# Hypertonic saline impedes tumor cell–endothelial cell interaction by reducing adhesion molecule and laminin expression

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***Background.*** *Hypertonic saline infusion dampens inflammatory responses and suppresses neutrophil-endothelial interaction by reducing adhesion molecule expression. This study tested the hypothesis that hypertonic saline attenuates tumor cell adhesion to the endothelium through a similar mechanism.*
***Methods.*** *Human colon cancer cells (LS174T) were transfected with green fluorescent protein and exposed to lipopolysaccharide, tumor necrosis factor-alpha, and interleukin-6 under hypertonic and isotonic conditions for 1 and 4 hours. Confluent human umbilical vein endothelial cells were similarly exposed. Cellular apoptosis and expression of adhesion molecules and laminin were measured by flow cytometry. Tumor cell adhesion to endothelium and laminin was assessed with fluorescence microscopy. Data are represented as mean ± standard error of mean, and an ANOVA test was performed to gauge statistical significance, with* $P < .05$ *considered significant.*
***Results.*** *Hypertonic exposure significantly reduced tumor cell adhesion despite the presence of the perioperative cell stressors (42 ± 2.9 vs 172.5 ± 12.4,* $P < .05$*), attenuated tumor cell β-1 integrin (14.43 vs 23.84,* $P < .05$*), and endothelial cell laminin expression (22.78 ± 2.2 vs 33.74 ± 2.4,* $P < .05$*), but did not significantly alter cell viability.*
***Conclusion.*** *Hypertonic saline significantly attenuates tumor cell adhesion to endothelium by inhibiting adhesion molecule and laminin expression. This may halt the metastatic behavior of tumor cells shed at surgery. (Surgery 2004;136:76-83.)*

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ADHESIVE INTERACTIONS BETWEEN TUMOR CELLS and the extracellular matrix (ECM) of vascular endothelium play a pivotal role in the process of metastatic initiation, affecting cell proliferation, survival, and migratory potential.[1,2] The tendency for surgery to engender a permissive environment that encourages tumor growth and dissemination has been attributed to cytokine-driven augmentation of tumor cell adhesive potential.[3,4] The intricate processes that culminate in firm tumor cell attachment to vascular endothelium involve multiple cell surface receptors, adhesion molecules, and cell signaling. Many of these individual processes are mediated by integrins, a ubiquitously expressed family of adhesion molecules. The integrins consist of heterodimeric transmembrane glycoproteins containing noncovalently linked α and β chains. Variable subunit association results in an array of integrin dimers,[5,6] categorized into subfamilies on the basis of the β chain.[7] Integrins play a primary role in invasive growth; however, integrin-mediated cell migration is also a prerequisite for many nonpathologic processes, such as transmigration of leukocytes and angiogenesis.[8]

Previous studies undertaken in our laboratory have demonstrated an augmentation of tumor cell β-1 integrin expression after exposure to endotoxin, which correlates with enhanced binding avidity.[3] Furthermore, specific functional blockade of β-1 integrin results in markedly diminished affinity for the ECM, implicating β-1 as the dominant subunit in adhesion. The extracellular

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domains of β-1 integrin recognize many ECM proteins as ligands, such as laminin, collagen, and fibronectin.[9]

The physiologic effects of a hypertonic environment on neutrophil cytotoxicity have rekindled interest in hypertonic saline (HTS) as a resuscitative agent in states of hyperinflammation.[10] The benefits of transient hyperosmolar resuscitation extend to the attenuation of receptor-mediated leukocyte functions, including adhesion molecule expression, and the suppression of neutrophil activation.[10-13] We have recently demonstrated attenuation of pulmonary injury in animal models of immunologic disarray with hypertonic therapy, attributed to a depressed neutrophilic response.[14-16]

The immunomodulatory effects of HTS may be ascribed to interference with neutrophil adhesive potential. Osmotic perturbation of neutrophils prevents lipopolysaccharide (LPS)-induced CD11b upregulation and results in shedding of L-selectin,[17] whereas intravital microscopy has demonstrated impaired adhesion to postcapillary venules.[18]

As the intracellular machinery responsible for regulated adhesion molecule expression is conserved across differing cell types, we hypothesized that HTS may inhibit tumor cell adhesion molecule expression in a manner analogous to its effect on neutrophils. This may diminish the potential for interaction between tumor cells and the ECM, and may represent an impediment to successful dissemination, preventing endothelial adherence of tumor cells shed at the time of surgical extirpation and facilitating their phagocytosis.

