

Fractalkine has anti-apoptotic and proliferative effects on human vascular smooth muscle cells via epidermal growth factor receptor signalling

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Aims

Fractalkine (CX₃CL1) is a membrane-bound chemokine that signals through the G protein-coupled receptor CX₃CR1 that is implicated in the development of atherosclerosis. We have previously reported that CX₃CR1 is expressed by primary human coronary artery smooth muscle cells (CASM), where it mediates chemotaxis towards CX₃CL1. We sought to determine the effect of CX₃CL1 on CASMC survival and proliferation and elucidate the signalling mechanisms involved.

Methods and results

CX₃CL1 significantly reduces staurosporine-induced apoptosis of CASMC, as quantified by caspase 3 immunostaining and Annexin-V flow cytometry. Furthermore, CX₃CL1 is a potent mitogen for primary CASMC and induces phosphorylation of extracellular signal-regulated kinase (ERK) and Akt, measured by western blotting. Inhibition of either ERK or phosphoinositide 3-kinase (PI3K) signalling abrogates proliferation, while only PI3K signalling is involved in the anti-apoptotic effects of CX₃CL1. We describe a novel and specific small molecule antagonist of CX₃CR1 (AZ12201182) which abrogates the mitogenic and anti-apoptotic effects of CX₃CL1 on CASMC. Pharmacological inhibition of the epidermal growth factor receptor (EGFR) blocks CASMC survival and DNA synthesis, indicating a previously undocumented role for EGFR signalling in response to CX₃CL1 involving release of a soluble EGFR ligand. Specifically, CX₃CL1 induces shedding of epiregulin and increases epiregulin mRNA expression 20-fold within 2 h. Finally, antibody neutralization of epiregulin abrogates the mitogenic effect of CX₃CL1.

Conclusion

We have demonstrated two novel and important functions of CX₃CL1 on primary human SMCs: anti-apoptosis and proliferation, both mediated via epiregulin-induced EGFR signalling. Our data have important implications in vascular pathologies including atherosclerosis, restenosis, and transplant accelerated arteriosclerosis, where the balance of SMC proliferation and apoptosis critically determines both plaque stability and vessel stenosis.

Keywords

Smooth muscle cell • Proliferation • Anti-apoptosis • Chemokine • Fractalkine • EGFR • Epiregulin • CX₃CR1 • CX₃CL1

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1. Introduction

Chemokines (*chemoattractant cytokines*) are low molecular weight proteins responsible for coordinating cell trafficking in both homeostatic and inflammatory contexts.¹ Around 50 chemokines have been cloned to date, and they are divided into four families (C, CC, CXC and CX₃C) based on the location of key structural cysteine residues. Fractalkine or CX₃CL1 is the only member of the CX₃C chemokine family and displays an unusual membrane-bound structure with the chemokine domain attached to the membrane via a mucin-like stalk where it is capable of mediating adhesion of cells expressing the G protein-coupled receptor CX₃CR1.² CX₃CL1 expression has been detected on activated endothelial cells,³ smooth muscle cells (SMCs),⁴ and macrophages⁵ and its expression is enhanced by inflammatory stimuli, including TNF α , IFN γ , and LPS.⁴ CX₃CL1 can be cleaved from the cell surface in response to inflammatory stimuli by TNF α -converting enzyme (TACE/ADAM17).⁶ Soluble CX₃CL1 is then able to act as a classical chemoattractant for leukocytes⁷ and also SMCs expressing CX₃CR1.⁸

CX₃CL1 is involved in the development of numerous inflammatory pathologies including rheumatoid arthritis⁹ and graft rejection.¹⁰ Several lines of evidence implicate CX₃CL1 in the pathogenesis of atherosclerosis. First, two non-synonymous single-nucleotide polymorphisms in CX₃CR1 (V249I and T280M) have been associated with an altered risk of coronary artery disease and stroke in large population-based studies.^{11,12} Secondly, *cx3cr1*^{-/-} *apoe*^{-/-} animals show smaller atherosclerotic lesions containing fewer macrophages at numerous anatomical sites,¹³ while *cx3cl1*^{-/-} or *+/-* *apoe*^{-/-} animals show smaller lesions at the brachiocephalic artery.¹⁴ Finally, CX₃CL1 and CX₃CR1 expression can be detected in human atherosclerotic plaques, while expression in normal areas of vessel is low.⁸ Work from our laboratory demonstrated the expression of CX₃CL1 in mononuclear cell infiltrates of coronary artery plaques, while CX₃CR1 was found localized to SMCs, particularly those in close contact with CX₃CL1-positive macrophages.⁸

Knockout studies have demonstrated a clear role for CX₃CL1 in leukocyte migration into nascent atherosclerotic plaques. However, functional roles for CX₃CR1 expression on human SMCs remain relatively unexplored. We sought to investigate whether CX₃CL1 is a survival factor and mitogen for human coronary artery SMCs (CASM) *in vitro*, and elucidate the signalling pathways required for these activities.

2. Methods

2.1 Materials

All reagents for cell culture were obtained from Invitrogen unless otherwise stated. All other reagents were purchased from Sigma Aldrich unless otherwise specified. Recombinant human CX₃CL1 chemokine domain was purchased from R&D systems, and CXCL8 and PDGF-BB were from Peprotech. EGF and bFGF were from Promocell. AG1478 and staurosporine were from Calbiochem, and GM6001 from Biomol. Anti-pERK-1/2, anti-pAkt, anti-cleaved caspase 3, and total Akt antibodies were from Cell Signalling Technology. ERK2 antibody was from Santa Cruz. Annexin-V FITC was from Becton Dickinson. ECL

Supersignal reagents and the BCA protein assay kit were from Perbio. Fluorescently conjugated secondary antibodies were from Jackson ImmunoResearch.

2.2 Cell culture

Primary human CASMC obtained from normal regions of vessel from five independent donors were obtained from Lonza and cultured according to manufacturer's instructions using SmGM-2 media. The HCM-601EB medial SMC line was grown in DMEM supplemented with 10% FCS and 750 μ g/mL G418 and has been described previously.¹⁵ All experiments were performed following 48 h serum-starvation in serum-free media (SFM, DMEM/0.1% BSA) unless otherwise stated.

2.3 Measurement of apoptosis

Apoptosis was quantified by immunostaining of cleaved caspase 3, detection of caspase 3/7 activity using the Caspase-Glo assay (Promega) and by annexin V/propidium iodide staining and flow cytometry according to manufacturer's protocols. Further details and representative FACS plots are provided in Supplementary material online.

2.4 [³H]-thymidine incorporation as a measure of cellular proliferation

Detailed experimental information can be found in Supplementary material online. Briefly, cells were grown in 96-well plates until 80% confluent, serum-starved for 48 h, then agonist stimulated for 24 h prior to the addition of 0.1 μ Ci/well methyl-[³H]-thymidine (specific activity 1 μ Ci/ μ L, GE Healthcare) for 4 h and scintillation counting.

