Published in final edited form as:

Acta Biomater. 2014 May ; 10(5): 1886–1896. doi:10.1016/j.actbio.2013.12.058.

Cellular Traction Stresses Mediate Extracellular Matrix Degradation by Invadopodia

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Abstract

During tumorigenesis, matrix rigidity can drive oncogenic transformation via altered cellular proliferation and migration. Cells sense extracellular matrix (ECM) mechanical properties with intracellular tensile forces generated by actomyosin contractility. These contractile forces are transmitted to the matrix surface as traction stresses which mediate mechanical interactions with the ECM. Matrix rigidity has been shown to increase proteolytic ECM degradation by cytoskeletal structures known as invadopodia that are critical for cancer progression suggesting that cellular contractility promotes invasive behavior. However, both increases and decreases in traction stresses have been associated with metastatic behavior. Therefore, the role of cellular contractility in invasive migration leading to metastasis is unclear. To determine the relationship between cellular traction stresses and invadopodia activity, we characterized the invasive and contractile properties of an aggressive carcinoma cell line utilizing polyacrylamide gels of different rigidities. We found that ECM degradation and traction stresses were linear functions of matrix rigidity. Using calyculin A to augment myosin contractility, we also found that traction stresses were strongly predictive of ECM degradation. Overall, our data suggest that cellular force generation may play an important part in invasion and metastasis by mediating invadopodia activity in response to the mechanical properties of the tumor microenvironment.

Keywords

traction stresses; rigidity; actomyosin contractility; invadopodia; degradation

1. INTRODUCTION

A myriad of biological processes such as embryogenesis [1], wound healing [2], and inflammation [3] rely on the ability of cells to migrate through the extracellular matrix (ECM). Migration is typically driven by actomyosin-generated contractile forces that are transmitted to the ECM as traction stresses (force per area) [4]. Traction stresses facilitate mechanical interactions between cells and the ECM and are used to probe the stiffness of the cellular microenvironment in a process known as rigidity mechanosensing [5]. In normal cells, the magnitude of these stresses is dictated by the resistance that is sensed by the cells

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in response to the mechanical properties of the surrounding matrix [6]. These interactions regulate the organization of the actin cytoskeleton and focal adhesions [7] and can lead to changes in gene expression [8]. Therefore, traction stresses have been implicated in mediating many cellular events including adhesion and migration [9], proliferation [10], differentiation [11], ECM remodeling [12], and mechanotransduction [13].

In cancer, tumor cell migration is fundamental to disease progression via invasion and metastasis. Increasing ECM rigidity during tumorigenesis is thought to drive oncogenic transformation by disrupting tissue homeostasis and morphology due to proliferation and the acquisition of a motile phenotype [14]. While matrix rigidity has been shown to activate mechanical signaling pathways via actomyosin contractility [15] and regulate cancer cell invasion in vitro [16], current studies conflict as to whether transformation to a malignant phenotype is correlated to increased or decreased traction stresses. A common metastatic cell line of H-ras transformed 3T3 fibroblasts have been shown to exhibit decreased traction stresses on soft substrates compared to control cells [17], whereas metastatic A3 sarcoma cells derived from rat K2 fibroblasts exerted larger tractions at the leading edge and increased in vitro invasion when compared to parental K2 cells [18]. Indra et al. have reported an inverse relationship between traction stresses and metastatic capacity utilizing isogenic murine breast cancer lines with increasing metastatic capacity [19]. In contrast, Kraning-Rush et al. used well-established human breast, prostate, and lung cancer cell lines to show a direct correlation between metastatic capacity and traction stresses in response to rigidity [20]. While it remains unclear how the magnitude of traction stresses dictates invasive migration leading to metastasis, these variations may be indicative of altered adhesive and contractile properties that may be required for different modes of migration depending on the characteristics of the local ECM [21].

To penetrate tissues, cancer cells can utilize cellular forces to mechanically reorganize the ECM to move along collagen fibers as well as to migrate through pores, defects, and pre-existing matrix tunnels [21, 22]. However, ECM penetration by cancer cells also requires proteolytic degradation for invasive migration given the existence of covalent cross-links in native tissues [22]. Actin-rich subcellular protrusions known as invadopodia facilitate this task in vitro due to their ability to localize proteinases including matrix metalloproteinases (MMP)-2, -9, and MT1-MMP to focally degrade the ECM at these structures [23]. These structures are thought to be a hallmark of invasive cells and provide them the ability to breach tissue barriers; therefore, they have been implicated in tumor cell invasion and metastasis [24]. Previous work has shown that matrix rigidity can regulate the number and activity of invadopodia in breast cancer cells [25, 26]. In particular, stiffer substrates with mechanical properties that corresponded to cancerous breast tissues induced more actively degrading invadopodia and corresponding ECM degradation. In addition, invadopodia activity has been found to be dependent on myosin II activity and the mechanosensing proteins FAK and p130Cas [25]. Although present in ring-like structures around a subset of invadopodia, myosin II was not present at these cytoskeletal structures suggesting that actomyosin contractility throughout the cell plays an important role in rigidity mechanosensing during proteolytic migration [25].

