Dynamic regulation of ROCK in tumor cells controls CXCR4-driven adhesion events

Amanda P. Struckhoff¹, Jason R. Vitko¹, Manish K. Rana¹, Carter T. Davis¹, Kamau E. Foderingham¹, Chi-Hsin Liu¹, Lyndsay Vanhoy-Rhodes², Steven Elliot², Yun Zhu², Matt Burow² and Rebecca A. Worthylake¹,*

¹Departments of Oral Biology and Pharmacology, LSU Health Sciences Center, New Orleans, LA, 70112, USA
²Department of Medicine, Tulane University Medical Center, New Orleans, LA 70112, USA

*Author for correspondence (bworth@lsuhsc.edu)

Summary

CXCR4 is a chemokine receptor often found aberrantly expressed on metastatic tumor cells. To investigate CXCR4 signaling in tumor cell adhesion, we stably overexpressed CXCR4 in MCF7 breast tumor cells. Cell attachment assays demonstrate that stimulation of the receptor with its ligand, CXCL12, promotes adhesion of MCF7-CXCR4 cells to both extracellular matrix and endothelial ligands. To more closely mimic the conditions experienced by a circulating tumor cell, we performed the attachment assays under shear stress conditions. We found that CXCL12-induced tumor cell attachment is much more pronounced under flow. ROCK is a serine/threonine kinase associated with adhesion and metastasis, which is regulated by CXCR4 signaling. Thus, we investigated the contribution of ROCK activity during CXCL12-induced adhesion events. Our results demonstrate a biphasic regulation of ROCK in response to adhesion. During the initial attachment, inhibition of ROCK activity is required. Subsequently, re-activation of ROCK activity is required for maturation of adhesion complexes and enhanced tumor cell migration. Interestingly, CXCL12 partially reduces the level of ROCK activity generated by attachment, which supports a model in which stimulation with CXCL12 regulates tumor cell adhesion events by providing an optimal level of ROCK activity for effective migration.

Key words: CXCL12, CXCR4, ROCK, Flow, Metastasis, Tumor recruitment

Introduction

Metastasis is the result of a complex set of events that culminate in tumor cell colonization of distal tissues, resulting in deadly consequences. During the metastatic process, tumor cells acquire the ability to survive and proliferate in a new tissue environment (Steeg, 2006). To access these new tissues, metastatic cells display migratory capacities not present in their normal cell counterparts (Friedl and Wolf, 2003; Wang et al., 2005). One of these migratory behaviors is the ability to be trafficked into secondary organs. Although the mechanisms that govern organ-selectivity of metastasis are multi-faceted, one important mechanism is the recruitment of hematopoietic cell migration. The chemokine receptor CXCR4 is a potent motomorphogen and is essential not only for hematopoiesis, but also for regeneration of multiple solid tissues (Kucia et al., 2005). The expression of CXCR4 is normally restricted to hematopoietic cells and committed stem cells; however, aberrant expression of this chemokine receptor has been shown to be correlated with tumor metastasis (Balkwill, 2004; Zlotnik, 2006). Significantly, blocking CXCR4 signaling inhibits breast tumor cell metastasis in an intravenous model system (Muller et al., 2001). It is thought that metastatic tumor cells can hijack the CXCR4 trafficking signals needed for normal tissue reorganizing activities, thereby gaining entry to distal organs as potential sites for colonization.

Rho GTPase signals are responsible for many cell migration activities (Heasman and Ridley, 2008; Titus et al., 2005; Wennerberg and Der, 2004). The Ser/Thr kinases, ROCKI and ROCKII (also known as ROCK-1 and ROCK-2), are RhoA effectors that have been associated with metastasis in animal models (Hakuma et al., 2005; Itoh et al., 1999; Lin et al., 2007; Nakajima et al., 2003a; Nakajima et al., 2003b; Narumiya et al., 2009; Ogawa et al., 2007; Takamura et al., 2001; Wong et al., 2009; Xue et al., 2007; Ying et al., 2006). Several lines of investigation implicate ROCKs in the invasive behavior of metastatic tumor cells (Cardone et al., 2005; Itoh et al., 1999; Joshi et al., 2008; Kamai et al., 2003; Kitzing et al., 2007; Li et al., 2006; Wilkinson et al., 2005). Recent studies have demonstrated that RhoA and ROCK promote invasion of matrix in response to CXCL12 (Amine et al., 2009; Azab et al., 2009; Molina-Ortiz et al., 2009). ROCKs also play a role in the regulation of invasive pseudopodia and/or invadopodia in metastatic tumor cells. ROCK activity is required for organization of the matrix surrounding advancing tumor cells to promote invasion in three dimensions (Provenzano et al., 2008) and ROCKs have been shown to be required for an ‘amoeboid’ mode of motility that is used by many metastatic cells to invade through three-dimensional matrix (Rosel et al., 2008; Sahai and Marshall, 2003; Torka et al., 2006; Wyckoff et al., 2006). Thus, one way that ROCKs are thought to contribute to metastasis is by stimulating invasiveness; either by the promoting invasive protrusions, or remodeling the matrix.

