by which this occurs has not been fully elucidated. Previously, we have demonstrated that downregulation of Axl expression is associated with the induction of mineralization in pericytes and that the addition of Axl–extracellular domain to pericytes reduces endogenous Axl phosphorylation and enhances mineralization. Furthermore, Son et al. have demonstrated that stabilization of Gas6 mRNA and maintenance of Gas6/Axl survival signaling is the mechanism by which statins protect human VSMCs from inorganic phosphate-induced apoptosis and calcification.

The purpose of this study was to test the hypothesis that Axl signaling directly regulates mineral deposition by VSMCs and to identify the signaling pathways involved. Specifically, we demonstrate that overexpression of Axl using recombinant adenoviruses encoding full-length human Axl inhibits matrix mineralization and this inhibition is dependent on activation of the PI3K signaling pathway. In addition, we show that overexpressing kinase-dead Axl significantly promotes mineralization and that this effect is dependent on apoptosis and the presence of an external phosphate source. This study provides new evidence that Axl signaling directly modulates matrix mineralization by VSMCs, suggesting that targeting this receptor may provide a novel therapeutic avenue by which vascular calcification can be regulated.

Materials and Methods

Cell Culture

VSMCs were isolated from adult bovine aortic explants and cultured in DMEM containing 10% FCS (10% FCS-DMEM). For experiments, VSMCs were seeded at 1 x 10^4 cells/cm² (day 0 [D0]) and maintained in 10% FCS-DMEM until confluence (D6), when calcification was induced by adding 5 mmol/L β-glycerophosphate (Sigma, UK). Recombinant Adenoviral Infection of VSMCs

VSMCs were infected at 80% confluence using (1) wild-type human Axl recombinant adenovirus (RAd) (pacAd5CMV/WT-Axl), encoding the full-length human Axl cDNA, (2) kinase-dead human Axl RAd (pacAd5CMV/KD-Axl) encoding the Axl cDNA with an arginine to lysine substitution at position 569, (3) β-galactosidase (Ad5/LacZ) recombinant adenovirus, (4) empty vector (Ad5/V5), (5) PBS alone (mock infected). A dose response of RAd infection was performed using a range of multiplicity of infection (0 to 500) to assess the most appropriate multiplicity of infection that should be used to achieve ~90% infection without any cell toxicity (Figure I in the online data supplement, available at http://circres.ahajournals.org). A multiplicity of infection of 300 was selected. Adenoviral vectors were generated by the Gene Transfer Core, University of Iowa, under the direction of Dr Beverly Davidson.

For mineralization experiments, cells were infected on D4 and induced to calcify 48 hours later (D6). Overexpression of the transgene was maintained by reinfection of the cultures on day 8 (D8) and day 15 (D15). In some experiments, 100 mmol/L wortmannin (Sigma) or DMSO control) was added 1 hour before addition of β-glycerophosphate (D6). zVAD.fmk (5 μmol/L; Calbiochem) (or DMSO control) was added at the same time as β-glycerophosphate. Each experiment was performed at least 3 times with triplicate cultures being used in every experiment.

Calcium Assay

Mineral deposition was confirmed by staining with 2% (wt/vol) Alizarin Red and quantified by dye elution. Alternatively, medium was supplemented with 0.52 μCi/mL of ^45CaCl2 and incubated for 48 hours and the level of incorporation assessed. Data are shown as the mean±SEM of at least 2 experiments performed in triplicate. Groups were compared using 1-way ANOVA; P<0.05 was considered statistically significant.

