Chemokines induce matrix metalloproteinase-2 through activation of epidermal growth factor receptor in arterial smooth muscle cells

Ravindra Kodali, Mustapha Hajjou, Adriane B. Berman, Meena B. Bansal, Shihong Zhang, Jiang Jin Pan, Alison D. Schecter

Abstract

Objective: Matrix metalloproteinases (MMP) are critical to smooth muscle cell (SMC) migration in vivo. MMP-2 dysregulation has been implicated in the pathogenesis of abnormal arterial remodeling, aneurysm formation, and atherosclerotic plaque structure and stability. The chemokine receptors CCR3 and CXCR4 are present and functional on SMC and are up-regulated in vascular diseases such as atherosclerosis. We sought to determine a potential mechanism for chemokine receptor-mediated effects on the vasculature by asking whether the chemokines eotaxin (CCL11), the ligand for CCR3, and stromal cell-derived cell factor (SDF-1, CXCL12), the ligand for CXCR4, induce MMP-2 in SMC. Studies were then performed to define the signaling pathways involved.

Methods and results: As determined by RT-PCR, Western blotting and zymography, SDF-1 and eotaxin induce MMP-2 mRNA, protein, and activity in SMC. An anti-CCR3 antibody and a CXCR4 antagonist blocked proMMP-2 induction by SDF-1 and eotaxin, the respective ligands for the chemokine receptors CXCR4 and CCR3, suggesting that the inductions by these chemokines are receptor-mediated. Receptor cross-talk between G-protein-coupled receptors (GPCR) and the epidermal growth factor receptor (EGFR) is a method of expanding the GPCRs’ signaling repertoire. We demonstrate, for the first time to our knowledge, that in SMC, chemokine induction of proMMP-2 is dependent on activation of the EGFR. Interestingly, by blocking the ligand binding domain of EGFR, we demonstrate that activation of EGFR by SDF-1 and eotaxin occurs through different cellular pathways.

Conclusion: The pro-inflammatory chemokines eotaxin and SDF induce proMMP-2 activation of EGFR through two different pathways. SDF and eotaxin, as regulators of proMMP-2 expression and by engaging in receptor cross-talk, may play critical roles in atherosclerosis, restenosis, and plaque rupture. These ligands and their respective receptors, CXCR4 and CCR3, therefore may serve as future potential therapeutic targets.

Keywords: Atherosclerosis; Cytokines; Matrix metalloproteinases

1. Introduction

Vascular smooth muscle cells (SMC) constitute the predominant cellular type of the arterial wall. The regulation of SMC migration, proliferation, and its response to proinflammatory signals are important in the pathogenesis of atherosclerosis and vascular remodeling. We [1] and others [2,3] have demonstrated previously that functional...
chemokine receptors are present on vascular SMC. Upon activation, SMC “switch” from a predominantly contractile to a synthetic secretory phenotype [4]. By secreting various chemokines and cytokines, activated SMC can both recruit macrophages and lymphocytes into the vessel wall as well as respond to these proteins in an autocrine fashion.

In the vasculature, matrix metalloproteinases (MMP) have been implicated in geometric remodeling [5], plaque formation [6], and restenosis [5], by facilitating the migration of vascular SMC through the internal elastic lamina into the intimal space, where they can proliferate and contribute to plaque formation. In addition, there is increasing evidence that acute coronary and cerebrovascular disease and a primary role of proMMP-2 in the disease as well. In patients with abdominal aneurysms, even in the vasculature remote from the aorta, elevation of proMMP-2 is detected, suggesting a systemic nature of aneurysmal disease and a primary role of proMMP-2 in the disease process [15]. Therefore, the regulation of proMMP-2 levels by chemokines is highly physiologically relevant.

Both chemokines and MMP have been localized to atherosclerotic plaques and SMC of injured vessels. In particular, the CC and CXC chemokines, eotaxin (CCL11) and stromal-derived cell factor (SDF-1, (CXCL12)), have been identified and associated with SMC in the plaque [16–18]. Eotaxin, in particular, is upregulated in SMC following vascular injury [19]. We have previously reported the receptors for eotaxin, namely, CCR3, and for SDF-1, CXCR4, to be present and functional on SMC.