These studies demonstrate that phenotypic changes in cancer cells alter their biophysical properties which may have significant implications for disease progression. Although invadopodia activity is rigidity-dependent and appears to rely on cellular contractility, it is unknown whether traction stresses mediate ECM degradation which is required for proteolytic migration. Therefore, our study is focused on determining whether traction stresses correlate with invadopodia-dependent ECM degradation by evaluating the response of an invasive head and neck squamous cell carcinoma (HNSCC) cell line to matrix rigidity. In order to accomplish our goal, we first characterized a set of polyacrylamide hydrogels

(PAAs) that could be utilized as the basis for both invadopodia and traction force assays that span the range of rigidities found for normal and tumor tissues that have been reported in the literature [14, 27]. For our HNSCC cells, we found that invadopodia expression and activity are dependent on matrix rigidity and specifically that ECM degradation is a linear function of the elastic modulus of our substrates. Traction stress analyses also revealed that cellular forces generated by the actin cytoskeleton are a linear function of the elastic modulus of our substrates. In order to determine if ECM degradation was indeed a direct function of traction stresses, we chemically induced actomyosin contractility to increase traction stresses with calyculin A, an inhibitor of myosin phosphatase. We found that increasing these forces led to an increase in ECM degradation as well. Using a linear correlation between ECM degradation and traction stresses, we were able to correctly predict the expected increase in ECM degradation based on the experimental results for the chemically induced increase in traction stresses. Overall, our data indicate that traction stresses directly correlate to ECM degradation and suggest that cellular mechanical forces may play an important role in rigidity mechanosensing to mediate invadopodia activity during proteolytic migration.

2. MATERIALS AND METHODS

2.1 Cell culture and reagents

SCC-61 cells, a HNSCC cell line, were used for these studies since they have previously been characterized in terms of invadopodia activity, ECM degradation, protease secretion, and aggressiveness in vitro and in vivo [28–30]. This cell line was originally derived from a patient with a highly aggressive and recurrent tumor of the tongue that infiltrated the oral cavity [31, 32]. They were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 20% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 0.4 µg/ml hydrocortisone (Sigma, St. Louis, MO). They were passaged by 0.25% trypsin (Invitrogen) removal and resuspended in medium prior to becoming completely confluent. Cells were counted with a hemacytometer then centrifuged and resuspended in the appropriate volume for experiments. Traction stresses were increased with calyculin A (Sigma), a potent myosin phosphatase inhibitor [33] that allows for continued myosin II activity.

2.2 Substrate synthesis

In order to utilize the PAAs in both the invadopodia and traction force assays so that the cells experience the same rigidities, they were modified from previously established methods [25, 26] to include embedded fibronectin [34, 35]. The embedded fibronectin provides the same density of ligand for cellular adhesion at the surface of the PAAs for the traction force assays (see Section 2.6). For the invadopodia assays, these PAAs are overlaid with additional matrix proteins to assess proteolytic ECM degradation (see Section 2.4). Briefly, soft, hard, and rigid PAAs were synthesized from varying ratios of 40% acrylamide/2% BIS-acrylamide (Bio-Rad, Hercules, CA) to yield final concentrations of 8%/0.05%, 8%/0.35%, and 12%/0.6%, respectively. Different volumes of a 1 mg/ml fibronectin (Invitrogen) stock solution were added to achieve final concentrations of 200 µg/ml, 215 µg/ml, and 230 µg/ml for the soft, hard, and rigid PAAs, respectively. Embedded fibronectin was conjugated (i.e., covalently bound) to the polymerizing PAAs by inclusion of a 10 mg/ml acrylic acid N-hydroxysuccinimide (NHS) ester (Sigma) solution at a final concentration of 0.1%. For traction force assays, 200 nm red fluorescent beads (carboxylate modified microspheres with excitation 580 nm/emission 605 nm, size of 0.201 ± 0.01 µm, and concentration of 4.5×10^{12} particles/ml; Invitrogen) were also included at a final ratio of 1:125.

2.3 Substrate rheology

The rigidities of the PAAs were measured as previously described using rheometry [25, 26]. In brief, the shear storage modulus, G' , for each substrate was measured at 0.05% constant strain at a frequency of 1 Hz. The elastic modulus, E , was estimated as $E=3G'$ assuming a Poisson's ratio of 0.5.

2.4 Invadopodia assays

This assay is a standard in vitro technique for assessing invadopodia expression and proteolytic ECM degradation [36–39]. As previously established [25, 26], PAAs (Section 2.2) were cast on activated (aminopropyltrimethoxysilane and glutaraldehyde) glass coverslips of 35 mm MatTek dishes (MatTek, Ashland, MA) and overlaid with 1% gelatin (cross-linked with 0.5% glutaraldehyde) and FITC-labeled fibronectin to assess ECM degradation of these layers (fibronectin contains a specific collagen binding domain that provides a strong link between the PAA and gelatin as well as the gelatin and FITC fibronectin). For each dish, 25,000 cells were plated in invadopodia medium which contained DMEM:RPMI 1640 (Invitrogen) with 5% NuSerum (Gibco, Carlsbad, CA), 10% FBS (Invitrogen), and 20 ng/ml EGF (Invitrogen) and incubated for 18 hours.