2.5 Ki67 staining as a measure of cell proliferation

Cells were grown in 4-well chamber slides and treated exactly as for the tritiated thymidine incorporation assay. Cells were fixed for 20 min in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100/PBS for 5 min, and blocked with 3% BSA/0.1% Triton X-100/PBS for 15 min. Cells were stained with mouse anti-human Ki67 antibody (Dako) for 1 h followed by goat anti-mouse FITC for 30 min. Nuclei were counterstained with DAPI and cells examined by fluorescence microscopy.

2.6 Signalling assays

Serum-starved cells grown in 6-well plates were pre-treated with or without antagonist for the indicated times before agonist addition and lysate preparation. Further details can be found in Supplementary material online.

2.7 Western blotting

Protein lysates (20 μ g) were resolved on a 10% SDS-PAGE gel, and transferred to Hybond-C nitrocellulose membrane (GE Healthcare). Blots were blocked with 10% w/v non-fat-dried milk/Tris buffered saline/0.05% Tween-20 (TBS-T) for 90 min. Blots were incubated with primary antibody overnight at 4°C, then incubated for 90 min with appropriate secondary antibodies which were detected using the Supersignal chemiluminescence kit and autoradiography. Western blots were stripped for 30 min at 50°C in stripping buffer (67 mmol/L Tris-HCl, 2% SDS, 100 mmol/L 2-Mercaptoethanol).

2.8 Real-time quantitative SYBR green RT-PCR

RNA was isolated using the RNeasy RNA isolation kit (Qiagen) according to manufacturer's instructions, reverse transcribed and PCR reactions carried out using Quantitect SYBR green mastermix (Qiagen) according to manufacturer's instructions. Further details can be found in Supplementary material online.

2.9 Epiregulin detection by flow cytometry

CASMC were grown in T25 flasks, serum-starved as above, and treated for the indicated times with PMA (phorbol-12-myristate-13-acetate) or CX₃CL1 and stained with anti-epiregulin antibody (clone 183 629, R&D systems) according to the manufacturer's protocol. See the Supplementary material online for more details.

2.10 Data analysis

All data were analysed using GraphPad Prism software (version 4.03). Statistical significance was assessed by one-way ANOVA and Bonferroni's, Tukey's, or Dunnett's post-test for comparisons between three or more groups.

3. Results

3.1 CX₃CL1 has anti-apoptotic effects on primary human SMCs via CX₃CR1

We first sought to establish whether CX₃CL1 could block apoptosis of primary human CASMC induced by staurosporine. Treatment with staurosporine (0.5 µmol/L) for 6 h induced a significant increase in the number of cells staining positively for cleaved caspase 3: around 35% of cells in the population were cleaved caspase 3⁺ ($P < 0.001$, Figure 1A and D). Cells stained with isotype control antibody showed no detectable staining (data not shown). In addition, cells treated with staurosporine showed the hallmarks of apoptosis, including nuclear condensation and fragmentation (Figure 1C and D). Pre-treatment with CX₃CL1 (30 nmol/L) for 30 min prior to the addition of staurosporine significantly blocked caspase 3 cleavage by around 50% ($P < 0.01$, Figure 1A).

To confirm these findings, the activity of caspases 3 and 7 was quantified using the Caspase-Glo 3/7 assay (Promega), which measures cleavage of a luminescent caspase substrate. Treatment with staurosporine induced caspase 3/7 activation ($P < 0.001$) which was significantly blocked by pre-treatment with 30 nmol/L CX₃CL1 ($P < 0.001$, Figure 1B).

Using a third assay to measure apoptosis induction, we quantified annexin-V staining by flow cytometry. Treatment with staurosporine (0.5 µmol/L) for 6 h induced a significant increase in the number of cells binding annexin-V FITC: around 50% of the cells in the population ($P < 0.001$, Figure 1E). This was significantly reduced by pre-treatment with CX₃CL1 (30 nmol/L) for 30 min prior to staurosporine treatment ($P < 0.05$, Figure 1E). To confirm that the effects of CX₃CL1 were mediated via the CX₃CR1 receptor, we utilized a specific small-molecule antagonist of the receptor AZ12201182 (see Supplementary material online, Tables S2 and S3 for information on potency and specificity). Cytotoxicity assays confirmed that the drug showed no detectable toxicity and did not have any pro-apoptotic effect in annexin-V binding assays at the dose used (Figure 1F and data not shown). Pre-

treatment of the cells for 1 h with 500 nmol/L AZ12201182 prior to the addition of CX₃CL1 abrogated the pro-survival effect ($P < 0.01$, Figure 1F), indicating that the anti-apoptotic effects of CX₃CL1 on primary human CASMC are mediated via CX₃CR1.

3.2 CX₃CL1 is a mitogen for primary human SMCs and the SMC line HCM-601EB

Since CX₃CL1 has pro-survival effects on SMCs, we next sought to determine whether CX₃CL1 could induce human SMC proliferation *in vitro*. Using primary CASMC in a tritiated thymidine incorporation assay to measure *de novo* DNA synthesis, CX₃CL1 induced a dose-dependent increase in DNA synthesis with an average proliferation index in five donors of 8.8 ± 1.42 in response to 30 nmol/L CX₃CL1 ($P < 0.01$, Figure 2A). The proliferation index varied between donors, ranging from 2.5- to 17-fold in response to 30 nmol/L CX₃CL1. Using a previously described CASMC line: HCM-601EB¹⁵ in the same assay, CX₃CL1 induced a dose-dependent increase in DNA synthesis with an average proliferation index of 23.1 ± 1.22 for a dose of 30 nmol/L CX₃CL1 ($P < 0.01$, Figure 2B).

These data were validated with a single dose of CX₃CL1 and a positive control (PDGF-BB) using two alternative methods of measuring proliferation: Ki67 staining and total cell counting. CX₃CL1 (30 nmol/L) was found to significantly increase both the total cell number and the number of Ki67⁺ cells (i.e. those in interphase) to a similar extent as 1 nmol/L PDGF-BB in CASMC ($P < 0.05$, Figure 2C; $P < 0.001$, Figure 2D).

To assess whether the mitogenic effects of CX₃CL1 are mediated via CX₃CR1, we used the small molecule antagonist AZ12201182 at a range of doses in a tritiated thymidine incorporation assay. Pre-treatment of cells with 10–500 nmol/L AZ12201182 for 1 h prior to the addition of CX₃CL1 significantly blocked DNA synthesis, with complete blockade of the CX₃CL1 response at 500 nmol/L ($P < 0.001$, Figure 2E). AZ12201182 had no effect on the DNA synthesis in response to CXCL8 (30 nmol/L) or 1% FCS, indicating a specific blockade of proliferation in response to CX₃CL1 (Figure 2F).