Immunoblotting

Cell layer/matrix extracts were prepared using either radioimmunoprecipitation assay (RIPA) lysis buffer, or Igepal lysis buffer (1% [vol/vol] Igepal CA-630 [Sigma], 50 mmol/L Tris, pH 7.2, 2 mmol/L EDTA; 50 mmol/L sodium fluoride) containing 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium pyrophosphate, 0.7 μg/mL pepstatin A, 1 μg/mL leupeptin. Samples were separated by 8% SDS-PAGE and immunoblotted as described using specific primary antibodies: goat anti-human Axl (1:5000, sc-1096), mouse anti-phosphotyrosine (PY99; 1:1000, sc-7020), mouse anti–β-tubulin (1:5000, sc-5274), goat anti-human Gas6 (1:1000, sc-1935) (Santa Cruz Biotechnology), rabbit anti-phospho subunit (1:1000, 06-497, Upstate Biotechnology), rabbit anti-phosphorylated Akt (1:1000, no. 9271), rabbit anti-total Akt (1:1000, no. 9272) (Cell Signaling), mouse anti-Gla (1:1000, 3570, American Diagnostica). Secondary antibodies were conjugated to horseradish peroxidase (Dako). Results were visualized by enhanced chemiluminescence and autoradiography (ECL Plus, GE Healthcare). Membranes were reprobed following submersion in 0.2 mol/L sodium hydroxide for 5 minutes at room temperature. To confirm equal loading, blots were stained with 10% India ink or immunoblotted for β-tubulin expression.

Immunoprecipitation

Confluent VSMCs (1x10 cm dish) were extracted into 1 mL of either RIPA or Igepal buffers. Tyrosine phosphorylated proteins were immunoprecipitated from RIPA extracts as described. Alternatively, RIPA extracts were incubated overnight with 4 μg/mL of anti-Axl antibody (sc-1096) and precipitated using 40 μL/mL protein G agarose (Roche) for 2 hours. Precipitates were then immunoblotted for p85. For studies in which the cells were infected with RAd, immunoprecipitations were performed 48 hours after infection. In these experiments, Igepal lysates were incubated for 1 hour with 4 μg/mL goat anti-human Axl (N terminal, AF154, R&D Systems) and precipitated using protein G agarose for 1 hour. Precipitates were analyzed by SDS-PAGE and immunoblotting. In some experiments, VSMCs were also treated with 10 μmol/L warfarin (Sigma) or vehicle control (acetone) for 24 hours preinfection and maintained with or without warfarin until analysis by immunoprecipitation. All experiments were performed at least 3 times.

Results

Axl Is Expressed and Phosphorylated in Confluent VSMCs and Is Downregulated When These Cells Mineralize Their Matrix

To determine whether endogenous Axl is active in confluent VSMCs, extracts were immunoprecipitated with an anti-phosphotyrosine antibody, PY99, and then immunoblotted for Axl. Figure 1A demonstrates that endogenous Axl can be immunoprecipitated with this antibody, suggesting that it is phosphorylated in confluent cells (lane 1). No bands were observed when the same sample was immunoprecipitated with mouse IgG and immunoblotted for Axl (lane 2).

Confluent VSMCs cultured in the presence of β-glycerophosphate for 7 days (ie, D6–13) deposited a calcified matrix localized to multilayered ridges that stain positive with Alizarin Red (Figure 1B, ii). VSMCs cultured in...
the absence of β-glycerophosphate did not calcify their matrix (Figure 1B, i). Western blot analysis of VSMC lysates collected 7 days after incubation with or without β-glycerophosphate (D13) demonstrated that endogenous Axl expression was markedly downregulated in these cells compared with controls (Figure 1C, compare lanes 1 and 2). Immunoblots were stripped and reprobed for β-tubulin to confirm equal protein loading (Figure 1C).