2.5 Immunofluorescence

The colocalization of actin and cortactin were used as a marker for invadopodia [25, 26]. F-actin was identified by staining with Alexa Fluor 546 phalloidin (Invitrogen). Cortactin was identified with an anti-cortactin mouse primary monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) and visualized with a 633 goat anti-mouse IgG secondary antibody (Invitrogen). Fluorescent images were captured on a Nikon Eclipse TE2000-E inverted microscope with a 40× Plan Fluor oil immersion lens. Metamorph software (Molecular Devices, Sunnyvale, CA) was used to manually count invadopodia and quantitate ECM degradation by manually thresholding areas of black that were devoid of any FITC-fibronectin signal [36–39]. Fibronectin at the surface of the PAAs was identified with an anti-fibronectin mouse monoclonal antibody (BD Biosciences, San Jose, CA) and visualized with a 488 goat anti-mouse IgG secondary antibody (Invitrogen).

2.6 Traction force microscopy

Traction force microscopy and the subsequent analyses to determine substrate deformations and cellular traction stresses were performed as previously described [17]. PAAs and beads were prepared as described above (Section 2.2). Similarly, cells were incubated in invadopodia medium for ~18 hours but then switched to pre-warmed L-15 medium (Mediatech) with the same supplements and equilibrated for 1 hour in a custom-built environmental microscope chamber at 37°C with high humidity. Images were captured with a 40× 0.75 NA Plan Fluor objective of multiple cells using an automated stage to mark their positions. At each position, a phase image of the cell of interest and a “stressed” image of the fluorescent beads at the surface of the PAA substrate were taken. Cells were then removed with Triton-X 100, and the “null” image of the fluorescent beads was taken of the relaxed PAA substrate.

2.7 Calculating substrate deformations and traction stresses

The computer program LIBTRC was obtained via academic license from Micah Dembo of Boston University whom has previously described the theory for calculating substrate deformations and traction forces [40]. Briefly, a pattern recognition algorithm based on the optical flow method was utilized to determine the deformations of the substrate (“stressed” image) caused by cellular traction forces based on changes in the bead distribution in comparison to the undeformed state (null image). Using the bead displacements and

polyacrylamide gel mechanical properties (E and Poisson's ratio), the surface traction stress vectors were calculated based on the Boussinesq solution for an isotropic, elastic half space utilizing the maximum likelihood method. The mean of the magnitude of these vectors were taken to determine an overall average traction stress for each cell [17].

2.8 Statistics

Data were evaluated for normality with the Shapiro-Wilk or Kolmogorov-Smirnov test. Data that passed the normality test were analyzed by the Student's t-test or a one-way ANOVA. Data that did not pass the normality test were analyzed by a Mann-Whitney or Kruskal-Wallis test. If significance was determined within a group, either a Tamhane post-hoc test or a Student's t-test/Mann Whitney test with Bonferroni correction was used for pairwise comparisons. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed with IBM SPSS Statistics 21 (IBM, Armonk, NY). All experiments were performed in either triplicate or quadruplicate.

3. RESULTS

3.1 Characterization of PAAs with fibronectin

PAAs were developed as the basis for both the invadopodia and traction force assays to ensure that cells experienced the same rigidities in both experiments. In order to create substrates with the same amount of protein at their surfaces, varying amounts of fibronectin were mixed into the soft, hard, and rigid PAAs. Fluorescence images using a fibronectin antibody revealed a uniform layer of protein both macroscopically and microscopically (Figs. 1A–C) as well as similar fluorescence intensities (Fig. 1D) at the surface of the PAAs. To confirm uniformity, we also found that the average standard deviations of the pixel fluorescence intensities for all of our images were quite small (<7% of the mean average fluorescence intensity values from Fig. 1D) and did not differ between substrates (Fig. 1E). This approach was utilized since preliminary experiments revealed that using the same concentration of fibronectin in each PAA yielded different amounts of fibronectin at the surface (Fig. S1). In addition, fluorescence intensity at the surface of the PAAs with the different concentrations of embedded fibronectin was confirmed with secondary antibody only experiments to be a result of specific fibronectin antibody staining at this plane and not non-specific interactions or autofluorescence on the top or inside the PAAs (Fig. S2). Finally, we only detected fluorescence signal at the surface of the PAAs indicating local staining at this plane and not inside the PAAs. These data verified that increasing concentrations of fibronectin resulted in the same amount of protein at the surface of the soft, hard, and rigid PAAs.

3.2 Mechanical properties of PAAs with fibronectin

Since incorporation of fibronectin into PAAs can change their mechanical properties [34], we performed rheometry to evaluate the rigidity of the PAAs with and without fibronectin (Fig. 2). In addition, PAAs containing fluorescent beads were also analyzed since they are mixed into the substrates with fibronectin for traction force assays. The elastic moduli of the control PAAs were consistent with previous reports [26]. Fluorescent beads had no effect on the rigidities of the PAAs when compared to controls. In contrast, the inclusion of fibronectin significantly decreased the elastic moduli of the PAAs when compared to controls. In addition, fluorescent beads did not impact the mechanical properties of PAAs containing fibronectin. Therefore, the elastic moduli of the PAAs with both fibronectin and fluorescent beads were chosen as the representative values for their rigidities (mean $\pm$ standard error): $1,023 \pm 60$, $7,307 \pm 649$, and $22,692 \pm 1,209$ Pa for the soft, hard, and rigid PAAs, respectively. These rigidity values (1–23 kPa) span the range of elastic moduli reported for normal and cancerous tissues of other tumor types [27].