3.3 CX₃CL1 induces ERK and Akt phosphorylation in human CASMC

We next investigated the signalling pathways involved in CX₃CL1-mediated proliferation, initially focusing on extracellular signal-regulated kinase (ERK): a serine/threonine kinase known to be a key regulator in proliferation, survival, and differentiation. CX₃CL1 stimulated ERK 1/2 phosphorylation within 10 min (Figure 3A). CX₃CL1-induced ERK phosphorylation was completely abolished following treatment with pertussis toxin (PTX) or a MAPK/ERK kinase (MEK) inhibitor U0126 (Figure 3A). We then assessed whether CX₃CL1 treatment induced phosphorylation of Akt: a key regulator in cell survival. CX₃CL1 stimulated Akt phosphorylation within 5 min and this was also completely blocked by PTX and a phosphoinositide 3-kinase (PI3K) inhibitor LY294,002 (Figure 3B). MTS assays demonstrated that neither PTX, LY294,002 nor U0126 had any detectable cytotoxicity at

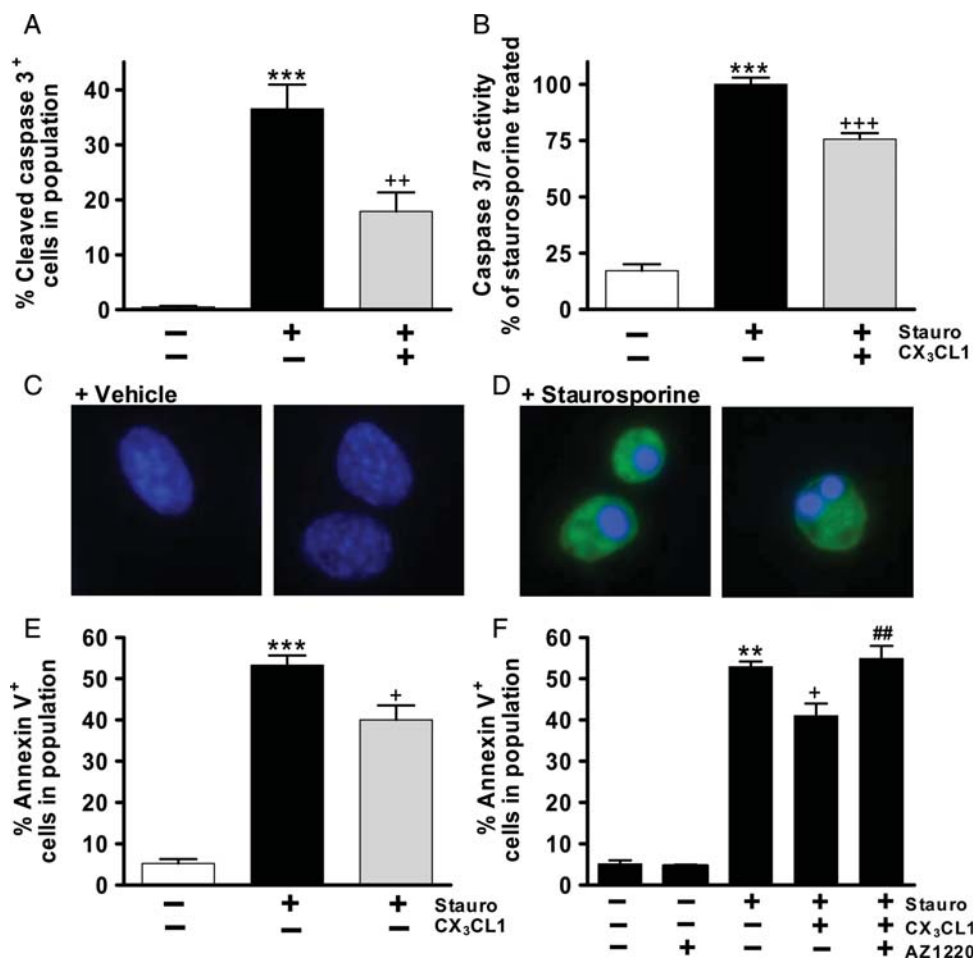


Figure 1 CX₃CL1 has anti-apoptotic effects on primary human CSMC that are blocked by the CX₃CR1 inhibitor AZ12201182. (A) CSMC were pre-treated ± 30 nmol/L CX₃CL1 for 30 min prior to the addition of 0.5 µmol/L staurosporine or vehicle (DMSO) for 6 h. Cells were stained with anti-cleaved caspase 3 antibody and nuclei stained with DAPI. Data expressed as percent cleaved caspase 3⁺ cells, 750–1300 cells counted per treatment. (B) Cells were treated as in (A) and after staurosporine treatment were incubated with Caspase-Glo 3/7 reagent to quantify caspase activity. (C and D) Representative images (× 1000 magnification) showing vehicle treated (C) and staurosporine treated (D) cells stained with anti-cleaved caspase 3 antibody and DAPI. (E) Cells were treated as in (A) and after staurosporine treatment were stained with annexin-V FITC and propidium iodide and analysed by flow cytometry to calculate the number of apoptotic cells (annexin-V⁺, propidium iodide⁻). (F) CSMC were pre-treated with 500 nmol/L AZ12201182 (AZ1220) then treated ± CX₃CL1 followed by staurosporine treatment as in (E). All data shown as mean ± SEM. Data analysed with one-way ANOVA and Tukey's *post hoc* test. ****P* < 0.001 relative to untreated, +*P* < 0.05, ++*P* < 0.01, +++*P* < 0.001 relative to staurosporine treated. ###*P* < 0.01 relative to CX₃CL1 treated. (A) Data from two donors, four independent experiments. (B) Data from three donors, four independent experiments. (E) Data from three independent experiments, three flasks per treatment in each experiment. (F) Data from two independent experiments, three flasks per treatment in each experiment.

the doses used (data not shown). This indicates coupling via a G_{αi}-dependent pathway, and the involvement of both ERK and PI3K in CX₃CL1 signalling in SMCs.

