Thromboxane prostanoid receptor stimulation induces shedding of the transmembrane chemokine CX3CL1 yet enhances CX3CL1-dependent leukocyte adhesion

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Tole S, Durkan AM, Huang YW, Liu GY, Leung A, Jones LL, Taylor JA, Robinson LA. Thromboxane prostanoid receptor stimulation induces shedding of the transmembrane chemokine CX3CL1 yet enhances CX3CL1-dependent leukocyte adhesion. Am J Physiol Cell Physiol 298: C1469–C1480, 2010. First published March 17, 2010; doi:10.1152/ajpcell.00380.2009.—In atherosclerosis, chemokines recruit circulating mononuclear leukocytes to the vascular wall. A key factor is CX3CL1, a chemokine with soluble and transmembrane species that acts as both a chemoattractant and an adhesion molecule. Thromboxane A2 and its receptor, TP, are also critical to atherogenesis by promoting vascular inflammation and consequent leukocyte recruitment. We examined the effects of TP stimulation on processing and function of CX3CL1, using CX3CL1-expressing human ECV-304 cells and primary human vascular endothelial cells. TP agonists promoted rapid shedding of cell surface CX3CL1, which was inhibited by pharmacological inhibitors or specific small interfering RNA targeting tumor necrosis factor-α-converting enzyme (TACE). Because it reduced cell surface CX3CL1, we predicted that TP stimulation would inhibit adhesion of leukocytes expressing the CX3CL1 cognate receptor but, paradoxically, saw enhanced adhesion. We questioned whether the enhanced ability of the remaining membrane-associated CX3CL1 to bind targets was caused by changes in its lateral mobility. Using fluorescence recovery after photobleaching, we found that plasmalemmal CX3CL1 was initially tethered but ultimately mobilized by TP agonists. TP stimulation provoked clustering of transmembrane CX3CL1 at sites of contact with adherent leukocytes. These data demonstrate that TP stimulation induces two distinct effects: a rapid cleavage of surface CX3CL1, thereby releasing the soluble chemoattractant, plus mobilization of the remaining transmembrane CX3CL1 to enhance the avidity of interactions with adherent leukocytes. The dual effect of TP allows CX3CL1 to recruit leukocytes to sites of vascular inflammation while enhancing their adhesion once recruited.

atherosclerosis; inflammation; endothelial cells

ATHEROSCLEROSIS is a chronic inflammatory disease involving the vascular wall, leukocytes, and platelets. Thromboxane A2 (TXA2), a prostanoid formed from the metabolism of arachidonic acid via the cyclooxygenase (COX) pathway, has long been implicated in the pathogenesis of atherosclerosis and induces platelet aggregation, vasoconstriction, and vascular smooth muscle cell (VSMC) proliferation (42). It is produced mainly by platelets but also by monocytes/macrophages, VSMC, and endothelial cells (37, 41, 42). TXA2 mediates its effects through the thromboxane prostanoid (TP) receptor, a G protein-coupled receptor expressed in diverse hematopoietic cell types, including platelets and monocytes, as well as in endothelial cells, fibroblasts, and VSMC (15, 41). COX inhibitors, which decrease TXA2 production, protect against vascular inflammation and acute vascular events in patients at high risk (3). Mice deficient in TP or treated with a TP antagonist show markedly decreased leukocyte accumulation within the vascular wall (5, 22).

In atherogenesis, chemokines recruit circulating leukocytes and other cells to the injured vascular wall. The chemokine CX3CL1 (fractalkine) plays a key role by recruiting monocytes, T lymphocytes, and other inflammatory cells (6, 24, 28, 29, 32, 40). Most chemokines are small, secreted proteins. CX3CL1 is one of only two chemokines that have soluble and membrane-anchored species (2, 10, 27). Soluble CX3CL1 consists of a chemokine domain and mucin stalk and is released from the plasma membrane following cleavage of the full-length protein by ADAM (a disintegrin and metalloproteinase)-10 and ADAM17/TACE (tumor necrosis factor-α-converting enzyme) metalloproteinases (12, 17, 43). The soluble species thus generated is chemoattractant for monocytes/macrophages, killer cells, T lymphocytes, and VSMC, all of which express CX3CR1, the unique receptor for CX3CL1 (10, 18, 26).

CX3CL1-CX3CR1-mediated cellular traffic is critically implicated in coronary atherosclerosis and stroke. In affected coronary arteries, CX3CL1 protein expression is increased throughout the vascular wall in endothelium, intima, media, and adventitia (26, 45, 47). CX3CL1 is also expressed in macrophages, foam cells, and VSMC and acts to recruit monocytes, memory T lymphocytes, and additional VSMC to the injured arterial wall (26, 45, 47). Thus, within the atherosclerotic lesion, CX3CL1 is expressed by and can recruit cells capable of secreting large amounts of extracellular matrix proteins, thereby favoring formation of stable atherosclerotic plaques (10, 18, 47). By recruiting monocytes to the injured vessels, CX3CL1 promotes formation of macrophage-rich, unstable plaques. Genetic targeting of CX3CL1 or CX3CR1 in atherosclerosis-prone mice highly protects them from vascular disease (6, 24, 40). In humans, gene polymorphisms in CX3CL1 that impair leukocyte chemotaxis and binding of the receptor to cell surface CX3CL1 protect against myocardial infarction, unstable angina, cardiac death, and stroke (13, 28, 32).

