

The role of the cytoskeleton in differentially regulating pressure-mediated effects on malignant colonocyte focal adhesion signaling and cell adhesion

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Increased extracellular pressure stimulates colon cancer cell adhesion by activating focal adhesion kinase (FAK) and Src. We investigated the role of the cytoskeleton in pressure-induced inside-out FAK and Src phosphorylation and pressure-stimulated adhesion. We perturbed actin polymerization with phalloidin, cytochalasin D and latrunculin B, and microtubule organization with colchicine and paclitaxol. We compared the effects of these agents on pressure-induced SW620 and human primary colon cancer cell adhesion and inside-out FAK/Src activation with outside-in adhesion-dependent FAK/Src activation. Cells pretreated with cytoskeletal inhibitors were subjected to 15 mmHg increased pressure and allowed to adhere to collagen I coated plates or prevented from adhesion to pacificated plates for 30 min. Phalloidin, cytochalasin D, latrunculin B and colchicine pretreatment completely prevented pressure-stimulated and significantly inhibited basal SW620 cell adhesion. Taxol did not inhibit pressure-induced colon cancer cell adhesion, but significantly lowered basal adhesion. Cytochalasin D and colchicine had similar effects in pressure-stimulated primary human malignant colonocytes. Phalloidin, cytochalasin D, latrunculin B and colchicine prevented pressure-induced SW620 FAK phosphorylation but not Src phosphorylation. FAK phosphorylation in response to collagen I adhesion was significantly attenuated but not completely prevented by these inhibitors. Although Src phosphorylation was not increased on adhesion, the cytoskeleton disrupting agents significantly lowered basal Src phosphorylation in adherent cells. These results suggest that both cytoskeleton-dependent FAK activation and cytoskeleton-independent Src activation may be required for extracellular pressure to stimulate colon cancer cell adhesion. Furthermore, the cytoskeleton plays a different role in pressure-activated FAK and Src signaling than in FAK and Src activation in adherent cells. We, therefore, hypothesize that cytoskeletal interactions with focal adhesion signals mediate the effects of extracellular pressure on colon cancer cell adhesion.

Abbreviations: ELISA, enzyme linked immunosorbent assay; ECM, extracellular matrix; F-actin, filamentous actin; FAK, focal adhesion kinase; NF-kappaB, nuclear factor kappaB; PBS, phosphate buffered saline.

Introduction

Wound recurrence after cancer surgery occurs in ~1% of patients (1), although distant tumor dissemination also occurs. The adhesion of circulating cancer cells is an early step in such metastasis. Tumor cells are often portrayed as passive participants in this process, moving through the surgical site, lymphatics or circulatory system until they happen to come into contact with endothelial cells or matrix proteins that match their adhesion receptors (2,3). However, we have previously demonstrated that modestly increased extracellular pressure or non-laminar shear stress activates focal adhesion kinase (FAK) and Src within colon cancer cell lines, and that these signals stimulate adhesion to matrix proteins or endothelial cells (4–6). Primary human colon cancer cells isolated directly from surgical specimens display similar force-activated adhesion and signals, and blocking these signals blocks the adhesion response (5). Furthermore, our previous work has demonstrated that pressure stimulates transplantable colon cancer cell adhesion to wounds in anesthetized mice via similar signals (7).

Malignant colonocyte adhesion may, therefore, be activated by physical forces generated by pressure, shear stress and turbulence within the peritoneal cavity during irrigation, laparoscopic air insufflation or surgical manipulation, and during passage through the venous or lymphatic systems. Such forces appear to increase $\beta 1$ integrin heterodimer binding affinity, since pressure-stimulated cells also display increased adhesive force, and a functional antibody to the $\beta 1$ integrin subunit blocks the effect (4) but $\beta 1$ integrins do not appear recruited to the cell surface after pressure (5). However, the manner by which these cells sense the mechanical stimuli and transduce them to FAK and Src in the focal adhesion complex is not well understood. We hypothesized that the cytoskeleton interactions with focal adhesion signals mediate the effects of extracellular pressure on colon cancer cell adhesion. In the present study, we therefore sought to test this hypothesis by studying the effects of cytoskeletal perturbation upon FAK and Src phosphorylation and increased adhesion in response to increased extracellular pressure.

Mechanical stimuli imparted by forces, such as deformation, pressure, shear stress and fluid flow, result in a variety of signaling responses and may play a significant role in physiological or pathological states as diverse as cardiac hypertrophy (8,9), atherosclerosis (10), inflammation (11), bone remodeling (12), the intestinal mucosal response to peristalsis and villus motility (13), and pulmonary barotrauma (14). Research in the diverse cell types involved in these conditions has variously implicated extracellular matrix (ECM) molecules (15,16), integrins (17–19) or cytoskeletal structures as mediators of mechanotransduction (20–22). Intracellular biochemical signals, including cation channels (23), G-protein-dependent pathways (24,25) and linkages between

the cytoskeleton and the phospholipase C or phospholipase A pathways (12) have also been theorized to be mechanosensors. Kumar and Boriek (26) in their paper have shown that the activation of nuclear factor kappaB (NF-kappaB) occurs by mechanical stretch in diaphragmatic myocytes from normal mice and higher activation of NF-kappaB was observed in muscles lacking dystrophin.

Ingber's 'stick and string' tensegrity model suggests that living cells are hard-wired via a complex of transmembrane matrix receptors, cytoskeleton filaments and nuclear scaffolds to respond immediately to external mechanical stresses (27,28). Accumulated evidence points to the cytoskeleton as a particularly theoretically attractive mechanosensor because of its contribution to a variety of cellular events, including cell motility (29), intracellular trafficking (30) and cell adhesion (31). For instance, stress fiber formation induced by hemodynamic forces, such as cyclic stretch and shear stress, has been reported to increase the adhesive strength of cells to substrates (32,33). In cerebral arteries, the actin cytoskeleton regulates pressure-induced depolarization and calcium influx (34).

The cytoskeleton, a complex of interconnected fibrillar elements, has been recognized as an important factor in mediating adhesion-independent and dependent signaling (35,36). In particular, changes in the organization of the actin cytoskeleton lead to remarkable changes in the tyrosine phosphorylation of several signaling proteins localized at the focal adhesion complex, and signaling within this complex may act on integrins via inside-out pathways. In association with integrin cytoplasmic tails, cytoskeletal proteins play important roles in regulating intracellular signaling events that affect the conformation of integrin extracellular domains and promote integrin clustering (37). Sampath *et al.* (38) have demonstrated that cytoskeletal interaction with the $\alpha 2$ integrin cytoplasmic tail after activation regulates leukocyte adhesion to endothelial cells. It has also been reported that disruption of the actin cytoskeleton alters the adhesive properties of platelets and neutrophils.

Since the actin cytoskeleton is recognized as a sensor important for transduction of mechanical forces, we sought to determine whether the actin cytoskeleton plays a role in pressure-induced inside-out signaling as well as pressure-induced adhesion. In the present study, we used the actin polymerization stabilizer phalloidin, the actin polymerization inhibitors, cytochalasin D and latrunculin B, and the microtubule inhibitor, colchicine, to study the requirement for malignant colonocyte cytoskeletal integrity in responding to increased extracellular pressure by increased adhesion. Since our previous work demonstrated that FAK and Src phosphorylation are required for this response, we also studied the potential role of the actin cytoskeleton in mediating pressure-induced FAK and Src phosphorylation, based on inside-out signaling, and compared this to the requirement for an intact actin cytoskeleton in outside-in (adhesion-mediated) FAK and Src activation in response to integrin-dependent adhesion to matrix.

