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Effects of Increased Ambient Pressure on Colon Cancer Cell Adhesion

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Abstract Forces such as strain modulate intestinal epithelial biology. Shear and pressure influence other cells. The effects of pressure on human colon cancer cells are poorly understood. Increasing ambient pressure for 30 min by 15 mm Hg over atmospheric stimulated adhesion to matrix proteins of four human colon cancer cell lines and primary cells from three human colon cancers, but not bovine aortic smooth-muscle cells. This effect was energy dependent and cation dependent (blocked by azide and chelation), accompanied by tyrosine phosphorylation of intracellular proteins including focal adhesion kinase, and blocked by tyrosine kinase inhibition (genistein, tyrphostin, and erbstatin) and a functional antibody to the β1 integrin subunit. Although pressure stimulated adhesion even in a balanced salt solution, baseline and pressure-stimulated adhesion were each substantially diminished in the absence of serum. These data suggest that relatively low levels of increased pressure may stimulate malignant colonocyte adhesion by a cation-dependent β1-integrin-mediated mechanism, perhaps via focal adhesion kinase–related tyrosine phosphorylation. In addition to elucidating another aspect of physical force regulation of colonocyte biology, these findings may be relevant to the effects of increased pressure engendered by colonic peristalsis, surgical manipulation, or laparoscopic surgery on colon cancer cell adhesion. J. Cell. Biochem. 78:47–61, 2000.

Key words: colonocyte; focal adhesion kinase; laparoscopy; matrix; physical forces; port site

Physical forces such as cyclic strain, shear stress, and pressure have been reported to affect several cell types [Buckley et al., 1988; Nollert et al., 1992; Almekinders et al., 1993; Bishop et al., 1993; Desrosiers et al., 1995; Leeves and McDonald, 1995; Patrick and McIntire, 1995; Stanford et al., 1995a, b; Carano and Siciliani, 1996; McDonald et al., 1996; Owens, 1996; Oluwole et al., 1997; Owan et al., 1997; Ballermann et al., 1998; Chien et al., 1998; Songu-Mize et al., 1998], whereas we [Basson et al., 1996a; Han et al., 1996, 1998a, b] have previously described the effects of cyclic strain at physiologically relevant levels on intestinal epithelial cell proliferation and differentiation in cultured cell lines. However, different physical forces would not necessarily be expected to have parallel effects [Oluwole et al., 1997]. The effects of increased pressure on human colonocytes are less well understood, although 40–120 mm Hg pressures have previously been reported to stimulate intestinal epithelial proliferation via a protein kinase C (PKC)-mediated pathway [Hirokawa et al., 1997] while increased biliary pressure engendered by bile duct ligation appears to promote biliary ductal epithelial proliferation [Slott et al., 1990].

Elevated pressures may occur in the colon in both physiologic colonic peristalsis and various clinical states. The high-amplitude propagating contractions that periodically move luminal contents from the ascending colon toward the sigmoid, for instance, generate luminal pressures in excess of 80 mm Hg for more than 10 s, while other nonpropagating contractions occur during seconds to minutes at lower amplitudes [O'Brien and Phillips, 1996; Camilleri and Ford, 1998]. The frequency, duration, and amplitude of colonic contractions appear to be increased by a low-fiber diet, even in randomized animal trials [Brodribb et al., 1979], and it is commonly believed that diverticular disease

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begins with increased intracolonic pressure sufficient to weaken the colonic wall over time. Low-fiber diets have also been reported to be associated with increased colon cancer [Howe et al., 1992], although some controversy now attends this observation [Fuchs et al., 1999; Potter, 1999], but this incidence may be more likely to be influenced in a substantial way by other better characterized consequences of low-fiber diets, such as changes in luminal short-chain fatty acid concentrations [Binder et al., 1994; Basson et al., 1998]. The colonic distension of a large-bowel obstruction or colonic pseudoobstruction is also likely to be accompanied by increased colonic intraluminal pressure. Finally, in addition to the luminal pressures engendered by such physiologic and pathophysiologic processes, intraperitoneal insufflation to 15 mm Hg above atmospheric pressure is now commonly used for the duration of laparoscopic intraabdominal procedures to distend the abdominal cavity and provide room in which to operate. Although some controversy attends the issue [Pearlstone et al., 1999], such intraperitoneal insufflation may be accompanied by increased implantation of shed tumor cells at the port sites through which laparoscopic instruments are inserted for cancer surgery, with subsequent grave clinical consequences [Cirocco et al., 1994; Martinez et al., 1995; Wexner and Cohen, 1995; Iuppa et al., 1996; Schaeff et al., 1998] , which animal data suggests may be obviated by performing laparoscopic surgery without gas insufflation [Bouvy et al., 1996; Mathew et al., 1997b; Watson et al., 1997; Bouvy et al., 1998] .

We therefore sought to investigate the effects of increasing atmospheric pressure on human colon cancer cell adhesion in a simple in vitro model, using a single sustained 30-min increase in pressure for all studies to simplify experimental design and data analysis. Although extrapolation from such in vitro models to any in vivo circumstance must be exceptionally cautious [Madri and Basson, 1992], we demonstrate here that increasing pressure by 10–15 mm Hg above atmospheric significantly stimulates colon cancer cell adhesion to a variety of matrix substrates. We characterized the effects of pressure on four different colon cancer cell lines and primary cells isolated from two different surgically resected human colon cancers to avoid concerns that the effects we would study were artifacts of a single established cell line. We then investigated concomitant tyrosine phosphorylation and focal adhesion kinase (FAK) autophosphorylation signal transduction events associated with pressure stimulation of colon cancer cell adhesion, by characterizing these signals and then by evaluating the effect of pharmacologic tyrosine kinase blockade on the stimulation of adhesion by pressure.

MATERIALS AND METHODS

Cells

All cells were maintained using standard cell culture techniques. Media used for cell culture during this study was 90% McCoy's 5a medium, 10% fetal bovine serum (FBS) for HT-29 cells; 45% RPMI, 45% Dulbecco's modified Eagle's medium (DMEM), 10% FBS for SW620 cells; 45% DMEM, 45% Ham's F-12, 10% FBS with 100 U/ml PenG, 100 mg/ml streptomycin, 15 mM HEPES, 2 mM glutamine, and 100 mg/ml gentamycin for SW1116 cells; and DMEM with 10% fetal calf serum, 10 mg/ml transferrin, 25 mmol/l glucose, 2 mmol/l glutamine, 1 mmol/l pyruvate, 15 mmol/l HEPES, 100 U/ml PenG, 100 mg/ml streptomycin for Caco-2 cells.