3.4 The mitogenic and anti-apoptotic effects of CX₃CL1 require G_{αi}, ERK, and PI3K signalling

To confirm whether anti-apoptosis and/or proliferation were dependent on ERK or PI3K signalling, we investigated the effect of U0126 and LY294,002 on caspase 3 cleavage and tritiated thymidine

incorporation. CX₃CL1-induced anti-apoptosis was blocked by the PI3K inhibitor (*P* < 0.05), but not the MEK inhibitor (Figure 3C). In contrast, the mitogenic effect of CX₃CL1 on DNA synthesis was completely blocked by both the MEK inhibitor and the PI3K inhibitor (*P* < 0.001, Figure 3D). Both DNA synthesis (*P* < 0.001, Figure 3E) and anti-apoptosis (*P* < 0.01, Figure 3C) were abrogated by pre-treatment with PTX, indicating both are mediated via a G_{αi}-dependent pathway. In contrast, PTX had no effect on DNA synthesis induced by PDGF-BB, indicating that the effects of PTX are specific (Figure 3F). Taken together, these experiments show that ERK and PI3K activity downstream of G_{αi} are necessary to drive

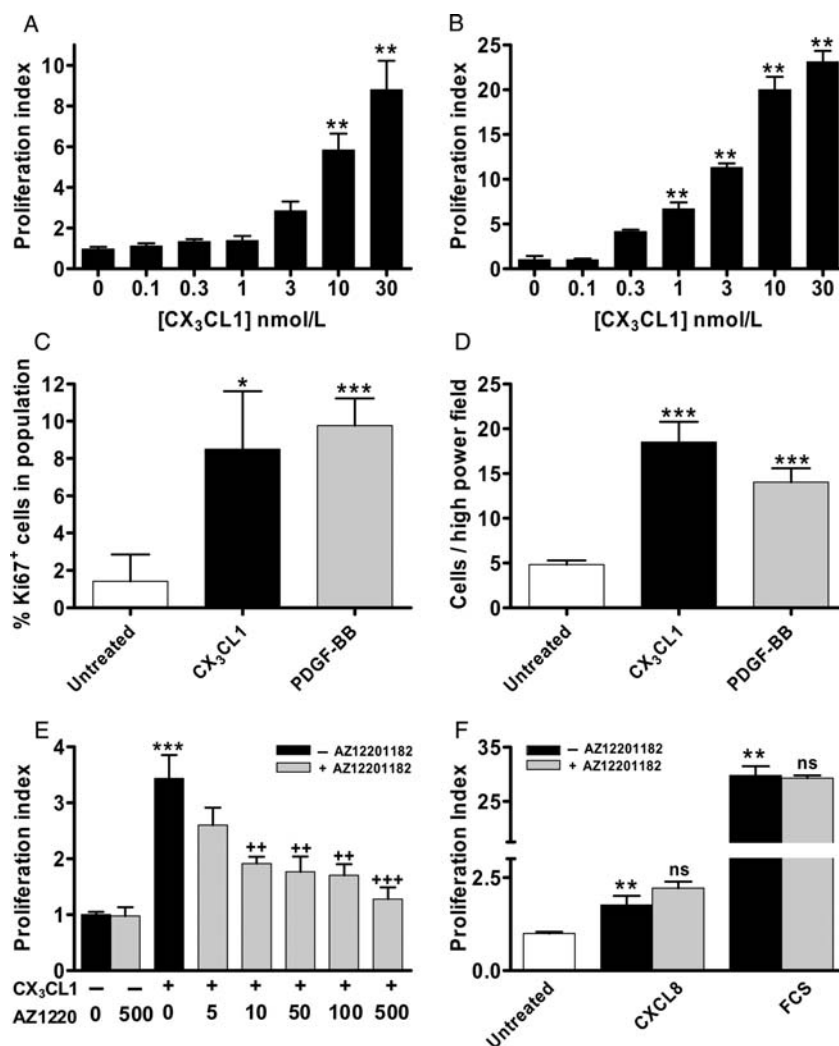


Figure 2 CX₃CL1 is a mitogen for primary human CASMC and this effect is blocked by the CX₃CR1 inhibitor AZ12201182. (A) CASMC were serum-starved for 48 h to induce quiescence prior to stimulation with CX₃CL1 for 24 h. A 4 h tritiated thymidine pulse was applied and incorporation measured by scintillation counting. Data expressed as proliferation index: fold change over untreated. (B) The SMC line HCM-601EB was treated with CX₃CL1 as in (A). (C) Cells were grown in chamber slides and treated as for part (A) with PDGF-BB (1 nmol/L) or CX₃CL1 (30 nmol/L). Cells were stained with anti-Ki67 antibody and DAPI, data are expressed as % Ki67⁺ cells in the population. (D) Cells were treated as in (C) and the number of cells per high power field quantified. (E) Cells were pre-treated for 1 h with the indicated dose of AZ12201182 (AZ1220) or vehicle, prior to treatment with CX₃CL1 as in part (A). (F) Cells were pre-treated for 1 h with 500 nmol/L AZ12201182 prior to treatment with 30 nmol/L CXCL8 or 1% v/v FCS and measurement of DNA synthesis as in part (A). All data shown as mean \pm SEM. Data analysed with one-way ANOVA and Dunnett's *post hoc* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 relative to untreated. ++*P* < 0.01, +++*P* < 0.001 relative to CX₃CL1 treated. (A) Data from five donors, greater than five independent experiments. (B) Data from greater than five independent experiments. (C and D) Data from three independent experiments. (E and F) Data from three independent experiments.

CASMC proliferation in response to CX₃CL1, while only PI3K activity is required for the anti-apoptotic effects of CX₃CL1.

3.5 CX₃CL1-induced DNA synthesis and anti-apoptosis requires epidermal growth factor receptor activity

A number of mitogens acting via GPCRs are known to transduce signals via transactivation of tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR). This can involve

intracellular transactivation of the EGFR by an activated GPCR or can be mediated via metalloprotease-dependent cleavage of a membrane bound EGFR ligand which is then able to activate nearby EGFRs.¹⁶ Phenylephrine, for example, binds to the α 1-adrenoreceptor and induces release of heparin-binding EGF-like growth factor (HB-EGF) and transactivation of the EGFR, leading to SMC proliferation.¹⁷ Two chemokines (CXCL12 and CCL11) have recently been shown to regulate the expression of matrix metalloproteases in SMCs via a mechanism involving EGFR activation.¹⁸