Materials and methods

Cell culture

SW620 cells were cultured at 37°C in 5% CO₂ in an equal mixture of DMEM and RPMI 1640 containing 5% fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM

HEPES, 100 U/ml penicillin G, 100 µg/ml streptomycin (Sigma, St Louis, MO) and 5 µg/ml transferrin (Roche Diagnostics, Indianapolis, IN).

Primary cells

Single cell suspensions of primary human colonocytes were isolated from resected tumors not required for pathological study by mincing and collagenase digestion as described in a previous work from our laboratory (39). Trypan blue exclusion was used to assay viability of isolated cells. At each isolation, >90% of cells excluded trypan blue. These studies were approved by the Human Investigations Committees of the John D. Dingell VA Medical Center and Wayne State University.

Pressure regulation

Ambient pressure was controlled using an airtight box with inlet and outlet valves, thumb screws, a pressure gauge and an O-ring for an airtight seal. The box was prewarmed to 37°C for 1 h to prevent internal temperature and pressure fluctuations. Temperature was maintained within ±2°C and pressure within ±1.5 mmHg as discussed in our previous reports (4,5,11).

Matrix precoating

Six-well plates were precoated with collagen I (12.5 µg/ml) in enzyme linked immunosorbent assay (ELISA) buffer as previously described (40), and the wells were rinsed with sterile phosphate buffered saline (PBS) prior to cell seeding.

Cell adhesion

Cells (100,000 cell/well) were allowed to adhere to collagen I coated 6-well plates for 30 min at 37°C under ambient and increased pressure (15 mmHg). After 30 min, non-adherent cells were gently washed away with warm PBS, and adherent cells were formalin fixed, hematoxylin stained and counted in 20 or more random high power fields per well using an Olympus microscope. Cells pretreated with the actin cytoskeleton disrupting agents cytochalasin D (0.5–5 µM) or latrunculin B (0.5–5 µM), the actin stabilizer phalloidin (10 µM), the microtubule disrupting agent colchicine (5–10 µM) or the microtubule stabilizer paclitaxol (10 µM) were treated similarly. For adhesion-dependent studies of cell signaling, cells were allowed to adhere to collagen I coated plates for 30 min and then lysed for western analysis.

Model

Initial studies were directed at demonstrating the effect of cytoskeletal modulation on pressure-induced colon cancer cell adhesion. For adhesion experiments, SW620 cells in suspension pretreated with various inhibitors were then subjected to ambient or 15 mmHg increased pressure for 30 min while they were allowed to begin to adhere to 6-well plates precoated with type I collagen. After 30 min, non-adherent cells were aspirated, and the remaining adherent cells were washed with PBS, fixed with formalin, stained with hematoxylin and counted.

We have previously demonstrated that pressure significantly increases FAK and Src phosphorylation prior to adhesion (inside-out signaling) and reducing FAK expression by FAK siRNA transfection or inhibiting FAK activation by FRNK transfection prevents the effect of pressure on colon cancer cell adhesion, (5) suggesting that FAK activation in response to pressure not only precedes adhesion but is required for it. Therefore, further studies were designed to determine the role of the cytoskeleton in pressure-induced inside-out signaling. For these studies, SW620 cells in suspension were subjected to ambient pressure or 15 mmHg increased pressure for 30 min in bacteriological plastic plates pretreated with 1% heat inactivated bovine serum albumin in PBS to prevent all adhesion. After pressure treatment, the suspended cells were collected by centrifugation. The cell pellet was washed with ice-cold PBS and lysed in lysis buffer.

Since pressure-induced inside-out signals may differ from the well-characterized outside-in integrin-ligand-mediated signaling pathways, we used signaling induced by adhesion to collagen I (rather than signaling preceding it) as a comparative model of outside-in signaling. Adhesion-induced signals were studied by placing SW620 cells in collagen I coated plates for 30 min. Non-adherent cells were removed by aspiration. Adherent cells were washed with ice-cold PBS and then scraped into lysis buffer prior to western blotting. In further studies, we sought to determine whether cytoskeletal inhibitors might alter pressure-induced FAK and Src signaling in cells that were already adherent. Cells pretreated with cytoskeletal inhibitors were plated on collagen I coated plates and subjected to pressure treatment for 30 min. Control plates were placed outside the pressure box at ambient pressure condition. After 30 min, adherent cells were collected as stated above for western analysis.

Western analysis

Cells pretreated with cytoskeleton inhibitors were plated on pacificated bacteriological plates or collagen I coated plates for pressure-induced or

adhesion-induced signal studies. Cells were lysed in lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 , 50 mM NaF, 10 mM sodium pyrophosphate, 2 $\mu\text{g}/\text{ml}$ aprotinin and 2 $\mu\text{g}/\text{ml}$ leupeptin, (pH 7.4)] and centrifuged at 10 000 g for 15 min at 4°C. The protein concentration of the supernatant was determined using bicinchoninic acid reagent (Pierce Chemical, Rockford, IL). Equal amounts of protein were resolved by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blotted with specific antibodies to FAK Tyr397 or SrcTyr416 (Transduction Laboratories, San Diego, CA; Cell Signaling, Beverly, MA) and visualized with the appropriate secondary antibody coupled to horseradish peroxidase. Bands were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech) and analyzed with a Kodak Image Station 440CF (Perkin Elmer, Boston, MA). Membranes were then stripped and reprobed with antibodies to total FAK or Src (Upstate, NY; Santa Cruz Biotechnology, CA) and appropriate secondary antibodies. All exposures used for densitometric analysis were within the linear range.

Statistical analysis

All data are expressed as mean \pm SE. Statistical analysis was performed using paired or unpaired t -tests as appropriate. A P -value of <0.05 was considered significant.

Results

Overall rationale

Numerous previous studies have demonstrated that integrin-ligand binding plays a significant role in subsequent FAK activation in many cells (outside-in signaling). Conversely, we have previously demonstrated that pressure significantly increases FAK and Src phosphorylation prior to adhesion (inside-out signaling). Since we previously reported that phalloidin, which modulates actin depolymerization, prevents the effect of pressure on colon cancer cell adhesion, we conducted a series of studies designed to more fully characterize the role of the cytoskeleton in pressure-induced adhesion and the signals which mediate this effect, and to contrast pressure-induced inside-out signals with adhesion-induced outside-in signaling.