There is a paucity of data about the relationship between chemokines and MMP in arterial SMC and the potential mechanism(s) of MMP-2 regulation in these cells. We therefore performed the following studies to determine whether the chemokines, eotaxin and SDF-1 induce MMP-2 and explored the relevant signaling pathways.

2. Methods

2.1. Cell culture and reagents

Human aortic SMC were either obtained from ATCC (Manassas, VA) or isolated from human thoracic aortas and cultured as previously described [20]. Murine [21] and rat [22] aortic SMC were prepared and cultured as described. Carrier-free recombinant human and mouse eotaxin and SDF-1 were purchased from Peprotech (Rocky Hill, NJ). Anti-MMP-2 antibody (Ab-3), AG1478, PD153035, U0126, LY294002 and the HB-EGF inhibitor, CRM-197, were from Calbiochem (La Jolla, CA). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). EGFR Ab, phospho-ERK1/2 (Thy 202/ Tyr 204) Ab and phospho-Akt (Ser 473) Ab were obtained from Cell Signaling (Beverly, MA). Anti-phosphotyrosine antibody (4G10) was purchased from Upstate (Charlottesville, VA). Dominant negative EGFR (CD533) and control (pRK5) vectors were the generous gifts of Dr. Liliana Ossowski, Mount Sinai School of Medicine. Vector pEGFP-N3 was purchased from Clonetech (Mountain View, CA). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki.

2.2. Transfection of SMC

All the transfection experiments were carried out using rat SMC, as the transfection efficiency was higher compared to human or mouse SMC. Rat SMC were grown to 80% confluence in 35 mm plates. SMC were transfected with plasmid (CD533) or control vector (pRK5) using “FuGENE 6 Transfection Reagent” following manufacturer’s protocol. Transfection efficiency was 30% as verified by FACS analysis using pEGFP-N3 vector and pRK5 control vectors. After 24 h transfection, cells were starved in serum free medium for 24 h before incubating with chemokines for 24 h. Conditioned media (CM) were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using an anti-human MMP-2 antibody.

2.3. Real-time PCR analysis for MMP-2 mRNA

Total RNA was extracted from serum-starved control and chemokine treated human or mouse SMC using Qiagen kit with on-column Dnase I treatment. One microgram of total RNA was used as template to make first strand cDNA by random priming using Promega RT System. The following primers were used: MMP-2 forward (for mouse): 5'-ACC CAG ATG TGG CCA ACT AC-3', MMP-2 reverse: 5'-TAC TTT TAA GGC CCG AGC AA-3'; MMP-2 forward (for human): 5'-CAAGTTTCCATTCCGCTTC-3', MMP-2 reverse: 5'-GTTCCCCACCAACAGTGGACA-3'. Duplicate real-time quantitative PCR was performed by monitoring
the increase in fluorescence of the SYBR Green dye using iCycler iQ (BioRad, Hercules, CA) according to the manufacturer’s instructions. The relative expression level of MMP-2 in stimulated cells was plotted as fold change compared with untreated cells. All measurements were done in triplicate. $2^{-\Delta\Delta C_T}$ method was used to determine relative changes in gene expression. RNA derived from human and mouse SMC had similar levels of MMP-2 induction.

2.4. Immunoprecipitation and Western blotting

SMC were cultured for 48 h in serum-free medium before treatment with carrier-free chemokines. For immunoprecipitations, SMC were treated for 2 min with either eotaxin (100 ng/ml) or SDF-1 (0.75 μg/ml) prior to lysis with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM Sodium fluoride, Protease inhibitors). EGFR antibody was incubated with 400 μg of proteins overnight and with protein A/G-plus agarose beads for 3 h. Beads were pelleted, washed, boiled in Laemmli buffer, and subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with a monoclonal antibody to phosphotyrosine (4G10, 1:2000). For ERK1/2 and Akt phosphorylation experiments, protein lysates were analyzed by Western blotting with monoclonal antibodies to ERK1/2, phospho-ERK1/2 (Thr 202/Tyr 204), Akt and phospho-Akt (Ser 473), respectively, and then detected by chemiluminescence detection (Pierce). For blocking experiments, SMC were pretreated, for 30 min to one h, with blocking agents and then treated with chemokines. For blocking experiments involving pertussis toxin (PTX), cells were pretreated with PTX (200 ng/ml) for 3 and 16 h, in separate experiments. Cell viability (>95%) after 16 h was assessed by Trypan Blue exclusion.