Recombinant Adenovirus-Mediated Overexpression of WT-Axl, But Not KD-Axl, Enhances Axl Phosphorylation

First, we determined the effects of recombinant adenoviruses encoding wild-type Axl (pacAd5CMV/WT-Axl) or kinase-dead Axl (pacAd5CMV/KD-Axl) on Axl expression and phosphorylation in VSMCs. Cells were infected with Ad5/BgII as a control. Initially, VSMCs were infected at preconfluence with mock, Ad5/BgII, pacAd5CMV/WT-Axl, and pacAd5CMV/KD-Axl and maintained for 48 hours before extracting protein. Samples were separated by SDS-PAGE and immunoblotted for Axl expression. Figure 2A shows that endogenous Axl was present in mock-infected (lane 1) and Ad5/BgII-infected (lane 2) VSMCs, migrating with an apparent molecular mass of approximately 140 to 150 kDa. Marked overexpression of Axl was confirmed in cells infected with pacAd5CMV/WT-Axl (lane 3) and pacAd5CMV/KD-Axl (lane 4) compared with controls (lanes 1 and 2). Equal protein loading was demonstrated by India ink staining (Figure 2A).

To investigate the effects of pacAd5CMV/WT-Axl and pacAd5CMV/KD-Axl on Axl phosphorylation, VSMCs infected with RAds were maintained for 48 hours in 10% FCS-DMEM before immunoprecipitating with anti-Axl antibodies and immunoblotting for phosphoryrosine (Figure 2B). VSMCs infected with pacAd5CMV/WT-Axl demonstrated a marked increase in levels of phosphorylated Axl (Figure 2B, lane 2) compared with mock-infected cells (Figure 2B, lane 1), whereas cells infected with pacAd5CMV/KD-Axl did not (Figure 2B, lane 3). Longer exposures of this blot confirmed basal levels of phosphorylated Axl in immunoprecipitates from mock-infected or pacAd5CMV/KD-Axl-infected VSMCs (data not shown). To determine whether phosphorylation of overexpressed WT-Axl was dependent on Gas6, immunoprecipitation experiments were conducted using Ad5/BgII- and pacAd5CMV/WT-Axl–infected VSMCs incubated with 10 μmol/L warfarin or vehicle control. This concentration of warfarin reduces Gas6 secretion and prevents its γ-carboxylation (supplemental Figure I). Figure 2C shows that warfarin treatment reduced the levels of phosphorylated Axl in Ad5/BgII-infected cells (compare lanes 1 and 2). In contrast, warfarin did not appear to reduce Axl phosphorylation in pacAd5CMV/WT-Axl–infected cells (Figure 2C, compare lanes 3 and 4), indicating that overexpressed WT-Axl is constitutively active.

Overexpression of WT-Axl and KD-Axl Modulates Matrix Mineralization

The results presented above demonstrate that overexpression of WT-Axl enhances the levels of tyrosine phosphorylated Axl in confluent VSMCs. In contrast, overexpression of KD-Axl increases levels of Axl protein but not its phosphorylation. Therefore, cells induced to overexpress WT-Axl and KD-Axl were used to investigate whether signaling downstream of Axl regulates matrix mineralization by VSMCs. VSMCs infected with pacAd5CMV/WT-Axl, pacAd5CMV/KD-Axl, and Ad5/BgII were treated with β-glycerophosphate 48 hours after infection, for an additional 7 days (Figure 3A). Preliminary studies demonstrated that under these conditions, Axl protein levels were reduced to control levels 5 days after the initial infection with pacAd5CMV/WT-Axl (D9) (supplemental Figure IC). In contrast, KD-Axl remained highly overexpressed at this time point (not shown). Therefore, to maintain overexpression of Axl, cultures were reinfected with RAd 4 days after initial infection (D8) (Figure 3A and B). Alizarin Red staining of cultures demonstrates that overexpression of WT-Axl inhibits matrix mineralization (Figure 3C, ii) compared with control VSMCs (Figure 3C, i). In contrast, overexpression of KD-Axl accelerates matrix mineralization (Figure 3C, iii) compared with controls (Figure 3C, i). To quantify the effects on
mineral deposition, levels of $^{45}$Ca incorporation into the cell layer were determined. The results shown in Figure 3D demonstrate that calcium incorporation is significantly inhibited when WT-Axl is overexpressed compared with control cultures infected with Ad5/Bgl II ($P < 0.001$). In contrast, a significant increase in $^{45}$Ca incorporation was demonstrated in cells overexpressing KD-Axl ($P < 0.001$). These results demonstrate that maintaining the overexpression of active Axl inhibits matrix mineralization by VSMCs cultured in the presence of $\beta$-glycerophosphate. In contrast, the overexpression of KD-Axl accelerates matrix mineralization, indicating it may inhibit the function of endogenous Axl. Therefore, to investigate whether overexpression of KD-Axl per se could overcome the requirement of $\beta$-glycerophosphate for VSMCs to mineralize their matrix, cells infected with pacAd5CMV/KD-Axl and Ad5/Bgl II were cultured in normal maintenance medium for up to 20 days. Reinfection of cells to maintain Axl overexpression was performed on D8 and D15 of the experiment (supplemental Figure III). Mineral deposition was not detected in the absence of $\beta$-glycerophosphate under any of the conditions investigated (supplemental Figure III), suggesting that overexpression of KD-Axl alone is not sufficient to induce mineral deposition by VSMCs.