In addition to a role in tumor invasiveness, ROCKs are also known to regulate cell migration in response to extracellular cues, including those mediated by chemokines (Moyer et al., 2007; Samaniego et al., 2007; Tan et al., 2006; Vicente-Manzanares et al., 2002). Regulation of integrin adhesion is an important mechanism by which chemokines regulate cellular recruitment. CXCL12, the ligand for CXCR4, is known as a ‘capture’ chemokine because of its ability to induce arrest of circulating hematopoietic cells by the vascular endothelium (Alon and Ley, 2008; Laudanna and Alon, 2006; Ley, 2003). Although tumor cell
recruitment is probably more complex than leukocyte recruitment and can involve interactions with platelets and other hematopoietic cells (Borsig, 2008; de Visser et al., 2006; Witz, 2008), tumor cell recruitment shares several features of leukocyte recruitment (Balkwill, 2004; Ben-Baruch, 2008; Dittmar et al., 2008; Miles et al., 2008). Notably, tumor cell trafficking can be directed by chemokines, and direct adhesive interactions between tumor cells and the endothelial ligands including selectins, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (1VCAM-1) have been shown to contribute to tumor cell arrest (Gassmann and Haier, 2008; Glinsky et al., 2003; Jones et al., 2007; Kobayashi et al., 2007; Zetter, 1993).

Subsequent to arrest, chemokines are further thought to regulate chemotaxis by spatially coordinating integrin adhesions, such that adhesion occurs at the front and de-adhesion occurs at the back end of the migrating cell (Imhof and Aurrand-Lions, 2004; Kinashi, 2007; Worthylake and Burridge, 2001). Integrins cluster together with many associated proteins in the cytoplasm to form adhesion complexes that contain both structural and signaling molecules that regulate adhesion behavior. The highly dynamic nature of these adhesion complexes controls the formation of nascent adhesions and their maturation to more stable integrin adhesions during initial attachment and subsequent cell migration. Thus, CXCR4 has the potential to regulate recruitment through two distinct adhesion mechanisms: initial arrest of circulating tumor cells, and subsequent reorganization of the adhesion complexes to support chemotaxis.

ROCKs are important regulators of integrin adhesions, promoting the development of large integrin adhesion complexes (Riento and Ridley, 2003). Several chemokines, including CXCL12, have been shown to regulate the activity of RhoA and ROCKs; however, their specific effect on ROCK activity is context dependent. In some systems, CXCL12 induces ROCK activity whereas in others the chemokine inhibits ROCK activity (Amine et al., 2009; Arakawa et al., 2003; Moyer et al., 2007; Tan et al., 2006; Vicente-Manzanares et al., 2002). These discrepancies can probably be explained by the use of different cell types, experimental conditions, and the time course examined. CXCL12 is known to induce rapid changes in cell adhesion, cell shape and cell migratory capacities, which correspond to underlying changes in signaling pathways (Kucia et al., 2004). During a dynamic process such as cell migration, it is very likely that the effect of CXCL12 on ROCK activity varies over time and might be further influenced by the particular cellular context. Therefore, CXCL12 regulation of ROCK activity is a potential means of controlling tumor cell adhesive events. However, studies to date have not specifically investigated the role of ROCK activity in CXCL12-induced adhesive events during the early phases of cellular attachment, which is thought to be an important first step in tumor cell recruitment.

To investigate the role of ROCK in CXCR4-driven tumor cell adhesion during recruitment, we overexpressed CXCR4 in MCF7 breast tumor cells and analyzed their behavior in response to adhesion stimulated by CXCL12. To more closely mimic the conditions of a circulating tumor cell, we specifically designed our experiments to examine the transition from suspension to adhesion. Our results show that CXCL12 promotes adhesion in two ways. First, it increases initial cellular attachment to both extracellular matrix and endothelial adhesion molecules. Second, it promotes the maturation of integrin adhesion complexes following attachment. Analysis of the role of ROCK activity in these processes reveals an unexpected biphasic role for ROCK activity during adhesion.

Initially, inhibition of ROCK activity is required to allow cellular attachment. Following attachment, re-activation of ROCK is required for maturation of adhesion complexes and subsequent migration. Interestingly, although CXCL12-induced adhesion maturation and migration require ROCK activity, CXCL12 reduces the level of ROCK re-activation. This suggests a model in which CXCL12 promotes adhesion maturation and migration by providing an optimal level of ROCK activity. Finally, we have examined tumor cell attachment under physiological flow conditions, and find that constitutively active ROCK blocks tumor cell attachment to an endothelial monolayer, highlighting the importance of the transient decrease in ROCK activity during tumor cell adhesion.