3.3 ECM degradation is dependent on matrix rigidity

To determine the role of substrate rigidity on invadopodia activity by SCC-61 cells, these cells were cultured overnight on the PAAs overlaid with fluorescently labeled matrix (gelatin and FITC fibronectin) to assess proteolytic ECM degradation. Samples were then fixed and immunostained for actin and cortactin which are markers for invadopodia (Fig. 3), and invadopodia and their associated ECM degradation were quantified (Fig. 4). We found that ECM degradation by SCC-61 cells significantly increased with increasing rigidity of the PAAs (Fig. 4A). Similarly, we also observed a statistical increase in the number of invadopodia actively degrading ECM (Fig. 4B) as well as the total number of invadopodia (Fig. 4C) in response to increasing PAA rigidity. Surprisingly, when we plotted the mean values of ECM degradation versus elastic modulus and performed a regression (Fig. 4D), we found an extremely linear relationship ($R^2 > 0.99$).

3.4 Cellular traction stresses are dependent on matrix rigidity

Contractile forces generated by the actomyosin cytoskeleton used to sense and respond to mechanical forces are transduced to the matrix surface as traction stresses [4]. Since our data indicated that invadopodia activity by SCC-61 cells was dependent on the elastic moduli of our substrates, we wanted to test whether contractility was also dependent on matrix rigidity. We measured cellular traction stresses exerted by SCC-61 cells as an indicator of contractility using traction force microscopy to determine whether overall cellular force generation was dependent on matrix rigidity (Fig. 5). Using the PAAs with embedded fibronectin and beads, we saw an increase in traction stresses as rigidity increased (Fig. 5A–C) that was statistically significant (Fig. 5D). Interestingly, when we performed a regression on the mean traction stress values as a function of the elastic modulus (Fig. 5E), we once again found a highly linear relationship ($R^2 = 0.99$). Therefore, these results indicated that actomyosin contractility by SCC-61 cells was a strong function of matrix rigidity.

3.5 Actomyosin contractility regulates ECM degradation

Since both ECM degradation and traction stresses increased with matrix rigidity, invadopodia activity may be mediated by cellular forces. Therefore, we wanted to determine if ECM degradation was dependent on traction stresses generated by actomyosin contractility. Calyculin A, a specific inhibitor of protein 1 and 2A phosphatases (PP1 and PP2a, respectively), has been utilized in many studies as a potent inhibitor of myosin phosphatase which allows for continued myosin light chain phosphorylation and thus augmented contractility and traction stresses [12, 41, 42]. Preliminary experiments verified that treatment with 0.1 nM calyculin A (Fig. S3A) did not significantly increase the size of SCC-61 cells on soft PAAs (Fig. S3B) but did significantly increase phosphorylation of myosin as measured by integrated fluorescence intensity (Fig. S3C) which has been established as a reliable method for phosphomyosin quantitation [43]. Therefore, we treated SCC-61 cells on soft PAAs without (Fig. 6A) and with (Fig. 6B) 0.1 nM calyculin A to increase actomyosin contractility. As expected, calyculin A treatment resulted in a statistically significant increase in traction stresses (Fig. 6C). We then performed our invadopodia assay on the soft PAAs without (Fig. 7A) and with (Fig. 7B) 0.1 nM calyculin and observed an increase in ECM degradation (Fig. 7C) as well. We also observed a statistical increase in the number of invadopodia actively degrading ECM (Fig. 7D) as well as the total number of invadopodia (Fig. 7E) in response to calyculin A.

3.6 Predicting ECM degradation with traction stresses

Our results have shown that ECM degradation by invadopodia appears to be dependent on actomyosin contractility. Since contractile forces generated by the actin cytoskeleton are transduced to the substrate surface as traction stresses, traction stresses could potentially

serve as a mechanical indicator for invadopodia activity. In order to determine whether traction stresses could be predictive of invadopodia activity, we first wanted to establish a correlation between these parameters. Since our results showed that both ECM degradation and traction stresses were linearly dependent on the mechanical properties of the substrates, we plotted the mean values of ECM degradation versus traction stresses to determine their relationship (Fig. 8A). Not surprisingly, we found a strong linear relationship between the two parameters ($R^2=0.98$). While these parameters were strongly related due to their dependence on the mechanical properties of the substrates, we needed to test whether we could use this relationship to predict ECM degradation based on traction stress levels independent of matrix rigidity. Since we previously showed that sustaining actomyosin contractility increases traction stresses as well as ECM degradation with calyculin A (Figs. 6 and 7), we used these experimental values to determine whether we could predict ECM degradation solely based on traction stress levels using the linear correlation derived in Fig. 8A. Interestingly, when we use our control and calyculin A traction stress values in this correlation, the predicted values for ECM degradation are within 4% of our experimental values (Fig. 8B).

4. DISCUSSION

ECM rigidity is thought to play a crucial role in activating cancer cells to become malignant and enhance tissue invasion through cellular migration [14] which has been reported to occur via proteolytic-dependent and -independent modes [21]. Migration is regulated by mechanical interactions between cells and the ECM that are mediated by traction stresses generated by actomyosin contractility [4]. Traction stresses are typically a function of ECM rigidity in normal cells since they are responsive to matrix mechanical properties and contribute to rigidity mechanosensing [5, 6]. However, the role of traction stresses in cancer cell migration is unclear since oncogenic transformation can alter the biophysical properties of cancer cells leading to diverse force profiles. For example, increases and decreases in traction stresses by metastatic cells in response to matrix rigidity have been implicated in regulating nonproteolytic migration [17–20]. In this study, we asked whether a relationship exists between traction stresses and ECM degradation since proteolytic migration is thought to rely on invadopodia activity which has been shown to be dependent on matrix rigidity and myosin II [25, 26]. Using SCC-61 cells, our results indicate that ECM degradation and traction stresses are significantly influenced by matrix rigidity. Furthermore, we have demonstrated that ECM degradation is a function of traction stresses. Overall, our data suggest that cellular contractile forces mediate invadopodia activity through mechanical interactions with the ECM to promote invasion and metastasis.

