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Hypertonic Saline Attenuates Colonic Tumor Cell Metastatic Potential by Activating Transmembrane Sodium Conductance

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Abstract. Hypertonic saline (HTS) suppresses tumor cell-endothelial interactions by reducing integrin expression. This translates into reduced adhesion, migration and metastatic potential. This study determined the relative contributions of hyperosmolarity and sodium-specific hypertonicity on the inhibitory effects of HTS, the intracellular pH and sodium responses to HTS and the role of cytoskeletal remodeling in these changes. Human colonic tumor cells (LS174T) were exposed to lipopolysaccharide under isotonic, hypertonic, sodium-free (*N*-methyl-D-glucamine), hyperosmolar (mannitol or urea), disrupted cytoskeletal (10 μg/ml cytochalasin D) conditions or in the presence of 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). β_1 integrin expression was measured flow-cytometrically. Intracellular sodium and pH were measured with confocal laser microscopic imaging. Statistical analysis was performed with analysis of variance, and $P < 0.05$ was considered significant. Data are represented as mean ± SEM. Hypertonic exposure attenuated integrin expression (62.03 ± 4.7% of control, $P < 0.04$). No discernible effect was observed with sodium-free or hyperosmolar solutions. HTS evoked a cellular alkalinization (by a mean 0.2 pH units) and an increase in cytosolic sodium concentration (by a mean 12.4 mM, $P < 0.001$) via upregulation of sodium-hydrogen exchange. Disassembly of actin microfilaments by cytochalasin D and antiporter inhibition with EIPA abrogated the effect of hypertonicity on integrin expression and intracellular sodium and pH ($P < 0.05$). HTS downregulates adhesion molecule expression via a hypertonic, sodium-specific, cytoskeletally mediated mechanism that involves activation of sodium-hydrogen exchange with associated changes in intracellular pH and sodium concentrations.

Key words: Hypertonic saline — Colonic tumor cell — Metastasis — Transmembrane sodium conductance

Introduction

Refined fluid and electrolyte balance is a pivotal component of modern surgical care, where hypertonic fluids exert beneficial cardiovascular, renal, pulmonary, gastrointestinal and immunomodulatory effects (Deb et al., 1999; Andrews et al., 2001; Umenishi, Narikiyo & Schrier, 2004; Shao et al., 2005; Toung et al., 2005). The propensity for hypertonic fluids to alter host responses to sepsis and inflammation has rekindled interest in them as resuscitative agents (Shields et al., 2003a, 2003b; Powers et al., 2005). In particular, HTS decreases neutrophil potency, an effect attributed to perturbation of adhesion molecule expression (Ciesla et al., 2001; Shields et al., 2003a, 2003b). However, it remains unknown whether the effects of hypertonic saline (HTS) are due to hyperosmolarity-induced cell shrinkage or electrochemical (ionic tonicity) influences on cellular processes. Rizoli, Rotstein and Kapus (1999) suggested that cell volume changes are central by demonstrating shedding of L-selectin from neutrophils under hypertonic conditions derived from both sodium chloride and sucrose. However, it is challenging to separate one from the other; Thiel et al., (2001) postulated that reduced cell volume is not sufficient to account for the inhibitory effect on leukocyte integrin upregulation.

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Cell volume is regulated, in part, by the maintenance of a transcellular sodium ion (Na^+) gradient via a balance between cellular accumulation of Na^+ by Na^+/H^+ exchange and $Na^+K^+2Cl^-$ cotransport and Na^+ extrusion by Na^+/K^+-ATPase pumps (Lang et al., 2003). In cells exposed to fluctuations in external osmolarity and tonicity (e.g., leukocytes and epithelial cells), the relative activities of such ion transport processes are linked closely to cytoskeletal remodeling (Cantiello et al., 1991). Increased extracellular tonicity alters cytoskeletal dynamics and signal transduction pathways such that phenotypic expression of surface molecules and membrane transport proteins is diminished (Krump, Nikitas & Grinstein, 1997; Kapus et al., 1999; Miyakawa et al., 1999).