**Results**

**CXCR4 stimulation increases MCF7 cell attachment**

Chemokines, such as CXCL12, are known to increase leukocyte arrest on the endothelium by stimulating integrin adhesion. In order to identify the role of the CXCR4-CXCL12 axis in tumor cell adhesion, we used cell attachment assays to evaluate whether CXCR4 activation stimulated MCF7 breast cancer cell adhesion. We expressed CXCR4 in MCF7 cells (MCF7-CXCR4) to a level equivalent to metastatic MDA-MB 361 cells (supplementary material Fig. S1). MCF7-CXCR4 cells labeled with CellTracker™ Green were put into suspension, incubated for 5 minutes with or without 10 nM CXCL12, and then plated onto 96-well plates coated with a panel of adhesion molecules for 20 minutes. We limited the time of attachment to 20 minutes to restrict our analysis to early adhesion events as a model for tumor cell attachment. We found that CXCL12 increased the attachment of MCF7-CXCR4 cells to collagen 1 (Fig. 1A). For comparison, the attachment of MCF7-vector-control cells is also shown. We found that overexpression of CXCR4 in MCF7 cells promotes adhesion to collagen 1 even in the absence of CXCL12 stimulation. The binding to collagen 1 was very efficient and was subsequently used to normalize the attachment data and serve as a reference point for comparison between different experiments. We next analyzed binding to laminin and several endothelial cell ligands expected to be important for tumor cell adhesion and found that CXCL12 stimulated adhesion to E-selectin, ICAM-1 and VCAM-1 (between 38% and 60% of plated cells attached depending on the ligand; Fig. 1B). This is an important result because it demonstrates that stimulation of CXCR4 can enhance adhesion to endothelial ligands which might be important for tumor cell migration during metastasis.

Because ROCK is known as a potent promoter of adhesion complexes, we next wanted to determine whether basal and CXCL12-induced early adhesion events were dependent on ROCK activity. We pretreated MCF7-CXCR4 cells with Y-27632, an inhibitor of ROCKI and II. (Note that we are not distinguishing between the two ROCK isoforms because the reagents used do not discriminate between them.) We found that inhibition of ROCK with Y-27632 increased MCF7-CXCR4 cell attachment to collagen 1 (30%) and ICAM-1 (45%). In addition, we found that the ROCK inhibitor increased cell attachment even over the levels observed with CXCL12 stimulation (Fig. 1C). Inhibition of ROCK similarly increased attachment to laminin (32%), VCAM (35%) and E-selectin (50%); supplementary material Fig. S2). Because ROCK activity is associated with the formation of robust integrin adhesions, the finding that ROCK activity was not only dispensable, but actually promoted tumor cell attachment was unexpected.
ROCK in CXCR4-driven tumor cell adhesion

CXCL12 enhances adhesion complexes in a ROCK-dependent fashion

Existing evidence for integrin adhesions being promoted by ROCK is largely based on studies that monitor the size of individual integrin adhesions as an experimental readout; whereas our initial experiments were specifically designed to analyze attachment of whole cells. Thus, we next examined the effect of CXCL12 stimulation on individual adhesion complexes within single cells. Because we are interested in the initial phase of tumor cell attachment, we used Total internal reflectance fluorescence (TIRF) microscopy to examine the formation of adhesion complexes, marked by GFP-paxillin during the first 20 minutes after cell plating.

In control cells, GFP-paxillin was clearly incorporated into adhesion complexes, visible by TIRF microscopy. By 20 minutes, cells were well spread and the adhesion complexes were distinct and visible, primarily at the cell periphery with a few located on the interior of the cell (Fig. 2A). We also observed that in addition to effects on adhesion complexes, CXCL12 stimulation led to increased cell protrusions and a less symmetric, more elongated morphology (Fig. 2A). CXCL12 stimulation enhanced adhesion complexes as measured by a 79% increase in the number of adhesion complexes, and a 144% increase in the area occupied by adhesions (Fig. 2B,C).

To determine the effect of ROCK on adhesion complexes, we included the ROCK inhibitor, Y-27632, during cell attachment. Inhibition of ROCK led to accumulation of numerous small adhesion complexes that were restricted to a thin band along the cell periphery (Fig. 2A). Although there was a striking effect on the morphology of the adhesions that form with Y-27632, the total area occupied by adhesions was increased by Y-27632 (Fig. 2B), which might explain why cell attachment does not require ROCK activity. The morphology of the adhesions induced by CXCL12 was blocked by Y-27632, indicating that ROCK activity was necessary to form larger adhesions (Fig. 2A). At this early time following adhesion (20 minutes), the cells treated with Y-27632 appeared larger with relatively regular edges; the asymmetry induced by CXCL12 stimulation is lost when ROCK is inhibited.
Thus, these findings show that inhibition of ROCK during cell attachment leads to small clusters of GFP-paxillin-containing complexes near the periphery, analogous to the ROCK-independent adhesions formed during membrane extension (Rottner et al., 1999). These observations led us to propose that whereas ROCK is dispensable for CXCL12-driven attachment and nascent adhesion formation, re-activation of ROCK is required for adhesion complex maturation.

**ROCK inhibition prevents formation of mature adhesion complexes**

To test the idea that the small adhesion complexes that accumulate upon ROCK inhibition are immature adhesion complexes, we used two markers of early and mature adhesion complexes: tyrosine-phosphorylated paxillin [Y(P)-Pax] and zyxin, respectively (Zaidel-Bar et al., 2007b). We found that adhesion complexes, marked by vinculin, are differentially marked by Y(P)-Pax and zyxin following treatment with Y-27632. In untreated, control cells, adhesion complexes formed within the first 20 minutes of attachment are positive for vinculin, Y(P)-Pax and zyxin (Fig. 3A). However, when cell attachment occurs in the presence of Y-27632, the adhesions are smaller (as visualized by vinculin staining) and positive for Y(P)-Pax, but negative for zyxin (Fig. 3B). We obtained a similar result when cells were stimulated by 10 nM CXCL12. The small adhesions that formed in CXCL12 cells treated with the ROCK inhibitor were positive for Y(P)-Pax, but negative for zyxin, indicating that the enhancement of adhesions promoted by CXCL12 requires maturation that is dependent upon ROCK activity. This result highlights the distinct requirements for ROCK activity during CXCL12-promoted adhesion: ROCK is dispensable for both initial cellular attachment and formation of nascent adhesions, but ROCK activity is required for adhesion maturation which is probably important for cell migration.