In order to assess both ECM degradation and contractility, we first developed a set of PAAs with tunable mechanical properties to utilize in both invadopodia and traction force assays. The goal was to ensure that cells seeded in each assay experienced the same rigidity. Previous methods have relied on conjugating matrix proteins to the PAA surface using reagents such as sulfo-SANPAH [9, 25] or acrylic acid NHS ester [44]. In our hands, we did not find that either of these methods produced a uniform coating of fibronectin on the PAA surfaces. Therefore, we chose to covalently bind fibronectin inside the PAAs using acrylic acid NHS ester as performed by other groups [34, 35]. In this manner, fibronectin is incorporated throughout the gel, including the surface, which allows for either direct attachment of cells as needed for the traction force assays or direct binding of ECM (gelatin with a layer of FITC fibronectin) as needed for the invadopodia assays. We would like to note that the thickness of this ECM layer has been previously reported as $\sim 1 \mu\text{m}$ [25], so it does not shield the cells from detecting the underlying PAA rigidities since such an effect occurs above 10–20 μm [45]. Our results show that by increasing the amount of fibronectin in each of the PAAs, we were able to ensure that the same amount of fibronectin was present

at the surface which is critical for exposing cells to the same ligand density on the different PAAs in the traction force assays.

Although we used the same formulations for the different PAAs as previously reported [25, 26], incorporation of fibronectin has been shown to alter the mechanical properties of PAAs [34]. Therefore, we measured the mechanical properties of our fibronectin embedded PAAs to determine the change in elastic modulus for each substrate. Despite the fact that we found a significant decrease in these properties, the elastic moduli values still spanned a wide range of rigidities that corresponded to normal and cancerous tissues reported for other tumor types such as varying grades of breast tumors [27]. These tissue rigidities are associated with changes in tissue density during tumorigenesis due to increases in cancer cell proliferation and packing, stromal deposition and crosslinking, new blood vessel formation and growth, and increased fluid pressures [55]. Factors that contribute to tumor density including tumor cell proliferation, desmoplasia, and angiogenesis are ubiquitous features of most cancers including HNSCCs [44]. Although head and neck tumor rigidities have not been reported in the literature, the similarities in the physical characteristics of HNSCCs [46] suggest that these tumors may have similar rigidities. Interestingly, these elastic moduli also corresponded to softer head and neck structures such as glands [47, 48], the soft palate [49], and muscles [50, 51]. PAAs spanning these rigidities allow for analyses with both our assays since this range yields detectable bead displacements for calculating traction forces [52]. In addition, a well-characterized HNSCC cell line [28–30] was used as a model since primary HNSCC cells freshly derived from human patients exhibit large numbers of invadopodia and ECM degradation in vitro [28].

Using the invadopodia assay, we found a significant increase in invadopodia numbers and ECM degradation in response to rigidity for SCC-61 cells. Interestingly, we found that ECM degradation (mean values) was a linear function of the elastic modulus of the PAAs that we tested suggesting that matrix rigidity had a specific effect on invasiveness via invadopodia activity. Our findings are consistent with previous reports of rigidity effects on proteolytic ECM degradation by CA1d breast carcinoma cells using PAAs spanning a similar rigidity range [25, 26]. For CA1d cells on glass substrates, phosphorylated forms of the mechanosensing proteins FAK and p130Cas have been shown to localize at actively degrading invadopodia and to be dependent on myosin II activity [25]. While these results suggest that ECM rigidity induces mechanical signaling at invadopodia to promote cellular invasion, it is unclear whether contraction occurs locally at invadopodia since myosin IIA was not always localized at actively degrading invadopodia [25]. Alternatively, mechanical activation of invadopodia may occur as a result of larger scale contractile forces generated by actomyosin activity elsewhere in the cell that are transmitted to these structures [53–55].

Since invadopodia activity by SCC-61 cells and other cancer cell types [25, 53] is dependent on matrix rigidity but oncogenic transformation appears to diminish traction stresses in some cases [17, 19], we first wanted to determine if SCC-61 cells exerted rigidity-dependent traction stresses. Using traction force microscopy, we found an increase in traction stresses in response to matrix rigidity that was also highly linear in nature with respect to mean values. Our findings are consistent with normal cells such as 3T3 fibroblasts and MDCK epithelial cells which have also been shown to have highly linear relationships between traction forces and substrate rigidity [56]. These trends are also consistent with the results found by Kraning-Rush et al. using other human cancer cell lines tested on different rigidities [20] but also conflict with reports by Indra et al. which found an inverse relationship using murine breast cancer cell lines [19]. These differences may result from specific phenotypic characteristics of the different cell lines in terms of their modes of migration. The more metastatic murine cells had less focal adhesions and decreased $\beta 1$ integrin activity yet showed increases in 3-D collagen invasion [19]. As noted by the

