Colon Cancer Cell Adhesion in Response to Src Kinase Activation and Actin-Cytoskeleton by Non-Laminar Shear Stress

Vijayalakshmi Thamilselvan, Ashish Patel, Jochem van der Voort van Zyp, and Marc D. Basson*

Departments of Surgery, John D. Dingell VA Medical Center and Wayne State University, Detroit, MI 48201

Abstract Malignant cells shed from tumors during surgical resection or spontaneous metastasis experience physical forces such as shear stress and turbulence within the peritoneal cavity during irrigation, laparoscopic air insufflation, or surgical manipulation, and within the venous or lymphatic system. Since physical forces can activate intracellular signals that modulate the biology of various cell types in vitro, we hypothesized that shear stress and turbulence might increase colon cancer cell adhesion to extracellular matrix, potentiating metastatic implantation. Primary human malignant colon cancer cells isolated from resected tumors and SW620 were subjected to shear stress and turbulence by stirring cells in suspension at 600 rpm for 10 min. Shear stress for 10 min increased subsequent SW620 colon cancer cell adhesion by $40.0 \pm 3.0\%$ ($n = 3$; $P < 0.001$) and primary cancer cells by $41.0 \pm 3.0\%$ to collagen I when compared to control cells. In vitro kinase assay (1.5 ± 0.13 fold) and Western analysis (1.34 ± 0.04 fold) demonstrated a significant increase in Src kinase activity in cells exposed shear stress. Src kinase inhibitors PP1 (0.1 μM), PP2 (20 μM), and actin-cytoskeleton stabilizer phalloidin (10 μM) prevented the shear stress stimulated cell adhesion to collagen I. Furthermore, PP2 inhibited basal ($50.0 \pm 2.8\%$) and prevented shear stress induced src activation but phalloidin pretreatment did not. These results raise the possibility that shear stress and turbulence may stimulate the adhesion of malignant cells shed from colon cancers by a mechanism that requires both actin-cytoskeletal reorganization an independent physical force activation of Src kinase. Blocking this pathway might reduce tumor metastasis during surgical resection. J. Cell. Biochem. 92: 361–371, 2004. Published 2004 Wiley-Liss, Inc.[†]

Key words: adhesion; force; matrix; mechanotransduction; signal transduction

Viable tumor cells can be recovered from the circulation of many patients with cancer, yet few of these tumor cells progress to implantation and metastasis [Aoudjit et al., 1998]. Such viable tumor cells can also be recovered from the portal venous circulation and peritoneal cavity of many patients during surgical resection of abdominal cancers at a rate far in excess of what would be expected from their subsequent clinical course [Hewett et al., 1996]. Thus, some factor or factors not yet identified must influence tumor cell adhesion, proliferation, or progression.

Cooke et al. have recently described a model to study the effects of shear stress and turbulence on human carcinoid BON cells, in which plates were slowly rotated to generate movement of the assay buffer [Kim et al., 2001a]. These investigators reported that such shear stress activates BON cell secretion of 5-HT by activating heterodimeric G protein-coupled receptors and mobilization of intracellular Ca^{2+}. They therefore further postulated that shear stress within the bowel lumen may activate 5-HT release in vivo by such a mechanism. Shear stress and turbulence are well-described features of vascular flow [Chien, 1976; Lehoux and Tedgui, 1998], and may also characterize the movement of intraperitoneal fluid during surgical irrigation and suction [Marshburn and Hulka, 1990].

Shear forces may influence on adhesion and other cellular functions in a variety of other cell

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*Correspondence to: Dr. Marc D. Basson, Chief, Surgical Service, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48201-1932.
E-mail: marc.basson@med.va.gov

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types. For instance, shear stress has been reported to promote adhesion through conformational activation of integrins [Tzima et al., 2001; Ashida et al., 2003] and physiological levels of shear stress induces new focal contacts in endothelial cells [Girard and Nerem, 1993], cytoskeleton reorganization in bovine aortic endothelial cells [Thoumine et al., 1995; Langille, 2001] and up-regulates human vascular endothelial cell integrin expression [Urbich et al., 2000]. Integrin-mediated tumor cell adhesion is important for the development of metastasis lesions [Haier et al., 1999]. We therefore hypothesized that shear stress or turbulence might act on colon cancer cells to alter tumor cell adhesion. We sought to test this hypothesis using human SW620 colon cancer cells. We induced shear stress by gentle stirring, and measured subsequent adhesion to type I collagen, the dominant collagen of the interstitial matrix. Since Src has been shown to correlate with tumor invasiveness and metastasis in many tumor types [Summy and Gallick, 2003], we sought to determine whether Src was activated by shear forces, and whether Src inhibition could block the effects of shear on SW620 colon cancer adhesion. Since shear induces actin polymerization in neutrophils [Okuyama et al., 1996], we also sought to determine whether the stabilization of actin polymerization by phalloidin could inhibit shear-induced tumor cell adhesion or Src activation.

METHODS

Cells and Culture Conditions

SW620 colon cancer cells were maintained at 37°C in 5% CO_2 in an equal mixture of DMEM and RPMI 1640 media containing 5% FBS, 20 mM glutamine, 10 mM HEPES buffer, 1 μM sodium pyruvate, 100 U/ml pencillin/streptomycin, and 0.525 μg/ml transferrin. Single cell suspensions of primary human malignant colon cancer cells were isolated from resected tumors by collagenase digestion [Emenaker and Basson, 2001]. More than 90% of cells excluded trypan blue at each isolation.