Actin cytoskeleton disruption significantly inhibits pressure-induced colon cancer cell adhesion

We previously demonstrated that a 15 mmHg increase in pressure for 30 min significantly increases colon cancer cell adhesion to collagen I via FAK and Src signaling in an actin cytoskeleton-dependent manner. Therefore, we used pharmacological inhibitors of actin dynamics to more specifically study the role of the actin cytoskeleton in pressure-stimulated SW620 cell adhesion. Phalloidin and cytochalasin D have been widely used to perturb actin association and dissociation. Phalloidin is an actin stabilizer that binds to filamentous actin (F-actin) and inhibits actin depolymerization whereas cytochalasin D inhibits actin polymerization by binding to the barbed ends of G-actin filaments (41). We first determined the effect of these specific actin cytoskeleton inhibitors on pressure-induced cancer cell adhesion. Consistent with our previous observations, increased extracellular pressure significantly enhanced colon cancer cell adhesion to collagen I ($30.0 \pm 4.0\%$, $P < 0.001$, $n = 12$), whereas phalloidin reduced basal adhesion by 13% and prevented pressure-induced colon cancer cell adhesion (Figure 1a). In the current study, cytochalasin D (5 μM) also completely prevented pressure-induced cancer cell adhesion and significantly lowered basal adhesion (by 87%; Figure 1a). We have previously demonstrated that our assays are sufficiently sensitive to detect pressure stimulation

of adhesion when adhesion is globally inhibited to an even greater degree than that seen with these agents (5,6).

To confirm the impact of actin modulation on cell adhesion, we used latrunculin B, a potent marine toxin that impairs the polymerization of actin filaments by sequestering G-actin monomers (42). As illustrated in Figure 1a, incubating colon cancer cells with latrunculin B completely prevented the effect of pressure even after significant inhibition of basal adhesion (by 90%; $P < 0.001$; $n = 3$). Thus, the increased adhesiveness of SW620 colon cancer cells in response to elevated extracellular pressure is dependent, in part, on the integrity of the actin cytoskeleton.

Microtubule depolymerization but not stabilization inhibits pressure-induced colon cancer cell adhesion

Microtubules play an important role in maintaining cell shape (43), integrin-dependent adhesion (44) and intracellular signaling cascades (45). Since microtubules are an integral part of the cytoskeleton, we next investigated the role of microtubule organization in pressure-induced colon cancer cell adhesion. Colchicine inhibits microtubule polymerization thereby disrupting microtubule integrity, whereas paclitaxol stabilizes microtubules. Pretreatment of SW620 cells with 10 μM colchicine significantly inhibited basal adhesion ($58.0\% \pm 2.0$; $P < 0.001$; $n = 3$) and completely prevented the stimulation of adhesion by pressure. In contrast, although microtubule stabilization by paclitaxol significantly inhibited basal adhesion (by 36.0%; $P < 0.001$; $n=3$), paclitaxol did not negate the pressure effect (Figure 1b).

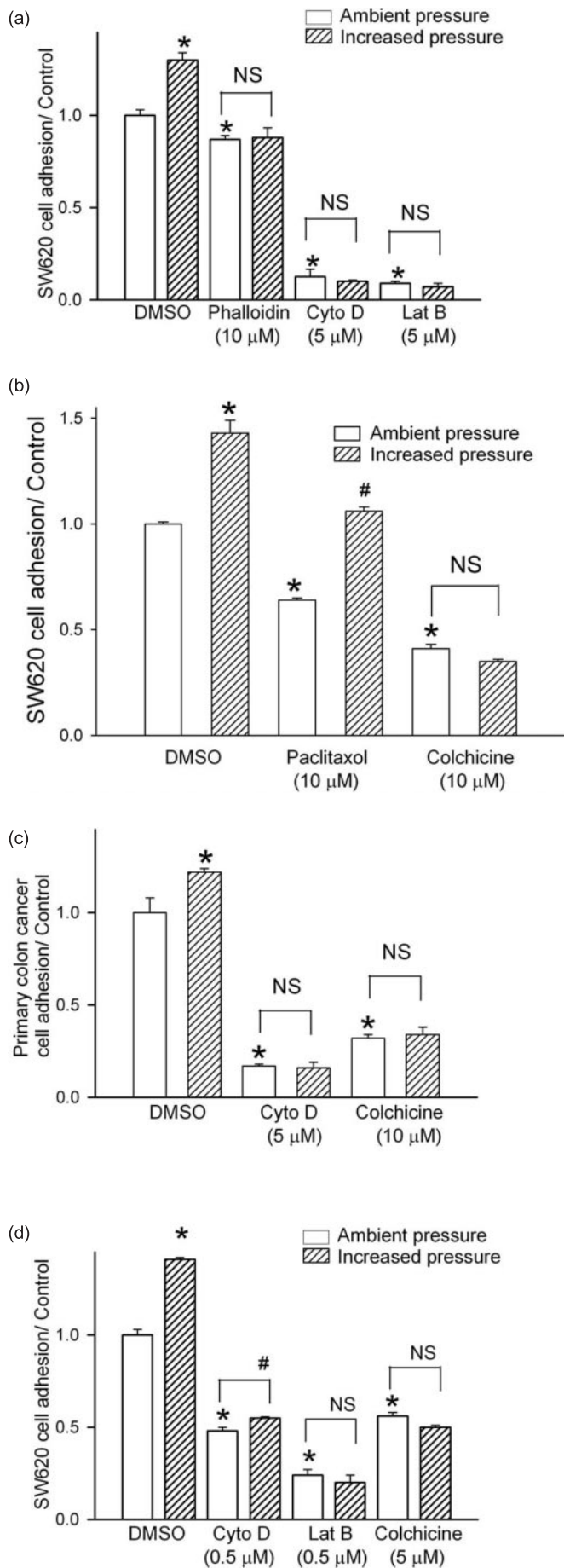
Disruption of cytoskeletal integrity alters pressure-induced adhesion in primary human colon cancer cells

We then proceeded to determine whether disruption of cytoskeletal integrity also affected pressure-stimulated adhesion of primary colon cancer cells isolated directly from surgical specimens. As illustrated in Figure 1c, pressure significantly increased primary human colon cancer cell adhesion. Pretreatment of primary cells with either cytochalasin D (5 μM) or colchicine (10 μM) completely prevented pressure-induced cancer cell adhesion and significantly inhibited basal adhesion to collagen I, confirming the importance of cytoskeleton integrity for pressure-stimulated cell adhesion. These results suggest that either cytoskeletal integrity is required for pressure to initiate the relevant intracellular signals or necessary for these signals to modulate integrin affinity.

Since a disparity in the effect of these drugs on cell adhesion at lower concentrations has been demonstrated in different cell types, we next studied their effect on adhesion at lower concentrations (46,47). As shown in Figure 1d, latrunculin B (0.5 μM) and colchicine (5 μM) completely prevented pressure-induced cell adhesion. However, although pressure-induced adhesion following pretreatment with cytochalasin D at 0.5 μM was significantly increased over that of treated cells at ambient pressure, adhesion remained significantly lower than that in vehicle-treated cells subjected to pressure. We used these lower concentrations for our subsequent studies of effects on pressure-modulated inside-out and adhesion-mediated signals.

Effect of cytoskeleton disrupting agents on pressure- or adhesion-induced FAK activation

We have previously demonstrated that pressure significantly increases FAK Tyr397 phosphorylation prior to adhesion, and



that reducing FAK by a specific siRNA or inhibiting FAK Tyr397 signaling by transfection with a dominant negative construct prevents the effect of pressure on adhesion (5). Since the cytoskeleton is critical to organizing the focal adhesion complexes that structure FAK–integrin association, we examined the effect of inhibiting actin polymerization on FAK phosphorylation in response to pressure.