Surgery induces cytoskeleton-adherens junction interactions, which contribute to inflammatory cascades by site-specific gene expression and to wound healing by maintenance of tissue architecture and cell polarity (Tuckwell, Weston & Humphries, 1993). While these processes govern normal cellular restitution, they also determine tumor invasiveness and metastatic potential such that aberrant adhesion-mediated signaling is a trait of malignant transformation. Inflammatory responses to surgery upregulate both adhesion molecules on colorectal cancer cells and their corresponding endothelial ligands (Andrews et al., 2001). Both laparotomy and air laparoscopy are accompanied by endotoxin (lipopolysaccharide, LPS) contamination of the peritoneal cavity and systemic endotoxemia via bacterial translocation across the gut (Watson et al., 1995). Furthermore, this translocation amplifies systemic inflammatory responses that provide a permissive environment for remote endothelial adhesion of shed tumor cells (Watson et al., 1995; Pidgeon et al., 1999; Andrews et al., 2001). Two of these studies showed a crucial role for LPS contamination following surgery in tumor growth and metastases (Watson et al., 1995; Pidgeon et al., 1999). Previous studies undertaken in our laboratory have demonstrated an augmentation of tumor cell β_1 integrin expression following exposure to LPS, which correlates with enhanced binding avidity (Andrews et al., 2001; Shields et al., 2004). This may initiate intravascular growth patterns with diverse angiogenic requirements - intravasation, extravasation and replacement – hallmarks of colorectal metastases (Vermeulen et al., 2001). HTS (7.5% NaCl) was previously found to attenuate tumor cell adhesion to the endothelium by inhibiting adhesion molecule and laminin expression (Shields et al., 2004). This study was designed to determine the effects of the specific constituents of HTS on cellular processes that govern tumor cell adhesion molecule expression.

Materials and Methods

Solutions

Balanced salt solutions contained 2 mM KCl, 1.5 mM K_2HPO_4, 1 mM $MgSO_4$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM $CaCl_2$ and 10 mM glucose, brought to pH 7.4 by the addition of either NaOH or HCl, depending on the desired sodium concentration. Isotonic saline solutions contained, in addition, 141 mM NaCl, while HTS solutions contained either 160, 180 or 200 mM NaCl. Sodium-free solutions contained either 141 mM *N*-methyl-D-glucamine (NMDG) base (isotonic) or 180 or 200 mM NMDG (hypertonic). The osmolarity of all solutions was measured with an osmometer (Micro-osmometer 3M0; Advanced Instruments, Needham Heights, MA). Equiosmolar solutions of mannitol and urea (280–400 mOsM/l) were employed to determine the contribution of osmolarity to the cellular response.

Cell Culture

The human colon cancer cell line LS174T (American Type Culture Collection, Rockville, MD) was used in all experiments. This is a P53 wild-type, E-cadherin mutant, colon epithelial cell line which stays unpolarized under cell culture conditions, possibly due to an inability to form junctional complexes (Baas et al., 2004). It was maintained in Eagle's minimum essential medium supplemented with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids and 1.0 mM sodium pyruvate (90%) plus fetal bovine serum (10%). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2, and medium was renewed three times weekly.

Determination of Adhesion Molecule Expression

β_1 integrin expression was determined by flow-cytometric analysis of immunofluorescence. Tumor cells were cultured in sodium-containing or sodium-free solutions at increments of tonicity or equiosmolar solutions of mannitol or urea before addition to the culture medium of LPS at a concentration of 100 ng/ml, an optimal dose for enhancement of integrin expression, simulating postoperative cell activation status, according to previous dose-response experiments (Andrews et al., 2001; Wang et al., 2003), for 24 h at 37°C in humidified 5% CO_2 conditions. The potential reversibility of the effects of HTS on tumor cell β_1 integrin expression was ascertained by resuspending tumor cells in an isotonic environment at 10 h, followed by further exposure to a hypertonic environment at 15 h. The contribution of a possible target of hypertonic shock, the cytoskeleton, was assessed by preincubation for 60 min with the helminthosporium-derived alkaloid cytochalasin D (1 mg/ml), a potent inhibitor of actin polymerization, before estimation of β_1 integrin expression. Functional coupling between the cytoskeleton and channel proteins impacts upon membrane ion transport via amiloride-sensitive sodium channels. The importance of sodium influx via the antiporter was determined by introduction of the Na^+/H^+ exchange (NHE) blocker 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA, 100 μM), an analogue with a higher specificity for NHE than amiloride. Every 2 h, 100 β_1 aliquots of the cell suspensions (1×10^6 cells/ml) were incubated with 15 μl (10 μg/ml) of either phycoerythrin (PE)-conjugated mouse anti-human monoclonal antibody specific for the β_1 integrin chain CD29 (Pharmingen, San Diego, CA) or a PE-conjugated IgG1 antibody for control samples, at 4°C for 30 min, and analyzed on a FACScan flow cytometer (Becton Dickinson Biosciences, Palo Alta, CA), for detecting the log of the mean channel fluorescence intensity with an acquisition

of FL1. A minimum of 10,000 events were collected and analyzed on CellQuest software (Becton Dickinson Biosciences).