Shear Model

Shear and turbulence were applied by stirring the experimental petri dish containing cell suspension with a Teflon coated stir bar (3.7 cm long and 1 cm diameter, weighing 7.4 g) at 600 rpm for 10 min at room temperature. Control plates were placed next to the stir plates but had no stir bar. After 10 min, cell suspension from control and experimental plates were collected and subjected to adhesion experiments and Western analysis.

Matrix Protein Precoating

Bacteriologic plastic culture dishes or six-well plates were precoated with collagen I (Sigma, St. Louis, MO) at saturating densities of 12.5 μg/ml using an ELISA-based buffer at 4°C and then washed three times with PBS as previously described [Basson et al., 1992].

Adhesion Assay

Adhesion experiments were carried out in SW620 and primary human isolated colon cancer cells. After 10 min of shear treatement, 100,000 cells/well were seeded into collagen-precoated bacteriologic plastic six well plates for adhesion studies. After an appropriate time (generally 10 min), nonadherent cells were gently washed away using 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. In some studies, cells were pretreated with the Src kinase inhibitors PP1 (0.1 μM) or PP2 (20 μM) or the actin polymerization stabilizer phalloidin (10 μM).

Western Analysis

Cells were collected after 10 min of shear treatment by centrifugation at 1,000 rpm for 3 min, washed with ice cold PBS, and lysed in a buffer with protease inhibitors (50 mM Tris, 150 mM NaCl, 10% Triton X-100, 1% deoxycholate, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin). Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce) and equal amounts of protein were resolved by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA and incubated with primary antibody specific to pSrc-416 (Cell Signaling) and then with secondary anti-rabbit IgG-horseradish peroxidase conjugated antibody. The membranes were then stripped and reprobed with an antibody that reacts with unphosphorylated Src (Santa Cruz) and the same secondary, as a control for protein loading.

Protein bands were detected using ECL plus chemiluminesence and analysed by Kodak Image station. Src phosphorylation was assessed as the ratio of phospho-Src to total Src band intensity. All exposures were within the linear range of exposure.

Src In Vitro Kinase Assay

Cells were collected after 10 min of shear treatment and rinsed with ice cold phosphate buffered saline (PBS) and harvested in lysis buffer with protease inhibitors (50 mM Tris, 150 mM NaCl, 10% Triton X-100, 1% deoxycholate, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin). After protein assay, 300 μg lysate was incubated with 2 μg anti-Src mAb (Upstate) together with 20 μl 10% protein A-Sepharose beads for 12 h at 4°C. The immunocomplexes were washed with PBS containing 0.2% Triton X-100 and twice with a kinase buffer (25 mM HEPES pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerol-phosphate, 0.1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 20 mM dithiothreitol). The kinase reaction was initiated by suspending the immunoprecipitates in 20 μl kinase buffer containing 1μCi [γ-^{32}P] ATP, 1 μl of 25 μM ATP, 0.2 μg enolase (Sigma). The reaction mixture was incubated for 20 min at 30°C and terminated with 6× SDS sample buffer. The proteins were resolved by 10% SDS–PAGE and autoradiograph for phosphorylated enolase.

Statistical Analysis

All experiments were performed in triplicate wells, and at least three separate experiments with similar results were performed in each case. Cell counts were normalized to basal cell adhesion. Statistical analysis was performed by unpaired t test, with a p value of <0.05 being set a priori as the level sought for statistical significance.

RESULTS

Model Validation

Initial calibration studies demonstrated a linear relationship between the dial setting on the stir plate and the revolutions per minute of the stir bar over a range of 100–750 rpm. Trypan blue exclusion studies demonstrated cell viability after stirring for 10 min at 375 rpm to be 97% and after 10 min at 750 rpm to be 94%. We accordingly chose a rate of 600 rpm for subsequent studies, applying shear stress and turbulence to cells placed in 100 mm petri dishes. No change in pH or pO_2 was observed within the culture medium with this intervention (not shown).

Effects of Shear on Subsequent Adhesion

Cells pretreated with shear at 600 rpm for 10 min and then allowed to adhere to collagen-precoated dishes exhibited significantly increased adhesion to collagen I over 10–60 min. Figure 1a shows a typical experiment, with data at each time point normalized to its respective control to compensate for the gradual increase in control cell adhesion over time. SW620 cell adhesion was enhanced after 10 min with shear and turbulence by $40.0 \pm 3\%$ ($n = 3$, $P < 0.001$). The effect decreased over time thereafter, but remained statistically significant for at least 60 min ($n = 3$, $P < 0.05$). Basal adhesion increases over time because the cells do not all adhere simultaneously or rapidly. This is a well-described phenomenon in the study of cell adhesion that probably reflects both increasing opportunity for the cells to contact the substrate over time and the recruitment of additional adhesion receptors after initial cell contact. Conversely, since cells are pretreated with shear force, which is not present during the actual adhesion in our model, the gradual decrease in the difference between control and shear-treated cells may be caused either by an acceleration by shear of the rate of cell adhesion, so that the untreated cells can eventually "catch up," or by a diminution in the previous effect of shear pretreatment over time. In addition, shear and turbulence pretreatment significantly increased primary human colon cancer cell adhesion by $41.0 \pm 3\%$ to collagen I (Fig. 1b).