Isolation of Primary Human Colon Cancer Cells

The primary human colonocytes used for these studies were isolated from resected tumor tissue not required for clinical pathological study as previously described [Emenaker et al., 1997; Basson et al., 1998]. Briefly, the specimen was opened in the operating room or in the pathology suite by surgical and pathology attending staff, and a piece of grossly malignant tissue was excised from within the tumor in such a way as to not compromise pathological assessment of depth of invasion or surgical margins. (Parallel histological confirmation of malignancy was performed clinically and subsequently verified.) Tissue was transported to the laboratory in sterile oxygenated phosphate-buffered saline (PBS) and thoroughly washed in fresh PBS with 2 mM dithiothreitol (DTT) until visible debris has been removed. Tumor tissue was then finely minced with a sterile scalpel or razor blade in warm, oxygenated DMEM containing 100 U/ml penicillin/streptomycin until a uniform cell suspension was achieved [Gibson et al., 1989]. Clumps of malignant cells were pelleted at 500 *g* in DMEM,

washed until clean in warm oxygenated DMEM containing 100 U/ml penicillin/streptomycin, and then treated with 0.15% (wt/vol) type IV collagenase (Sigma Chemical Co., St. Louis, MO) dissolved in DMEM in a 37°C incubator at 8% CO_2 for approximately 1 h or until a single suspension was achieved. Cells were pelleted at 500 *g*, washed three times in fresh, warm, oxygenated DMEM to remove collagenase, and then used for studies as described below. Viability was more than 90% for each isolation by trypan blue exclusion. Preliminary studies also demonstrated these preparations to be more than 90% epithelial by cytokeratin staining (data not shown). The protocol for these procedures was approved by the human investigations committees of Yale University and the CT VA Health Care System.

Matrix Precoating

Purified matrix proteins were purchased from Sigma Chemical Co. and precoated at saturating densities onto bacteriologic plastic culture dishes or six-well plates using an enzyme-linked immunosorbent assay–based coating buffer at 4°C and then washed three times with PBS as previously described [Basson et al., 1992].

Pacification

To saturate and block nonspecific binding sites for cell adhesion for some experiments, bacteriologic plastic dishes were treated for 1 h at 37°C with a 10% solution of bovine serum albumin that had previously been boiled for 10 min. Although the cells in general do not bind to bacteriologic plastic dishes, this process prevents any nonspecific binding or interaction. This was done only for the experiments shown in Figure 9.

Pressure Regulation

Ambient pressure was controlled in these studies using a modification of an apparatus previously used to study the effects of pressure on other cell types [Sumpio et al., 1994; Watase et al., 1997; Vouyouka et al., 1998] Briefly, cells are placed in an airtight Lucite box with inlet and outlet valves, thumb screws and an O-ring for achieving an airtight seal, and a pressure gauge for pressure measurement. The box is routinely prewarmed to 37°C for at least 1 h before each study to prevent fluctuations in internal pressure caused by temperature shifts of the pressurizing gas, and increased pressure at the appropriate target is achieved within 1 min. The gas itself is passed through tubing warmed to 37°C in a water bath to similarly maintain temperature control. Control cells for each experiment were similarly handled but not exposed to increased pressure. Preliminary studies demonstrated our ability to maintain constant temperature and pressure conditions within ±2°C for temperature and ±1.5 mm Hg for pressure using this method (data not shown).

Adhesion Studies

For studies using cultured cells, subconfluent flasks of cells were lightly trypsinized (2–3 min), resuspended in cell culture medium containing 10% serum (to inactivate the trypsin), and triturated using a 3-ml sterile cell culture autopipette to achieve a single-cell suspension. Cells were routinely counted via hemocytometer and their viability was assessed by trypan blue exclusion. Preparations yielding less than 90% viability (uncommon) were discarded. Similar precautions were used for suspensions of primary colon cancer cells isolated from surgical specimens. Equal aliquots of the single-cell suspension (500 μl of cells at 100,000 cells/ml) were seeded into six-well plates of matrix-precoated bacteriologic plastic for adhesion studies and matrix-precoated P-100 dishes for signaling studies. The plates were immediately placed in the prewarmed pressure box, which was pressurized and replaced in a 37°C incubator. Control cells for each experiment were similarly handled but not exposed to increased ambient pressure. In serum-free experiments, the trypsin was neutralized using 1 mg/ml soy trypsin inhibitor instead of serum. After an appropriate time (generally 30 min), nonadherent cells were gently washed away using warm Dulbecco's Modified 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. (Preliminary studies, not shown, yielded similar results with retrypsinization and counting via an automated Coulter counter, but we chose this technique to visually identify the cells that were being counted and to eliminate concerns about artifacts of cell clumping or fragmentation biasing the automated count-

ing.) For signal transduction studies, cells were lysed as described below rather than counted.

Western Blotting

Cells were harvested in a hypotonic lysis buffer containing 10 mmol/l Tris-HCl, pH 8.0, 5 mmol/l KCl, 1 mmol/l DTT, 1.5 mmol/l $MgCl_2$, 1 mmol/L EGTA, 100 mmol/l sodium orthovanadate, 100 mg/ml PMSF, 1 mg/ml aprotinin, 2 mg/ml leupeptin, and homogenized by 10 strokes with a type B pestle. Sample proteins are resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Tyrosine phosphorylated proteins were detected by incubating with anti-phosphotyrosine monoclonal antibody and a 1:3,000 final dilution of a peroxidase-coupled secondary antibody (Amersham) before visualization by ECL.

Immunoprecipitation of Focal Adhesion Kinase

Four hundred microgram protein samples of cell lysates were precleared for 1 h at 4°C with rabbit anti-mouse IgG (Sigma) and protein A sepharose CL4B (1:1 slurry in PBS; Pharmacia) and centrifuged (5,000 rpm for 2 min in Heraeus Instruments Biofuge 13). Supernatants were incubated with monoclonal antibody to FAK (Transduction Laboratories) for 2 hours at 4°C. Rabbit anti-mouse IgG and protein A sepharose CL4B were then added and immunoprecipitations were incubated for another hour at 4°C. The samples were then centrifuged at 5,000 rpm for 2 min and rinsed three times with 0.5 ml immunoprecipitation buffer (with centrifugation between rinses) before resolution on 3.5% stacking/7.5% resolving SDS polyacrylamide gels. Gels were transferred overnight at 4°C, 0.1 A onto Hybond ECL nitrocellulose (Amersham) before immunodetection. For immunodetection of phosphotyrosine, blots were blocked with 5% bovine serum albumin in wash buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20), rinsed, and incubated with a phosphotyrosine antibody conjugated to horseradish peroxidase (RC20; Transduction Laboratories). After rewashing, blots are detected via ECL method and Hyperfilm ECL (Amersham). For immunodetection of FAK itself, blots immunodetected for phosphotyrosine were then stripped in 100 mM Tris HCl, 0.0033% SDS, 2 mM β-mercaptoethanol at 50°C. Stripped blots were rinsed twice with PBS, and the adequacy of stripping was verified by autoradiographic reexposure before reblocking in 5% albumin in wash buffer, and immunodetection for FAK was performed similarly to the Western immunodetection described above.