Measurement of Cytosolic Sodium Ion Concentration ($[NA^+]$) and Intracellular PH

$[Na^+]$ measurements were acquired using confocal laser microscopic imaging of colonic cells loaded with fluorescent probes as described previously (Winter et al., 1999). In brief, cells were loaded with 5 μmol/l Na^+ green (Molecular Probes, Eugene, OR). Dye molecules were excited with an argon laser at a wavelength of 488 nm and emission was recorded in the wavelength range of 515–565 nm. Cells were exposed to HTS by perfusion. Images of the emitted fluorescence (reflecting Na^+ concentration, increased intensity equates to elevated Na^+ concentrations) were captured at 15-s intervals and digitized. Inhibition of membrane sodium ion transport was achieved with EIPA (100 μM). Results are expressed as mean ± standard error of the mean (SEM), with $n = 5$ cell recordings per experiment (experiments performed with replicates, $n \geq 3$). For pH measurements, 1×10^6 tumor cells were incubated with the pH-sensitive fluorophore 2-carboxyethyl-5(6)-carboxyfluorescein (BCECF, 10 μM) for 15 min at 37°C. The dye was excited with the same argon laser at 488 nm, with emission of 515–565 nm. Following baseline readings, tumor cells were cultured in saline buffer before incubation in isotonic (140 mmol/l Na^+) or hypertonic (180 mmol/l Na^+) medium. Increased intensity was indicative of alkaline pH. In two further experiments, EIPA (100 μM) was employed to inhibit NHE and cytochalasin D (10 μg/ml) was used to prevent actin polymerization before measurement of intracellular pH (experiment performed with replicates, $n \geq 3$). The system was calibrated *in vitro* using cation exchange ionophores for Na^+ (gramicidin D) or pH (nigericin) as described previously (Winter et al., 1999).

Data Analysis

All data are presented as mean values with SEM. Statistical analysis was performed using a two-way factorial analysis of variance, with time and treatment as independent variables. $P < 0.05$ was considered significant.

Results

To evaluate whether indiscriminate increases in extracellular tonic or osmotic strength inhibit adhesion signaling, quantitative analysis of integrin expression was performed on tumor cells exposed to media of varying osmolarity using either ionic (NaCl) or nonionic (mannitol or urea) molecules. Specifically, LPS-induced β_1 integrin expression was assessed at normal salinity (141 mM), with increments of ionic hypertonicity (160, 180 and 200 mM NaCl) or with mannitol or urea at equiosmolar (280 mOSM/l) and hyperosmolar (400 mOSM/l) concentrations.

Effect of Hypertonicity on Tumor cell β_1 Integrin Expression

HTS solutions (180 and 200 mM NaCl) caused a significant time- and concentration- dependent decline in integrin expression on the tumor cell surface, in keeping with our previous findings (Shields et al., 2004). The reversibility of HTS-mediated adhesion molecule inhibition was evaluated by resuspending tumor cells in fresh isotonic buffer 6 h after initial osmotic shock (Fig. 1). The restoration of normotonicity occasioned a gradual recovery in integrin expression, demonstrating the transient nature of the cellular effects of hypertonicity. Further exposure to sodium chloride-dependent hypertonic medium resulted in another precipitous decline in β_1 integrin expression (Fig. 1).

Effect of Hyperosmolarity on Tumor Cell β_1 Integrin Expression

In contrast to the inhibitory effects of HTS on integrin expression, neither mannitol- nor urea-containing medium induced significant diminution of adhesion molecule expression (Fig. 2). Mannitol exerts hyperosmolar effects, but the large molecular weight negates transmembrane traffic (unlike HTS) such that there are no direct intracellular effects. Rather, there are alterations in membrane water transport that cause secondary cell effects due to osmosis. Meanwhile, although urea equilibrates across the cell membrane, it does not evoke cell shrinkage (unlike HTS) (Rizoli et al., 1999). Therefore, these experiments suggest that HTS effects are due to transmembrane saline transport rather than simply the exertion of extra- or intracellular hyperosmolar influences.

Effect of Sodium-Free Hypertonicity on Tumor Cell β_1 Integrin Expression

In addition to causing a reduction in adhesion molecule expression, HTS may affect alterations in extra- and intracellular Na^+ concentration. To discern whether Na^+ transport across the membrane possessed significance, Na^+ was substituted with a nontransportable base (NMDG chloride) in the balanced salt solution. Tumor cell β_1 integrin expression was assessed at increments of NMDG chloride using similar osmolarity changes as the previous experiments. Incubation in this Na^+-free hypertonic medium did not result in significant integrin decay (Fig. 3). Thus, the failure of hyperosmolar and Na^+-free hypertonic solutions to engender a decline in adhesion molecule expression suggests a specific role for Na^+ transport in HTS effects.