2.5. Gelatin zymography

SMC cultured in serum-free media for 48 h, were treated with bovine serum albumen (BSA) free chemokines for 24 h. Control cultures were treated with equivalent amounts of DMEM alone. MMP activity in CM was determined by zymography as described previously [23].

2.6. Statistical analysis

Experiments were performed at least 3 times and values presented as the mean ± SD. Student’s $t$ test was used in the analysis of paired and unpaired means. Unless indicated, all values were significant at $p < 0.05$.

3. Results

3.1. Eotaxin and SDF-1 induce proMMP-2 in cultured SMC

Both eotaxin and SDF-1 have been detected by immunohistochemistry in the SMC rich regions of atherosclerotic lesions [16,17] but not in normal vessels. Given the critical role of MMP-2 in SMC biology, we examined whether these chemokines can regulate MMP-2 expression. Human (primary cultures and from ATCC) and murine SMC were

![Fig. 1. SDF-1 and eotaxin induce proMMP-2. Human SMC cultured in serum-free media for 48 h were treated for 24 h with either SDF-1 (0.75 μg/ml) or eotaxin (100 ng/ml). Conditioned medium (CM), normalized to cell number, were analyzed using gelatin zymography (A) or subjected to immunoblotting using an anti-MMP-2 antibody (B). Graph (C) represents the fold change of MMP-2 mRNA following 3 h treatment of human SMC with eotaxin (100 ng/ml) (**$p<0.001$) and SDF (750 μg/ml) (*$p<0.01$). Depicted results are average of five independent experiments using both human and murine SMC with similar results. Error bars represent standard deviation (SE). No differences were observed between murine and human SMC.](https://academic.oup.com/cardiovascres/article/69/3/706/273512/fig1)
treated with either eotaxin or SDF-1 at the indicated concentrations after being cultured in serum-free medium for 48 h and MMP-2 levels were assessed by Western blot and zymography. Both chemokines increased proMMP-2 activity (Fig. 1A) and protein levels (Fig. 1). The maximum induction of proMMP-2 was obtained with SDF-1 at 0.75 μg/ml and eotaxin at 100 ng/ml (Fig. 1B). ProMMP-2 protein was detectable at 24 h post treatment (data not shown). To determine whether chemokines affect MMP-2 mRNA levels, real-time PCR with primers for MMP-2 was performed. Both eotaxin and SDF-1 increased MMP-2 mRNA levels, in both human and mouse SMC (Fig. 1C), suggesting that these chemokines affect either transcription of MMP-2 or its mRNA stability in SMC. To determine whether the induction of proMMP-2 by eotaxin and SDF-1 occurred through their respective receptors, CCR3 and CXCR4, SMC were treated with either an anti-CCR3 antibody or AMD3100, a small peptide inhibitor of CXCR4. Treatment of murine SMC with two different concentrations of anti-CCR3 antibody (5 and 10 μg/ml) prior to eotaxin treatment resulted in a significant and incremental reduction of proMMP-2 protein (Fig. 2A). Treatment with equal concentrations of an irrelevant isotype-matched IgG had no effect. Treatment of human SMC with AMD3100, a selective and highly specific CXCR4 antagonist [24] (10 μM) prior to SDF-1 decreased proMMP-2 protein levels (Fig. 2B). Treatment of SMC with either AMD alone (Fig. 2B) or CCR3 Ab alone (data not shown) had no effect on proMMP-2 induction. These results suggest that the induction of proMMP-2 by eotaxin and SDF-1 is the result of chemokine binding to their respective receptors. All experiments were performed with both human and murine SMC. There were no significant differences in between species.

3.2. Induction of proMMP-2 protein by eotaxin and SDF-1 is \( \text{G}_{\alpha_i} \)-dependent

CCR3 and CXCR4 belong to the family of seven transmembrane spanning heterotrimeric G-protein-coupled receptors (GPCRs). Treatment of quiescent murine and human (ATCC) SMC with pertussis toxin, an inhibitor of the \( \text{G}_{\alpha_i} \) subunit, prior to chemokine exposure, decreased the expression of proMMP-2 (Fig. 2C), suggesting that the proMMP-2 induction by eotaxin and SDF-1 is dependent, in part, on \( \text{G}_{\alpha_i} \)-mediated signaling. Similar results were obtained regardless of the length (3 or 16 h) of pertussis toxin pretreatment. Cell viability after 16 h was >95%, as determined by Trypan Blue exclusion.