This study sought to determine whether exposure of tumor cells to hypertonic saline reduces their affinity for endothelial anchorage, and to test the hypothesis that suppression of adhesion molecule expression may be attributed to osmotic perturbation.

## MATERIAL AND METHODS

**Solutions.** Balanced salt solutions contained 2 mM KCl, 1.5 mM $K_2HPO_4$, 1 mM $MgSO_4$, 10 mM 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, 140 mM NaCl, whereas hypertonic saline solutions contained either 160 mM, 180 mM, or 200 mM NaCl.

**Cell culture, transfection, and subcloning.** The human colon cancer cell line LS174T (American Type Culture Collection, Rockville, Md) was used in all experiments. This 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%; fetal bovine serum, 10%. The cells were grown at 37° C in a humidified atmosphere with 5% $CO_2$, and medium was renewed 3 times weekly.

The cell line was transfected with the enhanced green fluorescent protein gene; a gain-of-function humanized mutant complementary deoxyribonucleic acid generated from the green fluorescent protein gene originally cloned from the bioluminescent jellyfish *Aequorea victoria* as previously described.[19] A retroviral vector construct carrying the gene sequence was used to transfect the cells. This technique has been used on many cell lines and has been shown to lead to stable transfection.[3,19]

Human umbilical vein endothelial cells (HUVECs) were isolated using collagenase treatment of umbilical veins and cultured on 2% gelatin-coated culture flasks (Becton Dickinson Labware, Rutherford, NJ) and maintained in complete medium 199 (Gibco-BRL, Grand Island, NY) supplemented with 20% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μl/ml), amphotericin B (0.25 μg/ml, heparin (16 U/ml), endothelial cell growth supplement (75 μg/ml), and glutamine (2 mmol/L). The cells were grown at 37°C in a humidified atmosphere with 5% $CO_2$ and subcultured by trypsinization with 0.05% trypsin–0.02% EDTA when confluent.

**Adherence of tumor cells to endothelium and extracellular matrix proteins.** HUVECs ($4 \times 10^4$/well) were cultured to confluence in 150 μl of culture medium in the wells of a 96-well cluster plate (Becton Dickinson Labware) coated with 2% gelatin. Confluence was confirmed on microscopy. Tumor cells at a concentration of $1 \times 10^6$ cells/ml were incubated in either an isotonic or hypertonic saline solution, before addition to the culture medium of LPS (Sigma Corp, Dublin, Ireland) at a concentration of 100 ng/ml, or tumor necrosis factor (TNF)-α (Sigma) at a concentration of 5 ng/ml, or interleukin (IL)-6 (Sigma) at a concentration of 25 ng/ml, for 1 or 4 hours at 37°C in a humidified atmosphere with 5% $CO_2$. After incubation, the tumor cells were extracted by centrifugation at 1500 rpm for 5 minutes. The pellet of cells was washed and resuspended in culture medium to a concentration of $1 \times 10^6$ cells/ml. One hundred microliters of the tumor cell suspension was then cocultured with the HUVEC monolayer or with ECM proteins laminin or fibronectin for 120 minutes. After each monolayer was vigorously washed twice with 150 μl Dulbecco modified Eagle medium (DMEM), the