It is widely recognized that important links exist between the thrombotic events and the vascular inflammation that define atherosclerosis. The precise nature of these links has not yet been well elucidated. TXA2, acting through TP, potentially in-
duces vascular inflammation, promoting leukocyte recruitment to the injured vascular wall. We studied whether TP mediates these effects by enhancing the exposure and activity of the chemokine CX3CL1. We found that TP stimulation causes rapid shedding of CX3CL1 from the cell surface yet, unexpectedly, promotes enhanced CX3CL1-dependent leukocyte adhesion. TP stimulation mobilizes remaining membrane-associated CX3CL1, allowing it to cluster at sites of contact with adherent leukocytes. The potential implications of these dual mechanisms of regulation of CX3CL1 function by TP for the development of vascular inflammation are discussed.

MATERIALS AND METHODS

Cell culture. ECV-304 cells were obtained from the American Type Culture Collection (Manassas, VA), and the generation of cells expressing green fluorescent protein-tagged CX3CL1 (ECV-CX3CL1-GFP) or untagged CX3CL1 (ECV-CX3CL1) has been described previously (8, 25). A cDNA construct encoding mCherry-tagged CX3CL1 was generated by PCR amplification of full-length CX3CL1 with EcoR1 and BamH1 sites at the NH2 and COOH terminus, respectively, and subcloning into the corresponding restriction sites of pmCherry-N1 vector (Clontech Laboratories, Mountain View, CA). The resulting CX3CL1-mCherry cDNA construct was verified by sequencing and was expressed in ECV-304 cells using FuGene HD transfection reagent according to the manufacturer’s specifications (Roche Diagnostics, Laval, PQ, Canada). Cells were grown in DMEM with 5% fetal calf serum. Human K562 erythroleukemia cells that stably express CX3CR1 (K562-CX3CR1) were cultured in RPMI supplemented with HEPES and 10% fetal calf serum (8, 10). Primary human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (La Jolla, CA) and cultured in endothelial cell growth medium (Clonetics) (10). Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers as previously described (8).

Antibodies and reagents. The following primary antibodies were used: goat anti-human CX3CL1 (R&D Systems, Minneapolis, MN), rabbit anti-Erk (extracellular signal-related kinase) and rabbit anti-phospho-Erk (Cell Signaling Technology, Danvers, MA), mouse anti-actin (Stressgen, Victoria, BC, Canada), mouse anti-GAPDH (Clontech Laboratories, Palo Alto, CA), mouse anti-calpain (Calbiochem-Novabiochem, La Jolla, CA), mouse anti-phospho-c-Raf (Cell Signaling Technology, Danvers, MA), mouse anti-phospho-MEK1/2, and rabbit anti-MEK1/2 were all obtained from Cell Signaling Technology. Rabbit anti-c-Raf was obtained from Santa Cruz Biotechnology. Mouse monoclonal antibody (Ab) directed against human TACE was a kind gift of Dr. Roy Black (Amgen, Shermann Oaks, CA). The following secondary antibodies were used: horseradish peroxidase-conjugated anti-mouse, anti-goat and anti-rabbit IgG, Cy3- and Cy5-conjugated anti-goat IgG, Cy5-conjugated anti-mouse IgG, and phycocerythrin-conjugated anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The anti-sheep IgG Ab was used to control for protein loading. Anti-actin or anti-GAPDH Ab was used to control for protein loading. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) recorded on X-ray film.

Flow cytometry. ECV-CX3CL1-GFP cells were grown in 10-cm dishes and treated with the TP agonists IBOP or U46619 for various time periods. Cells were lifted by briefly incubating with 5% EDTA at 4°C, washed, and fixed in 4% paraformaldehyde. Cells were incubated with 5% donkey serum followed by anti-CX3CL1 Ab for 1 h at room temperature. After further washing, cells were incubated with phycoerythrin-conjugated anti-goat IgG for 1 h at room temperature. Cells were suspended in PBS and analyzed using a fluorescence-activated cell scanner (FACScan; BD Biosciences, San Jose, CA).

Immunofluorescence staining. ECV-CX3CL1-GFP cells were grown on glass coverslips, fixed using 4% paraformaldehyde, washed, and blocked with 5% donkey serum at room temperature for 1 h. Cells were incubated with anti-CX3CL1 Ab (2.5 μg/ml) and anti-TACE Ab (2.7 μg/ml) at room temperature for 1 h followed by Cy3-conjugated anti-goat IgG and Cy5-conjugated anti-mouse IgG for 1 h. Cells were mounted onto glass slides and visualized using a spinning disk DMIRE2 confocal microscope (Leica Microsystems, Toronto, ON, Canada) equipped with a Hamamatsu backthinned charge-coupled device (EM-CCD) camera. Images were acquired using ×100 oil immersion and appropriate filters (8, 25).

Fluorescence recovery after photobleaching. Fluorescence recovery after photobleaching (FRAP) was performed as previously described (8, 25). Briefly ECV-CX3CL1-GFP cells or ECV-CX3CL1 cells transfected with GPI-GFP or FcγRIIA-GFP cDNA were grown on 25-mm coverslips, mounted into Attolight chambers, and incubated in RPMI containing 5% fetal calf serum and 25 mM HEPES at 37°C (8, 25). Live cells were analyzed by confocal microscopy. A 30-nm LASOS argon laser was used to irreversibly photobleach the entire juxtanuclear CX3CL1-containing compartment (8, 25). To prevent potential shedding of cell surface CX3CL1, cells were treated with the metalloprotease inhibitor GM6001 for 4 h before assays were performed. In other experiments, a 2-μm region of the plasma membrane was irreversibly photobleached. Recovery of fluorescence was measured over time. Control, nonbleached areas of the plasma membrane of the same cell were also serially monitored to quantify the amount of bleaching caused by repeated image acquisition. Results are expressed as the mean (±SE) normalized fluorescence recovery.