Pharmacologic Blockade

In some experiments, cells were treated with either 75 μg/ml genistein, 50 mM tyrphostin, or the appropriate vehicle control [0.02% dimethyl sulfoxide (DMSO)] during the adhesion study to inhibit intracellular tyrosine kinase activity.

Densitometry

All blot results were quantitated densitometrically using a Microtek IIXE ScanMaker and an IBM-based densitometric software package (SigmaScan/Image, Jandel Scientific). Multiple exposures were taken of each blot, and all densitometry was performed within linear range of the scanner and the film.

Statistical Analysis

All adhesion studies were done in paired format, in which cells from the same cell suspension were identically plated before allowing adhesion under control (ambient pressure) or increased pressure conditions. For each condition in each adhesion study (including variable pressure increases, variable temperature, or the presence or absence of a pharmacologic agent), data from at least three separate wells were normalized against control values from identically plated cells in at least three separate wells under control (ambient pressure) conditions as described above without any pharmacologic agent except, where appropriate and indicated, for a vehicle or IgG control. Preliminary experiments (not shown) showed no change in adhesion if cells are placed in the prewarmed and then sealed pressure box without increasing internal pressure, so it seems unlikely that any other physical characteristics of the box itself might cause the effects shown below. Unless specified, the results represent pooled data from at least four similar studies for each data point, all normalized against their respective controls. For SDS-PAGE studies, data from at least five similar blots were subjected to densitometric analysis as described above, normalized to respective control values, and pooled before statistical analysis.

Statistical analysis of all data was by unpaired *t*-test or analysis of variance. $P < 0.05$ was set a priori as the threshold for statistical significance.

RESULTS

Effect of Pressure on Solution pH and Po_2

Since the 15 mm Hg increase used for most of the studies shown below is relatively small in comparison to ambient atmospheric pressure (approximately 760 mm Hg), this increase in pressure exerts relatively slight effects on the Po_2 and pH of the solutions. For instance, in one study, cell culture medium maintained at 15 mm Hg increased pressure at 37°C in room air for 30 min and had a pH of 7.99 ± 0.01 and a Po_2 of 171.0 ± 10.8, whereas medium similarly maintained at ambient pressure had a pH of 7.96 ± 0.01 and a Po_2 of 181.8 ± 3.8 (n = 3, $P > 0.05$, n.s.). A balanced crystalloid solution such as D-PBS had a pH of 7.05 ± 0.002 and a Po_2 of 177.0 ± 4.1 under ambient pressure versus a pH of 7.07 ± 0.002 and a Po_2 of 179.2 ± 9.2 under increased pressure (n = 3, $P < 0.05$ for pH but not Po_2). Although we have previously reported that large extracellular pH changes alter Caco-2 colonocyte adhesion and motility [Perdikis et al., 1998], the small pH changes seen here, based on these previous studies, would not be expected to cause the substantial alterations in adhesion described below in response to increased pressure.

Pressure Stimulates Colon Cancer Cell Line Adhesion

Increasing ambient pressure for 30 min substantially and pressure-dependently stimulated human SW620 colon cancer adhesion to Matrigel (a basement-membrane-like extracellular matrix preparation purchased from Becton Dickinson). (Fig. 1). The threshold for this effect occurred at 10 mm Hg, and maximal effects occurred at 15 mm Hg. We found similar pressure-stimulated increases in adhesion to Matrigel for four different established human colon cancer cell lines, so the phenomenon is not limited to a single cell line (Fig. 2). Pressure stimulation of SW620 adhesion was also prevented by the metabolic inhibitor sodium azide and by performing the study at 2°C. (Fig. 3). This suggests that the response to pressure is energy dependent. Further studies (Fig. 4) also showed that pressure stimulates SW620 adhesion even when the study is performed in pure filtered nitrogen for 30 min. Pressure-stimulated adhesion also appeared independent of the matrix to which adhesion occurs for SW620 cells (Fig. 5A) and HT-29 cells (Fig. 5B).

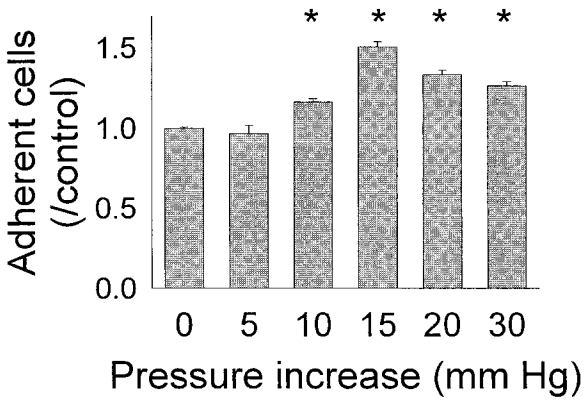

Fig. 1. Effect of varying ambient pressure on SW620 cell adhesion. Increasing ambient pressure for 30 min substantially and pressure-dependently stimulated human SW620 colon cancer adhesion to Matrigel (a basement-membrane-like extracellular matrix preparation). This effect achieved statistical significance at 10 mm Hg greater than atmospheric pressure and appeared to be maximal at 15 mm Hg. Experiments were performed in a paired fashion, comparing control (atmospheric) pressure to each increased pressure, and then normalized against control values before pooling and data analysis. (n ≥ 4 for each bar, $*P < 0.05$).