Several previous studies have demonstrated that integrin–ligand binding plays a significant role in subsequent FAK activation in many cells (48–50). We studied FAK phosphorylation induced by adhesion itself as a positive control for outside-in signaling, since this effect relates directly to integrin occupancy and is presumably independent of the signals required for the pressure effect through inside-out signaling.

Consistent with our previous studies, increased extracellular pressure-stimulated FAK phosphorylation in SW620 cells ($n = 3$, $P < 0.01$, Figure 2a). Pretreatment with the actin stabilizer phalloidin did not affect basal phosphorylation but completely prevented pressure-induced FAK phosphorylation ($n = 3$; Figure 2a). In contrast, phalloidin pretreatment did not affect basal or pressure-induced Src phosphorylation ($n = 3$; $P < 0.01$; Figure 2b).

We have previously reported that phalloidin pretreatment prevents pressure-induced colon cancer cell adhesion (5). In the present study, we have shown that cytochalasin D and latrunculin B, an actin destabilizer, each prevented the pressure-induced colon cancer cell adhesion to collagen I. Since all three compounds appeared to exert similar effects on cell adhesion as well as on focal adhesion complex signaling, we used cytochalasin D and latrunculin B in our further studies to investigate the differences between pressure-induced inside-out signaling and outside-in signaling in response to cell adhesion. As shown in Figure 3a, 15 mmHg for 30 min significantly increased FAK Tyr397 phosphorylation in non-adherent cells (hatched bar). Adhesion to collagen I *per se* induced greater FAK phosphorylation (cross hatched bar) than did pressure in non-adherent SW620 cells, but both effects were significant (pressure: 1.18 ± 0.04 ; adhesion 2.57 ± 0.18 ; $P < 0.05$; $n = 3$). Actin cytoskeleton disruption by cytochalasin D did not significantly alter basal FAK phosphorylation (open bar) but completely prevented pressure-induced FAK

Fig. 1. Actin cytoskeleton inhibition prevents pressure-induced SW620 cell adhesion. (a) A 15 mmHg elevation in pressure significantly increased colon cancer cell adhesion with cells at ambient pressure, ($n = 12$; $P < 0.001$). Disruption of actin cytoskeleton organization with phalloidin (10 μ M), cytochalasin D (5 μ M; cyto D) or latrunculin B (5 μ M; Lat B) lowered basal SW620 cell adhesion and prevented further pressure-induced adhesion to collagen I compared with vehicle-treated cells (DMSO). Results are expressed as mean \pm SE. Data shown are adhesion normalized to control, ($n = 3$, * $P < 0.05$ versus DMSO control for each; NS, not significant). (b) Although the microtubule stabilizer taxol inhibited basal cancer cell adhesion, taxol did not prevent pressure-induced adhesion ($n = 3$, * $P < 0.05$ versus DMSO control; # $P < 0.05$ versus taxol at ambient pressure). Microtubule polymerization inhibition by colchicine (10 μ M) inhibited basal adhesion and blocked pressure-stimulated adhesion. (c) In primary cells isolated from human tumors, cytochalasin D and colchicine each lowered basal adhesion and prevented pressure-induced adhesion ($n = 3$, * $P < 0.05$ versus DMSO control). (d) Pretreatment with cytochalasin D at 0.5 μ M decreased basal adhesion. Cytochalasin D (0.5 μ M) did not prevent pressure-induced adhesion, but did substantially attenuate the pressure effect. Latrunculin B (0.5 μ M) and colchicine (5 μ M) each lowered basal adhesion and each completely prevented the stimulation of SW620 colon cancer cell adhesion by increased extracellular pressure ($n = 3$, * $P < 0.05$ versus DMSO control, # $P < 0.05$ versus cytochalasin D at ambient pressure).

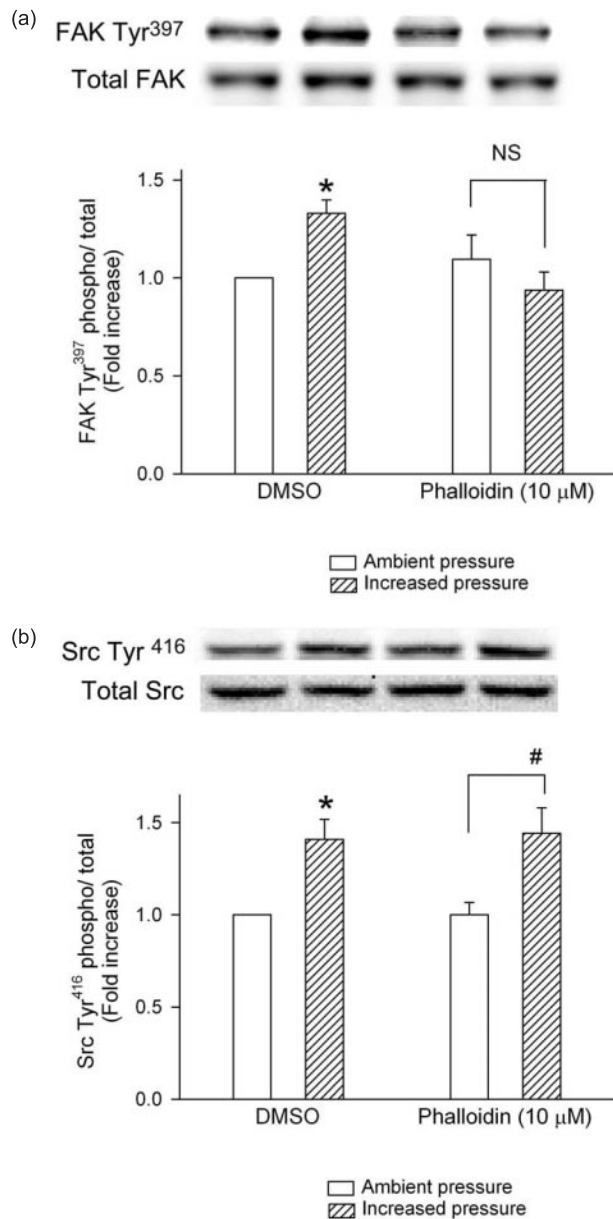


Fig. 2. Actin stabilization by phalloidin prevents pressure-induced FAK phosphorylation but does not affect Src phosphorylation. (a) Pressure significantly increased FAK Tyr³⁹⁷ phosphorylation in SW620 cells compared with DMSO-treated cells at ambient pressure ($n = 3$; $*P < 0.01$ versus DMSO control). Pretreatment with phalloidin (10 μ M) did not affect basal but prevented pressure-induced FAK phosphorylation compared with phalloidin-treated cells at ambient pressure. In this, and subsequent figures, the bar graphs summarize densitometric analysis of multiple independent but similar studies and representative western blots are shown above. (b) Pressure significantly increased Src Tyr⁴¹⁶ phosphorylation compared with DMSO control ($n = 3$; $P < 0.001$ versus DMSO control). Phalloidin pretreatment did not affect basal or pressure-induced Src phosphorylation compared with phalloidin-treated cells at ambient pressure.

phosphorylation in non-adherent cells (hatched bar). Although adhesion-induced FAK phosphorylation was significantly attenuated by cytochalasin D compared with vehicle-treated adherent cells (cross hatched bar), FAK phosphorylation was still significantly higher than phosphorylation in non-adherent cells treated with cytochalasin D (non-adherent: 0.88 ± 0.1 ; adherent cells: 1.63 ± 0.27 ; $P < 0.05$).