Acid-stripping experiments. The rate of delivery of internalized CX3CL1 back to the plasma membrane was assessed as previously described (25, 44). To prevent potential shedding of cell surface CX3CL1, cells were pretreated with the metalloprotease inhibitor GM6001 for 1 h before incubation with anti-CX3CL1 Ab for 1 h (43). Membrane-associated Ab was removed by acid wash (0.15M NaCl, 50 mM glycine, 0.1% BSA, pH 2.5) on ice for 2 min and then allowed to recover for various time periods in the presence of TP agonist. Cells were washed, fixed, and incubated with Cy3-conjugated secondary Ab. Images were captured using a Leica DMIRE2 microscope and OpenLab software (Improvision, Lexington, MA). Cell surface immunofluorescence intensity was measured using Velocity imaging software (Improvision, Waltham, MA).

Adhesion assays. Hemmer-Woodruff assays were performed as described elsewhere with minor modifications (8, 35, 36). Briefly, ECV-304, ECV-CX3CL1-GFP, or HUVEC cells were grown to confluence on 25-mm coverslips and treated with the TP agonist IBOP for 30 min. In some experiments, HUVEC were incubated with TNF-α (100 U/ml) for 4 h, followed by incubation with function-blocking anti-CX3CL1 Ab (1 μg/ml) for 30 min before addition of leukocytes. Control human K562 or K562-CX3CL1 cells were labeled with Alexa555-conjugated cholera toxin to easily visualize them. Cells (5 × 10^6) were incubated with either ECV-304 or ECV-CX3CL1-GFP
Fig. 1. Thromboxane prostanoid (TP) receptor stimulation decreases surface and total levels of CX3CL1. A: ECV-304 cells expressing green fluorescent protein-tagged CX3CL1 (ECV-CX3CL1-GFP) were incubated with U46619 (20 μM) for the indicated time periods. Cell lysates were harvested, and immunoblotting was performed using anti-CX3CL1 antibody (Ab; 0.2 μg/ml) and horseradish (HRP)-conjugated secondary Ab. To control for protein loading, blots were stripped and reprobed with anti-GAPDH Ab and HRP-conjugated secondary Ab. B: cells were incubated with IBOP (0.1 μg/ml), and immunoblotting was performed as described in A. To control for protein loading, blots were stripped and reprobed with anti-actin Ab and HRP-conjugated secondary Ab. C-E: experiments were performed as described in B. Band intensities for CX3CL1 were quantified and normalized to actin. Values are means ± SE for 4 separate experiments. *P < 0.05; †P < 0.01 vs. untreated. D: ECV-CX3CL1 cells were incubated with IBOP (0.1 μg/ml) for 30 min, and immunoblotting was performed as described in B. E: experiments were performed as described in D. Band intensities for CX3CL1 were quantified and normalized to actin. Values are means ± SE for 3 separate experiments. *P < 0.01. F: ECV-CX3CL1-GFP cells were incubated with U46619 (20 μM) for the indicated time periods, fixed, and labeled with anti-CX3CL1 Ab (2.5 μg/ml) and PE-conjugated secondary Ab, and cell surface CX3CL1 expression was measured using flow cytometry. Results are means ± SE expressed as a percentage of control fluorescence for 5 separate experiments. ‡P < 0.05. G: cells were incubated with IBOP (0.1 μg/ml) for 30 min after preincubation with the specific TP antagonist SQ29548 (10 μM for 15 min), and immunoblotting was performed as described in B. H: experiments were performed as described in G. Band intensities for CX3CL1 were quantified and normalized to actin. Values are means ± SE for 3 separate experiments. ¶P < 0.05.
cells using gentle rocking at 10 cycles/min. Nonadherent cells were washed away, and remaining cells were fixed and mounted onto slides. Using a Leica deconvolution microscope, we examined at least 50 high-power fields (×63) to count the number of adherent cells. Results represent the mean values (±SE) and were compared using Student’s t-test.

To examine localized changes in CX3CL1 expression at the point of contact with adherent leukocytes, ECV-304 cells were plated in six-well plates and grown to 50% confluence. Cells were transfected with CX3CL1-mCherry cDNA (1.0 µg) using FuGene HD (Roche Diagnostics). After 36 h, adhesion assays were performed by incu-
bating ECV-CX3CL1-mCherry cells with K562-CX3CR1 cells (5 \times 10^5 cells/well) in the presence or absence of IBOP (2.5 \mu g/ml). To prevent inadvertent shedding of CX3CL1 from the plasma membrane, ECV-CX3CL1-mCherry cells in some wells were incubated with the metalloprotease inhibitor GM6001 for 4 h before experiments were performed. To rule out any inadvertent effects of TP stimulation on cell surface expression and mobility of the CX3CL1 receptor CX3CR1, K562-CX3CR1 cells were fixed using 4% paraformaldehyde and washed with glycerin (100 mM) in PBS just before adhesion assays were performed. K562-CX3CR1 cells were allowed to adhere for 30 min at 37°C under gentle rocking conditions and then gently washed twice with PBS to remove nonadherent cells. Cells were fixed and permeabilized using 0.1% Triton, and K562-CX3CR1 cells were labeled by incubation with anti-Bcr Ab (2 \mu g/ml) followed by AlexaFlor 488-conjugated anti-mouse IgG secondary Ab (23). Spermiing disk confocal microscopy was performed, and Z-stack images were captured. XY or YZ dimensions of the Z-stack images were used to compare the localized fluorescence intensity CX3CL1-mCherry signal on transfected ECV-304 cells at the point of interface with adherent K562-CX3CR1 cells with the CX3CL1-mCherry fluorescent signal in an adjacent region of similar size in the plasma membrane, where no leukocyte was attached. Fluorescent signals were quantified using ImageJ software (National Institutes of Health), and the ratio of signal intensities between site of contact and site of no contact was measured for 24 randomly chosen cells for each treatment condition. Experiments were performed three separate times.