Effect of Actin Depolymerization on Cellular Response to Hypertonicity

It was conceivable that the inhibitory effects of HTS on tumor cell integrin expression stemmed from disruption of the actin polymerization/depolymerization cycle. To further investigate this aspect, cytoskeletal

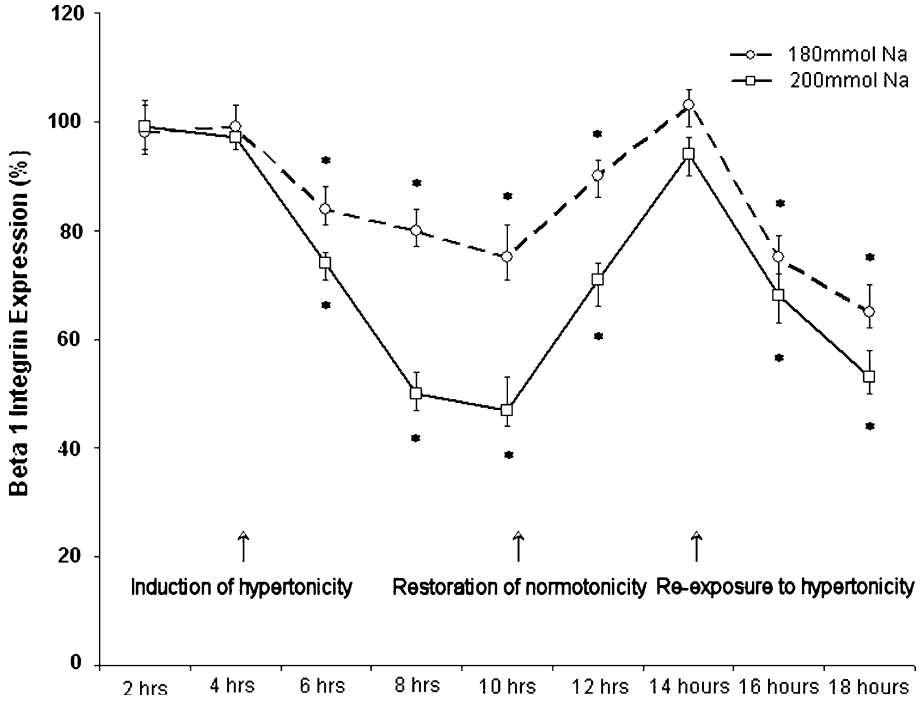

Fig. 1. Effect of repeated exposure to increased extracellular sodium concentration on tumor cell adhesion molecule expression. Tumor cells were exposed to LPS under isotonic conditions before suspension in hypertonic medium at 4 h, subsequent restoration to isotonicity at 10 h, followed by resuspension in a hypertonic environment at 15 h. There were 10,000 events/group analyzed by FACScan. Quantitative determination of β_1 expression was performed at 2-h intervals. Data are expressed as percentage of β_1 integrin at isotonicity (140 mmol/l Na^+) and represent experiments performed with replicates ($n \geq 3$/group). *$P < 0.05$ vs. 140 mmol Na^+.