Axl Downstream Signaling via PI3K-Akt Is Enhanced by Overexpression of WT-Axl But Not KD-Axl

Previous studies have shown that Axl acts via the PI3K pathway in VSMCs$^{18}$ and that phosphorylated Axl binds the p85 regulatory subunit of PI3K in NIH3T3 cells.$^{38}$ Therefore, to determine whether a similar interaction occurs in VSMCs, confluent cell lysates were immunoprecipitated with an anti-Axl antibody and then immunoblotted for p85 (Figure 4A). Accordingly, p85 was detected coprecipitating with Axl (Figure 4A, lane 1), demonstrating that endogenous Axl associates with PI3K in confluent VSMCs. No bands were observed in controls (Figure 4A, lane 2).

To investigate whether the enhancement of Axl phosphorylation induced by overexpressing WT-Axl results in increased signaling via the PI3K pathway, levels of p85 coprecipitation were assessed in pacAd5CMV/WT-Axl–infected VSMCs compared with pacAd5CMV/KD-Axl– and control-infected VSMCs. High levels of p85 were detected coprecipitating with Axl in immunoprecipitates from pacAd5CMV/WT-Axl–infected VSMCs (Figure 4B, lane 3) when compared with pacAd5CMV/KD-Axl samples (Figure 4B, lane 4). Longer exposures of these blots confirmed that basal levels of p85 coprecipitated with Axl in VSMCs infected with mock, Ad5/Bgl II, and pacAd5CMV/KD-Axl (data not shown). The blots were stripped and reprobed with an anti-Axl antibody that confirmed that equivalent amounts of Axl were immunoprecipitated from VSMCs overexpressing either WT-Axl or KD-Axl (Figure 4B). Basal levels of Axl were detected in control samples (lanes 1 and 2) after longer exposure times (data not shown).

Akt is a downstream target of PI3K and is phosphorylated in response to Axl activation in rabbit VSMCs.$^{18}$ To further investigate the downstream signaling molecules involved in Axl-mediated signaling in our cells, cell lysates were separated by SDS-PAGE and immunoblotted for phosphorylated Akt and total Akt. Figure 4C demonstrates that cells infected with pacAd5CMV/WT-Axl (lane 3) exhibit higher levels of phosphorylated Akt than either control cells (lanes 1 and 2) or cells infected with pacAd5CMV/KD-Axl (lane 4).