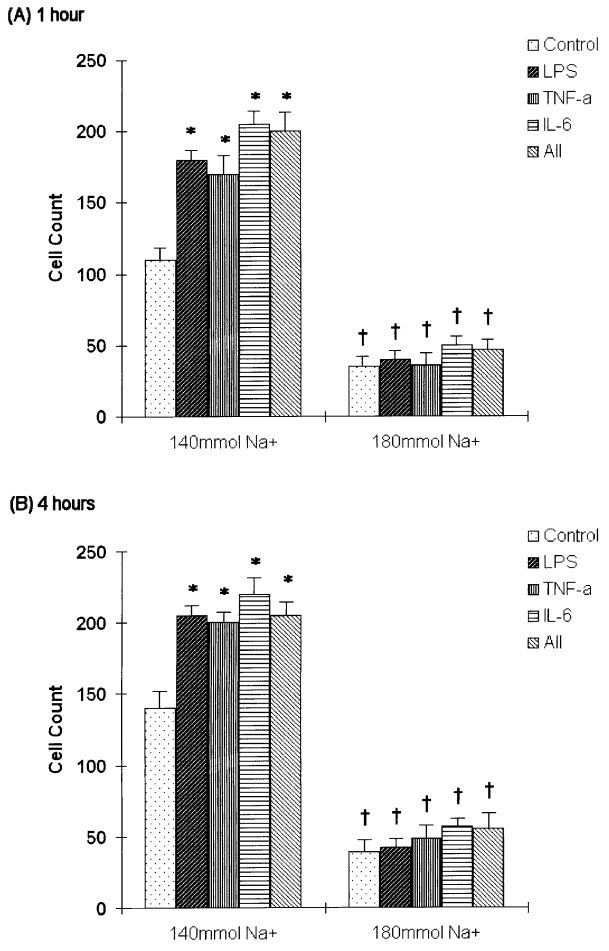


**Fig 1.** Effect of exposure to perioperative cell stressors and hypertonicity on number of tumor cells adherent to endothelium after 2 hours of incubation. Tumor cells were exposed to 1 of LPS, TNF-α, and IL-6, or all 3 cytokines, under hypertonic or isotonic conditions. **A,** Treatment for 1 hour before quantitative determination of cell adhesion. **B,** Treatment for 4 hours before quantitative determination of cell adhesion. Data are expressed as mean (SEM) and are representative of 5 separate experiments. *$P < .05$ vs control 140 mmol $Na^+$. †$P < .05$ vs corresponding 140 mmol $Na^+$ groups.

adherent tumor cells were counted under fluorescence microscopy by 2 observers who were blinded to the experimental manipulation.

In a separate experiment, HUVECs were cultured to confluence in the wells of a 96-well cluster plate coated with 2% gelatin as before. Each monolayer was then treated with 100 μl of 100 ng/ml LPS, or 5 ng/ml TNF-α, or 25 ng/ml IL-6 in isotonic or hypertonic medium for 1 or 4 hours. Untreated tumor cells were then cocultured with the treated HUVEC monolayer for 120 minutes, and after each plate was vigorously washed twice with 150 μl RPMI-1640, the adherent tumor cells were counted by 2 blinded observers under fluorescence microscopy using an excitation wavelength of 488 nm.

**Determination of adhesion molecule expression.** Tumor cells and HUVECs ($2 \times 10^6$/ml) were incubated in isotonic (140 mmol/L $Na^+$) or hypertonic (160 or 180 mmol/L $Na^+$) medium, before addition of LPS at a concentration of 100 ng/ml, for 24 hours. Every 2 hours, 100 μl of the cell suspension was incubated with 15 μl (10 μg/ml) of either phycoerythrin (PE)-conjugated mouse antihuman monoclonal antibody specific for the β-1 integrin chain, CD29 (PharMingen, San Diego, Calif), or a PE-conjugated IgG1 antibody for control samples, at 4°C for 30 minutes. HUVEC expression of laminin in a hypertonic environment was assessed after cell incubation in suspension with LPS. A primary mouse antihuman monoclonal antibody, 4C7 (Chemicon), was added and incubated at 4°C for 30 minutes. The samples were washed with PBS and reconstituted to their original concentration. A second PE-conjugated goat antimouse monoclonal antibody (Jackson ImmunoResearch, West Grove, Pa) was then added and expression of laminin assessed by flow cytometric analysis after incubation at 4°C for 30 minutes. A Becton Dickinson FACScan was used to analyze antibody binding.

**Assessment of apoptosis.** Tumor cells were treated with LPS (100 ng/mL) at 37°C in a humidified 5% carbon dioxide atmosphere before incubation in isotonic or hypertonic (160, 180, or 200 mM $Na^+$) medium. Determination of apoptosis of both quiescent and stimulated cells by flow cytometry was performed after 1, 6, 12, and 18 hours. Apoptosis was quantified according to the percentage of cells with hypodiploid DNA by use of the propidium iodide staining technique as previously described.[20] All measurements were performed using the same instrument settings.

**Data analysis.** All data are presented as mean values with standard error of mean (SEM). Statistical analysis was performed using ANOVA, and a $P$ value $< .05$ was considered significant.

## RESULTS

**Effect of hypertonicity on tumor cell adhesive potential.** Under fluorescence microscopy, we observed that exposure of tumor cells to perioperative endogenous and exogenous signaling molecules resulted in significantly enhanced adhesive potential, in keeping with previous studies undertaken in both our laboratory and elsewhere.[3,4] Affinity for endothelial binding increased