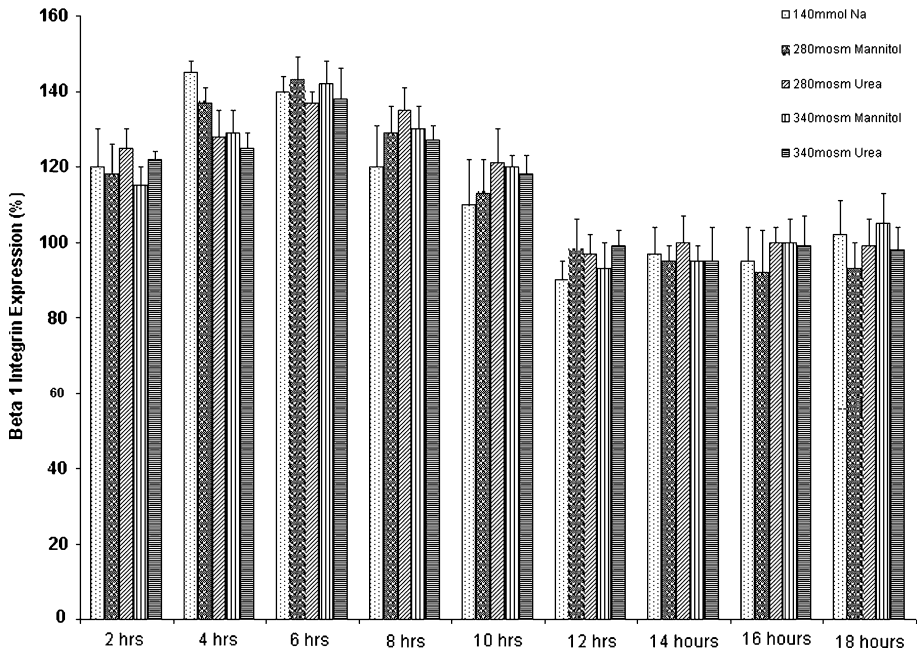

Fig. 2. Effect of exposure to mannitol or urea on tumor cell adhesion molecule expression. Tumor cells were exposed to LPS under hypertonic or isotonic conditions before quantitative determination of β_1 expression at 2-h intervals. There were 10,000 events/group analyzed by FACScan. Data are expressed as percentage of β_1 integrin at isotonicity (140 mmol/l Na^+) and represent experiments performed with replicates ($n \geq 3$/group).

alterations were disrupted using cytochalasin D, a helminthosporium-derived inhibitor of F-actin polymerization, prior to HTS exposure. Tumor cells thus exposed failed to respond to HTS by reducing integrin expression (Fig. 4).

Effect of Nhe Mechanism Blockade on Cellular Response to Hypertonicity

Interaction between the actin cytoskeleton and channel proteins is implicated in the regulation of membrane ion transport and cellular signaling. A reduction in cell volume is known to stimulate the NHE mechanism, which occasions intracellular alkalinization and enables a compensatory increase in cell volume. To further elucidate the role of the sodium cation in the determination of the cellular response to HTS, tumor cells were exposed to ionic hypertonicity in the presence of EIPA. Under conditions of maximal cytokine stimulation, the effect of HTS was abrogated by antiporter blockade (Fig. 4). This suggests that HTS-induced NHE activity is

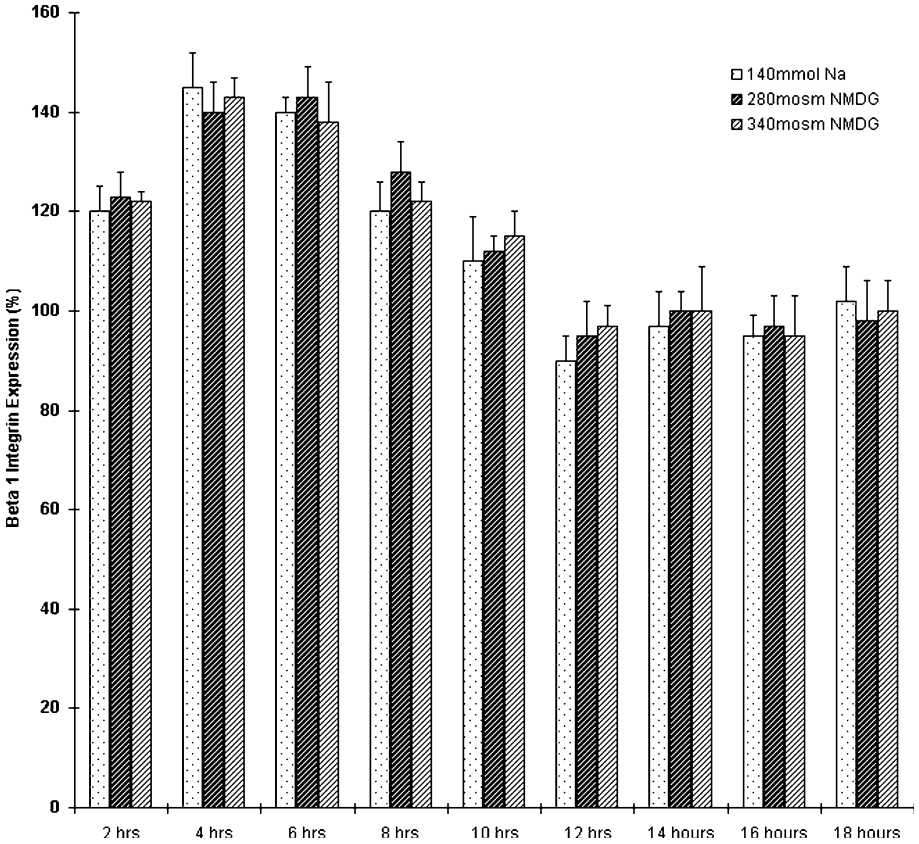

Fig. 3. Effect of exposure to increased extracellular osmolarity due to NMDG-chloride on tumor cell adhesion molecule expression. Tumor cells were exposed to LPS under hypertonic or isotonic conditions before quantitative determination of β_1 expression at 2-h intervals. There were 10,000 events/group analyzed by FACScan. Data are expressed as percentage of β_1 integrin at isotonicity (140 mmol/l Na^+) and represent experiments performed with replicates ($n \geq 3$/group).