Activation of PI3K Signaling Is Required for the Inhibitory Effect of Axl on Mineral Deposition

The data presented above demonstrate that maintaining Axl signaling through overexpression of WT-Axl prevents
VSMCs from mineralizing their matrix, whereas overexpressing KD-Axl, which does not enhance PI3K signaling, has the opposite effect. The increased association of phosphorylated Axl with PI3K and downstream Akt phosphorylation demonstrated in cells infected with pacAd5CMV/WT-Axl indicates that the inhibitory effect mediated by Axl is focused through the PI3K-signaling cascade. Therefore, to test whether inhibition of PI3K signaling would overcome the protective effect of Axl overexpression on mineralization, pacAd5CMV/WT-Axl–infected VSMCs were incubated with β-glycerophosphate with or without the specific PI3K inhibitor wortmannin for 7 days (D13), and mineral deposition was assessed. Figure 5 demonstrates that inhibition of PI3K signaling by wortmannin prevents the inhibition of mineralization by wild-type Axl (compare A and B).

Induction of Mineralization by Overexpression of Kinase-Dead Axl Requires Apoptosis

Overexpression of KD-Axl accelerates matrix mineralization, indicating it may inhibit the function of endogenous Axl in VSMCs. As previous studies have shown that Axl inhibits apoptosis in VSMCs and that apoptosis plays a role in mineralization, we used the general caspase inhibitor, zVAD.fmk, to test the hypothesis that the effect of KD-Axl is dependent on apoptosis. Figure 6 demonstrates that inhibition of caspase activity by zVAD.fmk significantly inhibits the increased mineralization induced by KD-Axl (Figure 6A, compare iii and iv; Figure 6B). Under these conditions, mineralization was not significantly different from controls (Figure 6B). These results suggest that KD-Axl acts by inhibiting the anti-apoptotic effect of endogenous Axl.
Discussion
This study demonstrates for the first time that Axl signaling inhibits the mineralization of VSMCs. Mineral deposition is inhibited if Axl expression, and, critically, downstream PI3K-Akt signaling, is maintained at high levels in VSMCs. Furthermore, overexpression of KD-Axl not only fails to inhibit mineral deposition but actively increases mineralization of VSMCs, and this is dependent on the presence of β-glycerophosphate and on apoptosis.

We show that endogenous Axl is expressed and phosphorylated in confluent VSMCs and that levels of Axl protein are markedly downregulated as the cells are induced to calcify. These findings are consistent with our previous study using pericytes and with a recent study in which downregulation of Axl and its ligand Gas6 during inorganic phosphate-induced calcification of human smooth muscle cells was observed. Positive and negative regulation of Axl and Gas6 expression is clearly important in a number of disease contexts, including vascular calcification; however, at present, only limited data are available on the major players that may be involved (eg, upregulated expression by angiotensin II and thrombin, mRNA stability, and ubiquitin-mediated degradation). Therefore, further studies to identify the mechanism(s) by which Axl and Gas6 expression are regulated in the vasculature are warranted.

Axl/Gas6 signaling has been attributed with a number of functions in VSMCs, including proliferation, migration, and upregulation of scavenger receptors. However, as Axl can also potentially function via Gas6-independent and even signaling-independent mechanisms, the role of Axl, and Axl signaling in particular, in preventing mineralization is unclear. Therefore, we have used RAd to overexpress wild-type and kinase-dead versions of Axl specifically to investigate the functional effects of Axl signaling on mineralization. We show that WT-Axl enhances the levels of active, phosphorylated Axl, whereas KD-Axl increases Axl protein levels but not its phosphorylation. The increased phosphorylation observed after overexpression of WT-Axl was not increased further by addition of recombinant Gas6 (results not shown) and was not attenuated by the presence of warfarin, suggesting that Axl is constitutively active in these cells, as reported by others.

Overexpression of WT-Axl significantly inhibits VSMC mineralization. Conversely, KD-Axl was not only unable to prevent VSMC mineralization but significantly increased mineralization compared with control VSMCs. The differing effects of WT-Axl and KD-Axl on VSMCs indicates that it is Axl signaling, rather than the presence of Axl per se, that plays a significant role in preventing mineral deposition. Therefore, we investigated whether overexpression of WT or KD-Axl affected downstream signaling events in VSMCs. Previous studies have shown that Axl activates several signaling pathways in a cell type–specific manner. In this study, we demonstrate that in VSMCs, endogenous Axl signaling is transduced via PI3K-Akt. Furthermore, WT-Axl (but not KD-Axl) overexpression enhances downstream signaling via this pathway. To assess the requirement of PI3K signaling for the effects of Axl on VSMC mineralization, we used the small-molecule, PI3K inhibitor wortmannin. Wortmannin prevented the inhibitory effect of WT-Axl overex-
Axl signaling will further define the mechanism(s) by which Axl exerts its effects.