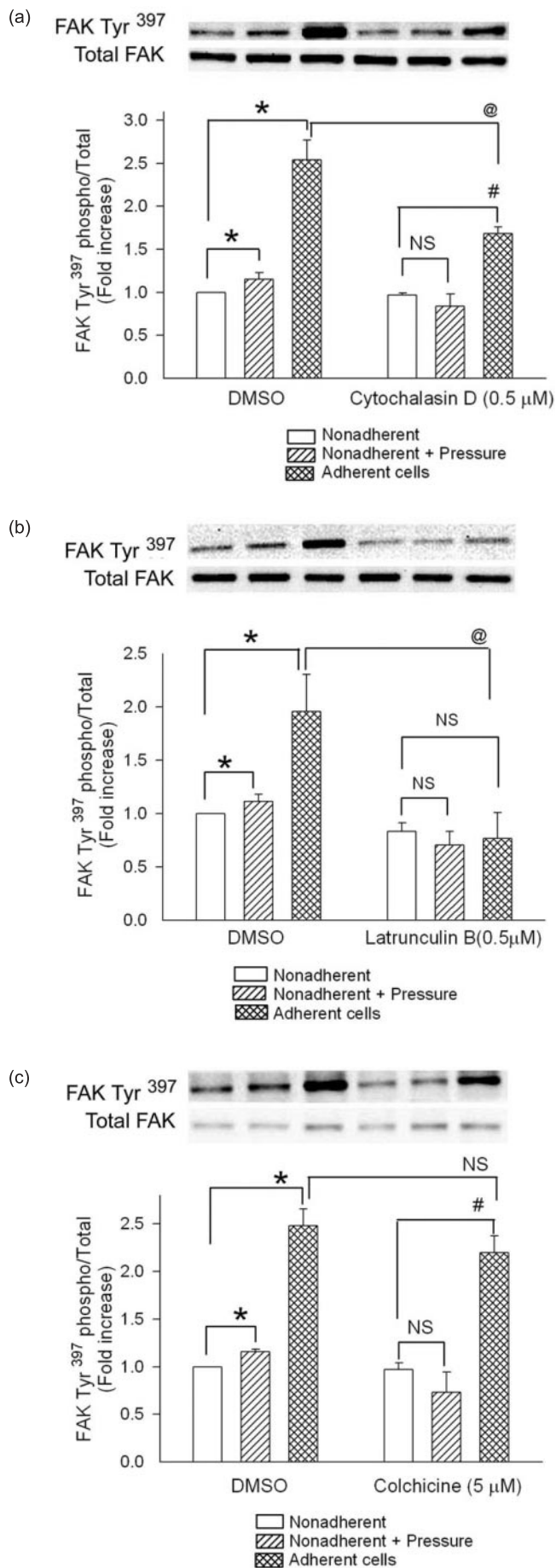
Similarly, pretreatment of SW620 cells with latrunculin B did not significantly alter basal FAK phosphorylation but completely prevented pressure-induced FAK phosphorylation in non-adherent cells (Figure 3b). In contrast to cytochalasin D, latrunculin B pretreatment also completely prevented adhesion-induced FAK phosphorylation. Our adhesion experiments suggested that paclitaxol did not prevent the effect of increased extracellular pressure on colon cancer cell adhesion. However, colchicine completely prevented pressure-induced colon cancer cell adhesion. We, therefore, used colchicine in further studies to characterize the potential role of microtubules in focal adhesion complex signaling in this system. Microtubule disruption by colchicine did not significantly alter basal FAK phosphorylation prevented pressure-induced FAK Tyr³⁹⁷ phosphorylation in non-adherent cells, and had no effect on adhesion-induced FAK phosphorylation (vehicle-treated adherent cells: 2.46 ± 0.19 ; colchicine-treated adherent cells: 2.24 ± 0.28 ; Figure 3c).

Src phosphorylation by pressure is independent of cytoskeletal integrity

Since Src is a prominent substrate for tyrosine phosphorylation by FAK, we next investigated Src phosphorylation in response to pressure and adhesion to collagen I. Src phosphorylation in non-adherent SW620 cells was significantly enhanced by 30 min exposure to 15 mmHg of increased pressure (Figure 4a–c), consistent with our previous observations (5). However, in contrast to the effects of adhesion on FAK phosphorylation, adhesion to collagen I did not increase SW620 Src Tyr⁴¹⁶ phosphorylation. In the aggregation analysis of all control and pharmacologic study data, we found that pressure stimulated Src phosphorylation in non-adherent cells by $18 \pm 2\%$ ($P < 0.009$, $n = 9$), whereas Src phosphorylation in adherent SW620 cells remained at $100.8 \pm 2\%$ of non-adherent ambient pressure controls. In parallel studies, adhesion to type IV collagen also failed to elicit Src phosphorylation in SW620 cells (data not shown). We were thus unable to compare the effects of cytoskeleton modulating agents on pressure-induced versus adhesion-induced Src phosphorylation in parallel with FAK Tyr³⁹⁷ phosphorylation. Pretreatment of SW620 cells with cytochalasin D, latrunculin B, or colchicine, enhanced basal Src phosphorylation in non-adherent SW620 cells and did not inhibit further pressure-induced Src phosphorylation. Interestingly, all three cytoskeleton disrupting agents significantly reduced basal Src phosphorylation in adherent cells, suggesting that pressure-induced Src phosphorylation may be independent of cytoskeleton integrity, but that maintenance of Src phosphorylation in adherent cells may require an intact cytoskeleton.

Pressure did not increase adhesion-induced FAK or Src signals in adherent cells

Our study was primarily directed at determining whether the cytoskeleton is required for pressure-induced colon cancer cell adhesion by modulating inside-out FAK and Src signals in suspended cells prior to cell adhesion. Since pressure-induced inside-out signals appear distinct from integrin–ligand-mediated outside-in signaling, we studied the effects of cytoskeletal-modulating agents on adhesion-induced outside-in signaling for comparison, as depicted in Figures 3 and 4. However, it then became interesting to investigate whether such agents might alter pressure-induced FAK and Src signaling in cells that were already adherent. Figure 5a demonstrates