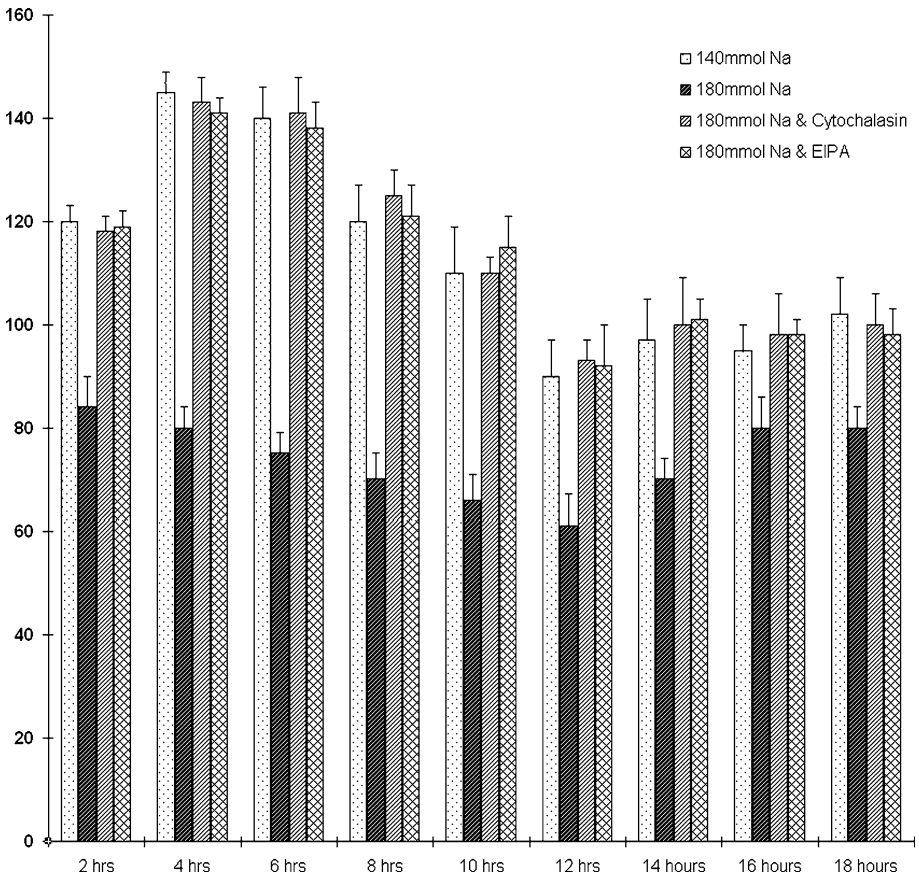

Fig. 4. Effect of exposure to increased extracellular sodium concentration in the presence of cytochalasin D or EIPA on tumor cell adhesion molecule expression. Tumor cells were exposed to LPS under hypertonic or isotonic conditions before quantitative determination of β_1 expression at 2-h intervals. There were 10,000 events/group analyzed by FACScan. Data are expressed as percentage of β_1 integrin at isotonicity (140 mmol/l Na^+) and represent experiments performed with replicates ($n \geq 3$/group).

central to the decline in adhesion molecule expression observed in hypertonic solutions.

Measurements of Cytosolic Ph

HTS induced a cytosolic alkalinization, indicated by an increase in BCECF fluorescence, corresponding with a pH change from 7.1 to 7.3 ($n = 25$, $P < 0.001$). HTS-induced alkalinization was significantly reduced by NHE inhibition with EIPA (100 μM) in separate experiments (pH change of < 0.1, $P < 0.001$ vs. HTS controls, $n = 25$). Similarly, cytochalasin D inhibited HTS-induced alkalinization (pH change of < 0.1, $P < 0.01$ vs. HTS controls, $n = 25$) (Figs. 5 and 6). These data suggest that HTS perfusion increases NHE activity, implying an increase in cytosolic Na^+ while H^+ ions are extruded from the cell, via cytoskeletal rearrangement.