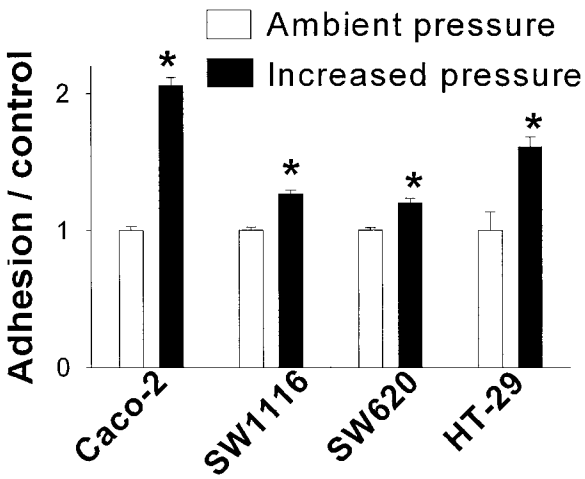

Fig. 2. Effects of 15 mm Hg increased pressure on adhesion of different colon cancer cell lines. Increasing ambient pressure by 15 mm Hg substantially stimulated the adhesion of human Caco-2, SW1116, SW620, and HT-29 cells to Matrigel. Experiments were performed in a paired fashion, comparing control (atmospheric) pressure (open bars) to increased pressure (shaded bars), and then normalized against control values before pooling and data analysis (n ≥ 4 for each bar, $*P < 0.05$).

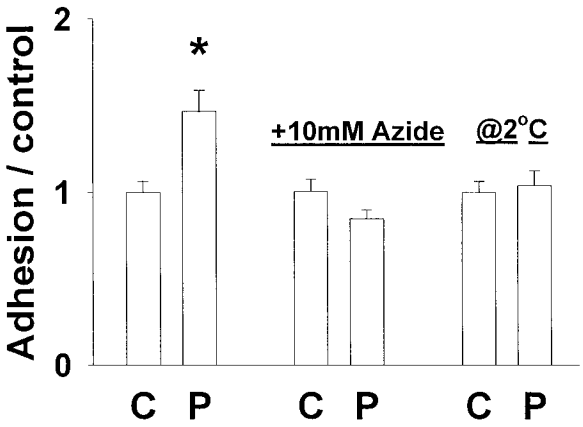

Fig. 3. Energy dependence of pressure-stimulated adhesion. In three separate series of paired experiments, human SW620 cells were permitted to adhere to Matrigel under control (C) conditions and conditions of 15 mm Hg increased pressure (P) in standard cell culture media at 37°C (first two bars), after the addition of 10 mM sodium azide (second two bars), and at 2°C (third pair of bars). Data were normalized against control adhesion in each study, and pooled for analysis. Although conditions of increased pressure stimulated SW620 cell adhesion to Matrigel (first pair of bars), both the metabolic inhibitor sodium azide and performing the experiment at 2°C prevented pressure-stimulated adhesion.

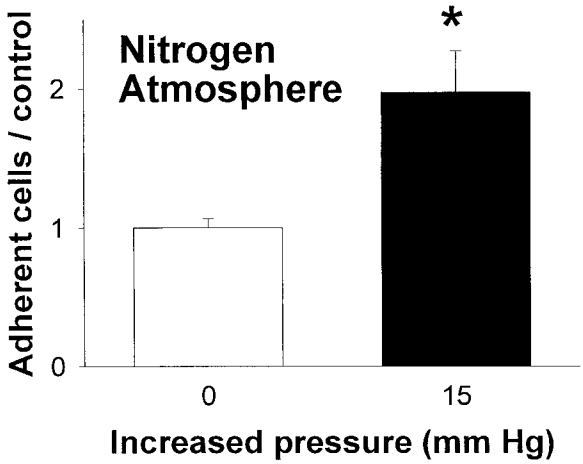

Fig. 4. Pressure stimulation of adhesion during gassing with nitrogen. SW620 cell adhesion to Matrigel was stimulated by conditions of 15 mm Hg increased pressure (shaded bar) as compared with adhesion under ambient pressure conditions (open bar) even when the gas used to generate this pressure was pure filtered nitrogen.

Effects of Pressure Occurred in Balanced Salt Solutions with Divalent Cations as Well as in Cell Culture Medium

Adhesion to Matrigel was substantially up-regulated in medium containing 10% serum

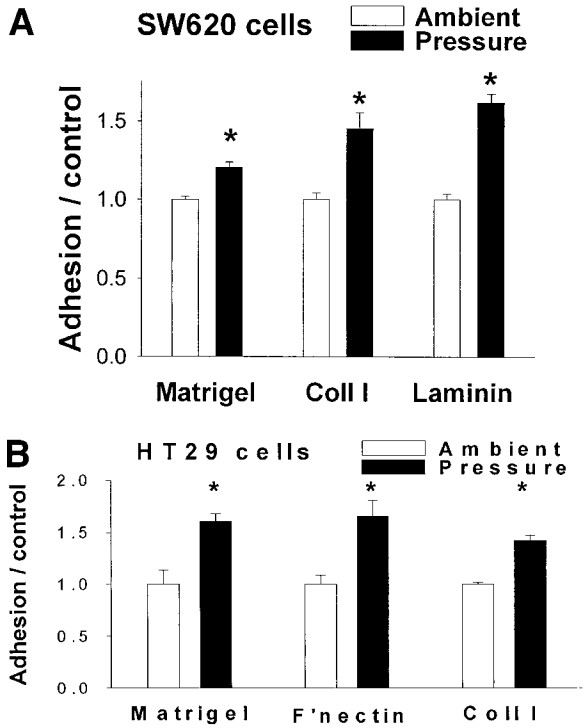

Fig. 5. Matrix independence of pressure-stimulated adhesion. Both for SW620 cells (**A**) and for HT-29 cells (**B**), 15 mm Hg increased pressure (shaded bars) appeared to stimulate adhesion in comparison to ambient pressures (open bars) to Matrigel, type I collagen (Coll I), laminin, and fibronectin (F'nectin).

compared with adhesion in D-PBS (phosphate buffered saline supplemented with 1 mM $CaCl_2$ and $MgCl_2$) (Fig. 6A, first two unshaded bars) However, adhesion *was* increased by pressure in *both* cell culture medium and D-PBS. (Fig. 6A) SW620 adhesion to Matrigel was only slightly further reduced when the divalent cations are removed from D-PBS to form PBS. However, no increase in SW620 adhesion was observed in response to increased ambient pressure in PBS without divalent cations. (Fig. 6A, last two bars). Similarly, adding 1 mM EDTA and 1 mM EGTA to the D-PBS to chelate the cations blocked pressure-stimulation of adhesion (Fig. 6B), further suggesting that divalent cations may be required for pressure-stimulated adhesion.