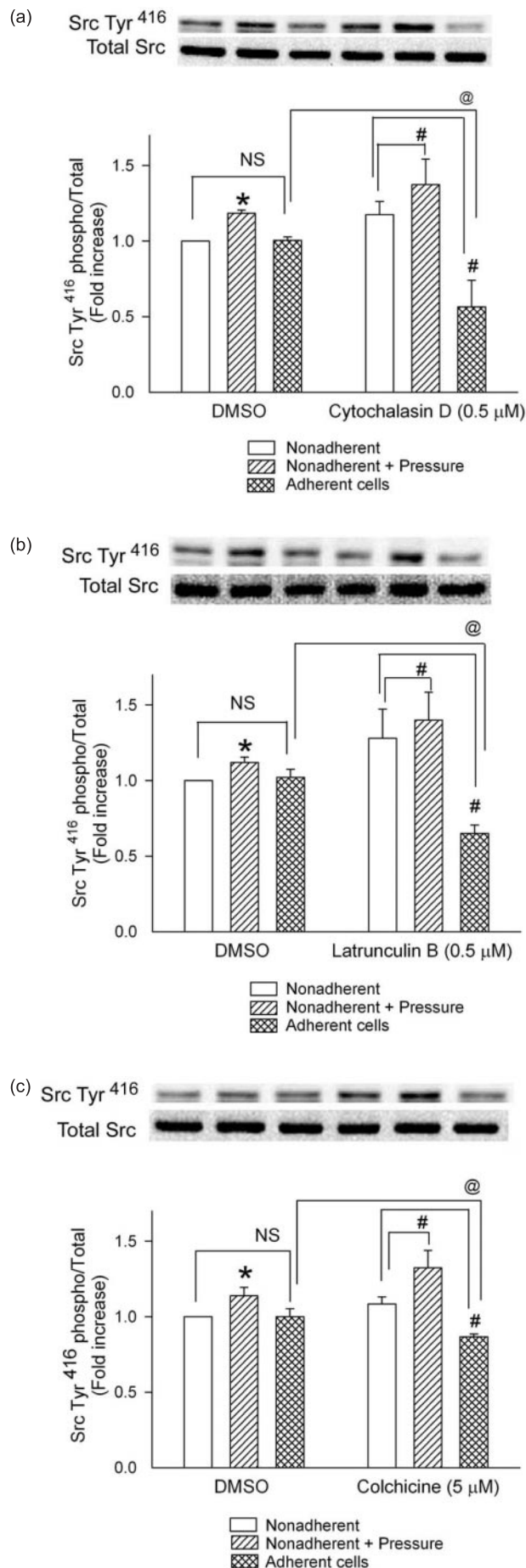
that pressure did not have any significant effect on FAK-397 phosphorylation in cells in which FAK had already been activated by adhesion, and that such adhesion-induced FAK activation was inhibited by cytochalasin D and latrunculin B similarly in pressure-treated adherent cells and adherent cells under ambient pressure conditions ($n = 6, P < 0.05$). Colchicine pretreatment significantly decreased adhesion-induced FAK phosphorylation in pressure-treated adherent cells ($n = 6, P < 0.05$).

In contrast, pressure actually slightly but statistically significantly decreased Src phosphorylation in adherent cells ($n = 6; P < 0.05$; Figure 5b). In general, cytochalasin D, latrunculin B and colchicine each decreased Src activation in pressure-treated adherent cells similar to adherent cells under ambient pressure conditions. However, despite the decrease in Src phosphorylation observed in adherent cells treated with these agents, exposure to increased extracellular pressure resulted in a further decrease in Src phosphorylation in adherent cells treated with cytoskeletal inhibiting agents just as we had observed in adherent cells not treated with cytoskeletal-modulating agents.

Discussion

In a previous study, we reported that 15 mmHg increased pressure enhances SW620 and primary human colon cancer cell adhesion to collagen I via ‘inside-out’ FAK and Src signals. Furthermore, stimulation of cell adhesion by increased extracellular pressure was prevented by phalloidin stabilization of the actin cytoskeleton (5). We also previously reported that pressure stimulates adhesion of a transplantable murine colon cancer to surgical wounds in intact mice via a similar mechanism (7). In the present study, we used specific pharmacological inhibitors of actin and microtubule dynamics to demonstrate that the cytoskeleton is a crucial mediator of pressure-stimulated FAK phosphorylation and subsequent

Fig. 3. Actin or microtubule disruption prevents pressure-induced FAK activation. **(a)** Pressure significantly increased FAK Tyr³⁹⁷ phosphorylation in SW620 cells compared with DMSO-treated non-adherent cells. FAK phosphorylation in response to adhesion was greater than that observed in response to pressure. Pretreatment with cytochalasin D (0.5 μM) did not affect basal phosphorylation but prevented pressure-induced FAK phosphorylation. However, although cytochalasin D treated adherent cells exhibited significantly greater phosphorylation than non-adherent cells, cytochalasin D significantly attenuated adhesion-induced FAK phosphorylation compared with DMSO-treated adherent cells ($n = 3; *P < 0.05$ versus DMSO control; $^{\#}P < 0.05$ versus cytochalasin D non-adherent; $^{\textcircled{\#}}P < 0.05$ versus DMSO adherent controls). **(b)** Pressure significantly increased FAK Tyr³⁹⁷ phosphorylation in SW620 cells compared with DMSO-treated non-adherent cells. FAK phosphorylation in response to adhesion was greater than that in response to pressure. Pretreatment with latrunculin B (0.5 μM) did not affect basal phosphorylation but prevented both pressure- and adhesion-induced FAK phosphorylation. Pretreatment with latrunculin B significantly inhibited adhesion-induced FAK phosphorylation compared with DMSO-treated adherent cells ($n = 3; *P < 0.05$ versus DMSO control; $^{\textcircled{\#}}P < 0.05$ versus DMSO adherent controls). **(c)** Pressure significantly increased FAK Tyr³⁹⁷ phosphorylation in SW620 cells compared with DMSO-treated non-adherent cells. FAK phosphorylation in response to adhesion was greater than that in response to pressure. Pretreatment with colchicine (5 μM) did not affect basal FAK phosphorylation but prevented pressure-induced FAK phosphorylation in non-adherent cells. Colchicine pretreatment did not affect adhesion-induced FAK phosphorylation compared with FAK phosphorylation in DMSO-treated adherent cells. However, adhesion-induced FAK phosphorylation was greater than FAK phosphorylation in colchicine-treated non-adherent cells. ($n = 3, *P < 0.05$ versus DMSO control; $^{\#}P < 0.05$ versus colchicine-treated non-adherent cells).



increased cell adhesion. The increased adhesion we describe here in response to pressure by necessity follows the pressure-stimulated FAK phosphorylation we describe since the FAK studies were done in suspended cells prior to adhesion. We have previously reported that reducing FAK expression by FAK siRNA transfection or inhibiting FAK activation by FRNK transfection prevents the effect of pressure on colon cancer cell adhesion (5), suggesting that FAK activation in response to pressure not only precedes adhesion but is required for it. In contrast, pressure-induced Src phosphorylation, which is also required for the effect of pressure on adhesion, was not affected by actin cytoskeleton perturbation. Interestingly, focal adhesion complex signaling that has been induced by adhesion ('outside-in' signaling) displays a different pattern of sensitivity to these agents.

Chronic exposure to stimuli such as mechanical strain and shear stress may influence adhesion in cells by altering adhesion receptor expression (51,52). However, during acute exposure, there is insufficient time for alteration of receptor expression. Indeed, we have previously reported that integrin surface expression is unchanged in colon cancer cells after 30 min of increased pressure. The mechanisms by which mechanical force can influence adhesion on this rapid time scale are less clear. Vortex-mediated mechanical stress stimulates THP-1 monocyte adhesion by calmodulin and inositol 1,4,5-trisphosphate-mediated Ca^{2+} releases (53), while Rap-1 activation by turbulence stimulates megakaryocyte adhesion (54). We have reported that pressure stimulates colon cancer cell adhesion without changing integrin surface expression, in parallel with an increase in integrin-mediated binding force.