**ROCK activity is required for migration**

To test whether ROCK activity was required for tumor cell migration in response to CXCL12, we monitored migration by phase contrast microscopy over a 4 hour time course (See Materials and Methods for details). MCF7-CXCR4 cells were plated onto collagen-1-coated MatTek plates in the presence or absence of CXCL12 and images were collected every 2.5 minutes for 4 hours. Fig. 4A and B show the tracks of a representative group of cells (full movies are included in supplementary material Movies 1-4). Measurement of migration by net displacement distance shows that the migration activity of the CXCL12-treated cell population was increased relative to that of the control cell population (P=0.001). The CXCL12-induced increase in migration activity was significantly attenuated in the presence of Y-27632 (P=0.014), demonstrating that ROCK activity is required for efficient cell migration. Interestingly, we found that Y-27632 did not block membrane activity because there were numerous, active membrane extensions (Fig. 4A and supplementary material Movies 3 and 4), but the morphology was abnormal. The most striking effect of CXCL12 stimulation was the increase in the percentage of cells that moved long distances. Fig. 4C shows the distribution of net cell displacement for the populations of cells under each treatment condition. Whereas 19% of the CXCL12-treated cell population migrated >80 μm, none of the control cell population migrated this far. The data also demonstrate that ROCK activity is required for CXCL12-enhanced migration, because none of the cells treated with both CXCL12 and Y-27632 migrated >80 μm.

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**Fig. 3. ROCK inhibition prevents formation of mature adhesion complexes.** Immunofluorescence images of MCF7-CXCR4 cells plated for 20 minutes on collagen-1-coated coverslips (A). MCF7 cells were detached from culture plates and incubated at 37°C for 5 minutes with 10 nM CXCL12 (B), 10 minutes with 10 μM Y-27632 (C) or 10 minutes with Y-27632 followed by 5 minutes with CXCL12 (D). Cells were then plated onto collagen-1-coated coverslips in treatment media. After adhering for 20 minutes, cells were fixed with paraformaldehyde and incubated with vinculin, zyxin or FITC-conjugated phosphotyrosine (Y118) paxillin. White boxes indicate regions of the cells shown enlarged above the respective panel. Pretreatment with Y-27632 resulted in small, peripheral adhesion complexes. These small Y-27632-induced adhesion complexes were positive for Y(P)-Pax, but negative for zyxin, indicating that ROCK activity is necessary for adhesion maturation. Inhibition of ROCK by Y-27632 also prevented the formation of mature adhesions in the presence of CXCL12. Scale bars: 10 μm.
Biphasic ROCK activity during tumor cell attachment

Because initial CXCL12-stimulated tumor cell attachment is promoted by ROCK inhibition, but subsequent events, such as maturation of adhesion complexes and enhanced cell migration, are blocked by ROCK inhibition, we examined the kinetics of ROCK activity during the transition from suspension to adhesion. To measure ROCK activity, we used phosphorylation of two downstream targets of ROCK signaling, myosin phosphatase target subunit (MYPT) and cofolin, as readouts for ROCK activity during early adhesion events. As shown in Fig. 5A, cell attachment induced a brief but pronounced decrease in ROCK activity within 5 minutes of cell attachment. Following this brief decrease in activity, ROCK signaling was re-activated to basal levels in response to attachment.

It is known that CXCL12 and other chemokines can regulate RhoA and ROCK activity, however, the kinetics of regulation are dependent upon the specific experimental system used (Arakawa et al., 2003; Moyer et al., 2007; Tan et al., 2006; Vicente-Manzanares et al., 2002). To determine how CXCL12 affected the ROCK activity during tumor cell attachment, MCF7-CXCR4 cells were pretreated with 10 nM CXCL12 5 minutes prior to plating onto collagen-1-coated dishes, and the phosphorylation state of MYPT and cofolin were measured as indicators of ROCK activity. We found that CXCL12 decreased the overall levels of ROCK activity, although the pattern of ROCK activity in response to attachment was retained (Fig. 5A). Quantification of the blots is provided in supplementary material Fig. S4A,B. We next measured the levels of GTP-RhoA using an ELISA-based activity assay (Fig. 5B,C). As with ROCK activity, cellular attachment induced a transient decrease in RhoA activity, followed by re-activation. Pretreatment with 10 nM CXCL12 decreased the activity of RhoA at early time points of tumor cell adhesion, mirroring the effect of CXCL12 on ROCK activity. These findings are different from that observed in adherent cell cultures, where CXCL12 has been shown to enhance RhoA and ROCK activity (Amine et al., 2009; Azab et al., 2009; Molina-Ortiz et al., 2009). For comparison, we performed analogous assays in steady state adherent cells and found that CXCL12 increased ROCK activity in this context, consistent with other studies (supplementary material Fig. S5). Thus, the influence of CXCL12 on ROCK activity is dependent upon the specific conditions.
**CXCR4 stimulation increases MCF7-CXCR4 cell attachment under flow conditions**

Together our data show that CXCL12 induces attachment to a wide range of adhesive ligands, including both extracellular matrix and endothelial cell adhesion molecules. The goal of the study was to determine if CXCR4-CXCL12 signaling could influence adhesive activity of tumor cells transitioning from suspension to adhesion. Thus, we measured the adhesion of the MCF7-CXCR4 cells under physiological flow conditions following stimulation with 10 nM CXCL12.