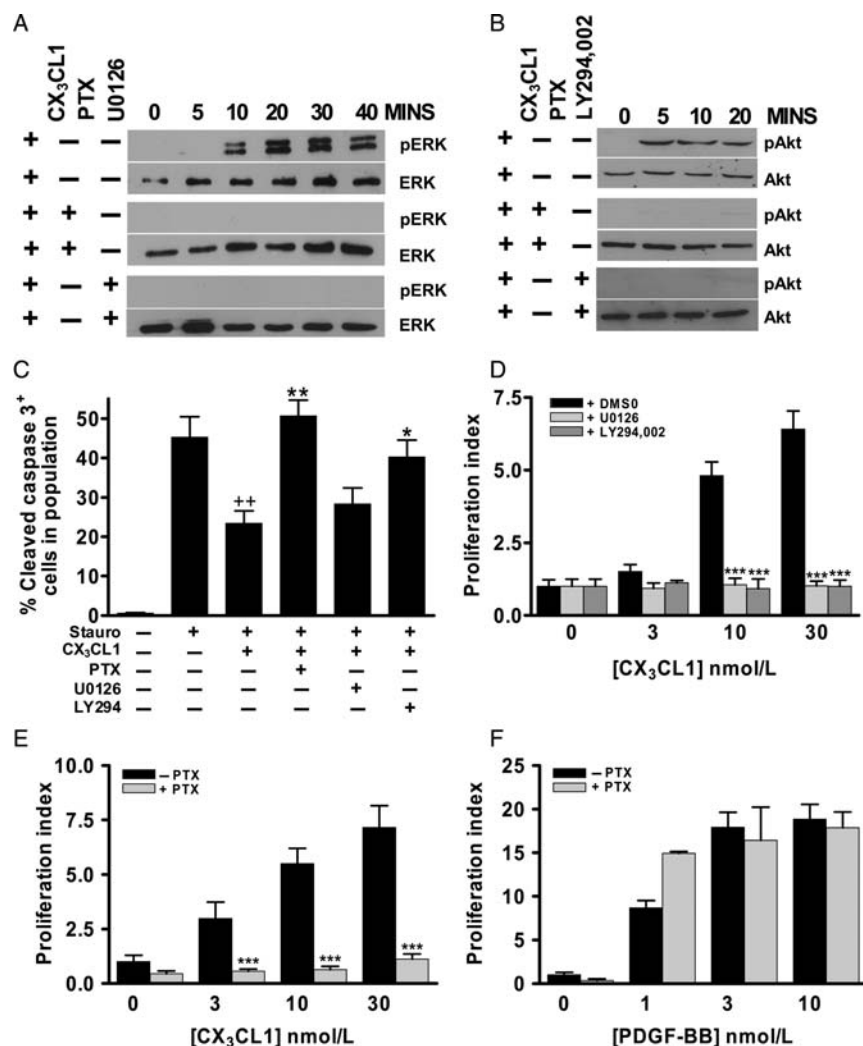


Figure 3 The mitogenic and anti-apoptotic effects of CX₃CL1 require G_{αi}, ERK, and PI3K signalling. (A) CASC were serum-starved for 48 h, then pre-treated ± 200 ng/mL pertussis toxin (PTX) for 2 h or U0126 (10 µmol/L) for 1 h, prior to the addition of 30 nmol/L CX₃CL1 for the indicated times. Cell lysates were prepared, and western blotted for pERK before stripping and re-probing with total ERK antibody. (B) CASC were treated as in (A), and pre-treated with PTX or LY294,002 (10 µmol/L) for 1 h before CX₃CL1 stimulation and western blotting for pAkt and total Akt. (C) CASC were pre-treated for 2 h with PTX or for 1 h with U0126/LY294,002 prior to the addition of 30 nmol/L CX₃CL1 followed by staurosporine treatment for 6 h and staining as in Figure 1A. (D) A tritiated thymidine incorporation assay was carried out as in Figure 1 following 1 h pre-treatment with U0126, LY294,002, or vehicle (DMSO). (E and F) CASC were pre-treated with PTX for 2 h prior to the addition of CX₃CL1 or PDGF-BB, before tritiated thymidine incorporation was measured as described in Figure 2. Data shown as mean ± SEM. Data analysed with one-way ANOVA and Bonferroni's multiple comparison *post hoc* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 relative to CX₃CL1 treated. ++*P* < 0.01 relative to staurosporine treated. (A and B) Data are representative of three independent experiments from three donors. (C) Data from two donors, three independent experiments. (D–F) Data from three donors, three independent experiments.

We investigated whether the EGFR is involved in mediating the anti-apoptotic and mitogenic effects of CX₃CL1 using pharmacological inhibitors. We used the EGFR kinase inhibitor AG1478 to block both the intracellular and extracellular pathways of EGFR activation and the broad-spectrum metalloprotease inhibitor GM6001 to block the extracellular EGFR activation pathway. To ensure the inhibitors were specific for the EGFR, CASC were treated with the ligands EGF, bFGF, and PDGF-BB. AG1478 completely blocked DNA synthesis in response to EGF (*P* < 0.001), but had no effect on the response to PDGF-BB or bFGF, indicating

a specific blockade of the EGFR alone (Figure 4A). MTS assays demonstrated that neither AG1478 nor GM6001 showed any detectable cytotoxicity at the doses used (data not shown). Using cleaved caspase 3 staining, cells were pre-treated with either AG1478 or GM6001 for 30 min, then CX₃CL1 (30 nmol/L) was added for 30 min before the addition of staurosporine for 6 h. Both drugs blocked the pro-survival effect of CX₃CL1 (*P* < 0.05, Figure 4B), indicating the involvement of the EGFR and the extracellular activation pathway. In a tritiated thymidine incorporation assay, both AG1478 and GM6001 again completely