authors, these findings are consistent with an amoeboid mode of migration in which cells utilize cortical actomyosin contractility in a non-adhesive manner to physically move ECM fibers without strong traction forces [57]. In contrast, the metastatic cell lines used by Kraning-Rush et al. are all known to form invadopodia [58–60] suggesting a mesenchymal mode of migration dependent on strong adhesions and proteolysis [57]. Therefore, typical rigidity responses would be expected by cells using a mesenchymal mode of migration. While we found that ECM degradation and traction stresses linearly increased with matrix rigidity, we needed to determine if invadopodia activity was indeed dependent on actomyosin contractility. Thus, we treated the SCC-61 cells with calyculin A to augment myosin II activity on soft PAAs. As expected, treatment with calyculin A increased traction stresses exerted by SCC-61 cells. We also found an increase in invadopodia numbers and associated ECM degradation with calyculin A indicating that invadopodia activity was dependent on the overall contractile forces generated inside the cell.

Calyculin A is a potent and specific PP1 and PP2a protein phosphatase inhibitor that inhibits myosin light chain phosphatase to allow for sustained myosin II activity [33]. Given that off-target effects are possible, we attempted to minimize this by using an extremely low concentration of 0.1 nM that still elicited a measureable effect. While other major known proteins susceptible to calyculin A inhibition may be affected such as Raf, MEK, and Akt, inhibition of these kinases would have had either no effect (Raf/MEK pathway is not required for invadopodia formation [61]) or would have decreased invadopodia numbers (Akt is required for invadopodia formation [62]). Calyculin A has been used in numerous studies to augment actomyosin contractility [12, 41, 42] and has advantages over other chemicals that augment contractility such as okadaic acid which requires much higher concentrations [33]. In addition, okadaic acid and other inhibitors such as microcystin-LR and cantharidin may result in more off-target effects due to greater activity toward PP2a protein phosphatases. In preliminary experiments, we attempted to use higher concentrations of calyculin A; however, the increased cellular force production caused the cells to eventually come off of the soft PAA surfaces. Studies on the hard and rigid PAAs were not possible since higher concentrations of calyculin A would have been required to detect statistically significant differences in bead displacements and thus traction stresses due to the higher moduli of these substrates. We also attempted to decrease actomyosin contractility with blebbistatin; however, the resulting decrease in bead displacements were difficult to ascertain (i.e., below the threshold of detectability or statistical certainty). Many of these limitations can be attributed to the use of carcinoma cells which in general exert relatively small traction stresses when compared to other highly contractile cells such as fibroblasts.

Given that invadopodia activity appeared to be dependent on contractile forces, we wanted to find out how strong this relationship was by determining whether we could predict ECM degradation based on experimental traction stress values. Therefore, we plotted ECM degradation as a function of traction stresses resulting in a strong linear relationship as expected given the strong correlation of these quantities to matrix rigidity. Since we had shown that increasing traction stresses with calyculin A led to an increase in ECM degradation, we used these experimental traction stress values to test whether we could predict ECM degradation. Interestingly, we were able to predict ECM degradation within 4% of experimental values indicating that invadopodia activity is strongly influenced by actomyosin contractility and suggests that rigidity mechanosensing via the actin cytoskeleton may play a pivotal role in proteolytic migration.

In adhesion-dependent cells, sensing of mechanical rigidity is thought to occur via focal adhesions which typically grow in size as a function of traction stresses [63]. Interestingly, focal adhesion size does not correlate with ECM degradation by both CA1d breast

carcinoma and SCC-61 cells on different rigidities [64] suggesting that an additional mechanism may contribute to cancer cell responses to matrix rigidity. Recently, Trichet et al. have shown that focal adhesions of the same size can exert varying traction forces depending on substrate rigidity [54]. Their work suggests that rigidity sensing is mediated by a global cellular mechanism reliant on larger scale contractile forces generated by actomyosin activity in addition to local mechanosensing at focal adhesions. Hoffecker et al. have also shown that rigidity sensing occurs across the length of the cell [55]. These conclusions emphasize the role of the actin cytoskeleton and are supported by other studies that have identified actin filaments as force-generating mechanotransducers [63]. While further studies are required to determine how rigidity signals are internalized to produce proteolytic degradation on a molecular level, our data are consistent with the notion that contractile forces generated by actomyosin contractility play an important role in the physical mechanism responsible for inducing invadopodia activity leading to proteolytic migration.