CX3CL1 shedding experiments. Small interfering RNA (siRNA) directed against human ADAM10, human TACE, and control nontargeting siRNA were purchased from Santa Cruz Biotechnology. ECV-CX3CL1 cells were electroporated with siRNA by nucleofection (Amaxa, Gaithersburg, MD) on day 0 and day 2. After the second electroporation, cells were plated in six-well tissue culture plates at a density of 1 \times 10^5 cells/well and cultured for 48 h. Cells were incubated with IBOP (2.5 \mu g/ml) for 30 min at 37°C, and conditioned medium was collected. Cells were washed once with PBS and lysed by adding 0.5 ml of RIPA buffer. Protease inhibitor cocktail (Sigma) was added (1:100) to both harvested conditioned medium and cell lysis buffer to prevent protein degradation. Supernatants and cell lyses were cleared by centrifugation at 13,000 rpm at 4°C for 10 min. With the use of fresh samples, CX3CL1 was detected for each experimental condition. Values are means \pm SE for 3 separate experiments. For full-length and cleaved CX3CL1, detected for each experimental condition. Values are means \pm SE for 3 separate experiments. For full-length and cleaved CX3CL1: P < 0.01, untreated vs. IBOP; P < 0.0001, IBOP vs. IBOP + TAPI-2. C: cells were electroporated with TACE small interfering RNA (siRNA), ADAM10 siRNA, or control, nontargeting (scrambled) siRNA on days 0 and 2. After 48 h, cells were lysed, harvested, and immunoblotting was performed using anti-TACE and anti-ADAM10 Ab. D: cells were electroporated with the indicated siRNA and incubated with IBOP (30 min), conditioned medium was collected, and cell lysate was harvested. Protease inhibitor cocktail was added to conditioned medium and to cell lysis buffer to prevent protein degradation. Supernatants and cell lysates were cleared by centrifugation. With the use of fresh samples, CX3CL1 was measured using a CX3CL1 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s specifications. Three independent experiments were performed, and the data are expressed as the mean (±SE) percentage of soluble CX3CL1 released into the medium in relation to the total amount of CX3CL1 (soluble and cell associated).

RESULTS

Stimulation of the TP receptor decreases surface and total levels of CX3CL1. When ECV-CX3CL1-GFP cells were treated with two distinct TP agonists, total levels of CX3CL1, measured by immunoblotting, fell after just 10 min, with the nadir observed at 30 min (Fig. 1, A–C). When experiments were performed using cells that express untagged CX3CL1, TP stimulation provoked a similar fall in the levels of CX3CL1 (Fig. 1, D and E; P < 0.05). Although TP stimulation promoted a significant decrease in both total and cell surface CX3CL1, the magnitude of the changes was not identical, presumably because the inherent differences in the two methodologies used resulted in a differential ability to detect CX3CL1. We further found that the specific TP antagonist SQ29548 prevented loss of CX3CL1, verifying the specificity of the response (Fig. 1, G and H). Thus TP activation promotes reduction of surface and total levels of CX3CL1.

TP stimulation induces cleavage of CX3CL1 by TACE. We questioned whether TP stimulation results in shedding of CX3CL1 from the plasma membrane, a process mediated by ADAM10 and TACE (12, 17, 43). Consistent with this notion, the metalloprotease inhibitors TAPI-2 and GM6001, which inhibit both ADAM10 and TACE, prevented TP-induced loss of CX3CL1 (Fig. 2A; P < 0.02; data not shown for GM6001). To verify these findings, we performed immunoblotting using an antibody directed against GFP, conjugated to the cytoplasmic domain of CX3CL1 (Fig. 2B). TP stimulation resulted in loss of full-length CX3CL1 (P < 0.01) and appearance of a small cell-associated species of CX3CL1 (P < 0.01) after shedding of the extracellular domain of the chemokine (Fig. 2B). These events were prevented by TAPI-2 (Fig. 2B). To determine whether ADAM10 and/or TACE induced the ob-
erved proteolysis of CX3CL1, we used siRNA to selectively knock down expression of each protease (Fig. 2C) and measured soluble levels of CX3CL1 generated. In the presence of nontargeting siRNA or ADAM10 siRNA, TP stimulation promoted release of soluble CX3CL1 (Fig. 2D, P < 0.03). In the absence of TACE, TP failed to promote shedding of CX3CL1 (Fig. 2D). In keeping with these observations, CX3CL1 and TACE, which did not colocalize on the cell surface under basal conditions, coassociated after 5 min of TP stimulation, yielding a Pearson’s correlation coefficient (r) greater than 0.5 (Fig. 2E; basal: r = 0.429 ± 0.042, P > 0.05; IBOP: r = 0.645 ± 0.031, P < 0.01). After TP stimulation, the increase in soluble CX3CL1 detected in the medium was not the same as the decrease in the amount of cell-associated chemokine. Although the magnitude of the individual changes is statistically significant, there is likely differential sensitivity of the two assays used to detect soluble and cell-associated CX3CL1 present. It is further possible that some of the soluble CX3CL1 released by proteolytic shedding could be bound to the surface of cells or to the underlying extracellular matrix, resulting in a failure to detect it by ELISA.