MCF7-CXCR4 cells were pumped through a laminar flow chamber in the presence or absence of CXCL12, as described in the Materials and Methods. We found that the flowing tumor cells attached to the collagen 1 under shear stresses ranging from 5.0 to 40 μN/cm². Based on published data, we selected 20 μN/cm² for subsequent analysis (Liang and Dong, 2008; Ngo et al., 2008). Our data shows that CXCL12 leads to a marked, 2.8-fold (180%) increase in the number of cells attached under flow (Fig. 6A). In fact, the increase is significantly more than what was observed in the static attachment assays (26%; Fig. 1). This is probably because shear stress provides a more stringent environment for attachment, and the requirement for CXCL12 signaling becomes more essential. Inhibition of ROCK activity with Y-27632 did not block adhesion induced by CXCL12, similar to what we observed with the static adhesion assays in Fig. 1. Microscopic analysis of the morphology of the newly adherent cells (Fig. 6B) showed that CXCL12 enhances cell spreading, whereas Y-27632 impairs spreading under flow conditions. Thus, CXCL12 and Y-27632 both promote attachment under flow, but only CXCL12 supports cell spreading.
ROCK in CXCR4-driven tumor cell adhesion

We have used collagen 1 matrix as an initial surface to measure adhesion under flow conditions because we find it to form a robust surface for MCF7-CXCR4 cell adhesion and it provides an important condition for comparing our static attachment assay results. However, to more closely mimic the conditions for tumor cell recruitment, we performed analogous experiments in which the lower surface of our laminar flow cell was composed of an endothelial cell monolayer. A confluent monolayer of endothelial cells (HUVECs) was stimulated with TNFα for 3 hours prior to the introduction of MCF7-CXCR4 cells (see Materials and Methods for details). In these experiments, the MCF7-CXCR4 tumor cells were fluorescently labeled with CellTracker™ Green prior to the attachment assay, so they could be easily discerned from the endothelial monolayer (Fig. 7B). Fig. 7A shows that stimulation of suspended tumor cells with 10 nM CXCL12 increased the number of tumor cells attached to the activated endothelium by 2.3-fold. Thus, tumor cells expressing CXCR4 have a greatly enhanced chance of adhering to activated endothelium when stimulated with CXCL12. Inhibition of ROCK with Y-27632 also increased attachment to the endothelium by nearly twofold, and did not block CXCL12-induced attachment (Fig. 7A), showing that ROCK activity is not required for initial tumor cell attachment under flow conditions.

Inhibition of ROCK activity is required for MCF7 cell attachment

The observations that ROCK activity is dispensable for tumor cell attachment, coupled with the decrease in ROCK activity during early adhesion time points, led us to hypothesize that the transient decrease in ROCK activity that occurs following tumor cell adhesion is required for cell attachment. To test the hypothesis that attachment under flow conditions requires the inhibition of ROCK, we expressed a fluorescently tagged constitutively active mutant of ROCK (GFP-CA ROCK) in MCF7-CXCR4 cells and measured the attachment of the active-ROCK-transfected cells as compared with GFP control transfectants under flow conditions. Fig. 6C shows that GFP-CA ROCK expression reduced attachment to collagen 1 by 51%, as compared with GFP controls, and prevented an increase in attachment in response to CXCL12. The morphology of the GFP-CA ROCK cells that did attach to the substratum was relatively round and unspread, even in the presence of CXCL12,
demonstrating that too much ROCK activity prevents both attachment and spreading in response to CXCL12 (Fig. 6D).

We then examined attachment to endothelial cell monolayers and found that tumor cells expressing GFP-CA ROCK reduced basal attachment by 27%, as compared with GFP controls. Strikingly, GFP-CA ROCK expression completely blocked attachment in response to CXCL12 (Fig. 7C). These results demonstrate that CXCL12 promotes the binding of flowing tumor cells to an activated endothelial monolayer in a manner that requires inhibition of ROCK. This is an important result because it demonstrates that inhibition of ROCK is required for tumor cell attachment. In other words, not only is ROCK activity dispensable for cellular attachment, but inhibition of activity is required for attachment (Figs 6, 7).