Measurements of Cytosolic Sodium Concentration

In order to test the hypothesis that intracellular concentrations of sodium ions ($[Na_i^+]$) were increased by HTS, cells loaded with a Na^+ green were imaged in separate experiments. Basal fluorescent intensity under isotonic conditions corresponded with an $[Na_i^+]$ of 15.7 ± 0.8 mM ($n = 30$). When perfused with HTS (180 mM/l), the $[Na_i^+]$ increased to a maximal 28.1 ± 2.4 mM within 10 min ($n = 30$, $P < 0.001$). This did not occur when tumor cells were treated with cytochalasin D (17.3 ± 1.1 mM, $P = 0.8$ vs. HTS controls, $n = 30$) or EIPA (16.5 ± 0.7 mM, $P < 0.01$ vs. HTS controls, $n = 30$) prior to HTS exposure (Figs. 7 and 8).

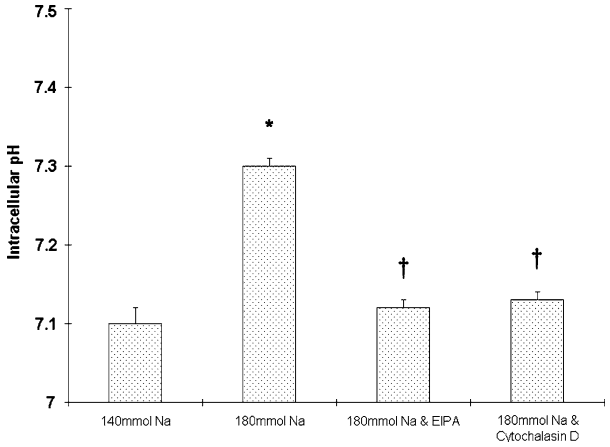

Fig. 5. Role of NHE and cytoskeletal interactions in HTS-induced intracellular akalinization. Exposure to HTS (180 mmol/l Na^+) evoked a significant rise in fluorescent intensity (cellular alkalinization). This was inhibited by blocking NHE activity prior to hypertonic exposure with EIPA (100 μM) or cytochalasin D (10 μg/ml) (all $n = 25$; $^*P < 0.001$ vs. basal readings [140 mmol/l Na^+], $^\dagger P < 0.01$ vs. HTS controls [180 mmol/l Na^+]).

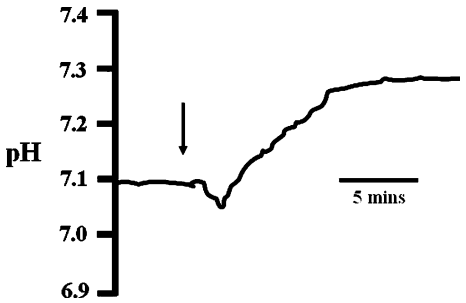

Fig. 6. Representative experimental recordings of the effect of HTS on intracellular pH. HTS (180 mmol/l Na^+) induced a rapid rise in the fluorescent intensity of BCECF, expressed as equivalent pH measurements, within cells imaged with confocal laser microscopy.

Discussion

The majority of deaths from cancer are attributable to metastatic disease (Hanahan & Weinberg, 2000). A hematogenous metastasis arises following complex interactions between a circulating tumor cell and the microvascular endothelium. Detachment of cells from the primary tumor accompanies surgical intervention (Hu & Chow, 2000; Dowdall et al., 2002) and may culminate in remote tumor cell adhesion and subsequent intravascular proliferation (Al-Mehdi et al., 2000). Adhesion molecules and their corresponding ligands are central to this process, resulting in stable engagement of detached tumor cells in precapillary arterioles, safeguarding the cell from apoptosis and phagocytosis. The rather precarious intravascular foothold that the early tumor cell colony enjoys may render it susceptible to perioperative therapies that alter the endothelial microenvironment, particularly those that diminish adhesive potential.

The present study describes a number of novel findings with regard to the cellular effects of HTS, most notably that while NaCl-dependent hypertonicity causes a decline in β_1 integrin expression, Na^+-free hypertonic or hyperosmotic solutions fail to induce a similar reduction in adhesion molecule expression. This suggests that HTS exerts cellular effects via Na^+-dependent events rather than by chloride (Cl^-) shifts, its overall tonicity (ionic potential) or its osmolarity (osmotic potential).