Gas6 may also influence mineralization via multiple mechanisms. In addition to its role in stimulating Axl, Gas6 may directly inhibit crystal formation by binding to phosphatidyserine on apoptotic bodies via its Gla domain, which is also critical for the activation of Axl by Gas6. Interestingly, lower levels of Gas6 have been detected in the sera of patients undergoing warfarin treatment, and warfarin treatment causes focal calcification of the elastic lamellae of major arteries and aortic valves. This phenotype has been attributed to the inhibition of γ-carboxylation of matrix Gla protein by warfarin; however, our results suggest it may also result from the loss of functional γ-carboxylated Gas6.

To conclude, these results demonstrate that Axl signaling inhibits mineral deposition by vascular cells, and they highlight the potential importance of Axl/Gas6 signaling in regulating vascular calcification. Identifying the upstream regulators and downstream mediators of these signaling molecules is therefore of crucial importance for our understanding of the development and progression of this pathological process. Furthermore, as it is now becoming recognized that Axl can be activated both by itself and by other factors in addition to Gas6, with extensive crosstalk between Axl and other signaling pathways, it is important to fully delineate the mechanism by which Axl exerts its pronounced effects.

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Disclosures
None.

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Optimisation of infection of VSMCs with recombinant adenoviruses

A dose response of recombinant adenoviral infection was carried out using a range of MOI (0, 10, 50, 100, 300 & 500) in order to assess the most appropriate MOI that should be used to achieve >90% infection and high levels of over-expression of Axl without any cell toxicity. Online Figure 1A shows that the number of VSMCs infected with Ad5/LacZ increased with increasing MOI. Maximum infection was seen with MOI ≥ 300. VSMCs were also infected with pacAd5CMV/WT-Axl using the same range of MOI. Cell lysates were prepared 48 h post-infection, separated by SDS/PAGE and analysed by western blotting using a specific anti-Axl antibody. Online Figure 1B shows that marked over-expression of Axl was detected in VSMCs infected with pacAd5CMV/WT-Axl with MOI ≥ 100. Maximal expression was obtained with MOI ≥ 300. On the basis of these experiments, an MOI =300 was selected for the studies presented in this paper.

To determine whether over-expression of Axl was maintained throughout the experiments, VSMCs were infected with pacAd5CMV/WT-Axl and Ad5/BglII, treated with β-glycerophosphate 48 h after infection (D6) and cells were maintained in culture until D10. Cell extracts were prepared every 24 h after infection and analysed for Axl expression by western blotting (Online Figure 1C). These studies demonstrated that Axl protein was highly over-expressed days 1-4 post-infection (compare Online Figure 1Ci and 1Cii, lanes 2-4); levels were reduced to control 5 days after the initial infection with pacAd5CMV/WT-Axl (compare Online Figure 1Ci and 1Cii, lanes 5 & 6). Therefore, cultures were re-infected with RAd 4 days after initial infection in order to maintain over-
expression of Axl during the course of the experiments presented in this paper.