Although many factors are likely to contribute to the recruitment of tumor cells to specific organs during metastasis, one mechanism is that adhesion promoted by CXCL12 plays a fundamental role in the process (Balkwill, 2004; Ben-Baruch, 2008; Dittmar et al., 2008; Miles et al., 2008; Witz, 2008). CXCL12 is a known ‘capture’ chemokine for hematopoietic cell recruitment, and as such functions as a gatekeeper for cellular trafficking into specific tissues. The aberrant trafficking characteristics of malignant tumor cells are often thought to be via appropriation of mechanisms normally used during

**Discussion**

To better understand the signaling mechanisms that promote metastasis, we designed a system to examine the role of ROCK signaling in CXCR4-driven breast tumor adhesive events. Interestingly, we found that ROCK plays a biphasic role in the regulation of adhesion during tumor cell attachment. Initially, ROCK inhibition is required for cell attachment; but subsequently, re-activation of ROCK is required for maturation of adhesion complexes and migration (Model, Fig. 8). These two distinct requirements for ROCK activity during adhesive events are reflected in the kinetics of ROCK activity. Specifically, analysis of ROCK activity following adhesion shows two distinct phases: a rapid initial decrease in ROCK activity followed by subsequent re-activation (Fig. 5). Interestingly, CXCL12 reduces the activity levels of ROCK at both phases. This suggests that CXCL12 signaling acts in concert with attachment signals to fine-tune ROCK activity for optimal tumor cell adhesive events, as too much or too little activity blocks tumor cell behavior. To more accurately model the environment of tumor cell recruitment, we performed adhesion assays under conditions of shear stress and examined adhesion to extracellular matrix and endothelial cell monolayers (Figs 6, 7). Under conditions of shear stress, the ability of the chemokine CXCL12 to stimulate tumor cell adhesion and spreading was even more pronounced than in the static adhesion assays (Figs 1, 6). Further analysis of cell attachment under flow conditions showed that expression of active ROCK blocked attachment, demonstrating that not only is ROCK activity dispensable for cellular attachment, but inhibition of activity is required for attachment (Figs 6, 7).

**Fig. 7. Role of ROCK activity in attachment to endothelial monolayers under flow.** MCF7-CXCR4 cells were processed as in Fig. 2 for the attachment assay. Cells were then flowed over endothelial (HUVEC) monolayers that had been stimulated with TNFα (see Materials and Methods) in a Bioptechs FCS2 flow chamber, at a rate of 20 μN/cm², for 20 minutes at 37°C. Tumor cells were distinguished from the endothelial monolayer by labeling with CellTracker Green, and phase-contrast and fluorescent images of 10 separate fields per sample were collected. (A) Graphs depict the number of attached cells per experimental condition, normalized to the control condition, and represent the average from three independent experiments. Note that CXCL12 significantly increases the attachment of tumor cells by the endothelium. (*indicates sample pairs for which P<0.05 and **sample pairs for which P<0.01; Student’s t-test.) (B) Representative images of control and CXCL12-treated MCF7-CXCR4 cells attached to the endothelial monolayer that were used for quantification. The fluorescent images of the tumor cells are overlaid on the phase contrast image of the endothelial monolayer. Scale bar: 200 μm. (C) Graphs depict the number of attached cells per experimental condition, normalized to the control condition, and represent the average from three independent experiments. Note that expression of GFP-CA ROCK significantly decreases attachment of tumor cells to the endothelium in both unstimulated and CXCL12 treatment conditions.
Thus, our results extend the previous findings by demonstrating integrin engagement; however, these studies did not measure the RhoA activity linked to adhesion complexes. We found that CXCL12-induced migration requires ROCK activity; an initial tumor cell attachment is followed by effective ROCK activity. At the second stage, ROCK activity levels increase, necessary for subsequent cell migration. In the situation where CXCL12-CXCR4 signaling is stimulated, overall ROCK activity levels are lower. Thus, we propose that CXCL12 modulates levels of ROCK activity regulated by attachment, to provide an optimal level of ROCK activity necessary for tumor cell migration.

Although inhibition of ROCK was necessary for initial tumor cell attachment, subsequent re-activation of ROCK was required for downstream responses to CXCL12. Paxillin-containing adhesion complexes were enhanced by CXCL12 through a mechanism that required ROCK activity (Fig. 2). Adhesion complexes can contain at least 150 different proteins, involving both structural and signaling components (Zaidel-Bar et al., 2007b). Investigations into regulation of integrin adhesion complexes have shown that several cytoskeletal and signaling systems are coordinated to control adhesion dynamics. Integrin adhesions are regulated by the structure of the matrix outside the cell, as well as actin and microtubule dynamics, myosin II contractility, and signaling modifiers within the cell (Broussard et al., 2008; Gupton and Waterman-Storer, 2006; Webb et al., 2004) (Choi et al., 2008; Even-Ram et al., 2007; Vicente-Manzanares et al., 2007). The interplay between these systems leads to the formation of nascent adhesions in the lamellipodium, followed by maturation of the nascent adhesions into larger, dynamic complexes which support forward movement of the lamella to advance cell motility (Giannone et al., 2007; Gupton and Waterman-Storer, 2006; Vicente-Manzanares et al., 2007). Our results show that ROCK activity is required for the maturation of nascent adhesions in response to CXCL12 and subsequent cell migration (Figs 3, 4). The requirement for ROCK activity during adhesion maturation and cell migration is reflected in the activity assays that show ROCK is re-activated following the initial decrease in activity (Fig. 5). Together, our data show that although ROCK activity is inhibited during cell attachment, its activity must return for CXCL12 to regulate adhesion complexes and cell migration.