Apposite adhesion molecule expression and bidirectional signaling facilitates attachment of tumor cells to the endothelium, a key event in tumor migration and metastatatic initiation (Al-Mehdi et al., 2000). These processes require considerable cytoskeletal remodeling for the conformational membrane alterations and overall cellular plasticity required for tumor cell viability. Cell regulation changes in response to osmotic or hypertonic exposure alter the conformation of polymerized F-actin in the cytoskeleton (Hallows, Packman & Knauf, 1991;

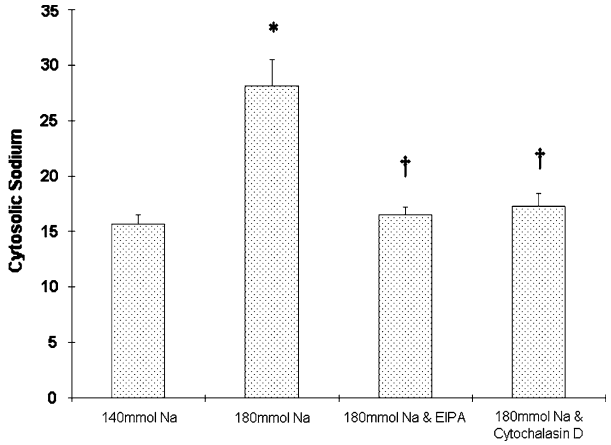

Fig. 7. Role of NHE and cytoskeletal interactions in the HTS-induced rise in cytosolic sodium. Exposure to HTS (180 mmol/l Na^+) evoked a significant rise in cytosolic sodium. This was inhibited by blocking NHE activity prior to hypertonic exposure with EIPA (100 μM) or cytochalasin D (10 μg/ml) (all $n = 30$; $^*P < 0.001$ vs. basal readings [140 mmol/l Na^+], $^\dagger P < 0.01$ vs. HTS controls [180 mmol/l Na^+]).

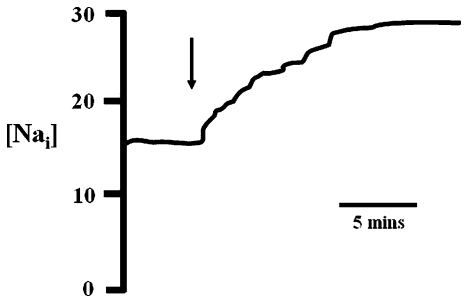

Fig. 8. Representative experimental recordings of the effect of HTS on cytosolic Na^+. HTS (180 mmol/l Na^+) induced a rapid rise in cytosolic sodium, expressed as absolute values, within cells imaged with confocal laser microscopy.

Hallows et al., 1996). Such cytoskeletal remodeling is a key component of hypertonic effects in neutrophils (Rizoli et al., 2000). Neutrophil shedding of L-selectin under hypertonic (sodium chloride) and hyperosmolar (sucrose) conditions suggested that hypertonicity-induced shedding was cell volume-dependent via cytoskeletal changes (Rizoli et al., 1999). The finding here that an environment rendered hypertonic by a sodium-free solution (NMDG) or hyperosmolar by mannitol/urea does not influence adhesion molecule expression implies that the effects of HTS are not occasioned by cell shrinkage in colonic tumor cells.

As evidenced by cytosolic Na^+ measurements, HTS exposure induced a net influx of Na^+. Osmotic water loss and cell shrinkage may have accounted, in part, for a rise in intracellular Na^+. However, HTS induction of a cytosolic alkalinization by upregulating NHE activity also increases intracellular Na^+ because it exchanges extracellular Na^+ for intracellular H^+ ions with a fixed stoichiometry of 1:1 (Aronson, 1985). Intracellular Na^+ transport in colonic epithelia is known to be mediated by membrane activity of NHE and Na^+ channels and this is maintained in colonic tumor cells (Charney et al., 1981; Kurtin & Charney, 1984). Similarly, cytoskeletal regulation of transcellular Na^+ transport is well-described (Smith et al., 1991; Rotin et al., 1994). In the current study, disruption of cytoskeletal regulation with cytochalasin D inhibited both HTS-induced cytosolic Na^+ increases and cellular alkalinization, while inhibition of NHE activity attenuated the intracellular rise in Na^+ concentration and the integrin response to hypertonicity. Therefore, it seems that the Na^+-specific antiadhesion influences exerted by HTS on colonic tumor cells are due to both cytoskeletal remodeling/signaling and an internally directed transmembrane Na^+ flux involving NHE.

In conclusion, HTS causes downregulation of colonic tumor cell adhesion molecule expression via cytoskeletal and NHE signals. Cellular responses to hyperosmolar perturbation with membrane-impermeable mannitol, permeable urea or a sodium-free hypertonic solution do not elicit such downregulation. HTS induces cytoplasmic alkalinization of tumor cells with a corresponding increase in cytosolic Na^+ concentration by upregulating NHE activity. This demonstrates a sodium-specific mode of action for HTS-induced cellular changes that translates into a potential mechanism by which the metastatic potential of tumor cells shed from a colorectal cancer during surgery or chemotherapy may be minimized simply through HTS infusion.

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