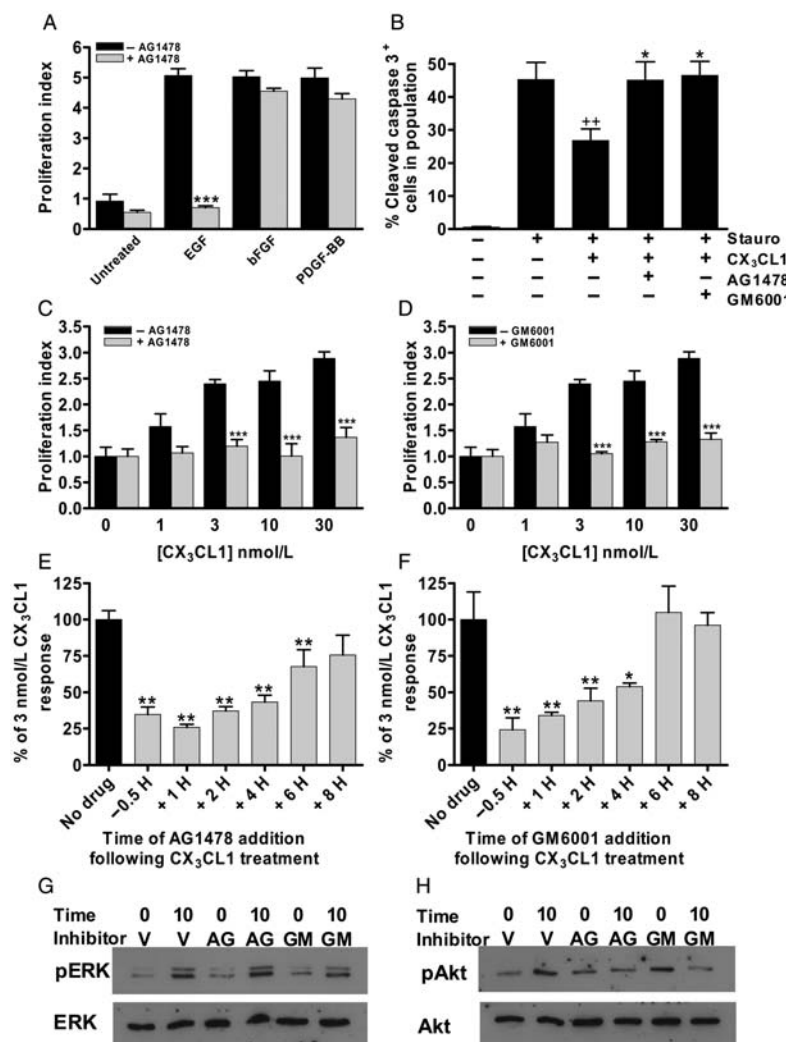


Figure 4 The mitogenic and anti-apoptotic effects of CX₃CL1 are mediated via EGFR activation. (A) CASMC were serum-starved for 48 h, then pre-treated for 30 min with AG1478 (1 μ M) prior to the addition of EGF (100 pmol/L), FGF (100 pmol/L), or PDGF-BB (1 nmol/L) and DNA synthesis quantified. (B) CASMC were pre-treated for 30 min with AG1478, GM6001 (10 μ M), or vehicle (DMSO), then treated for 30 min \pm 30 nmol/L CX₃CL1 before the addition of 0.5 μ M staurosporine for 6 h. Cleaved caspase 3 staining was performed as in Figure 1A. (C and D) CASMC were treated with AG1478, GM6001, or DMSO as in (B) prior to the addition of CX₃CL1 for 24 h and quantification of DNA synthesis. (E and F) CASMC were treated with AG1478 for the indicated times either prior to or following CX₃CL1 addition. Data shown as percent of 3 nmol/L CX₃CL1 response. (G) Serum-starved CASMC were pre-treated with or without vehicle (V, DMSO), AG1478 (AG; 1 μ M), or GM6001 (GM; 10 μ M) for 1 h, prior to the addition of 30 nmol/L CX₃CL1 for the indicated times. Cell lysates were prepared and western blotted for pERK and total ERK2 expression. (H) Cells were treated as in (G) and western blotted for pAkt and total Akt. All data shown as mean \pm SEM. Data analysed with one-way ANOVA and Dunnett's or Bonferroni's multiple comparison *post hoc* test. * P < 0.05, ** P < 0.01, *** P < 0.001 relative to CX₃CL1 treated. ++ P < 0.01 relative to staurosporine treated. (A–F) Data from two donors, three independent experiments. (G and H) Data are representative of four independent experiments from two donors.

abrogated DNA synthesis in response to CX₃CL1 (P < 0.001, Figure 4C and D, respectively) showing a requirement for EGFR activation in proliferation.

We then sought to establish the time-course of EGFR involvement in CX₃CL1 signalling. Primary CASMC were treated with AG1478 or GM6001 either 30 min prior to, or 0–8 h after the addition of CX₃CL1 and DNA synthesis measured. It was found that the addition of AG1478 could be delayed until 6 h after agonist addition and still have an inhibitory effect on DNA synthesis, whereas after 8 h this effect was lost

(Figure 4E). Similarly, GM6001 still inhibited DNA synthesis when added 4 h after CX₃CL1 treatment (Figure 4F). This implies that EGFR signalling occurs during the first 4–6 h of CX₃CL1 addition.

To confirm whether EGFR signalling was required to induce ERK and Akt activation, we used the EGFR inhibitor AG1478 and the metalloprotease inhibitor GM6001 in signalling assays. CX₃CL1 stimulated ERK phosphorylation within 10 min and this was unaffected by pre-treatment with either inhibitor (Figure 4G), indicating that ERK signalling occurs directly via CX₃CR1. In contrast, Akt