3.3. SDF and eotaxin differentially activate the epidermal growth factor receptor

Activation of the EGFR is important in the regulation of SMC functions such as growth, proliferation, and response to injury [25]. Other GPCRs have been shown to activate EGFR [26]. To determine whether SDF-1 or eotaxin activate the EGFR, SMC were treated with each respective chemokine, and then assessed for phosphorylation of the EGFR by immunoprecipitation with an anti-EGFR antibody followed by immunoblotting with an anti-phosphotyrosine antibody. Both eotaxin and SDF-1 caused an increase in tyrosine phosphorylation of the EGFR (Fig. 3A) that was detectable at 2 min. To examine whether EGFR kinase activity was necessary for proMMP-2 induction, murine and human SMC were pretreated with AG1478 (Fig. 3B) and PD153035 (Fig. 3C), two specific EGFR kinase inhibitors.
Fig. 3. SDF-1 and eotaxin induce proMMP-2 protein through EGFR phosphorylation. (A) SDF-1 and eotaxin transactivate EGFR. Serum-deprived murine SMC were treated with either SDF-1 (0.75 μg/ml) or eotaxin (100 ng/ml) for 2 min. Cell lysates subsequently were immunoprecipitated with anti-EGFR antibody and immunoblotted with anti-phosphotyrosine antibody (4G10). Membranes were stripped and immunoblotted with anti-EGFR antibody. In (A), the lane labeled control (C), represents baseline phosphorylation state of untreated, quiescent SMC and the lane labeled DMEM represents SMC treated with DMEM alone for 2 min. (B and C) Inhibition of EGFR kinase blocks proMMP-2 induction by SDF-1 and eotaxin. Serum-starved murine SMC were pretreated for 30 min with AG1478 [(B) 1 μM] and PD153035 [(C) 5 μM], prior to treatment with SDF-1 or eotaxin. All immunoblots were treated with an anti-MMP-2 antibody. (D) Graph represents densitometric analysis of (C).

Fig. 4. SDF-1 and eotaxin induce proMMP-2 through differential transactivation of EGFR. (A) Role of HB-EGF and external EGFR binding. Serum-deprived murine and human SMC were pretreated for 1 h with CRM-197 (100 ng/ml) prior to stimulation with eotaxin (100 ng/ml) or SDF-1 (0.75 μg/ml). Following 24 h incubation, conditioned media were analyzed by SDS-PAGE and immunoblotting. (B) and (C) Examination of the role of the extracellular EGFR domain in MMP-2 induction. Serum-starved murine SMC were treated for 30 min with neutralizing anti-EGFR antibody (B) or transfected with control vector pRK5 (C; rows 1 – 3) or dominant negative (DN) EGFR CD533 (C; rows 4 – 6) before treatment with either SDF-1 (0.75 μg/ml) or eotaxin (100 ng/ml). CM were analyzed by immunoblotting with an anti-MMP-2 antibody. (D) and (E) are graphs derived from densitometric analysis of (B) and (C), respectively.
Both inhibitors blocked proMMP-2 induction by SDF-1 and eotaxin suggesting that signaling through EGFR is involved in proMMP-2 induction by CCR3 and CXCR4. There were no differences detected in findings between murine and human SMC.

It has been recently established that EGFR activation can proceed through either ligand-dependent or ligand-independent mechanisms (reviewed in Ref. [25]). To examine the mechanism(s) through which eotaxin and SDF-1 induce EGFR activation, murine and human SMC were pretreated with either a ligand blocking anti-EGFR antibody, or with CRM-197 (100 ng/ml), which specifically prevents binding of HB-EGF to the EGFR. CRM-197 had no effect on eotaxin induction of proMMP-2, but completely blocked the induction of proMMP-2 by SDF-1 (Fig. 4A). Pretreatment of SMC with the anti-EGFR antibody significantly reduced the level of proMMP-2 induction by SDF-1, but had no effect on the induction of proMMP-2 by eotaxin (Fig. 4B). An irrelevant isotype matched antibody had no effect. These results demonstrate that the activation of EGFR and the subsequent induction of proMMP-2 by SDF-1 proceed via an extracellular ligand-dependent EGFR pathway whereas the activation of EGFR by eotaxin occurs in a ligand-independent manner. To confirm the involvement of EGFR activation in proMMP-2 induction, we transfected rat SMC with control vector pRK5 (Fig. 4C lanes 1–3) or dominant negative (DN) EGFR CD533 [27] (Fig. 4C lanes 4–6). There was no induction of proMMP-2 by either eotaxin or SDF in dominant negative (DN) EGFR transfected cells. In contrast, SMC transfected with control vector had a robust chemokine induction of proMMP-2 levels. Densitometric analyses of the antibody and dominant negative experiments are depicted in Fig. 4D and E.