β1 Integrin Blockade Prevents Pressure-Stimulated Adhesion

The cation dependence of pressure-stimulated adhesion raised the question of whether this adhesion might be integrin mediated. We therefore compared the effects of adding 1

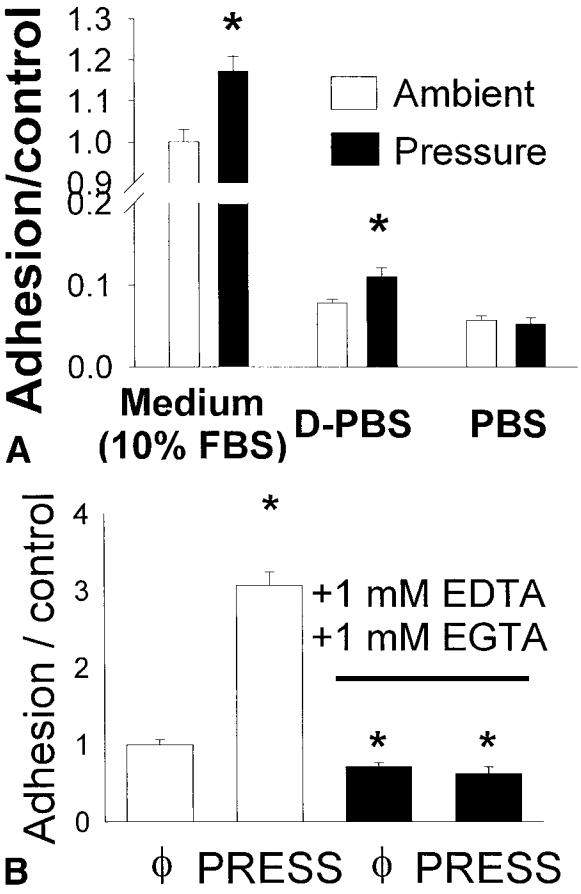

Fig. 6. Role of cations in pressure-stimulated adhesion. Adhesion to Matrigel was substantially upregulated in medium containing 10% serum compared with adhesion in D-PBS (phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and $MgCl_2$) (**A**, first two unshaded bars). However, adhesion *was* increased by pressure in *both* cell culture medium and D-PBS (A, first two shaded bars). SW620 adhesion to Matrigel under ambient pressure conditions was only slightly further reduced when the divalent cations are removed from D-PBS to form PBS. However, no increase in SW620 adhesion was observed in response to increased ambient pressure in PBS without divalent cations (A, last two bars). Similarly, adding 1 mM EDTA and 1 mM EGTA to the D-PBS to chelate the cations blocked pressure stimulation of adhesion (**B**), further suggesting that divalent cations may be required for pressure-stimulated adhesion. "0" represents ambient pressure and "PRESS" represents 15 mm Hg increased pressure.

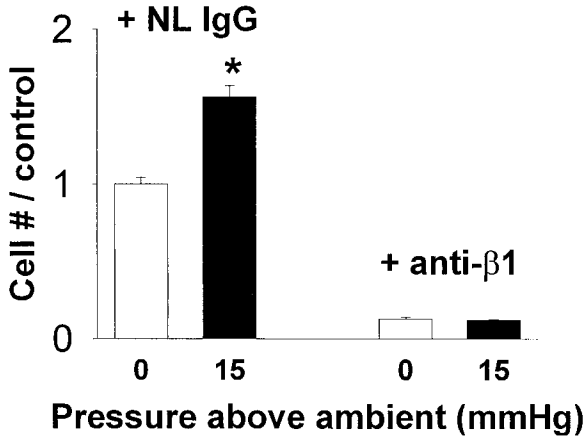

Fig. 7. Effects of a functional blocking antibody to the β1 integrin subunit on pressure-stimulated adhesion. In comparison to 1 mg/ml normal mouse IgG (+NL IgG), 1 mg/ml of a functional blocking antibody to the β1 integrin subunit (+anti-β1) substantially inhibited but did not prevent SW620 adhesion to type I collagen during 30 min under ambient pressure conditions (open bars). Increased adhesion was observed in response to 15 mm Hg increased pressure (shaded bars) in the presence of normal mouse IgG, but no increase was observed in the presence of antibody to the β1 integrin subunit.

mg/ml normal mouse IgG (as a nonspecific control) during SW620 adhesion to type I collagen under ambient and increased pressure conditions with the effects of adding 1 mg/ml of a functional blocking antibody to the β1 integrin subunit (clone P4C10, GIBCO). Antibody to the β1 integrin subunit inhibited 85–90% of SW620 adhesion to type I collagen during 30 min under ambient pressure conditions (suggesting that 10–15% is either mediated by a non-β1 integrin heterodimer or by nonintegrin mechanisms). However, no further increase was observed in adhesion in response to increased pressure in the presence of antibody to the β1 integrin subunit. (Fig. 7).

Tyrosine Kinase Inhibitors and Tyrosine Phosphoprotein Phosphorylation

The tyrosine kinase inhibitors genistein (75 μg/ml), tyrphostin (50 μM), and erbstatin analog (10 μM) prevented pressure stimulation of SW620 colon cancer cell adhesion to Matrigel as compared with a DMSO vehicle control. Indeed, in cells treated with tyrphostin, a statistically significant decrease in adhesion was observed with the application of 15 mm Hg increased pressure (Fig. 8a, $n \geq 6$, $P < 0.001$ for pressure stimulation with DMSO vehicle control and pressure inhibition with tyrphostin treatment). We have also observed blockade of pressure stimulation of adhesion by tyrosine kinase inhibition in HT-29 cells (not shown). This inhibition of pressure-stimulated adhesion by tyrosine kinase inhibitors suggests that pressure stimulation of colon cancer cell adhesion might require a tyrosine kinase signal. Indeed, Western blotting for tyrosine phosphoproteins (Fig. 8A) demonstrated substantial