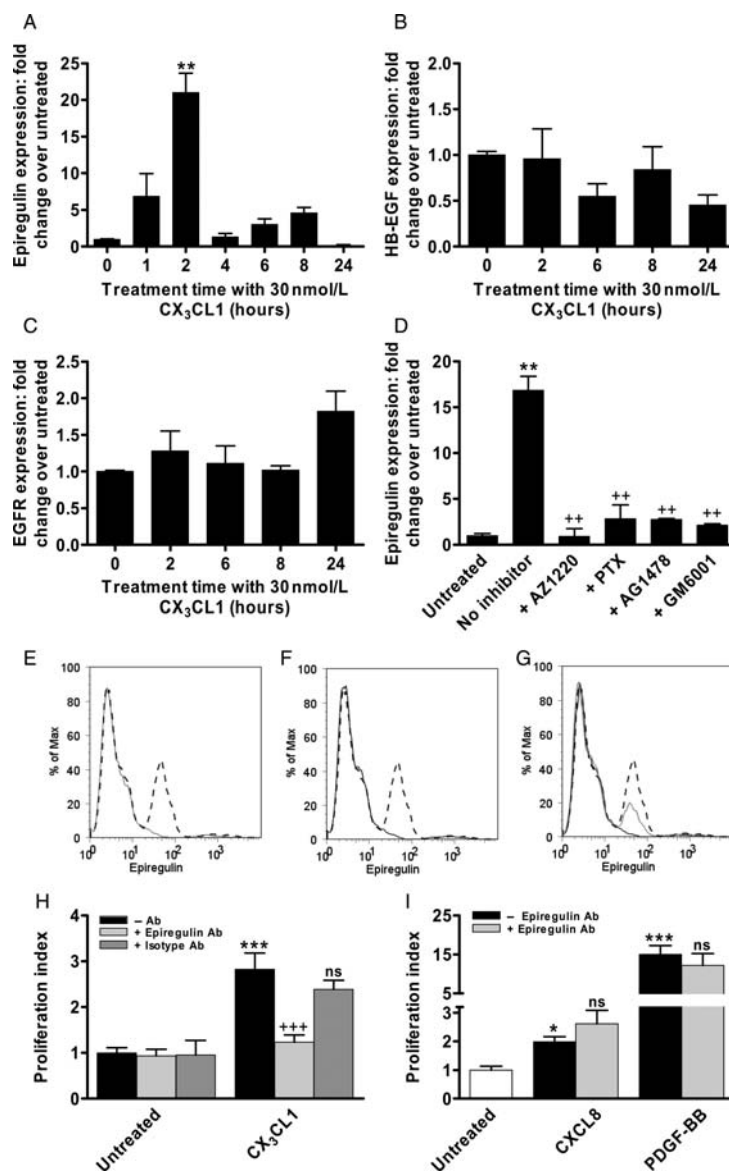


Figure 5 CX₃CL1 induces shedding of epiregulin within 10 min, leading to *de novo* synthesis of epiregulin mRNA and protein after 2 h. (A–C) Cells were serum-starved for 48 h prior to treatment with 30 nmol/L CX₃CL1 for the indicated times. Real-time SYBR green RT–PCR was performed for the indicated genes and the housekeeping gene GAPDH. Copy number for each gene was quantified from a genomic DNA standard curve, and data normalized by dividing by copy number of GAPDH. Data are expressed as fold change over untreated. (D) Cells were pre-treated for 1 h with AG1478 (1 μmol/L), PTX (250 ng/mL), GM6001 (10 μmol/L), or AZ12201182 (500 nmol/L) prior to the addition of CX₃CL1 for 2 h and mRNA quantitation of epiregulin as in (C). (E) CASMC were stained with anti-epiregulin antibody (dashed black line) or isotype control (solid grey line) and analysed by flow cytometry. (F) CASMC were treated with vehicle (dashed black line) or 200 nmol/L PMA (solid black line) for 30 min prior to staining with anti-epiregulin antibody by flow cytometry. (G) CASMC were treated with vehicle (dashed black line) or 30 nmol/L CX₃CL1 for 10 min (solid black line) or 2 h (solid grey line) and analysed for epiregulin expression as in (F). (H) CASMC were pre-treated for 1 h with anti-epiregulin antibody or isotype control (20 μg/mL) prior to the addition of CX₃CL1 (30 nmol/L) and quantitation of DNA synthesis. (I) CASMC were pre-treated with antibody as in (H) prior to the addition of PDGF-BB (1 nmol/L) or CXCL8 (30 nmol/L) and quantitation of DNA synthesis. All data shown as mean ± SEM. Data analysed by one-way ANOVA and Dunnett's or Tukey's *post hoc* test. ****P* < 0.001, ***P* < 0.01 relative to untreated, +++*P* < 0.001, ++*P* < 0.01, ns is not significant relative to CX₃CL1 treated. (A–C) Data from three donors, four independent experiments. (D) Data from two independent experiments. (E–G) Data are representative of two independent experiments, three flasks per treatment in each experiment. (H) Data from three independent experiments. (I) Data from two independent experiments.

signalling in response to CX₃CL1 was blocked by both inhibitors at the 10 min time-point (Figure 4H), indicating that Akt is activated via the EGFR.

3.6 CX₃CL1 induces synthesis of the extracellular EGFR ligand epiregulin

Since the broad-spectrum metalloprotease inhibitor GM6001 was able to block CX₃CL1-induced DNA synthesis and anti-apoptosis, we hypothesized that CX₃CL1 signalling involved release of an extracellular EGFR ligand within the first 6 h of CX₃CL1 treatment. Using real-time SYBR green PCR, we analysed the expression of the EGFR ligands epiregulin, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, TGF α , and EGF over a time-course of CX₃CL1 treatment. CX₃CL1 induced a 20-fold up-regulation in epiregulin mRNA within 2 h ($P < 0.01$, Figure 5A), but had no effect on mRNA expression of HB-EGF or other EGFR ligands (Figure 5B and data not shown). In addition, levels of the EGFR itself were unaffected by CX₃CL1 treatment over the time-course analysed (Figure 5C).

We next investigated the pathways required for the observed upregulation in epiregulin mRNA. Pre-treatment with the CX₃CR1 inhibitor AZ12201182 or pertussis toxin abrogated the induction of epiregulin by CX₃CL1 at the 2 h time-point, confirming the specificity of the response ($P < 0.01$, Figure 5D). In addition, treatment with both AG1478 and GM6001 completely blocked the increase in epiregulin mRNA, indicating that EGFR activation via the extracellular pathway is required for this effect ($P < 0.01$, Figure 5D).

To confirm whether epiregulin protein expression was affected by CX₃CL1 treatment, we performed flow cytometry to detect cell-associated epiregulin at early (10 min) and later (2 h) time-points after CX₃CL1 treatment. Untreated cells showed substantial epiregulin expression compared with the isotype control, though not all cells within the population expressed epiregulin (Figure 5E, dashed line). To confirm whether epiregulin shedding could be detected by flow cytometry, we treated cells with 200 nmol/L PMA, which is a broad-spectrum activator of metalloproteases and previously shown to induce epiregulin shedding.¹⁹ Treatment with PMA resulted in loss of epiregulin expression within 30 min (Figure 5F, solid line), indicating cleavage and release of epiregulin from the cell. Similarly, CX₃CL1 treatment for 10 min resulted in loss of epiregulin expression detectable by flow cytometry (Figure 5G, solid black line), indicating a rapid shedding from the cell in response to CX₃CL1. After 2 h CX₃CL1 treatment, there was a re-appearance of epiregulin expression (Figure 5G, solid grey line), indicating *de novo* synthesis of epiregulin protein, confirming our findings at the mRNA level. These data show that CX₃CL1 induces both the rapid shedding of epiregulin and regulates epiregulin expression over a longer time-course.

To confirm whether epiregulin shedding is essential for the mitogenic effect of CX₃CL1, we used a neutralizing antibody against CX₃CL1 in the tritiated thymidine incorporation assay. Anti-epiregulin antibody but not isotype control abrogated the induction of DNA synthesis by CX₃CL1 ($P < 0.001$, Figure 5H). DNA synthesis in response to PDGF-BB and CXCL8 were unaffected by the addition of anti-epiregulin antibody, confirming the specificity of the blockade (Figure 5I). Thus, CX₃CL1 induces

shedding of epiregulin leading to EGFR activation and proliferation of CSMC.

4. Discussion

We have demonstrated for the first time that CX₃CL1 has proliferative and anti-apoptotic effects on primary human SMCs which are mediated via the EGFR. Furthermore, we identify critical roles for ERK and PI3K signalling and identify the EGFR ligand epiregulin as an autocrine/paracrine growth factor regulated by CX₃CL1.

4.1 Apoptosis

The novel finding that CX₃CL1 is a survival factor for VSMCs has important implications for the development of vascular disease and supports recent data showing a role for CX₃CL1 in murine monocyte survival. Landsman *et al.*²⁰ demonstrated that deletion of either CX₃CL1 or CX₃CR1 results in a significant reduction of circulating monocytes in mice: particularly those of the Gr1^{low} 'resident' subset. When fed a high fat diet, these mice develop smaller lesions which contain more apoptotic cells than those in wild-type mice: implying a direct and pro-atherogenic effect of CX₃CL1 on monocyte survival.