In the present study, we examined the effect of cytoskeletal modulation on basal and pressure-stimulated adhesion. Our finding that pharmacological disruption of the actin cytoskeleton inhibits basal colon cancer cell adhesion is consistent with reports in HT-29 colon cancer cells (55), HeLa (56) and HT-1080 human fibrosarcoma cells (57). However, much lower concentrations of cytochalasin D or latrunculin B have also been reported to enhance unstimulated platelet binding to fibrinogen, B lymphocyte adhesion to ICAM-1 and sickle cell reticulocyte adhesion to VCAM-1, possibly by releasing cytoskeletal restraints and activating integrins (58–60).

More specifically, we also found that pressure-stimulated increases in SW620 cell adhesion were completely prevented by alterations in actin polymerization. Disruption of the actin cytoskeleton by cytochalasin D or doxorubicin detaches endothelial cells from a substrate under shear stress (61,62), but as these agents are toxic and induce apoptosis (63,64), such detachment may not necessarily reflect specific changes in adhesiveness. The direct effects of these agents on force-stimulated adhesion are less well understood. Cytochalasin D has been reported to inhibit platelet aggregation induced by

Fig. 4. Cytoskeletal inhibitors do not affect pressure-induced Src Tyr416 phosphorylation but significantly inhibit adhesion-mediated Src phosphorylation. Pressure significantly induced Src activation in non-adherent cancer cells compared with non-adherent cells at ambient pressure. Adhesion to collagen I did not significantly stimulate Src phosphorylation in SW620 cells. Cytochalasin D (a), latrunculin B (b) and colchicine (c) each enhanced basal Src phosphorylation but did not inhibit pressure-induced Src phosphorylation. All three drugs significantly inhibited adhesion-mediated basal Src phosphorylation compared with drug-treated non-adherent cells or DMSO-treated adherent cells. ($n = 3$; * $P < 0.05$ versus DMSO control; # $P < 0.05$ versus drug-treated non-adherent cells; @ $P < 0.05$ versus DMSO-treated adherent cells).

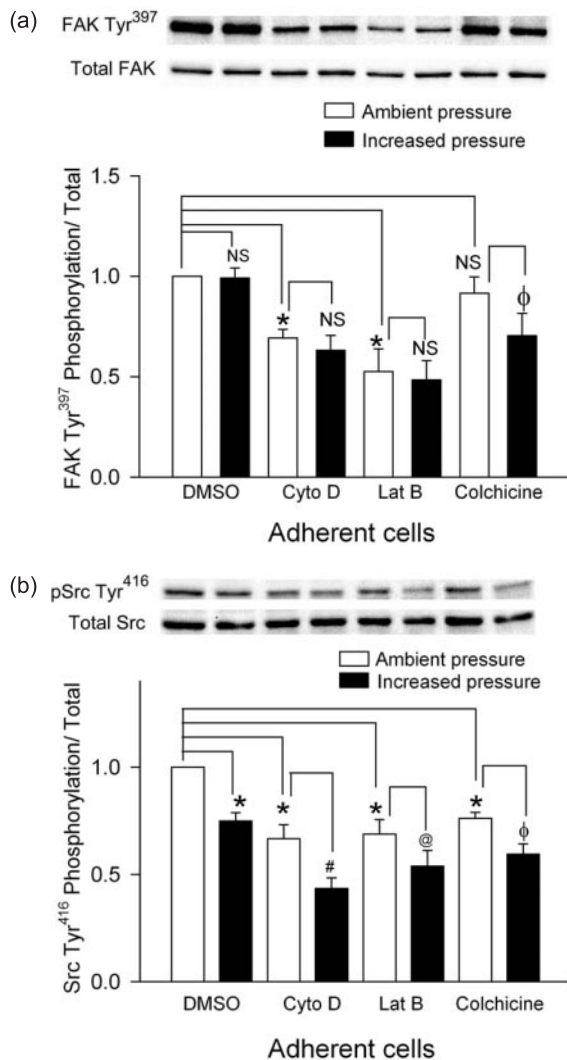


Fig. 5. Pressure does not increase adhesion-induced FAK or Src signals in adherent cells. (a) 15 mmHg increased pressure did not significantly affect FAK phosphorylation in adherent cells compared with DMSO-treated adherent cells at ambient pressure. Pretreatment with cytochalasin D (0.5 μ M) or latrunculin B (0.5 μ M) significantly decreased basal FAK phosphorylation but did not affect FAK phosphorylation in pressure-treated adherent cells compared with drug-treated adherent cells at ambient pressure. Colchicine pretreatment did not significantly decrease FAK phosphorylation in adherent cells compared with DMSO-treated adherent cells. However, pressure significantly decreased FAK phosphorylation in colchicine-treated adherent cells compared with colchicine-treated adherent cells at ambient pressure ($n = 6$; * $P < 0.05$ versus DMSO control; $\oplus P < 0.05$ versus colchicine-treated adherent cells). (b) Pressure significantly decreased Src phosphorylation in adherent cells compared with adherent cells at ambient pressure. Pretreatment with cytochalasin D (0.5 μ M), latrunculin B (0.5 μ M) or colchicine (5 μ M) significantly decreased Src phosphorylation in adherent cells at ambient pressure. In addition, Src phosphorylation was further decreased by extracellular pressure in drug-treated adherent cells similar to the effects of extracellular pressure in adherent cells exposed only to the DMSO vehicle control ($n = 6$; * $P < 0.05$ versus DMSO control; # $P < 0.05$ versus cytochalasin D-treated adherent cells; $\oplus P < 0.05$ versus latrunculin B-treated adherent cells; $\oplus P < 0.05$ versus colchicine-treated adherent cells).

shear (65), consistent with our observations, but both cytochalasin D and latrunculin A were reported by another investigator to actually enhance platelet aggregation in response to shear (47). These disparities may reflect differences in cell type, timing, dosing or specificity of the inhibitor, or the physical force studied.

Microtubules are stress-bearing struts that allow more efficient contractions of actin filaments. The microtubular system regulates intracellular architecture, cell shape and proliferation (45). Basal SW620 adhesion was significantly inhibited by colchicine and taxol, consistent with reports of similar decreases in adhesion in monocytes (66) and A2058 melanoma cells (67). Conversely, others have reported that the depolymerization of microtubules by colchicine or stabilization of microtubules by taxol increases leukocyte adhesion by increasing the lateral mobility of $\beta 2$ integrin heterodimers (44). Pharmacologic disruption of microtubule polymerization increases HT29 adhesion to purified matrix proteins *in vitro* (68), but has been reported to inhibit HT29 adhesion in a hepatic perfusion model (69). Transformed hepatic Ito cell adhesion to laminin was inhibited by colchicine but not by taxol (70), highlighting the heterogeneity of responses in different cell types.