3.4. Activation of Akt, PI3 kinase and ERK1/2 MAPK pathways are necessary for the eotaxin and SDF inductions of proMMP-2

Downstream effectors of active EGFR are the activated MAPK and Akt pathways. These cascades couple EGFR activation to gene transcription. Given the differences we found in the modes of EGFR activation by CCR3 and

![Fig. 5. ERK 1/2, PI3-kinase and Akt Activation are involved in chemokine induction of proMMP-2. Quiescent murine SMC were stimulated for the indicated times with SDF-1 (0.75 μg/ml) or eotaxin (100 ng/ml). Cells were lysed directly into Laemmli buffer and subjected to immunoblotting using either an anti-phospho-ERK1/2 antibody (A) or an anti-phospho-Akt antibody (C). The membranes were stripped and blotted with anti-ERK1/2 antibody or Akt antibody, respectively. (B) Serum starved murine SMC were pretreated with U0126 for 30 min before treating with eotaxin and SDF. CM were immunoblotted with an anti-MMP-2 antibody. (D) Inhibition of PI3-kinase blocks proMMP-2 induction by SDF-1 and eotaxin. Serum-starved murine SMC were pretreated for 30 min with LY294002 (5 μM) or Wortmannin (1 μM) prior to chemokine treatment. Conditioned media were analyzed by immunoblotting with an anti-MMP-2 antibody. (E) Serum-deprived murine SMC were incubated with Wortmannin (1 μM) for 30 min then treated with either SDF-1 (0.75 μg/ml) or eotaxin (100 ng/ml) for 1 min. Cell lysates subsequently were immunoprecipitated with anti-EGFR antibody and immunoblotted with anti-phosphotyrosine antibody (4G10). Membranes were then stripped and immunoblotted with anti-EGFR antibody. Graph (F) represents densitometric analysis of (B). Results are representative of three independent experiments using murine SMC. Similar results were observed with human SMC.

CXCR4, we wanted to determine whether there were differences in the signaling pathways downstream of the EGFR such as Akt and extracellular signal-regulated kinases 1 and 2 (ERK1/2). Western blots were performed with antibodies to phospho and total ERK1/2. Both chemokines caused a time-dependent increase in phosphorylated ERK1/2 (Fig. 5A). Total ERK1/2 levels were not affected. To confirm that MAPK was necessary for proMMP-2 induction via chemokines, SMC [human (ATCC) and murine] were treated with UO126 (5 μM), an inhibitor of the ERK1/2 phosphorylation. UO126 inhibited proMMP-2 induction by both eotaxin and SDF-1 (Fig. 5B,F).

Another downstream pathway linked to EGFR activation is the Akt pathway [28]. In parallel experiments as those described above for ERK1/2, lysates from SMC [human (ATCC) and murine] treated with either eotaxin or SDF-1 were analyzed by Western blots with antibodies to phospho- and total Akt. Both eotaxin and SDF-1 resulted in an increase in the phosphorylated Akt species at 1 min. Total Akt levels were not affected. To determine that Akt activation was EGFR dependent, lysates from DN-EGFR and empty vector transfected rat SMC following eotaxin and SDF exposure were assessed for an increase in Akt phosphorylation levels by Western blot. Inhibition of EGFR, by expression of the DN-EGFR, inhibited Akt phosphorylation (Fig. 5C), whereas transfection with control vector did not. To determine PI3-kinase involvement in proMMP-2 induction SMC [human (ATCC) and murine] were treated with either transforming growth factor-β, platelet-derived growth factor, or basic fibroblast growth factor. There may be several reasons for the discrepancy between the cited studies and the present one. First, the aforementioned studies use different sources of SMC, the former [23] studied venous, while the latter [29] examined rabbit SMC. Another possibility is that, in SMC, the chemokine regulatory effects on proMMP-2 are unique.