Our results show that ROCK has a biphase response to adhesion: a transient decrease, followed by a return to activity levels greater than or equal to baseline. This is similar to the pattern of RhoA activity in response to integrin engagement (Arthur et al., 2000; Ren et al., 1999) where a transient decrease in RhoA activity was shown to be necessary for cell spreading, although the effects on cell attachment were not assessed. Arthur et al. identified activation of p190RhoGAP as a mediator of the decrease in RhoA activity (Arthur et al., 2000). Interestingly, CXCL12 decreases the overall levels of ROCK activity, while retaining the transient decrease pattern induced by adhesion (Fig. 5B). One possibility is that CXCR4 signaling activates tyrosine kinases, such as Src or Arg, that activate p190RhoGAP, leading to inhibition of RhoA-GTP loading and lower ROCK activity (Bradley et al., 2006; Chang et al., 1995; Fincham et al., 1999). In this way, CXCR4 could act in concert with integrin signaling to modulate ROCK activity to regulate tumor cell adhesion events that support motility.

We found that CXCL12-induced migration requires ROCK activity; however, CXCL12 reduces the level of ROCK activity generated by adhesion. This seemingly paradoxical result suggests that CXCL12 leads to an optimal level of ROCK activity to enhance migration (Model, Fig. 8). In the context of current models for leading edge behavior during migration (Choi et al., 2008; Giannone et al., 2007; Gupton and Waterman-Storer, 2006), CXCL12 might fine-tune ROCK activity to regulate cycles of lamellipodial and adhesion dynamics, favoring advance of the lamella and persistent migration. Further studies are designed to probe how CXCR4 regulates lamellipodial and adhesion dynamics, as a means to understanding mechanisms by which modulation of ROCK activity contributes to tumor cell recruitment.
Metastasis is clearly an important health concern and CXCR4 and ROCK have each been identified as contributing factors. ROCK is a multifunctional kinase: it receives input from a wide range of extracellular signals and is responsible for cellular responses including regulation of the actomyosin cytoskeleton, cell growth and survival, and gene transcription. The mechanisms by which extracellular signals direct ROCK to act at the right place and at the right time to achieve the appropriate biological outcome are presently undefined. Likewise, the effects of CXCR4 are pleiotropic.

Although ROCK and CXCR4 might impinge on multiple steps of tumor cell metastasis, our studies implicate tumor cell adhesion to the endothelium as an effect of aberrant CXCL12 signaling, and identify ROCK-dependent regulation of cell adhesion events as a possible mechanism by which CXCR4 promotes tumor cell recruitment and metastasis. Continued investigation into how CXCR4 regulates ROCK signaling to promote tumor cell adhesion and recruitment has the potential for identifying target molecules for blocking CXCR4-driven metastasis.

Materials and Methods

Reagents

Recombinant ICAM-1, VCAM-1, CXCL12 and TNFα were purchased from R&D Systems (Minneapolis, MN). Anti-phospho-myosin phosphorylation (MYPT; Th696) was purchased from Biotica (Billericia, MA). Anti-phospho-ERK (1:2000) and anti-phospho-cofilin (Ser3,-cofilin,-ERK, actin and -MYPT (all at 1:1000) were obtained from Cell Signaling (Danvers, MA). Y-27632, anti-zyxin (1:100) and anti-vinculin (1:400) were obtained from Sigma-Aldrich (St Louis, MO). FITC-labeled phospho-paxillin antibody (Tyr118; used at 1:50 dilution) was purchased from Biosource (Camarillo, CA). CellTracker cell-permeable dye and Lipofectamine 2000 were both purchased from Invitrogen (Carlsbad, CA). Collagen I and reduced-growth-factor buffer (1:400) were obtained from Sigma-Aldrich (St Louis, MO). Microaqueduct slides (Bioptechs) were coated with collagen I (10 μg/ml) and then incubated alone or with 10 nM CXCL12 for 5 minutes at 37°C.

Fluorescent reporter GFP was used to visualize cell adhesion at the endothelial surface. To this end, MCF7-CXCR4 cells were transfected with nGFP-DEST vector alone, or nGFP-CA ROCK (transfection described above) and used in flow-based attachment assays after 48 hours. MCF7-CXCR4 cells were flowed through the chamber at a shear stress of 2 dynes/cm² for 20 minutes at 37°C, after which images from ten regions of the chamber were taken and the number of attached cells per field were counted manually. Images were acquired with a 10x (NA=0.45) objective on an Olympus IX81 microscope, equipped with a Hamamatsu OrcaER camera, run by Slidebook software.

Rock activity assay (Immunoblot)

To assess ROCK activity during adhesion, cells were serum-starved for 2 hours, then detached using 0.05% trypsin, pooled and centrifuged at 800 g for 5 minutes, and then incubated alone or with 10 nM CXCL12 for 5 minutes at 37°C. Cells were then plated in incubation medium onto collagen-1-coated (10 μg/ml) culture dishes and allowed to adhere for 5-60 minutes at 37°C. The cells were then washed with PBS to remove any unfixed cells and the remaining adherent cells were lysed with ice-cold modified RIPA buffer with protease and phosphatase inhibitors (Sigma). Equal amounts of protein were loaded on 10% polyacrylamide gels, transferred to PVDF membranes, blocked for 1 hour with 5% milk-TBS, and incubated with phophospecific antibodies to MYPT or cofilin.