Src Activation

We used an in vitro kinase assay (Fig. 2a) and Western analysis for phosphorylated Src (Fig. 2b) to determine whether Src was activated in the cells exposed to shear and turbulence, compared to control cells. A typical gel from an in vitro kinase assay is shown in Figure 2a together with the densitometric analysis of phosphorylated enolase from seven such similar experiments. Densitometric analysis revealed a 1.5 ± 0.13 fold increase in Src activation ($n = 7$, $P < 0.01$) in shear-treated cells compared to control by in vitro kinase assay. In

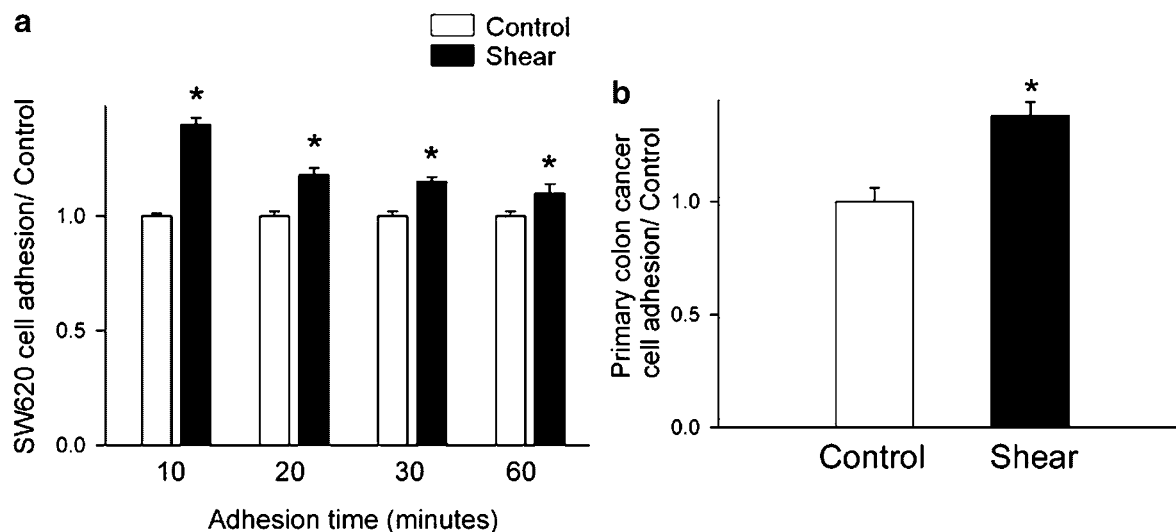

Fig. 1. Shear stress induces SW620 colon cancer cell adhesion to collagen. **a**: Cells preexposed to non-laminar shear stress (closed bars) for 10 min exhibited increased adhesion to collagen I compared to control cells (open bars) at 10–60 min after shear stress had been terminated. Results are normalized to their respective controls and expressed as mean ± SE. The shear effect on adhesion persisted for up to 60 min (*$P < 0.001$; n = 3). **b**: Shear and turbulence pretreatment for 10 min significantly increases primary human colon cancer cell adhesion to collagen I compared to primary colon cancer cells without shear treatment (*$P < 0.001$, n = 3).