Since TP can activate a number of signaling pathways, we studied the intracellular mechanisms whereby TP causes shedding of CX3CL1. TP promoted rapid phosphorylation of Erk (Fig. 2F, P < 0.01) and its upstream effectors, MEK1/2 (Fig. 2G, P < 0.01) and c-Raf (Fig. 2G, P < 0.05) (7). Erk inhibition prevented TP-induced loss of surface CX3CL1 (Fig. 2H, P < 0.05). Overall, TP stimulation causes cleavage of plasmalemmal CX3CL1 by TACE, rather than by ADAM10.

**TP stimulation does not affect recycling of CX3CL1.** Because CX3CL1 recycles between the plasma membrane and an endocytic compartment, we questioned whether TP stimulation might redistribute the chemokine from the superficial to the intracellular pool (25). To prevent inadvertent shedding of CX3CL1 from the cell surface, cells were preincubated with GM6001 and endocytosis was examined by FRAP, irreversibly photobleaching the entire CX3CL1-containing intracellular compartment, as previously described (25, 43). Recovery of fluorescence, an index of internalization of CX3CL1 from the plasma membrane, was recorded over time (25). Fluorescence of the plasma membrane pool remained largely unaffected by the bleaching procedure and was also monitored. Figure 3 depicts a cell before, immediately after, and 5 min after bleaching. Note that the plasmalemmal fluorescence decreased as the juxtanuclear fluorescence recovered, consistent with delivery of unbleached CX3CL1-GFP from the former to the latter compartment. The juxtanuclear CX3CL1-GFP compartment recovered to 21.5 ± 3.1% of its original fluorescence by 170 s and was unchanged by TP stimulation (Fig. 3D).

To test whether TP stimulation affects delivery of internalized CX3CL1 back to the plasma membrane, shedding of the chemokine was inhibited using GM6001, and the endomembrane pool was loaded by incubation with CX3CL1 Ab at 37°C. Ab bound to the surface was stripped by a short exposure to an acidic solution. Cells were allowed to recover, and reinsertion of CX3CL1 in the plasma membrane was determined by quantifying the reappearance of Ab (25). Fluorescence at the cell surface increased progressively after acid stripping, confirming that CX3CL1 within the endomembrane compartment traffics back to the plasma membrane (Fig. 4, A–E, P < 0.02). TP stimulation did not affect this traffic (compare Fig. 4, C and D; Fig. 4E).

**TP stimulation enhances, not inhibits, CX3CL1-dependent leukocyte adhesion.** We predicted that TP stimulation, which induces rapid loss of CX3CL1 from the cell surface, would impair adhesion of CX3CR1-expressing leukocytes. Minimal binding of K562 cells, which do not express CX3CR1, to either ECV-304 or ECV-CX3CL1-GFP cells was seen (data not shown). Likewise, there was very little adhesion of K562-CX3CR1 cells to ECV-C304 cells, which do not express CX3CL1 (Fig. 5A) (10). K562-CX3CR1 leukocytes effectively adhered to ECV-CX3CL1-GFP cells (Fig. 5A). The presence of plasmalemmal CX3CL1 increased leukocyte adhesion fourfold (Fig. 5A, P < 0.0000001). Unexpectedly, TP stimulation increased rather than decreased adhesion of K562-CX3CR1 leukocytes to ECV-CX3CL1-GFP cells (Fig. 5A, P < 0.05). TP stimulation did not increase adhesion of K562-CX3CR1 cells to untransfected ECV-304 cells, ruling out upregulated levels of other adhesion molecules as the cause of the enhanced leukocyte binding observed (Fig. 5A) (10).

To verify that the observed responses were not an idiosyncratic feature of the transfected cell lines, we performed experiments performed using primary human vascular endothelial cells. Upon TNF-α stimulation, endogenous expression of CX3CL1 increased fivefold (Fig. 5B, P < 0.01). TP stimulation caused a rapid fall in total levels of CX3CL1 (Fig. 5B,
Despite reducing the overall amount of the chemokine CX3CL1, TP stimulation mobilizes plasmalemmal CX3CL1 and induces clustering of CX3CL1 at sites of contact with adherent leukocytes. In principle, increased leukocyte binding to CX3CL1 could occur despite reduced CX3CL1 levels if TP stimulation mobilized and redistributed plasmalemmal CX3CL1, allowing the chemokine to move to sites of contact with CX3CR1 on leukocytes. TP stimulation could thereby increase the overall avidity of ligand-receptor interactions, strengthening adhesion between CX3CR1-expressing leukocytes and CX3CL1-expressing adherent cells.

To test this notion, an area of CX3CL1-GFP in the plasma membrane was irreversibly photobleached and recovery of fluorescence was serially observed (Fig. 6A, prebleaching; Fig. 6B, immediately after bleaching; Fig. 6C, 5 min after bleaching). As a control, CX3CL1-expressing cells were transfected with cDNA encoding freely mobile GPI-GFP, and recovery of fluorescence was similarly observed (Fig. 6, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA encoding the single transmembrane protein, FcRIIA-GFP). FcRIIA receptors were highly mobile, as indicated by the rapid and near complete recovery of fluorescence after photobleaching (Fig. 6H). These data demonstrate that lateral diffusion of transmembrane CX3CL1 within the membrane is highly impeded, rendering the chemokine largely immobile.