In contrast to the effects of these agents on basal adhesion, the stimulation of adhesion by pressure was prevented by colchicine but not affected by taxol, suggesting that destabilizing the microtubules in suspended cells prevents the effects of pressure, whereas further stabilization of the microtubules has no such effect. Although the effects of these agents on force-modulated adhesion have not previously been investigated, colchicine and taxol have been reported to have disparate, and even opposite, effects in other cells in response to other stimuli when other, non-adhesion, endpoints have been studied (71,72). Thus, although the finding that force-stimulated adhesion is sensitive to microtubule disruption but not microtubule stabilization was not predictable, it is also not implausible, based on extant literature.

Taken together with the available literature, our observations suggest that the function of the cytoskeleton in outside-in adhesion-induced FAK and Src signaling may be fundamentally different from the function of the cytoskeleton in inside-out pressure-induced FAK and Src activation. In our studies, pressure-induced FAK activation was blocked by cytochalasin D, latrunculin B and colchicine, consistent with reports that cytochalasin D inhibits FAK phosphorylation in response to repetitive deformation in osteoblasts (73) and hypoosmotic stress in HepG2 cells (74). In contrast, adhesion-induced FAK activation was inhibited by latrunculin B, attenuated by cytochalasin D and not at all affected by colchicine. These observations are also consistent with previous reports of cytochalasin effects on adhesion-induced integrin-mediated FAK phosphorylation in platelets (49) and NIH 3T3 fibroblasts (75). Our observation that inhibiting microtubule polymerization with colchicine did not inhibit FAK phosphorylation in adherent cells is consistent with a previous report in which nocodazole did not inhibit and indeed induced hyperphosphorylation of FAK in HT-29 cells after static adhesion to ECM (69). Thus, microtubules, in particular, may be significant in pressure-induced FAK activation, but less important for adhesion-induced FAK activation. Therefore, the present data demonstrate that actin organization plays a significant role in pressure-induced FAK phosphorylation, suggesting that the actin cytoskeleton acts as a mechanosensor to transduce pressure-initiated mechanical signals to the focal adhesion complex, thereby modulating integrin adhesiveness to the ECM.

FAK interacts with Src in the focal adhesion complex, and we have previously reported that Src activation is also essential for the stimulation of adhesion by pressure (5) or shear stress (6).

Upon activation and autophosphorylation at tyrosine 397, FAK associated with c-Src by binding to its SH2 domain (76). We also found that cytochalasin D, latrunculin B and colchicine tended to amplify basal Src tyrosine phosphorylation in suspended cells at ambient pressure. This is consistent with previous reports in quiescent R22 smooth muscle cells (77) and NIH 3T3 cells (78) that such cytoskeleton disrupting drugs can stimulate basal Src activity. Src activation by pressure in suspended cells was not affected by any of the cytoskeletal modulators that we studied. This result contrasts with the report by Liu *et al.* (79) that cytochalasin D inhibits strain-induced c-Src translocation in fetal rat lung cells. Liu *et al.* (79) studied a different cell type and a different physical force, and assessed Src translocation rather than phosphorylation. Perhaps more importantly, those studies were conducted in already adherent cells. Taken together with an extensive literature on integrin-mediated signaling (75,80,81), our present observations suggest that matrix-dependent adhesion is likely to affect signaling in the focal adhesion complex substantially; so force-activated focal adhesion signals may be very different in cells already stimulated by adhesion to matrix proteins. We have previously reported that the effects of repetitive strain in colonocytes depend critically on integrin–matrix interactions which are not found in suspended cells (13).

We had intended to contrast the sensitivity to cytoskeletal perturbation of force-mediated Src activation with that of adhesion-mediated force activation, as we had done for FAK. However, although Src phosphorylation in response to adhesion to matrix proteins is well described in other cells (75,80), we were unable to demonstrate increased Src phosphorylation by western blot in SW620 cells after adhesion to either type I or type IV collagen. Cary *et al.* (82) similarly reported that plating NIH 3T3 cells on fibronectin did not increase Src phosphorylation. Although the absence of detectable Src phosphorylation after adhesion by western blot prevented the comparison we wished to make, this disparity further emphasizes the difference between adhesion-related signaling and force-activated inside-out signaling. SW620 adhesion to collagen I does increase FAK phosphorylation on Tyr576, a Src specific phosphorylation site (data not shown), suggesting that Src may either be activated by adhesion at a level not detectable by phosphorylation studies or that Src may be induced to associate more closely with FAK after adhesion in these cells.

In contrast to our observations of Src phosphorylation in suspended cells exposed to increased extracellular pressure, Src activation in response to adhesion was substantially inhibited by all three cytoskeletal modulators studied. This again illustrates the difference between pressure-induced inside-out signaling and adhesion-induced outside-in signaling. Thus, in contrast to the patterns observed for FAK activation, outside-in adhesion-induced Src activation seems highly dependent on a functional cytoskeleton, whereas pressure-induced Src activation may not require cytoskeletal function, at least as far as determined by the present studies.

Furthermore, the absence of an effect of pressure on FAK-397 phosphorylation in adherent cells suggests that adhesion-induced FAK activation overshadows the effects of pressure in these cells. The similar effects of cytoskeletal modulation on adherent cell FAK phosphorylation in ambient pressure and increased pressure conditions is again consistent with the concept that adhesion-induced FAK activation dominates in this setting. However, our studies of Src-416 phosphorylation yielded somewhat different results. Although adhesion itself did

not affect Src-416 phosphorylation, adhesion appeared to reverse the effects of pressure on Src. Furthermore, although cytoskeletal modulation inhibited Src activation in adherent cells regardless of extracellular pressure, the reversed pressure effect on Src persisted in adherent cells treated with any of these modulators. This result contrasts with a previous report that pressure activates Src slightly in SW620 cells which had previously been maintained on a collagen matrix for 48 h before the application of pressure (83), and suggests the likely complexity of interaction between adhesion regulation of Src and pressure regulation of Src. Although the cytoskeleton seems important for basal Src phosphorylation in adherent SW620 cells, the cytoskeleton may be less important for inside-out pressure-modulated Src phosphorylation, whether increased in suspended cells or decreased in newly adherent cells. The mechanisms by which recent adhesion reverses the pressure effect await further study, but this reversal may reflect crosstalk with some other transient or rapid signal event induced by acute adhesion and integrin occupancy.

In conclusion, our data demonstrate that actin and microtubule integrity greatly influence pressure-induced colon cancer cell adhesion. In particular, modulating the balance of actin polymerization by specific pharmacological inhibitors prevents pressure-induced FAK phosphorylation, thereby inhibiting colon cancer cell adhesion. Similarly, disrupting microtubule polymerization with colchicine prevents pressure-induced FAK phosphorylation and subsequent cancer cell adhesion. Both adhesion-induced FAK activation and pressure-induced Src activation displayed different patterns of sensitivity to these agents. These results illustrate a dichotomy between the role of the cytoskeleton in mediating inside-out and outside-in signaling, and suggest that FAK and Src are activated differently in response to pressure. Taken together with our previous observations (5), these results suggest that both cytoskeleton-dependent FAK activation and cytoskeleton-independent Src activation may be required for extracellular pressure to stimulate colon cancer cell adhesion.

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