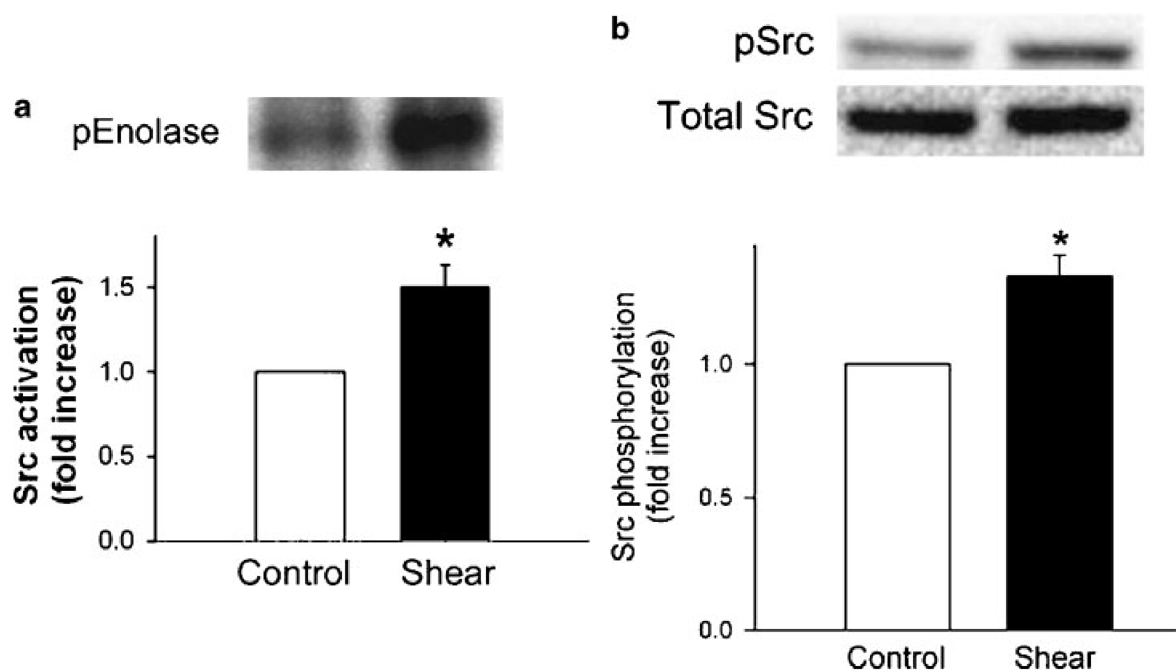

Fig. 2. Shear stress activates Src kinase in colon cancer cells. **a**: Src kinase activity was measured by Src in vitro kinase assay using enolase as the substrate (a). The **top panel** shows an autoradiograph of phosphorylated enolase from one of seven similar experiments. The **bottom panel** summarizes the densitometric analysis of all seven experiments. Shear stress for 10 min increased Src kinase activity in colon cancer cells (closed bar) compared to control cells treated similarly but without shear stress (open bars) (*$P < 0.001$: n = 7). Results are normalized to control and expressed as mean ± SE of fold increase in Src activation. **b**: Western analysis of shear stress induced Src phosphorylation is shown in b. The **top panel** represents a typical Western blot for pSrc-416 and total Src. The **bottom panel** represents the densitometric analysis of three similar experiments. Shear stress for 10 min increased Src phosphorylation in colon cancer cells (closed bar) compared to control cells (open bars) (*$P < 0.01$, n = 3).

parallel experiments conducted separately on different cells, Western blot analysis demonstrated that shear treatment for 10 min produced a similar 1.34 ± 0.04 fold increase in the phosphorylation of Src at tyrosine 416, which is commonly used as an indicator of Src activation [Harvey et al., 1989] (Fig. 2b, $P < 0.01$, n = 3).

Effects of Src Blockade

The effect of PP1 on shear and turbulence stimulated adhesion is shown in Figure 3. Pretreatment with the Src kinase inhibitor PP1 (0.1 μM) prevented the shear and turbulence-induced increase in cell adhesion to collagen I without affecting basal adhesion, although control cells treated with the DMSO vehicle exhibited a $34.0 \pm 4.0\%$ (n = 9, $P < 0.003$) increase in adhesion when exposed to shear and turbulence (Fig. 3).

We obtained similar results with a closely analogous Src kinase inhibitor, PP2. PP2 (20 μM) significantly inhibited basal adhesion by $76.1 \pm 3.1\%$ ($P < 0.001$, n = 3) and prevented shear induced SW620 cell adhesion to collagen I (Fig. 4a). In contrast, control DMSO-treated cells exhibited significantly increased cell adhesion to collagen I after shear treatment ($37.6 \pm 5.0\%$, $P < 0.01$, n = 3). In parallel studies, PP2 both significantly inhibited basal Src activation by $50.0 \pm 2.8\%$, and prevented the induction of Src phosphorylation by shear and turbulence for 10 min (Fig. 4b, n = 3, $P < 0.01$). In addition, PP2 pretreatment significantly inhibited basal adhesion by $34.0 \pm 4.1\%$ and prevented shear and turbulence induced primary colon cancer cell adhesion (Fig. 4c, n = 3, $P < 0.001$).

Effect of Phalloidin on Shear Induced Adhesion and Src Activation

Phalloidin pretreatment did not affect basal adhesion when compared to control cells treated with the DMSO vehicle. However, shear-induced SW620 cell adhesion was prevented by pretreatment with phalloidin (Fig. 5a, n = 3). However, phalloidin treatment did not inhibit or prevent basal or shear stress induced Src activation (Fig. 5b, n = 3). In addition, phalloidin pretreatment significantly inhibited basal adhesion by $22.0 \pm 1.9\%$ and prevented shear and turbulence induced primary colon cancer cell adhesion (Fig. 5c, n = 3, $P < 0.001$).

DISCUSSION

We recently reported that increased extracellular pressure stimulates colon cancer cell adhesion in a manner sensitive to tyrosine