Contractile activity has also been shown to be important in proteolytic migration by non-cancer cells. Similar to invadopodia, podosomes are actin-rich structures used by migrating normal cells such as osteoclasts and macrophages to degrade the ECM [65]. Not only have the spatial and temporal properties of podosomes been shown to be dependent on matrix mechanical properties [66], these structures also exert rigidity-dependent traction stresses that rely on myosin tension and thus act as local and dynamic mechanosensors [67]. However, these properties may be cell-type dependent since a loss of actomyosin-generated cellular tension induces the formation of podosomes in other cell types [68, 69]. Regardless, both invadopodia and podosomes contain adhesion proteins that typically participate in rigidity mechanosensing [70]; however, ascertaining whether invadopodia are tension-generating structures may be technically challenging since invadopodia are quite small and many cancer cells contain both invadopodia and focal adhesions [53]. In addition, a significant number of cancer cell types are more deformable than control cells due to decreases in actin filaments [71] which would suggest the potential for decreased cytoskeletal tension and thus less contractile forces. However, differences in cellular mechanical properties and responses to matrix rigidity have been shown to be dependent on cell type, the type of oncogenic transformation, and matrix architecture [72, 73]. While further investigation is clearly required to determine the exact role of cytoskeletal tension in invasion and metastasis, our data would suggest that contractile forces generated by the actin cytoskeleton are transmitted to invadopodia to regulate ECM degradation in some cancer cell types. Stabilization of invadopodia by such a mechanism would be similar to the role actomyosin contractility plays in focal adhesion maturation [15]. Given the presence of adhesions rings surrounding invadopodia, actomyosin contractility could promote invadopodia maturation and stabilize these structures for delivery of proteinases for ECM degradation [64] since they have lifetimes of approximately 1 hour [74].

While the underlying molecular mechanism remains unclear, proteins that regulate cellular force production such as Rho, ROCK, and MLCK have all been implicated in promoting ECM degradation by invadopodia [25] and may be affected by invasive signaling pathways involving growth factors and adhesion proteins [75]. These regulators of actomyosin contractility have also been suggested as clinical molecular markers for metastasis [20]. Our results provide mechanistic insight into this relationship since we have established that ECM degradation via invadopodia activity is highly dependent on traction stress levels indicating a strong role for global actomyosin contractility. While more experiments are required, one fascinating question is whether this relationship between ECM degradation and traction stresses is consistent among similar and/or different tumor cell types (i.e., would the regression slopes be the same but the curves be shifted up or down or would they be different?). Previous studies have shown increased ECM degradation over similar rigidity

ranges using CA1d breast carcinoma cells [26]. Intriguingly, if we perform a regression on this data, we also find a highly linear relationship between mean values for ECM degradation and the elastic moduli of PAAs ($R^2 > 0.99$). In fact, the slope for the CA1d data (0.0005554) is not statistically different from the slope for the SCC-61 data ($p > 0.32$) indicating a similar invasive rigidity response in a completely different cancer cell type. Interestingly, CA1ds are Ras-transformed cells that have been serially passaged through immunocompromised mice to create a highly invasive cell line [76], whereas SCC-61 cells were derived from a human tumor that contain a mutation in the catalytic subunit of phosphoinositide-3-kinase [77]. Although it remains to be determined if this relationship is universal or affects only certain cancer cells with specific properties, these data suggest the potential for targeting actomyosin contractility to inhibit the spread of cancer cells. In fact, myosin II has recently been proposed as a potential therapeutic target since it acts as a convergence point for many signal transduction pathways [78] including various local mechanosensing pathways which are interconnected and ultimately alter actomyosin contractility [79]. Therefore, our study may provide further relevance given our emphasis on the contractile activity of the actin cytoskeleton which may connect intracellular rigidity signals to invadopodia and promote malignant behavior through [25, 26] proteolytic ECM degradation [53].

5. CONCLUSIONS

In this study, we have shown that cellular traction stresses and ECM degradation by invadopodia are dependent on matrix mechanical properties in an invasive HNSCC cell line. Furthermore, we tested the correlation between traction stresses and ECM degradation and showed that traction stresses are predictive of ECM degradation indicating that cellular contractile forces mediate invadopodia activity. In addition, our data suggest that mechanical interactions with the ECM provide rigidity signals that are transmitted through the actomyosin network to invadopodia. Overall, these findings suggest a potentially important role for cellular force generation in proteolytic migration by invasive cancer cells as well as the possibility of targeting the contractile machinery for therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Micah Dembo of Boston University for licensing of the LIBTRC software as well as his assistance in its utilization for calculating traction stresses. The authors would also like to express their sincere gratitude to Sasha Doust for establishing LIBTRC in the laboratory and generating preliminary data as well as Scott Guelcher for assistance in measuring the mechanical properties of the PAAs. Finally, we would also like to thank Alissa Weaver for her critical reading of this manuscript. Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award Number K25CA143412 (Parekh). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional funding was provided by the Department of Otolaryngology.

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Figure 1.

PAA with embedded fibronectin. Fibronectin was incorporated in PAAs at concentrations of (A) 200 $\mu\text{g/ml}$, (B) 215 $\mu\text{g/ml}$, and (C) 230 $\mu\text{g/ml}$ and yielded a uniform coating on the substrate surfaces as identified by immunofluorescence with representative wide-field images shown. Insets represent zoomed 10 $\times$ 10 μm areas of each substrate. (D) Quantitation of total fluorescence intensity of fibronectin at the substrate surfaces resulted in statistically similar values for the different concentrations. (E) Quantitation of standard deviation of pixel fluorescence intensities for each substrate surface. Data are presented as box and whisker plots with red lines indicating means, black lines indicating medians, and whiskers

representing the 10th and 90th percentiles. n=60 for each substrate from three independent experiments.

Figure 2.

Rigidities of PAAs embedded with fibronectin. PAA elastic moduli were significantly reduced with the incorporation of fibronectin but not fluorescent beads. Shear elastic moduli (G') were measured with rheometry at a constant strain of 0.05% and a frequency of 1 Hz and converted to elastic moduli, $E=3G'$, assuming a Poisson's ratio of 0.5 [25, 26]. Data are presented as mean $\pm$ standard error. * indicates $p < 0.05$. $n=4-21$, $4-16$, and $4-20$ for the soft, hard, and rigid PAAs, respectively.