In this study, CX₃CL1-induced Akt phosphorylation and the activity of PI3K were shown to be essential for blockade of apoptosis. In contrast, ERK signalling was not associated with the anti-apoptotic effects of CX₃CL1. Our data are in agreement with those of Vantler *et al.*²¹ who systematically demonstrated that only PI3K signalling is required for the anti-apoptotic effects of growth factors on SMCs. The downstream mechanism by which CX₃CL1 blocks apoptosis is still unknown, but could involve rapid induction of anti-apoptotic proteins. Indeed, blockade of staurosporine-induced apoptosis by EGF in an esophageal carcinoma cell line was shown to require induction of the Bcl-2 family member Mcl-1.²²

4.2 Proliferation

Using assays to measure *de novo* DNA synthesis, Ki67 expression, and cell counting, CX₃CL1 (30 nmol/L) was shown to have mitogenic activity equivalent to that obtained with 1 nmol/L PDGF-BB: a potent SMC mitogen. To our knowledge, only a few chemokines have been shown to have a role in human SMC proliferation, including CCL2 (MCP-1) and CXCL8 (IL-8; Figure 2F).^{23,24} Thus, CX₃CL1 joins a select few chemokines which have this property, suggesting it has a key role in pathologies involving SMC proliferation. In preliminary experiments, we have also shown that CX₃CL1 induces DNA synthesis in saphenous vein SMCs, implicating CX₃CL1 as a mitogen for SMCs of both venous and arterial origin (data not shown). The dose of CX₃CL1 used in this study is in line with other published studies investigating functional effects of chemokines on SMCs and corresponds to the dose used to obtain peak SMC chemotaxis in a previous publication from our laboratory.^{8,18,24}

In contrast to anti-apoptotic effects, the mitogenic function of CX₃CL1 depends on both ERK and PI3K activation since both the MEK inhibitor U0126 and the PI3K inhibitor LY294,002 are able to block DNA synthesis. However, our signalling assays

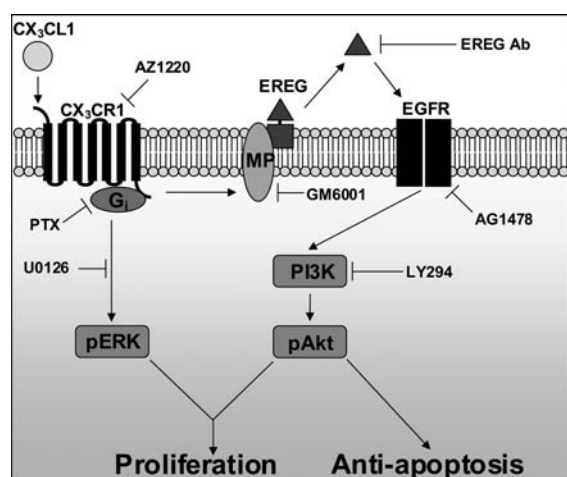


Figure 6 Proposed signalling mechanism downstream of CX₃CR1 in CASC: proliferation, but not anti-apoptosis, requires both ERK and PI3K activation. The effects of CX₃CL1 in primary human CASC are mediated entirely via the G_{αi}-coupled receptor CX₃CR1 (Figures 1F, 2E and 3C and E). Once activated, CX₃CR1 induces phosphorylation of ERK which is blocked by the MEK inhibitor U0126 (Figure 3A). Activation of ERK does not involve the EGFR (Figure 4G), but is essential for proliferation (Figure 3D). CX₃CR1 activation generates an additional signal leading to metalloproteinase activation and release of the EGFR ligand epiregulin from the cell (Figure 5G) and this is blocked by the inhibitor GM6001. Epiregulin activates the EGFR leading to PI3K activation and subsequent Akt phosphorylation (Figures 3B and 4H). EGFR activation can be blocked with the kinase inhibitor AG1478 and with a neutralizing epiregulin antibody. PI3K activation via the EGFR, but not ERK activation, is essential for the anti-apoptotic effect of CX₃CL1 (Figures 3C and 4B). PI3K activation is also required for proliferation and without the dual activation of both ERK and PI3K pathways, proliferation will not proceed (Figures 3D, 4C and D and 5H). Abbreviations: AZ1220=AZ12201182, PTX=pertussis toxin, EREG=epiregulin, LY294=LY294,002, MP=metalloproteinase. Arrow indicates activation and flat-ended arrow indicates inhibition.

clearly demonstrate that ERK and PI3K exist in separate pathways: ERK is directly downstream of CX₃CR1, while PI3K activation is dependent on epiregulin acting via the EGFR (Figure 6). Our data are consistent only with a model whereby dual activation of both ERK and PI3K is essential for proliferation to proceed, whereas anti-apoptosis does not require ERK activation.

4.3 EGFR involvement

Previous studies have demonstrated the activation of both ERK and Akt in response to CX₃CL1 in other cell types.²⁵ However, this is the first time that EGFR activity has been shown to be involved in CX₃CR1 signalling and essential for both mitogenic and anti-apoptotic activity. The EGFR has been implicated in the proliferation of several transformed cell lines in response to chemokines, and is involved in the regulation of matrix metalloproteinase

expression in primary SMCs by CCL11 (eotaxin) and CXCL12 (SDF-1α).¹⁸ We have shown that CX₃CL1 induces rapid shedding of the EGFR ligand epiregulin and subsequently induces further epiregulin synthesis via EGFR activation. Epiregulin is a potent mitogen for rat aortic SMCs and is induced by the GPCR agonists endothelin-1, angiotensin II, and thrombin.²⁶ Furthermore, epiregulin is an autocrine/paracrine de-differentiation factor for SMCs and is expressed in human atherosclerotic plaques and balloon-injured rat arteries, but not in normal vessels.²⁷ Intriguingly, PMA-induced epiregulin shedding in mouse embryonic fibroblasts is dependent on ADAM17 (TACE),¹⁹ the metalloproteinase required for CX₃CL1 cleavage from SMCs.⁶ Our work identifies a mechanism by which CX₃CL1 signalling may be amplified to induce SMC proliferation and survival and contribute to vascular pathology.

4.4 CX₃CL1 as a therapeutic target

We show for the first time that CX₃CR1 activation can be blocked by the novel and specific antagonist AZ12201182. This compound was found to inhibit the pro-survival and mitogenic effects of CX₃CL1, and block the induction of epiregulin mRNA in response to CX₃CL1. This compound may prove to be a useful tool for further analysis of CX₃CR1 function, and suggests the possibility of therapeutic CX₃CR1 blockade by small molecule inhibitors.

In conclusion, we provide evidence of a novel function for CX₃CL1 in human SMC anti-apoptosis and proliferation, via a mechanism involving ERK, PI3K, Akt, and the EGFR. This has important implications in vascular pathologies, including atherosclerosis, restenosis, and transplant accelerated arteriosclerosis, where the balance of SMC proliferation and apoptosis critically determines both plaque stability and vessel stenosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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