**Warfarin inhibits γ-carboxylation of Gas6 produced by VSMCs**

To confirm that the γ-carboxylation inhibitor warfarin was able to prevent γ-carboxylation of Gas6, VSMCs were incubated for 24 h with acetone (vehicle) or 10 μmol/L warfarin. VSMCs were then incubated in serum-free medium for a further 48 h in the continued presence of acetone or warfarin. Conditioned medium was collected and concentrated using Amicon ultra-15 filters (5,000 MWCO) (Millipore, USA). Concentrated conditioned medium was analysed by SDS/PAGE and immunoblotting using an anti-Gla residue antibody (1:1000, #3570, American Diagnostica Inc) (Online Figure 2, top panel). The blot was then stripped and re-probed with a polyclonal anti-human Gas6 antibody (1:1000, sc-1935, Santa Cruz, USA) (Online Figure 2, middle panel). Coomassie blue staining (Invitrogen) on a parallel SDS/PAGE gel (Online Figure 2, bottom panel) confirmed equal protein loading. Online Figure 2 shows that γ-carboxylation of Gas6 was markedly reduced in the presence of warfarin (compare lanes 1 and 2, top panels). A small reduction in Gas6 secretion was also detected in the presence of warfarin (compare lanes 1 and 2, middle panel). This experiment was repeated 3 times with identical results. These data confirm that warfarin prevents the production of active, γ-carboxylated Gas6 by VSMCs.

**KD-Axl does not induce mineralisation in the absence of β-glycerophosphate**

VSMCs infected with RAd/Empty or RAd/KD-Axl were maintained for 15 days (D4-20) in normal media without β-GP. Calcium incorporation was measured over the final 48 h.
Mineral deposition was not detected in the absence of β-glycerophosphate under any of the conditions investigated (Online Figure 3). However, if after 20 days in normal medium VSMCs were incubated with β-GP, mineral deposition occurred rapidly (3-4 days) (data not shown). These data suggest that increased phosphate levels are required for mineralisation and that over-expression of KD-Axl cannot overcome this requirement.

**FIGURE LEGENDS**

**Online Figure 1.** Infection of VSMCs with Ad5/LacZ or pacAd5CMV/WT-Axl using a range of MOI. A) Micrographs of X-Gal-stained VSMCs 48 h after infection with Ad5/LacZ at different MOI, mock control (i); 10 (ii); 50 (iii); 100 (iv); 300 (v); 500 (vi) (bar=200 µm). B) Immunoblot of cell layer extracts (15 µl/lane) prepared 48 h after RAd infection with pacAd5CMV/WT-Axl at different MOI, 10 (lane 2); 50 (lane 3); 100 (lane 4); 300 (lane 5); 500 (lane 6) and mock control (lane 1); using anti-human Axl goat polyclonal. Blots were also immunostained for β-tubulin as a loading control. C) Immunoblots of cell layer extracts (10 µg/lane) prepared every 24 h after RAd infection, over a 6 day period (D5-D10) (lanes 1-6) during which cells were treated with 5 mmol/L β-glycerophosphate on D6 until D10 (lanes 2-6), from VSMCs infected with pacAd5CMV/WT-Axl (i) & Ad5/BglI (ii) and using anti-Axl polyclonal. Over-expression of Axl is reduced from D9 (lane 5) onwards, therefore re-infection was performed on D8 (lane 4) 4 days after initial infection to maintain over-expression throughout the experiment (see Online Figure 3B). Blots were also immunostained for β-tubulin as a loading control.
**Online Figure 2.** VSMCs were incubated with acetone (lane 1) or 10 μmol/l (lane 2) warfarin for 24h in normal medium then 48h in serum-free medium. Conditioned media was collected and analysed by SDS/PAGE and either immunoblotting using anti-Gla residue (top panel) and anti-Gas6 (middle panel) antibodies or by coomassie blue staining (bottom panel). Molecular weight markers are indicated on the right-hand side.

**Online Figure 3.** VSMCs infected with RAd/Empty or RAd/KD-Axl were maintained for 15 days (D4-20) in normal media. (A) Diagram of experimental plan. (B) $^{45}$Calcium deposition by VSMCs. The level of radioactivity incorporated into the cell layer from day 18-20 was counted and expressed as mean ±SD (n=6).
Online

Figure 1

A

B

C