The present study shows that the CC and CXC chemokines, eotaxin and SDF-1, induce a concentration and time dependent increase in proMMP-2 protein in both human and murine arterial SMC. Using quantitative PCR, SDF and eotaxin cause an increase in MMP-2 mRNA levels in SMC [23]. Similarly, Fabunmi et al. [29] reported no increase of proMMP-2 mRNA or protein levels in SMC following treatment with either transforming growth factor-β, platelet-derived growth factor, or basic fibroblast growth factor. There may be several reasons for the discrepancy between the cited studies and the present one. First, the aforementioned studies use different sources of SMC, the former [23] studied venous, while the latter [29] examined rabbit SMC. Another possibility is that, in SMC, the chemokine regulatory effects on proMMP-2 are unique.

Fig. 6 depicts a simplified signaling schematic, based on the series of experiments described above. These studies suggest that EGFR activation by these chemokines differ and although downstream effectors overlap, there is potential to intervene at more proximal junctures to inhibit chemokine induced proMMP-2 synthesis.

4. Discussion

This report describes the induction of proMMP-2 mRNA, protein, and activity in SMC by the proinflammatory chemokines, eotaxin and SDF-1. To our knowledge, this is the first report demonstrating that chemokines induce MMP-2 through an EGFR-dependent mechanism and, in particular, that CCR3 and CXCR4 activate EGFR through distinct cellular pathways. ProMMP-2 is expressed constitutively at low levels in SMC. A previous study, using interleukin-1 and tumor necrosis factor α, showed no effect on proMMP-2 mRNA levels in SMC [23]. Similarly, Fabunmi et al. [29] reported no increase of proMMP-2 mRNA or protein levels in SMC following treatment with either transforming growth factor-β, platelet-derived growth factor, or basic fibroblast growth factor. There may be several reasons for the discrepancy between the cited studies and the present one. First, the aforementioned studies use different sources of SMC, the former [23] studied venous, while the latter [29] examined rabbit SMC. Another possibility is that, in SMC, the chemokine regulatory effects on proMMP-2 are unique.

The present study shows that the CC and CXC chemokines, eotaxin and SDF-1, induce a concentration and time dependent increase in proMMP-2 protein in both human and murine arterial SMC. Using quantitative PCR, SDF and eotaxin cause an increase in MMP-2 mRNA levels, suggesting that the increase of proMMP-2 protein occurs at the transcriptional level. Although it has been shown that MMP-2 can be induced by overexpression of connective tissue growth factor [30] and mechanical stretch [31] in vascular SMC, there is no report of EGFR mediated regulation of MMP-2 levels.

Transactivation of EGFR has been shown to have important physiological consequences in SMC [25]. GPCRs such as the angiotensin II, thrombin, and endothelin-1 receptors are critical for vascular homeostasis [32,33]. EGFR transactivation plays an important role in their intracellular signaling [34–36]. Until recently, EGFR transactivation was thought to proceed solely by intracellular events. However, recent studies have indicated that EGFR activation also can occur as a result of matrix-dependent release of the membrane-tethered EGFR ligands, such as heparin-binding EGF-like growth factor (HB-EGF) [37]. Several recent studies have supported the importance of HB-EGF in SMC biology. Zhang et al. [38], studying the role of α1-adrenoceptor stimulation on SMC growth, reported that aortic SMC, isolated from HB-EGF−/− and wild-type mice, displayed significantly different growth phenotypes, supporting an important role for juxtacrine activation of the EGFR by