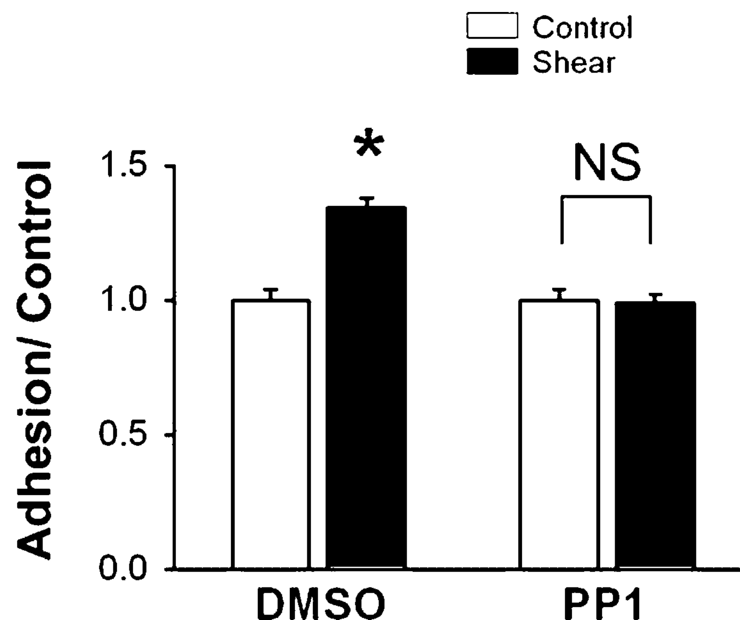

Fig. 3. Src kinase inhibitor PP1 prevents shear stress induced colon cancer cell adhesion. Cells treated with a DMSO vehicle demonstrated significantly increased adhesion following shear stress (closed bars) as compared with control cells not exposed to shear stress (open bars) (*$P < 0.001$—shear stress vs. control; n = 9) However, pretreatment with the Src kinase inhibitor PP1 (0.1 μM) prevented this increase (NS represents not significant). PP1 pretreatment did not affect basal colon cancer cell adhesion compared to the cells treated with the DMSO vehicle. Results are normalized to control and expressed as mean ± SE.