Analysis of adherent cells (supplementary material Fig. S5) MCF7-CXCR4 cells were plated 2 days prior to the experiment, then serum starved for 2 hours prior to the addition of 10 nM CXCL12 for the indicated times. Following CXCL12 treatment, cells were then washed with PBS and lysed with ice-cold modified RIPA buffer with protease and phosphatase inhibitors. Equal amounts of protein were loaded onto polyacrylamide gels for immunoblot analysis. For analysis of suspension cells (supplementary material Fig. S5) MCF7-CXCR4 cells were suspended as for the attachment assays, incubated for 10 minutes, then 10 nM CXCL12 was added for the indicated times. To demonstrate that the phosphorylation of MYPT and cofilin were dependent upon ROCK activity, we performed the same assay in the presence of Y-27632 (supplementary material Fig. S5). Following incubation, cells were pelleted at 50 g for 5 minutes and lysed with ice-cold modified RIPA buffer with protease and phosphatase inhibitors (Sigma). Equal amounts of protein were loaded onto polyacrylamide gels for immunoblot analysis.

RhoA and Rac activity assays

To measure Rho and Rac GTP levels, cells were suspended and then re-plated as described for the ROCK activity assay (see above) in the presence or absence of 10 nM CXCL12. Rho and Rac activity were measured by G-LISA assay (Cytoskeleton, Boulder, CO), with strict adherence to the manufacturer’s guidelines. The G-LISA reports levels of active RhoA and Rac normalized by protein input levels. Additionally, equivalent amounts of cellular lysates were taken from Rho activity lysates and were analyzed by immunoblotting to ensure Rho expression did not change during the time frame of the experiment.

Immunocytchemistry

MCF7-CXCR4 cells were plated onto collagen-1-coated coverslips (10 μg/ml) and allowed to adhere at 37°C for 20 minutes with 10 μM Y-27632. As a control, we also tested an alternative ROCK inhibitor, H1152 (supplementary material Fig. S3). Following the adhesion period, cells were washed with ice-cold PBS and then fixed with 4% paraformaldehyde in PBS. After washing three times with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS, washed once with PBS, and 4% normal goat serum was used as a blocking agent. Fixed cells were then incubated with primary antibodies (anti-phospho-paxillin (1:50), anti-vinculin (1:400) or anti-zyxin (1:100)) for 2 hours, followed by a 1-hour incubation with the appropriate secondary antibody (goat anti-rabbit or anti-mouse Alexa Fluor 488 or Alexa Fluor 594; Jackson ImmunoResearch, West Grove, PA). In some experiments, cells were stained for F-actin with Alexa Fluor 594-phalloidin. Coverslips were then mounted onto glass slides using Prolong AntiFade Gold (Invitrogen). Images were captured using an Olympus IX81 with a 60x (NA=1.4) oil immersion objective, equipped with a Hamamatsu EM camera, and processed using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Images were further processed using Adobe Photoshop, using only linear adjustments of the signal.

Total internal reflectance fluorescence (TIRF) microscopy

Cells were transfected overnight with GFP-paxillin with Lipofectamine 2000 transfection reagent (Invitrogen) at a ratio of 1:3 (μg/μl) DNA/Lipofectamine according to manufacturer’s protocol. The following day, cells were starved in SFM for 2 hours, detached from plates, and re-plated for 20 minutes on collagen-1-coated Bioptechs Delta T dishes. During attachment and imaging, cells were maintained at 37°C using a Bioptechs Delta T temperature-controlled stage adapter and heated lid. Images were collected on an Olympus IX71 microscope with a 60x TIRF objective (NA=1.49). Samples were excited with an argon laser (488 nm) and fluorescent images captured with a Hamamatsu OrcaER digital camera, using Slidebook Software.

Assessment of cell motility

MCF7-CXCR4 cells were detached, pretreated with CXCL12 (10 nM, 5 minutes), Y-27632 (10 μM, 10 minutes) or Y-27632 (10 μM, 10 minutes) followed by CXCL12 imaging.
(10 nM, 5 minutes), and plated onto collagen-1-coated MatTek dishes. Phase-contrast imaging of the cell cultures was performed using a Leica DMi8 inverted microscope and a color camera. After an additional 20 minutes of incubation, the cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. The actin cytoskeleton was stained with Alexa Fluor 488-phalloidin, and the nuclei were stained with DAPI. The images were acquired using a Leica TCS SP8 confocal microscope with a 63x/1.40 oil objective lens, and the cells were imaged in sequential images to minimize photobleaching. The data was analyzed using ImageJ software to quantify the actin stress fibers and the number of focal adhesions per cell.

**FACS analysis**

Cell suspensions were prepared from the cell cultures using TrypLE Express. The cells were washed with PBS and resuspended in PBS containing 2% FBS. The cells were stained with anti-CXCR4 antibodies and analyzed by flow cytometry using a BD CantoII flow cytometer, and the mean fluorescence intensity was used for comparison of cell surface CXCR4 expression.

**We appreciate all of the input from the members of the Cell Adhesion and Migration Group at LSU Health Sciences Center. In addition, we would like to thank the Cell Biology Core Facility for the gentle and efficient cell density measurements, Stephanie Cormier and Dahui You for the flow cytometry analysis, and Don Mercante for the statistical analyses. The project was supported by grant number 5R01GM082762 from the National Institute of General Medical Sciences,esda.

**References**