Figure 3. Invadopodia-associated ECM degradation by SCC-61 cells on PAAs. Overnight culture of SCC-61 cells on PAA substrates overlaid with 1% gelatin/FITC fibronectin resulted in a marked increase in ECM degradation in response to matrix rigidity. Representative, wide-field fluorescence images are shown of immunostained SCC-61 cells on soft, hard, and rigid PAAs. Invadopodia were identified by colocalization of actin and cortactin, and these markers are colocalized with black, degraded areas of ECM indicating active invadopodia (white arrows).

Figure 4.

Quantitation of invadopodia numbers and associated ECM degradation by SCC-61 cells on PAAs. (A) Degradation area/cell (mean values of 2.24, 6.43, and 16.01 μm^2 and median values of 0, 1.10, and 4.38 μm^2 for soft, hard, and rigid PAAs, respectively), (B) active invadopodia/cell (mean values of 0.53, 2.37, and 5.21 and median values of 0, 0, and 1 for soft, hard, and rigid PAAs, respectively), and (C) total invadopodia/cell (mean values of 2.35, 6.02, and 9.45 and median values of 2, 5, and 7 for soft, hard, and rigid PAAs, respectively) statistically increase as rigidity increases. (D) Replotting mean ECM degradation versus mean elastic modulus for each PAA and performing a regression yielded a strong relationship between these parameters ($R^2 > 0.99$). Data are presented as box and whisker plots with red lines indicating means, black lines indicating medians, whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for (A–C) while data in (D) are presented as mean $\pm$ standard error (significance not shown). $n = 207$, 197, and 195 for the soft, hard, and rigid PAAs, respectively, from five independent experiments.

Figure 5.

Traction force microscopy and subsequent analyses of SCC-61 cells on PAAs.

Representative traction maps of SCC-61 cells cultured overnight on (A) soft, (B), hard, and (C) rigid PAAs. (D) Quantitation revealed a statistically significant increase in traction stresses (which are the means of the traction magnitudes over the continuous traction fields, i.e., cell areas) in response to matrix rigidity (mean values of 37.23, 171.30, and 783.69 Pa and median values of 30.44, 147.00, and 622.70 Pa for soft, hard, and rigid PAAs, respectively). (E) Replotting the mean traction stress versus the mean elastic modulus for each PAA and performing a regression yielded a strong relationship between these parameters ($R^2=0.99$). Data are presented in (D) as a box and whisker plot with the red line indicating the mean, the black line indicating the median, the whiskers representing the 10th and 90th percentiles, and * indicating $p<0.05$ while data in (E) are presented as mean $\pm$ standard error (significance not shown). $n=86$, 68, and 56 for the soft, hard, and rigid PAAs, respectively, from four to five independent experiments.

Figure 6.

Traction force microscopy and subsequent analyses of SCC-61 cells treated with calyculin A on soft PAAs. Representative traction maps of SCC-61 cells cultured overnight and treated with (A) DMSO and (B) 0.1 nM calyculin A for 5 minutes. (C) Quantitation revealed a statistically significant increase in traction stresses (which are the means of the traction magnitudes over the continuous traction fields, i.e., cell areas) in response to calyculin A when compared to DMSO controls (mean values of 46.87 and 156.06 Pa and median values of 30.10 and 38.71 Pa for the DMSO and calyculin A treatments, respectively). Data are presented in (C) as a box and whisker plot with the red line indicating the mean, the black line indicating the median, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$. $n = 63$ and 65 for the DMSO and calyculin A treatments, respectively, from four to five independent experiments.

Figure 7.

Invadopodia-associated ECM degradation by SCC-61 cells treated with calyculin A on soft PAAs. Representative wide-field fluorescence images of SCC61 cells cultured overnight on soft PAAs overlaid with 1% gelatin/FITC fibronectin and treated with (A) DMSO and (B) 0.1 nM calyculin A. Quantitation revealed statistically significant increases in (C) ECM degradation/cell (mean values of 3.36 and 5.33 μm^2 and median values of 0 and 0 μm^2 , respectively) (D) active invadopodia/cell (mean values of 0.70 and 1.63 and median values of 0 and 0, respectively), and (E) total invadopodia/cell (mean values of 3.44 and 5.27 and median values of 3 and 4, respectively) when comparing DMSO controls to calyculin A treatment groups. Invadopodia were identified by colocalization of actin and cortactin, and these markers are colocalized with black, degraded areas of ECM indicating active invadopodia (white arrows) in (A, B). Data are presented in (C–E) as a box and whisker plot with the red line indicating the mean, the black line indicating the median, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$. $n = 149$ and 167 for the DMSO and calyculin A treatments, respectively, from three independent experiments.

Figure 8.

Predicting ECM degradation based on traction stress levels using a linear correlation. (A) Since both ECM degradation and traction stresses were strongly dependent on matrix rigidity, the mean values of these parameters were replotted against each other. A regression yielded a strong correlation between mean ECM degradation and mean traction stress values ($R^2=0.98$). (B) We used the experimental DMSO and calyculin A traction stress data from Figs. 6 and 7 and successfully predicted the corresponding ECM degradation values within 4% error of the experimental values.