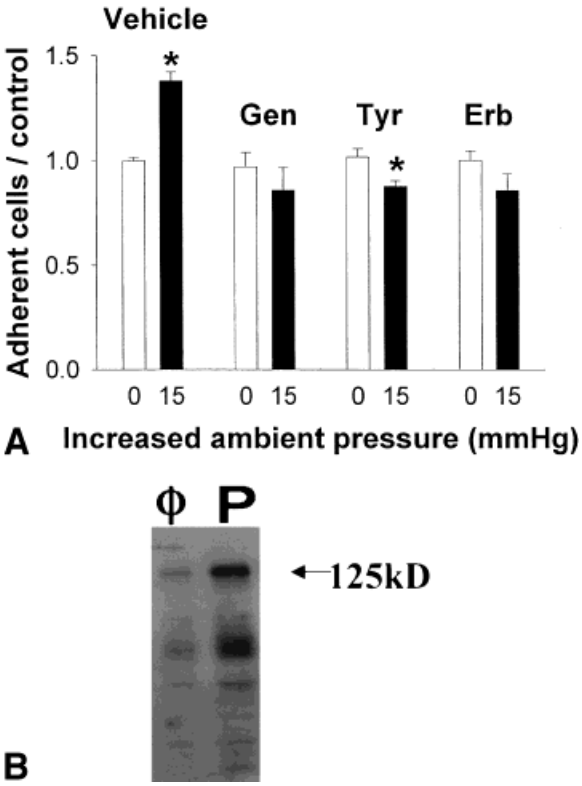

Fig. 8. Tyrosine kinase inhibition and tyrosine phosphorylation. The tyrosine kinase inhibitors genistein (Gen) (75 μg/ml), tyrphostin (Tyr) (50 μM), and erbstatin (Erb) analog (10 μM) prevented pressure stimulation of SW620 colon cancer cell adhesion to Matrigel as compared with a dimethyl sulfoxide vehicle control ($n \geq 6$, $^*P < 0.001$) (**A**). Western blotting for tyrosine phosphoproteins (**B**) demonstrated substantial upregulation of tyrosine phosphorylation in bands of several different molecular weights (from five similar studies), including a band of approximately 125 kD. "0" represents ambient pressure and "P" represents 15 mm Hg increased pressure.

upregulation of tyrosine phosphorylation in bands of several different molecular weights (from five similar studies), including a band of approximately 125 kD (Fig. 8B). Equivalency of loading across the lanes of the gel was routinely verified by Coomassie Blue staining (not shown).

Pressure Stimulates FAK Phosphorylation Independently of Adhesion

The 125-kD band, which increased tyrosine phosphorylation in response to pressure, was of particular interest because FAK is a 125 kD-tyrosine kinase associated with integrins in focal contacts. FAK autophosphorylates on activation and mediates integrin signaling and signal transduction by other physical forces in

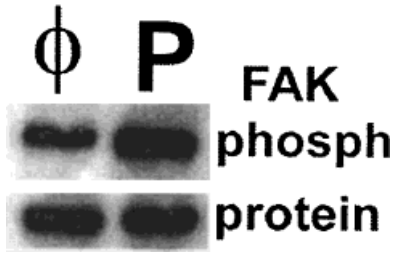

Fig. 9. Effects of pressure on focal adhesion kinase (FAK) tyrosine phosphorylation. Increasing pressure by 15 mm Hg stimulated FAK tyrosine phosphorylation (top) without altering FAK protein levels (bottom) in SW620 cells during exposure to pressure in pacificated bacteriologic plastic dishes to which adherence did not occur (one of four similar experiments). Densitometric analysis of all four studies demonstrated an increase in the ratio of phosphorylated FAK band intensity to total FAK band intensity in pressure-treated cells of 63.0 ± 11.0% as compared with the phosphorylated FAK/total FAK ratio in cells maintained under ambient atmospheric conditions. ($n = 4$, $P < 0.007$). "0" represents ambient pressure and "P" represents 15 mm Hg increased pressure.

other cell types. Thus, we asked whether increased pressure activates FAK in SW620 cells. Cell suspensions were subjected to ambient or 15 mm Hg increased pressure before lysis, BCA protein assay, immunoprecipitation for FAK, SDS-PAGE, and Western blotting with antibody to tyrosine phosphoproteins to visualize only the tyrosine phosphorylated (and therefore active) FAK. Initial experiments (not shown) appeared to suggest that pressure was associated with increased FAK phosphorylation. However, these experiments were unable to distinguish between FAK phosphorylation related to pressure itself and FAK phosphorylation as a consequence of increased (pressure-induced) adhesion. We therefore repeated these studies in bacteriologic plastic dishes pretreated with heat-inactivated bovine serum albumin to ablate nonspecific binding and prevent cell adhesion. Indeed, cell adhesion did not occur during these subsequent studies (data not shown). In this setting, increasing pressure by 15 mm Hg stimulated FAK tyrosine phosphorylation without altering FAK protein levels (Fig. 9). Thus, in suspended cells that were not presented with any substrate for adhesion, the ratio of FAK phosphorylation to FAK protein increased by 63.0 ± 11.0% after 30 min at a pressure 15 mm Hg above atmospheric pressure ($n = 4$, $P < 0.007$). Because FAK autophosphorylates on activation, this ratio may serve as an indicator of

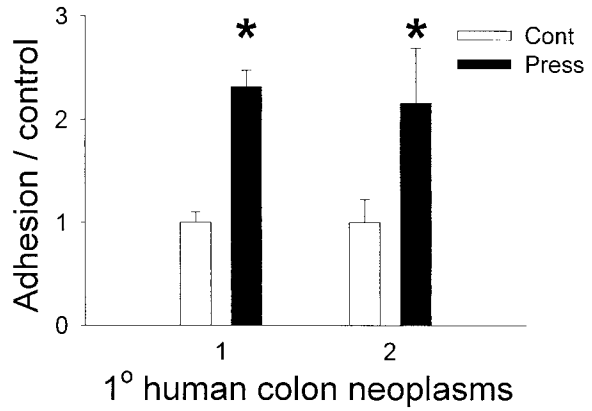

Fig. 10. Effects of pressure on the adhesion of primary human colon cancer cells. A 15 mm Hg increase in pressure promoted adhesion to type I collagen in colon cancer cells freshly isolated from two different primary human colon cancers by mincing and collagenase digestion (although with different magnitudes of effect) (n = 3, $*P <$ for each). "Cont" represents control adhesion at ambient pressure and "Press" represents adhesion at 15 mm Hg increased pressure.

FAK activity in human colonocyte cell lines [Liu et al., 1998, 1999].

Pressure-Stimulated Adhesion Occurs in Primary Human Colon Cancer Cells as Well as in Established Cell Lines

Although we had replicated pressure-stimulated adhesion in four different cell lines, established cell lines could differ in their adhesion under cell culture conditions from colon cancer cells from an actual in vivo tumor. To meet this objection, we isolated colon cancer cells from surgically resected human colon cancers by mincing and collagenase digestion. These cells were 90–95% viable by trypan blue exclusion and 90–95% epithelial by cytokeratin staining (not shown). A 15 mm Hg increase in pressure promoted adhesion to type I collagen in colon cancer cells from two different primary human colon cancers (Fig. 10, n = 3, $P < 0.05$ for each). Interestingly, we also evaluated the effects of a 15 mm Hg increase in pressure for 30 min on primary bovine aortic smooth-muscle cell adhesion to type I collagen. These cells, which differed in cell type and species from the other cells we studied, did not cause stimulation of cell adhesion by pressure (data not shown).