![Fig. 6. Schematic depiction of divergent signaling pathways involved in proMMP-2 induction by eotaxin and SDF. Eotaxin and SDF activate EGFR to induce MMP-2 through distinct mechanisms. Both chemokines activate PI3-kinase, ERK1/2 and Akt.](image-url)
HB-EGF in SMC. HB-EGF can also induce LOX-1 expression and play an integral role in foam cell transformation, cellular dysfunction, and proliferation of SMC [39]. Given the data supporting the physiologic importance of the mechanism of EGFR activation, we studied CCR3 and CXCR4-dependent activation of the EGFR. Our data demonstrate that not only is the activation of EGFR via CXCR4 and CCR3 integral for the induction of proMMP-2, since the inhibition of EGFR phosphorylation (by both DNN and EGFR kinase inhibitor) abolished the effect of both eotaxin and SDF on proMMP-2 protein levels, but also that the induction of proMMP-2 by SDF requires HB-EGF. The importance of ligand-mediated activation of the EGFR by CXCR4 was demonstrated by two methods. First, we showed a ligand-blocking anti-EGFR antibody blocked induction of proMMP-2 by SDF. Second, the HB-EGF inhibitor, CREM127, blocked SDF-1 but not eotaxin proMMP-2 induction. Together, these data support the conclusion that the binding of SDF to CXCR4 activates EGFR through a ligand-dependent process, whereas the induction of proMMP-2 protein by eotaxin is EGFR ligand-independent. 

In general, phosphorylation plays an essential role in kinase activation. The MAPK pathway, a critical downstream pathway of EGFR activation [40], is implicated in both chemokine receptor signaling and in the regulation of MMP-2 [41]. Our data show that, in SMC, proMMP-2 induction by SDF-1 and eotaxin depends on the activation of ERK1/2 MAPK. Both SDF-1 and eotaxin elicited a time-dependent increase in ERK1/2 phosphorylation and U0126, a MEK inhibitor, blocked the induction of proMMP-2 by both chemokines. The serine/threonine kinase Akt, also known as protein kinase B, is an anti-apoptotic protein through which various survival signals suppress cell death induced by growth factor withdrawal, cell cycle discordance, and detachment of cells from their extracellular matrix [42,43]. Akt can be activated by some stresses such as heat, hyperosmotic stress, and H2O2. It has previously been shown [28,44] that EGFR is involved in Akt activity. In this report, we demonstrate that Akt phosphorylation by both eotaxin and SDF-1 requires functional EGFR. These data suggest that, in SMC regulation of proMMP-2 levels by chemokines, EGFR plays a role in Akt activity as well.

Both of the chemokines, and their respective receptors, used in the present studies are highly expressed in the atherosclerotic plaque [17]. Eotaxin is located predominantly in SMC of the plaques [17] and SDF-1 is expressed on endothelial cells, SMC, and macrophages [16]. We have demonstrated that both CCR3 [19] and CXCR4 [45] are present and functional on arterial SMC. In addition, eotaxin is induced in the intima in response to injury [19]. Therefore, in the pathologic states where MMP-2 has been implicated, such as atherosclerosis and vascular remodeling, these chemokines may act in an autocrine fashion on SMC and contribute to these processes. Like most MMPs, MMP-2, secreted as an inactive proenzyme, requires proteolytic removal of a terminal propeptide domain for its activation. In particular, membrane type 1 MMP (MT1-MMP), a cell-surface associated MMP activator, is involved in this process. In the present study, the chemokines induced the proform of MMP-2 in SMC. However, MT1-MMP has been identified in the atherosclerotic plaque and associated with SMC [46]. These authors hypothesized that colocalization of MT1-MMP and proMMP-2 in SMC could play a critical role in normal vascular homeostasis and contribute to the pathologic remodeling observed in the atherosclerotic plaque. Other activators of proMMP-2 such as Factor Xa and integrin αvβ3 are also present on SMC in the atherosclerotic plaque [47–49] and could act as potential MMP-2 activators in vivo. Therefore, the induction of proMMP-2 in SMC in the plaque by chemokines, could be activated in a paracrine fashion by local MT1-MMP and Factor Xa.

SMC are an important source of proMMP-2. Given the co-localization of SDF-1, eotaxin, and their receptors in the SMC of atherosclerotic plaques and on activated SMC of injured vessels, the potential for autocrine activation is significant. To our knowledge, this is the first report demonstrating that proinflammatory chemokines, eotaxin and SDF-1, induce MMP-2 through differential activation of EGFR and involve HB-EGF. An improved understanding of the relationship between chemokines and the signaling pathways for proMMP-2 expression in SMC should provide new directions for the development of novel therapeutic interventions to prevent the pathologic vascular remodeling associated with atherosclerotic plaque growth, vulnerability, and intimal hyperplasia following vascular injury.

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