IBOP treatment significantly enhanced mobility of plasmalemmal CX3CL1, resulting in fluorescence recovery of 50.5 ± 1.0% at 55 s postbleaching, compared with 26.3 ± 2.5% recovery in the absence of IBOP (Fig. 6G, P < 0.05). After TP-induced ectodomain shedding of CX3CL1, we reasoned that it might not be possible to distinguish between reappearance of GFP fluorescence due to movement of full-length CX3CL1 or to

In keeping with these results, TP stimulation significantly enhanced proteolytic release of soluble CX3CL1 (P < 0.005, Fig. 5C). The enhanced shedding of CX3CL1 was prevented when activation of TACE was blocked (P < 0.005, Fig. 5C). When HUVEC were incubated with TNF-α, adhesion of K562-CX3CR1 cells increased by 30% (Fig. 5D, P < 0.005). In this instance, preincubation of endothelial cells with specific function-blocking anti-CX3CL1 Ab significantly decreased the number of bound leukocytes (Fig. 5D, P < 0.005). Similarly, TNF-α activation increased binding of primary PBMC 10-fold (Fig. 5E, P < 0.001). Although TP stimulation resulted in loss of cell-associated CX3CL1, K562-CX3CR1 cell adhesion increased by 70% (Fig. 5D, P < 0.005) and PBMC adhesion increased by 50% (Fig. 5E, P < 0.001). Preincubation of endothelial cells with anti-CX3CL1 Ab prevented TP-induced leukocyte adhesion, confirming the observed increase in binding was CX3CL1 dependent (Fig. 5D, P < 0.01; Fig. 5E, P < 0.001). Using a specific antagonist of TP, SQ29548, we verified that the observed increase in leukocyte adhesion was directly related to TP stimulation (Fig. 5F, P < 0.005). Blocking activation of TACE using the Erk inhibitor U0126 also prevented TP-induced increase in leukocyte adhesion (Fig. 5F, P < 0.001). Together, these data demonstrate that TP stimulation increases leukocyte adhesion to cell surface CX3CL1 despite reducing the overall amount of the chemokine present on the plasma membrane.

TP stimulation mobilizes plasmalemmal CX3CL1 and induces clustering of CX3CL1 at sites of contact with adherent leukocytes. In principle, increased leukocyte binding to CX3CL1 could occur despite reduced CX3CL1 levels if TP stimulation mobilized and redistributed plasmalemmal CX3CL1, allowing the chemokine to move to sites of contact with CX3CR1 on leukocytes. TP stimulation could thereby increase the overall avidity of ligand-receptor interactions, strengthening adhesion between CX3CR1-expressing leukocytes and CX3CL1-expressing adherent cells.

To test this notion, an area of CX3CL1-GFP in the plasma membrane was irreversibly photobleached and recovery of fluorescence was serially observed (Fig. 6A, prebleaching; Fig. 6B, immediately after bleaching; Fig. 6C, 5 min after bleaching). As a control, CX3CL1-expressing cells were transfected with cDNA encoding freely mobile GPI-GFP, and recovery of fluorescence was similarly observed (Fig. 6, D–F). CX3CL1-GFP fluorescence recovered poorly compared with GPI-GFP (Fig. 6, G and H). For CX3CL1-GFP, only 25.8 ± 2.6% of fluorescence of the bleached area recovered after 60 s, compared with 85.0 ± 2.9% of GPI-GFP (Fig. 6H). As another control, ECV-CX3CL1 cells were transfected with DNA encoding the single transmembrane-spanning protein, FcRIIA-GFP, conjugated to GFP (FcRIIA-GFP). FcRIIA receptors were highly mobile, as indicated by the rapid and near complete recovery of fluorescence after photobleaching (Fig. 6H). These data demonstrate that lateral diffusion of transmembrane CX3CL1 within the membrane is highly impeded, rendering the chemokine largely immobile.

IBOP treatment significantly enhanced mobility of plasmalemmal CX3CL1, resulting in fluorescence recovery of 48.2 ± 6.2% at 55 s postbleaching, compared with 26.3 ± 2.5% recovery in the absence of IBOP (Fig. 6G, P < 0.05). After TP-induced ectodomain shedding of CX3CL1, we reasoned that it might not be possible to distinguish between reappearance of GFP fluorescence due to movement of full-length CX3CL1 or to
movement of any COOH-terminal cleavage fragments retained within the plasma membrane. To prevent potential shedding of CX3CL1, cells were pretreated with the metalloprotease inhibitor GM6001. Accordingly, GM6001 did not affect basal mobility of CX3CL1 (Fig. 6H). In the presence of GM6001, TP similarly mobilized CX3CL1. The bleached area recovered to 55.5 ± 3.5% by 1 min, significantly greater than the 27.7 ± 2.5% recovery observed in the absence of TP stimulation (Fig. 6H, P < 0.001). At all time points after 20 s, TP facilitated recovery of fluorescence of plasmalemmal CX3CL1 (Fig. 6H,
$P < 0.03$ at each point). Thus TP stimulation enhances lateral mobility of CX3CL1, which is normally tethered within the plasma membrane.