DISCUSSION

Like intestinal epithelial cell–matrix interactions during growth [Carroll et al., 1988; Benya et al., 1993; Basson et al., 1996b; Wolpert et al., 1996; Simon-Assmann et al., 1998] and motility [Kedinger, 1994; Santos et al., 1997; Liu et al., 1998; Pai et al., 1998; Dieckgraefe and Weems, 1999], repetitive strain initiates intracellular signals that alter intestinal epithelial phenotype and proliferation [Basson et al., 1996a; Han et al., 1996, 1998a, b]. However, different physical forces may have different effects [Oluwole et al., 1997], and pressure on nonadherent cells may differ substantially from strain on adherent cells. Indeed, 30 min of repetitive 10% deformation at 10 cycles per minute does not stimulate Caco-2 adhesion to collagen I (unpublished observations), even though strain at these parameters alters other aspects of Caco-2 biology [Han et al., 1998a, b] Substantially higher and longer pressure increases stimulate intestinal epithelial proliferation and DNA synthesis in culture, but the mechanisms responsible for this effect are unclear [Hirokawa et al., 1997]. These data suggest that at least one physical force, pressure, also alters integrin-mediated cell–matrix interactions. This is consistent with previous reports in other cell types that physical forces can modulate cytoskeletally associated signals [Oluwole et al., 1997; Chien et al., 1998; Hughes and Pfaff, 1998], which could conceivably alter adhesiveness. However, neither stimulation of epithelial cell adhesion by pressure nor effects of pressure on adhesion of other cell types at these relatively low pressures have previously been described, whereas bovine aortic smooth-muscle cells do not exhibit increased adhesiveness in response to 30 min of 15 mm Hg pressure in this fashion. Although a sustained pressure increase during 30 min differs substantially from the irregularly fluctuating pressures that occur during normal colonic function or surgical manipulation, the effects of such patterns are likely to be difficult to reproduce and to analyze in culture. Results derived in this simpler pressure model may therefore offer a paradigm for pressure modulation of colonocyte biology. In addition, the application of a sustained 15 mm Hg pressure increase to malignant colonocytes resembles the intraperitoneal insufflation to 15 mm Hg pressure over baseline atmospheric pressures currently used for most laparoscopic cancer surgery. The effects and mechanisms described here could pertain to laparoscopic port site recurrence, as well as to effects of pro-

longed aggressive surgical tumor manipulation during difficult open or laparoscopic surgical procedures.

We observed that increasing ambient pressure to a relatively small degree (by approximately 15 mm Hg) significantly increased colon cancer cell adhesion to matrix proteins. The preliminary demonstration of a similar phenomenon in primary cells from surgically resected human colon cancers suggests that this phenomenon is independent of cell line adaptations to continuous culture.

Alternate explanations might include Po_2 and pH changes, gas flow turbulence, endotoxin injection into the medium from external gases, and changes in the size of gas microbubbles in the cell suspension to which cells might adhere, thus inducing strain as well as pressure. Gassing less than 15 mm Hg pressure with room air did not substantially change Po_2. That pressure stimulated adhesion even during gassing with pure nitrogen would also seem inconsistent with a Po_2 explanation. The extracellular pH changes were relatively small compared to those that substantially alter human colon cancer cell adhesion [Perdikis et al., 1998]. Gas filters to remove suspended endotoxin did not alter the effect (not shown). We cannot rule out the possibility that this pressure effect might involve changes in microbubble size in the cell suspension, because degassed solutions will acquire new gas microbubbles as cells are resuspended. Nevertheless, these observations suggest that increased extracellular pressure, either directly or via another physical phenomenon such as microbubble size changes, promotes colon cancer cell adhesion.

The once novel concept that physical forces could affect biology without extracellular soluble messengers has now been validated in cells as diverse as endothelial [Nollert et al., 1992; Patrick and McIntire, 1995; Oluwole et al., 1997; Ballermann et al., 1998; Chien et al., 1998] and smooth muscle cells [Owens, 1996; Songu-Mize et al., 1998], fibroblasts [Almekinders et al., 1993; Bishop et al., 1993; Desrosiers et al., 1995; Leeves and McDonald, 1995; Carano and Siciliani, 1996], osteoblasts [Buckley et al., 1988; Stanford et al., 1995a, b; McDonald et al., 1996; Owan et al., 1997], and intestinal epithelial cells [Basson et al., 1996a; Han et al., 1996; Hirokawa et al., 1997; Han et al., 1998a, b]. Physiologic pressure increases may influence other cell types. For instance, 60 mm Hg pressure increases modulate macrophage cytokine secretion [Sawyer et al., 1998], and 105 mm Hg pressure increases stimulate vascular smooth-muscle cell proliferation [Watase et al., 1997] and endothelial cells [Sumpio et al., 1994]. Directly applied pressure stimulates monocyte motility [Singhal et al., 1997], whereas 40 mm Hg hydrostatic pressure upregulates endothelial cell adhesion and clusters focal adhesion complex proteins during 12 h [Thoumine et al., 1995]. By contrast, a 20-minute 30 mm Hg pressure pulse rearranges the osteosarcoma cell cytoskeleton and increases adhesion [Haskin et al., 1993].

Interestingly, the far more extreme alterations in ambient pressure (>1,500 mm Hg) associated with undersea diving cause platelet aggregation to the point of relative thrombocytopenia [Moon et al., 1992], whereas in vitro studies of neutrophils at very high pressures (>2,200 mm Hg) suggest that hyperbaric oxygen inhibits human neutrophil β2-integrin-mediated adhesion by impairing cyclic guanosine 3′,5′-monophosphate synthesis [Thom et al., 1997]. Although pressure inhibited rather than stimulated neutrophil adhesion, both the cell type and pressure magnitude differ substantially from our experiments, whereas clinical diving studies have typically used not only far greater pressures but also a complex schedule for inducing and recovering from increased pressure to avoid deleterious consequences of rapid resurfacing. Thus, the diving model is not strictly analogous. Intravascular turbulence and microbubbles may also affect intravascular biology in this setting [Ikeda et al., 1989; Hills and James, 1991; James, 1993].

Published data in epithelial cells is consistent with epithelial sensitivity to pressure. Pressure at substantially longer duration and intensity (40–120 mm Hg) has been reported to stimulate intestinal epithelial proliferation via a PKC-mediated pathway [Hirokawa et al., 1997], whereas increased biliary pressure by bile duct ligation promotes biliary ductal epithelial proliferation [Slott et al., 1990]. Although different physical forces are likely to exert different effects [Oluwole et al., 1997], repetitive strain modulates Caco-2 colonocyte proliferation and differentiation in an amplitude-dependent manner via tyrosine kinase and PKC signals, and blockade of either the tyrosine kinase or PKC signals prevents these

effects [Basson et al., 1996a; Han et al., 1996, 1998a, b].