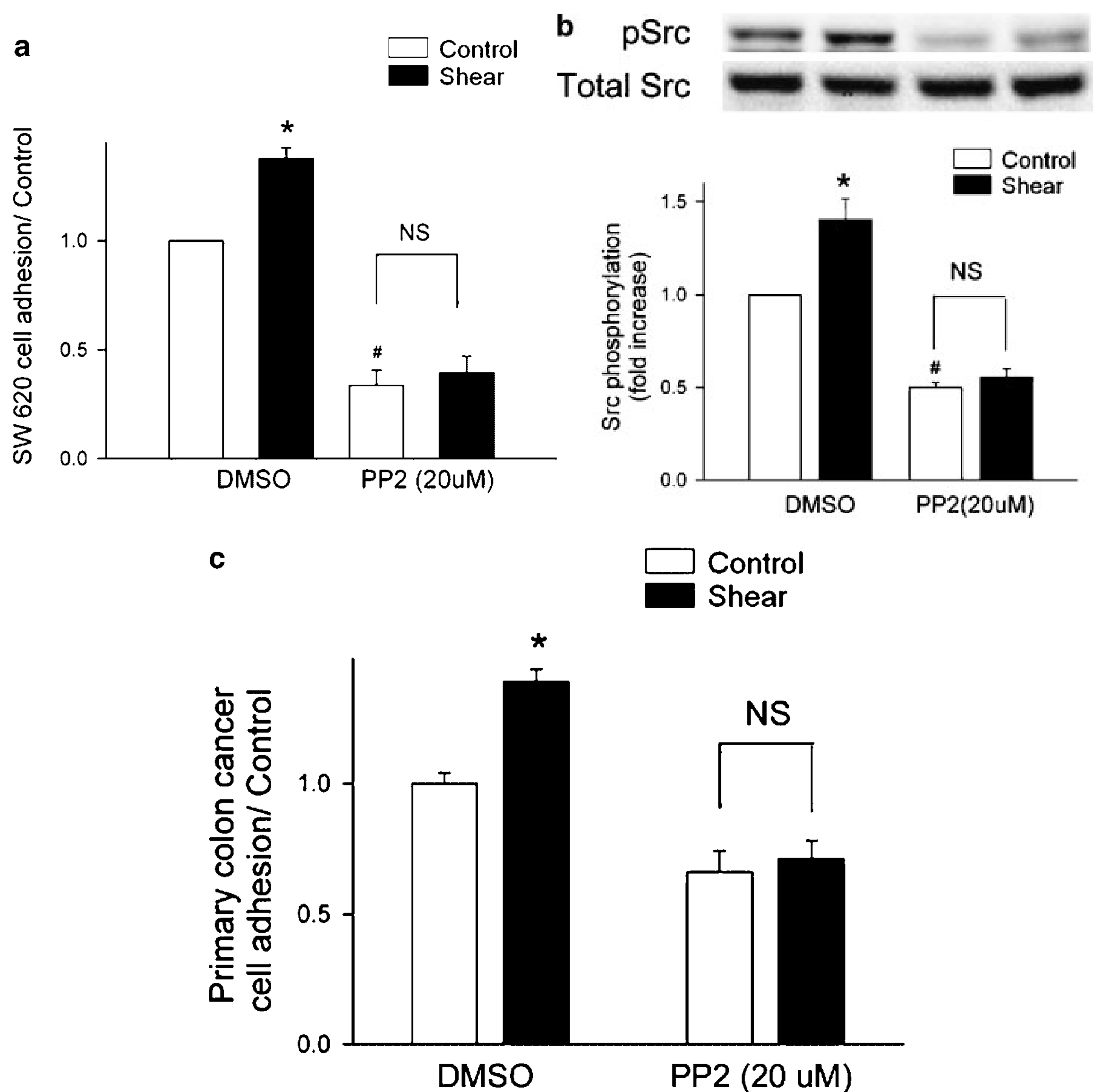

Fig. 4. Src kinase inhibitor PP2 prevents shear stress induced Src activation and colon cancer cell adhesion. **a**: PP2 pretreatment significantly inhibits basal adhesion compared to DMSO-treated controls ($^{\#}P < 0.01$, n = 3) and prevented shear-induced adhesion compared to PP2-treated controls (NS, not significant). Shear stress significantly increased DMSO-treated colon cancer cell adhesion compared to DMSO-treated control cells ($^{*}P < 0.01$, n = 3). Results are normalized to control and expressed as mean ± SE. **b**: PP2 (20 μM) significantly inhibits basal Src phosphorylation compared to DMSO-treated control cells and prevented the induction of Src phosphorylation by shear and turbulence treatment for 10 min ($^{\#}P < 0.01$, PP2 control vs. DMSO control; NS-not significant, PP2 treated shear vs. PP2 treated control, n = 3). Cells treated with a DMSO vehicle exhibited shear-induced Src phosphorylation compared to DMSO-treated control cells ($^{*}P < 0.01$, DMSO shear vs. DMSO control, n = 3). The **top panel** represents a typical Western blot for pSrc-416 and total Src. The **bottom panel** represents the densitometric analysis of 3 similar experiments. Results are normalized to control and expressed as fold increase in the ratio of pSrc to total Src after exposure to shear and turbulence. **c**: PP2 pretreatment significantly inhibits basal and prevented shear induced primary colon cancer cell adhesion ($^{\#}P < 0.001$, NS, not significant, n = 3).

kinase inhibitors, and that this effect is blocked by a functional antibody to the β1 integrin subunit [Basson et al., 2000]. However, different physical forces may exert different effects. Shear and turbulence are among a variety of forces that act on colon cancer cells intraoperatively or within the vasculature. The results presented here suggest that shear stress and turbulence stimulated subsequent colon cancer cell adhesion to collagen I and this effect appeared mediated by Src since Src phosphorylation and kinase activity were increased by