We directly examined whether TP stimulation promotes clustering of CX3CL1 at sites of contact with CX3CR1 on adherent leukocytes, by comparing the fluorescent signal of CX3CL1 at these contact points to that of CX3CL1 at adjacent sites where no leukocyte was tethered. We reasoned that TP stimulation could conceivably affect mobility of CX3CR1 receptor present on the surface of leukocytes, resulting in enhanced adhesion between these cells and CX3CL1-expressing cells. To selectively study the effects of TP stimulation on clustering of plasmalemmal CX3CL1, leukocytes were prefixed, effectively converting them to “beads” coated with CX3CR1 (4). After TP stimulation, the ratio of contact site CX3CL1 to non-contact site CX3CL1 was greater than in unstimulated cells (Fig. 6, I and J; control: $1.1 \pm 0.1$ vs. TP stimulated: $1.7 \pm 0.2$; $P < 0.005$). A ratio greater than one was seen in approximately one-half (46%) of unstimulated cells. After TP stimulation, 83% had a ratio greater than one, indicating clustering of CX3CL1. Collectively, these results demonstrate that TP stimulation promotes rapid shedding of plasmalemmal CX3CL1 but that despite decreased cell surface levels of the chemokine, CX3CL1-dependent adhesion of leukocytes is enhanced due to mobilization and clustering of the chemokine at contact sites with adherent leukocytes. In this manner, although net cell surface exposure of CX3CL1 is decreased, the overall avidity of interactions between leukocytes and CX3CL1 on the surface of adherent cells is actually strengthened.

**DISCUSSION**

The principal aim of this study was to examine regulation of CX3CL1 by TP. For these studies, we used CX3CL1-expressing ECV-304 cells and primary human vascular endothelial cells. Although derived from bladder epithelial carcinoma cells, ECV-304 cells have many endothelial features, including the propensity to grow in monolayers of flattened cells, formation of moderately tight intercellular junctions, and expression of the endothelial markers Flt-1 and von Willebrand factor (14, 16, 39). Since the identification of CX3CL1, ECV-304 cells have been used extensively to study distribution, cleavage, and function of the chemokine, because the surface levels of CX3CL1 mirror those seen in cytokine-activated primary vascular endothelial cells and because expression of other cellular adhesion molecules is minimal (10, 17, 25, 43). We previously showed that addition of a GFP tag alters neither distribution nor subcellular traffic of CX3CL1 (25). To confirm the key findings of the current study, assays were performed using primary human vascular endothelial cells.

Overall exposure of CX3CL1 at the cell surface reflects the balance between shedding and recycling. Thus, in principle, the TP-induced decrease in CX3CL1 could result from 1) increased endocytosis, 2) decreased traffic of internalized CX3CL1 back to the plasma membrane, and/or 3) increased shedding from the cell surface. TP stimulation did not acutely alter recycling of the chemokine. Sustained TP stimulation (4–24 h) has been shown to augment expression of the vascular adhesion molecules, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin (19, 31). The resultant increase reflected enhanced nuclear factor (NF)-κB-dependent transcription and translation (19, 31). ICAM-1 expression is significantly reduced in genetically targeted mice lacking TP, again illustrating the ability of TP to increase adhesion molecule expression over time (22). Likewise, prolonged TP stimulation enhances expression of the chemokine CCL2 (monocyte chemoattractant protein-1, MCP-1), also through protein kinase C and NF-κB (20). To our knowledge, ours is the first study to examine how TP activation posttranslationally regulates cell surface exposure of an endothelial adhesion molecule or chemokine.

Blocking TACE, but not ADAM10, attenuated TP-induced shedding of CX3CL1 from the cell surface. Erk inhibition similarly blocked CX3CL1 shedding, consistent with studies demonstrating that TP engagement promotes protein kinase C activation and Erk phosphorylation (11, 30). Erk, in turn, phosphorylates the cytoplasmic domain of TACE at Thr735 and Ser119, thereby activating the protease (7, 9). Blocking this phosphorylation prevents activation of TACE and ectodomain shedding of its biological substrates (7, 9). Overall, our results demonstrate that TP stimulation causes rapid loss of CX3CL1 from the cell surface by promoting TACE-induced, but not...
ADAM10-induced, shedding of CX3CL1, rather than by interfering with recycling of the chemokine.

Despite inducing proteolysis of approximately one-half the CX3CL1 present, TP augmented CX3CL1-dependent leukocyte adhesion. Although unexpected, this is not inconceivable, since the overall binding of CX3CR1 receptor on the leukocyte is determined by two distinct factors, namely, the density of CX3CL1 ligand present and the mobility of CX3CL1 within the plasma membrane. We hypothesized that TP stimulation must increase the avidity of remaining CX3CL1 for its receptor. In