If pressure stimulates colon cancer cell adhesion, how might this effect occur? The observation that pressure-stimulated adhesion requires both energy (blocked by azide) and extracellular cations (blocked by chelating agents) suggested the further hypothesis that pressure-stimulated adhesion was integrin dependent, although extracellular chelating agents might also alter intracellular cation concentrations. Studies in primary cells and two different cell lines (SW620 and HT-29) did not show matrix dependence, so it seems unlikely that pressure acts on a matrix-specific integrin heterodimer. However, global blockade of all integrin heterodimers containing the β1 integrin subunit prevented pressure-stimulated adhesion. Although these studies might be criticized because adhesion itself was dramatically inhibited by this antibody, the D-PBS/PBS studies (Fig. 6) suggest we could detect pressure-stimulated adhesion at similarly low adhesion levels. The involvement of specific β1 integrin heterodimers awaits elucidation. However, our data would be consistent with the hypothesis that pressure-stimulated adhesion is mediated by β1 integrin binding to matrix and can be blocked by a functional antibody.

Both integrin affinity (a change in integrin conformation that increases ligand affinity) and integrin avidity (changes in integrin distribution and clustering) can alter adhesiveness [Hughes and Pfaff, 1998]. Which occurs here is not yet clear, although we could not show that pressure alters surface pools of the β1 integrin subunit (Sanders and Basson, unpublished data). Regulation of affinity and avidity are incompletely understood, but may involve tyrosine phosphorylation [Shattil et al., 1992], tyrosine-phosphorylated adapter proteins such as paxillin [Turner, 1998], small GTP-binding proteins [Barry et al., 1997; Hughes et al., 1997; Clark et al., 1998; Keely et al., 1998], and PI 3-kinase [Shimizu et al., 1995; Hughes and Pfaff, 1998]. Although adhesion activates FAK in many cells, direct evidence that FAK modulates adhesion in an "inside-outside" fashion is still lacking [Cary and Guan, 1999].

Indeed, the effects of strain and other forces in other cell types have been postulated to be regulated by rapid strain-stimulated chemical and mechanical intracellular and extracellular signals, including those mediated by FAK [Guan, 1997; Shy and Chien, 1997; Takada et al., 1997], PKC [Mills et al., 1997; Han et al., 1998a; Songu-Mize et al., 1998], MAPK [Liang et al., 1997; Reusch et al., 1997; Ingram et al., 1999], G proteins [Dash et al., 1995; Renshaw et al., 1996; Barry et al., 1997; Hughes et al., 1997; Chien et al., 1998; Clark et al., 1998], soluble second messengers [Oluwole et al., 1997; Chien et al., 1998], direct remodeling of cytoskeleton or patterns of cell–matrix interactions [Ingber, 1997], and autocrine/juxtacrine extracellular growth factor release [Miyamoto et al., 1996; Rozengurt and Rodriguez-Fernandez, 1997; Rusnati et al., 1997; Chien et al., 1998; Sumpio et al., 1998]. The concept of tensegrity [Ingber, 1997] has formed the basis for a paradigm of mechanical signal transduction in response to such physical forces as shear stress, osmotic swelling, repetitive strain, and pressure [Stamenovic et al., 1996; Oluwole et al., 1997; Chien et al., 1998], in which mechanical molecular linkages between integrins and focal adhesion proteins, cytoskeletal filaments, and nuclear scaffolding may permit mechanical signal transduction parallel to "conventional" soluble signal cascades [Maniotis et al., 1997].

The mediation of pressure-stimulated adhesion in colon cancer cells is likely to be equally complex. Pressure stimulation of colon cancer cell adhesion was accompanied by intracellular tyrosine phosphorylation and blocked by pharmacologic tyrosine kinase inhibitors. Such inhibitors may have nonspecific effects, but the fact that three tyrosine kinase inhibitors with different mechanisms of action and specificities [Levitzki, 1992; Di Salvo et al., 1994; Constantinou and Huberman, 1995; Gazit et al., 1996] exert the same blocking effect supports the interpretation that tyrosine kinase inhibition rather than nonspecific effects is involved in this blockade. Thus, intracellular tyrosine kinases may be important for pressure-stimulated adhesion. Pressure activates FAK independently of adhesion, and this focal-adhesion-associated tyrosine kinase has been implicated in inside-out and physical force signal transduction in other cell types [Guan, 1997; Hanks and Polte, 1997; Li et al., 1997] as well as in mediating intestinal epithelial cell-matrix interactions [Liu et al., 1998; Sanders et al., 1997]. Proof that FAK regulates pressure-stimulated adhesion and identification of other

tyrosine phosphoproteins phosphorylated in response to pressure await further study, but these data are consistent with that hypothesis.

The observation that the pressure parameters in this study are consistent with laparoscopic colon cancer surgery may also be of interest. Such surgical procedures may involve operating times of 1–4 hours and generally use pressures of 15 mm Hg above atmospheric to expand the abdominal cavity sufficiently to maneuver surgical instruments placed through trochars into the abdomen. Some have suggested that colon cancer cells may tend to implant at the sites through which these trochars are passed into the abdominal cavity after such procedures [Cirocco et al., 1994; Jones et al., 1995; Martinez et al., 1995; Wexner and Cohen, 1995; Iuppa et al., 1996; Koster et al., 1996; Mathew et al., 1997a, b; Wu et al., 1997; Lee et al., 1998; Schaeff et al., 1998]. That animal models suggest this may be decreased by performing laparoscopic surgery without gas insufflation [Bouvy et al., 1996, 1998; Mathew et al., 1997b; Watson et al., 1997] has been attributed to decreased tumor cell aerosolization but would also be consistent with the hypothesis that increased pressure stimulates colon cancer cell adhesion to the matrix proteins of the port site. We also observed potentiation of both basal and pressure-stimulated adhesion by serum-containing culture media compared with a simple balanced electrolyte solution, D-PBS. Whether this adhesion-promoting cofactor is serum or some component of serum or indeed some other component of the cell culture medium awaits further studies. However, serum collecting in port sites during surgery might potentiate pressure-stimulated adhesion to port site matrix proteins, as opposed to matrix proteins elsewhere in the peritoneal cavity.

There are tremendous differences between the simple model described here and the irregular pressure rhythms induced by colonic peristalsis or the complex in vivo phenomena required to generate a clinically significant tumor implant after surgery. However, these data do suggest that pressure changes of magnitudes consistent with those observed physiologically may substantially alter "inside-out" signal transduction in colonic epithelial cells and thus stimulate colonocyte adhesiveness to matrix proteins.

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