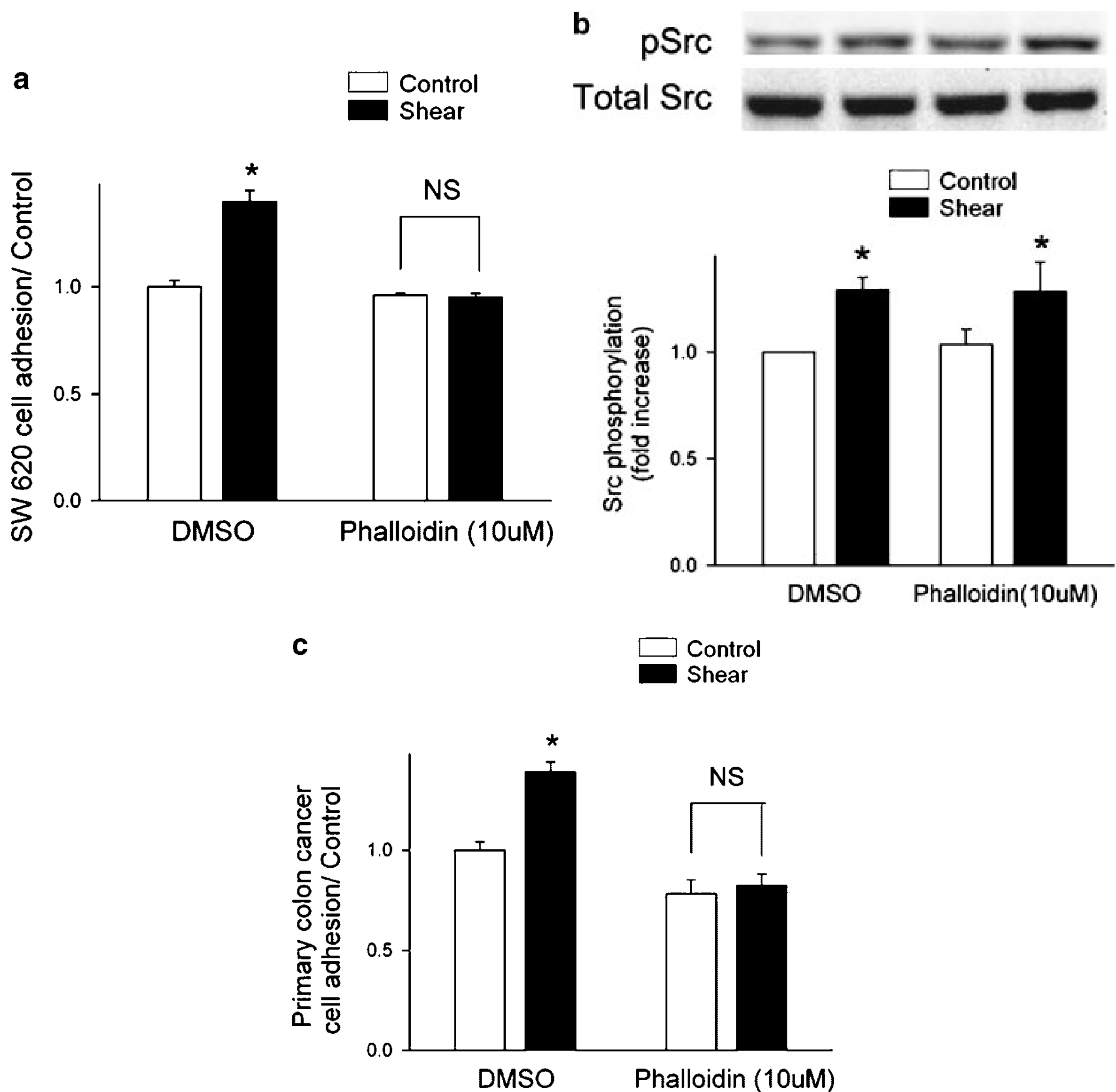

Fig. 5. Phalloidin pretreatment significantly prevents shear induced colon cancer cell adhesion but not Src phosphorylation. **a**: Phalloidin (10 μM) pretreatment prevented shear-induced SW620 colon cancer cell adhesion compared to phalloidin-treated control (NS, not significant). Shear stress for 10 min significantly increased DMSO-treated colon cancer cell adhesion compared to DMSO-treated controls (*$P < 0.01$, n = 3). Phalloidin pretreatment did not affect basal colon cancer cell adhesion compared to the cells treated with the DMSO vehicle. Results are normalized to control and expressed as mean ± SE. **b**: Phalloidin pretreatment did not inhibit basal or shear-induced Src phosphorylation compared to DMSO treated control and phalloidin treated control (n = 3). Shear significantly increased Src phosphorylation in DMSO-treated cells compared to DMSO treated control cells (*$P < 0.01$, n = 3). **c**: Phalloidin pretreatment significantly inhibits basal and prevented shear induced primary colon cancer cell adhesion ($^{\#}P < 0.001$, NS, not significant, n = 3).

the stimulus and the Src kinase inhibitors PP1 and PP2 prevented the effect. Furthermore, phalloidin-pretreatment prevented stimulation of cell adhesion by shear and turbulence independently of Src activation by shear.

Longer term shear stress and turbulence have previously been reported to influence the adhesion of endothelial cells [Urbich et al., 2000] and other cells normally found within the bloodstream, including platelets [Hindriks et al., 1992], monocytes [Ashida et al., 2003; Sorescu et al., 2003], neutrophils [Abbassi et al., 1993], and megakaryocytes [Kim et al., 2001a]. Although the mechanisms of such effects are not clear, signals including NF kappaB- in monocytes [Sorescu et al., 2003], inositol 1,4,5-triphosphate-sensitive calcium release in monocytic THP-1 cells [Ashida et al.,

2003], and Rap1 in megakaryocytes, [Kim et al., 2001a] may mediate the promotion of adhesion by laminar shear stress in these particular cell types. However, there has not been any demonstration that such putative regulatory signals form an integrated pathway or generalize among cell types. Thus, whether such other signals might play a role in a signal cascade upstream or downstream of Src in colon cancer cells in response to shear stress awaits further study. The role of Src activation in the regulation of adhesion by shear stress in such cells has not previously been studied. Interestingly, in one such study, Urbich et al. [2000] reported that shear stress for 24 h induced HUVEC adhesion by stimulating synthesis of the fibronectin receptor integrin $\alpha5$ and $\beta1$subunits. However, 20 min of preexposure to shear stress did not increase HUVEC cell adhesion in their study, presumably because there was not sufficient time to induce integrin synthesis. This result differs from our findings, which occur after 10 min of shear and turbulence and thus presumably are independent of integrin synthesis.

Shear stress associated with laminar flow may promote cancer cell adhesion by different mechanisms. First, shear stress appears to activate endothelial cells so that they may serve as better substrates for the subsequent adhesion of breast cancer cells [Gomes et al., 2003]. Second, pretreatment of endothelial cells with IL-1 also increases Colo 201 colon cancer cell adhesion under low flow conditions [Yoshida et al., 1999]. Third, laminar flow promotes the adhesion of lung cancer cells [Bastida et al., 1989] and both promotes the adhesion of HT29 colon cancer cells and stabilizes already formed adhesions [Haier and Nicolson, 2000]. Indeed, one report suggests that Src may be involved in the effects of such laminar flow [Haier et al., 2002]. However, these investigators have not studied the effects of pretreatment of cancer cells with shear stress on subsequent adhesion or demonstrated the persistence of such an effect.

More importantly, laminar flow may produce very different effects from non-laminar turbulent or circular flow such as we studied here [Davies et al., 1986]. For instance, vascular biologists have long argued that laminar flow protects against atherogenesis while non-laminar circular flow may actually stimulate atherogenic effects [Berk et al., 2002]. In one striking example, laminar flow inhibits TNF induced VCAM-1 expression, while non-laminar flow stimulates it [Yamawaki et al., 2003]. In the present study, we investigated the effects of shear stress associated with non-laminar circular flow.