Fig. 6. TP stimulation mobilizes plasmalemmal CX3CL1 and induces clustering of CX3CL1 at sites of contact with adherent leukocytes. With the use of ECV-CX3CL1-GFP cells, fluorescence recovery after photobleaching (FRAP) was performed as described in MATERIALS AND METHODS. A: confocal image of single cell (×100). B: a region within the plasma membrane was irreversibly photobleached (see arrow). C: same cell 5 min after photobleaching. D–F: as a control, the membrane mobility of glycosylphosphatidylinositol (GPI)-linked GFP in transfected ECV-CX3CL1 cells was determined in the same manner. G: cells were incubated with IBOP (0.1 μg/ml) for 30 min, and fluorescence recovery of the photobleached area was monitored over time. Values are means ± SE from 9 separate experiments per condition. P < 0.05, untreated vs. IBOP at all time points after 11 s. H: recovery of fluorescence of the photobleached area was measured over time. In some experiments, ECV-CX3CL1-GFP cells were preincubated with GM6001 (20 μM for 2 h), to inhibit cleavage of CX3CL1 from the cell surface, and then incubated with IBOP (0.1 μg/ml) for 30 min. In some experiments, ECV-CX3CL1 cells were transfected with cDNA encoding GFP-conjugated FcγRIIA. Recovery of fluorescence after photobleaching of untreated ECV-CX3CL1-GFP, GM6001-treated ECV-CX3CL1-GFP, and GM6001-treated ECV-CX3CL1-GFP stimulated with IBOP, GPI-GFP, or FcγRIIA-GFP was measured. At each time point, fluorescence intensity in the bleached region was normalized to the mean control unbleached membrane fluorescence intensity. Values are means ± SE from 5–9 separate experiments per condition. I and J: ECV-304 cells were transfected with CX3CL1-mCherry cDNA. Adhesion assays were performed by incubating ECV-CX3CL1-mCherry cells for 30 min with K562-CX3CR1 cells (5 × 10⁵/well) in the presence (J) or absence (I) of IBOP. To prevent unwanted shedding of CX3CL1, in some wells ECV-CX3CL1-mCherry cells were preincubated with GM6001. To rule out potential effects of TP stimulation on cell surface clustering of CX3CR1, K562-CX3CR1 cells were first fixed using 4% paraformaldehyde and then washed with 100 mM glycine. At the end of the adhesion assay, cells were fixed and permeabilized, and K562-CX3CR1 cells were visualized by labeling with anti-BCR Ab and Alexa488-conjugated secondary Ab. Z-stack images are from spinning disk confocal microscopy (×63). K: experiments were performed as described in I and J. XY or YZ dimensions of the Z-stack images were used to compare the localized fluorescence intensity of CX3CL1-mCherry at sites of interface with adherent K562-CX3CR1 cells with the signal in an adjacent, similar-size region in the plasma membrane, where no leukocyte was attached. With the use of ImageJ software, the ratio of fluorescent signal intensities between the site of contact and site of no contact was measured for 24 randomly chosen cells for each treatment condition. Values are means ± SE from 3 separate experiments. *P < 0.005 vs. control.
principle, this could occur through mobilization of plasmalemmal CX3CL1 and subsequent clustering of the chemokine at points of contact with adherent leukocytes. Accordingly, plasmalemmal CX3CL1 was relatively immobile under basal conditions but became highly mobile following TP stimulation. We previously reported that plasmalemmal CX3CL1 is also immobile in other cell types under basal conditions (8). TP stimulation promoted clustering of CX3CL1 at contact interfaces with bound leukocytes. Similarly, Kobayashi et al. (22) reported less leukocyte rolling and adherence in mice lacking TP, perhaps due to the absence of TP-induced clustering of CX3CL1 on the surface of the vascular wall. Our results are reminiscent of a study demonstrating that isoprostane iPF2α-III, a known agonist of TP, enhances neutrophil adhesion to endothelial cells despite no increase in expression of endothelial adhesion molecules (1, 46). Although the authors postulated that release of an unknown endothelial activating factor was responsible, isoprostane activation might also increase mobility of plasmalemmal adhesion molecules, thereby strengthening the avidity of interactions between neutrophils and endothelial cells.

Our results differ somewhat from those of Hundhausen et al. (17), who reported that the synthetic phorbol ester PMA inhibited adhesion of THP-1 monocytic cells to CX3CL1-expressing adherent cells. Several possible explanations for this apparent discrepancy exist. First, THP-1 cells express other adhesion receptors whose levels and function may be modulated by PMA stimulation. In our study, we purposely used CX3CR1-expressing K562 cells, which do not express adhesion molecules such as L-selectin (10). Thus any adhesion observed was presumably attributable to interactions between leukocyte CX3CR1 and CX3CL1 on the surface of adherent cells. Stimulation by PMA and TP may have differential effects on the cell. TP receptors couple to Gq/G11, with most functioning attributed to signaling via the αq- rather than βy-subunits (21). Thus stimulation by PMA or TP can activate phospholipase C, generating inositol triphosphate (IP3) and diacylglycerol (DAG), increasing intracellular calcium concentration, and activating protein kinase C (21). In primary human cells, TP also couples to Gq and to G12/13 and is linked to cAMP (15, 21). Thus the TP receptor has the potential to utilize at least four distinct intracellular signaling pathways. Mobilization of CX3CL1 by TP may reflect activation of these pathways. In our study, TP activation consistently increased CX3CL1-dependent leukocyte adhesion, even to primary human vascular endothelial cells expressing endogenous CX3CL1, highlighting the physiologic relevance of these observations. This is the first description of regulation of CX3CL1 exposure and mobility by a G protein-coupled receptor.

An intriguing question is how CX3CL1 is anchored to the plasma membrane in the first place. Previously, we reported that CX3CL1 does not directly interact with the actin cytoskeleton (8). Recent work has demonstrated an integral role for dendritic cell (DC) CX3CL1 in the accumulation of lipid rafts at DC-NK cell synapses (34). However, we found that in adherent cells, plasmalemmal CX3CL1 does not associate with lipid rafts and, accordingly, that cholesterol depletion does not alter the mobility of the chemokine (8). Future studies are needed to investigate 1) whether CX3CL1 indirectly associates with the actin cytoskeleton by one or more Triton-soluble adaptor proteins, 2) whether CX3CL1 is “fenced” into discrete regions of the membrane, and 3) precisely how TP uncouples CX3CL1 from its membrane anchor (33, 38).

TP plays a complex role in vascular inflammation. Given the timeframe of events that we investigated, it appears that early in the inflammatory cascade, TP stimulation induces shedding of endothelial CX3CL1 to release the soluble chemoattractant species, thereby recruiting circulating leukocytes. Slightly later, the balance is subtly altered to facilitate adhesion of leukocytes thus recruited to the area of vascular injury. Regulation of CX3CL1 by TP in this manner may represent a link, heretofore unexplored, between the events accompanying the platelet aggregation and activation and those accompanying the vascular inflammation that, collectively, contribute to atherogenesis. The dynamic interplay among these individual events in vivo remains to be elucidated.

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REFERENCES

REGULATION OF CX3CL1 BY TP