We demonstrated that pretreating SW620 colon cancer cells and primary human colon cancer cells to shear stress from non-laminar circular flow increased colon cancer cell adhesion to collagen I. This effect persisted for up to 60 min after 10 min of exposure to shear stress. Shear tended to increase colon cancer cell adhesion by approximately 40–50% in our various studies. Although larger effects might seem more impressive, a moderate difference in adhesiveness may well have significant biological effects (and significant implications for a patient who may or may not progress to metastatic disease). Changes in cancer cell adhesion of this magnitude have previously been described and felt to be important in response to other stimuli by several other authors [Bastida et al., 1989; Haier and Nicolson, 2001; Mine et al., 2003]. Moreover, preexposing SW620 colon cells to such shear stress and turbulence not only increased adhesiveness but also increased Src kinase activity in a durable fashion. Src is a non-receptor protein kinase that plays central roles in the control of cell growth and differentiation. Src has been reported to be activated by different mechanical forces in other cell types. For example, stretch has been shown to activate c-Src in fetal rat lung cells and a different mechanical force, pressure, also activate Src in mesenteric arteries [Rice et al., 2002]. Consistent with our findings, Okuda et al. [1999] described activation of c-Src in response to shear in HUVEC, although they did not assess the effect of this Src activation on endothelial adhesion [Okuda et al., 1999]. This effect was inhibited by PP1 and obliterated by transfection of endothelial cells with kinase-inactive Src. However, whether shear stress activates Src is actually controversial within the established literature. For instance fluid shear stress induced big mitogen activated protein kinase-1 (BMK-1) activity in bovine aortic endothelial cells dependent on other tyrosine kinases and on calcium mobilization, but independently of c-Src, and PP1 or kinase inactive src did not inhibit shear stress induced BMK-1 activity in that report [Yan et al., 1999].

c-Src is a major component of focal adhesion complex and regulates focal adhesion formation and/or cytoskeletal rearrangement. Various cell signaling molecules, such as focal adhesion kinase, pp60Src or paxillin, and cytoskeletal components, such as actin or microtubules, are reported be involved in tumor cell adhesion [Haier and Nicolson, 2001]. Activation of Src family kinases has been identified in many human cancers [Summy and Gallick, 2003]. Indeed, Src expression and activity is increased in more than 80% of human colon cancers [Windham et al., 2002]. Furthermore, such increased Src activity may correlate with the malignant potential of cancer cells [Summy and Gallick, 2003] while Src overexpression leads to enhanced invasive cell behavior in vitro [Pories et al., 1998]. Racchia et al. have reported that the c-Src kinase inhibitor, pyrrolopyrimidine reduced adhesion, motility, and invasion of prostate cancer cells [Recchia et al., 2003].

We demonstrated that phalloidin pretreatment prevented the stimulation of colon cancer cell adhesion to collagen I by shear and turbulence. Shear-induced stress fiber formation and focal contact reorganization play an important role in endothelial cell adhesion [Wechezak et al., 1989], and shear also induces actin polymerization in human neutrophils [Okuyama et al., 1996]. Actin filaments of the membrane cytoskeleton are required to protect endothelial cells from hemodynamic injury resulting from shear stress, and even a small disturbance in actin dynamics inhibits shear-dependent cell alignment [Schnittler et al., 2001]. Actin filament turnover may also play an important role in the well studied system of platelet aggregation, although the precise mechanism appears complex. Low concentrations of cytochalasin D have been reported to induce platelet adhesion, while higher concentrations of cytochalasin D actually inhibit agonist-induced platelet adhesion. The same authors found that jasplakinolide, an actin stabilizer, prevents the induction of platelet adhesion by low dose cytochalasin D [Bennett et al., 1999]. Haier and Nicolson [1999] reported that cytochalasin D inhibits the stimulation of adhesive interactions to ECM by laminar flow in another colon cancer line, the HT-29 line. In addition, phalloidin may inhibit pressure-induced cancer cell adhesion to collagen I [Thamilselvan and Basson, 2004]. Taken together with our present data, these results suggest that the actin cytoskeleton may be crucial for cellular responses to physical forces as diverse as laminar and non-laminar flow and constant pressure.

Phalloidin pretreatment did not affect basal Src activation or the activation of Src by shear stress. A functional actin cytoskeleton appears required for some but not all cell signals in response to various stimuli, For instance, consistent with our present findings, others have reported that treatment with cytochalasin D does not inhibit Src activation by bombesin in Swiss 3T3 cells [Rodriguez-Fernandez and Rozengurt, 1996]. Cytochalasin D also did not inhibit ERK activation by bombesin in the same cells [Seufferlein et al., 1996]. Conversely, cytochalasin D has been reported to inhibit adhesion-induced activation of FAK and Src in astrocytes [Cazaubon et al., 1997] and MAPK in REF52 fibroblasts [Chen et al., 1994], as well as hypoosmotic stress induced activation of FAK in HepG2 cells [Kim et al., 2001b]. Cytochalasin D inhibits both cell spreading and ERK activation following adhesion of bovine airway smooth muscle cells to fibronectin, but does not inhibit growth factor-induced ERK activation in adherent cells [Heuertz et al., 1997]. Ingber has postulated that the actin cytoskeleton may act as a mechanical linkage that itself induces tensegrity-type signals, while it is also possible that the actin cytoskeleton is involved in organizing the distribution of the relevant kinases within the cell [Ingber, 1997a,b]. More precise definition of the manner in which the actin cytoskeleton is involved in more conventional cell signaling therefore awaits further study.

Thus, taken together with these other observations in the literature, our results suggest that brief non-laminar shear stress and turbulence may stimulate colon cancer cell adhesion to matrix proteins such as collagen I in a durable fashion by activating Src and actin cytoskeletal reorganization. These findings support a role for shear stress in the regulation of colon cancer cell adhesion in the process of metastasis. The persistence of this effect for at least an hour after shear stress has been stopped and the demonstration that non-laminar turbulent flow produces this effect suggests as well that the turbulence associated with irrigation of body cavities containing shed tumor cells during surgical resection may activate the adhesion of these cancer cells to surgical sites even after the irrigation has stopped.

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