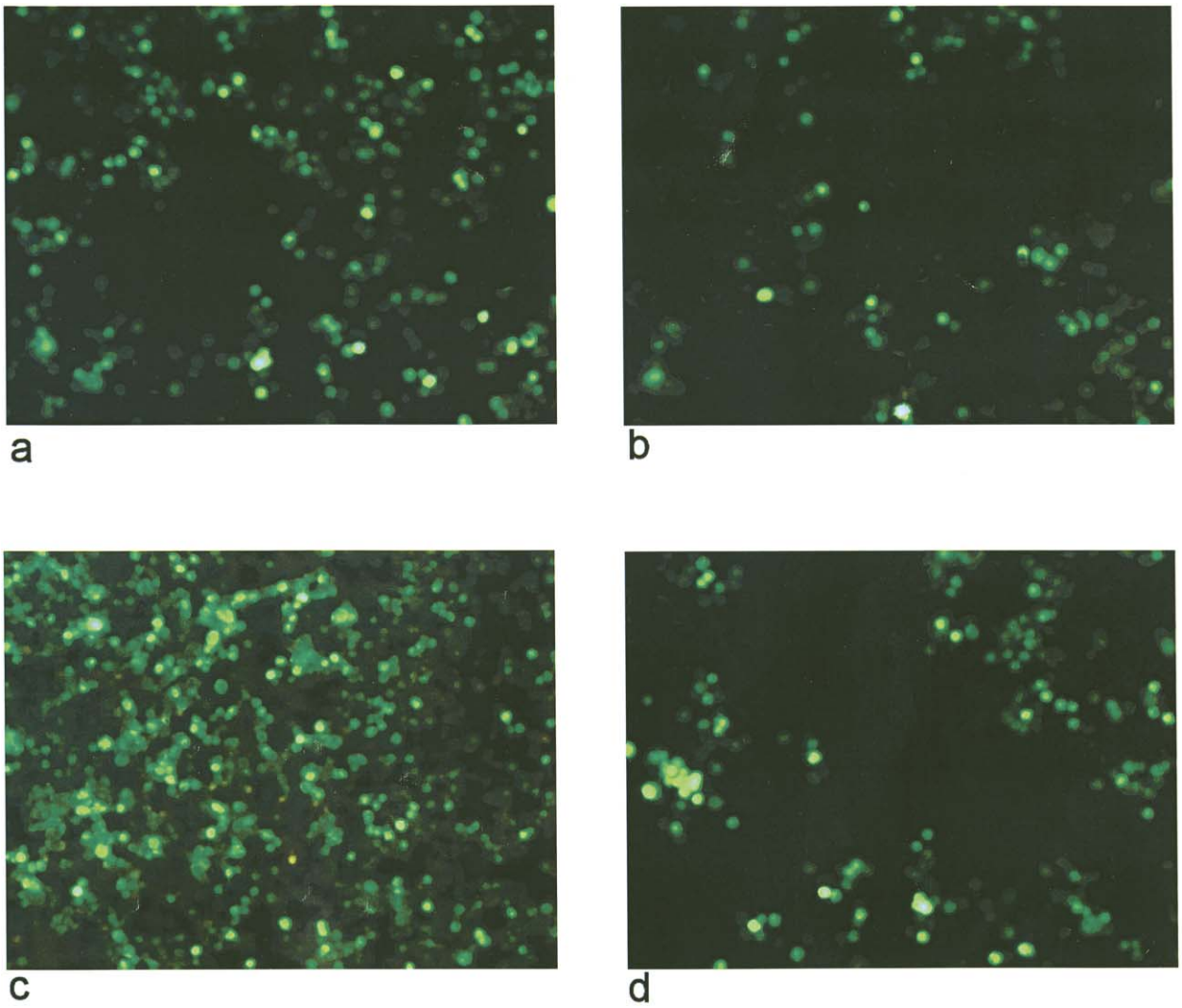


**Fig 2.** Fluorescence photomicrographs (×40) of activated fluorescing tumor cells adherent to endothelial monolayer. **A**, Unstimulated cells in isotonic environment. Baseline adhesion. **B**, Unstimulated cells in hypertonic environment. Markedly reduced cellular adhesion. **C**, Adhesion of cells exposed to lipopolysaccharide under isotonic conditions. Dense adhesion of tumor cells to endothelium. **D**, Adhesion of cells exposed to lipopolysaccharide under hypertonic conditions. Significant attenuation of adhesive potential of tumor cells.

with prolonged exposure to the inflammatory cytokines, further confirming the potent prometastatic effects of operative stress. However, tumor cells subjected to hypertonic shock, occasioned by exposure to 180 mM $Na^+$, exhibited a significantly diminished capacity to form anchoring bonds to endothelium at either time point (Figs 1 & 2).

Similarly, exposure of endothelium to inflammatory cytokines resulted in significantly enhanced tumor cell adhesion that was completely abrogated by incubation in an environment rendered hypertonic by the addition of sodium chloride (Fig 3). The significant effect of IL-6 alone on tumor cell adhesion is a finding in agreement with previous reports.[21] This may have added significance, as surgical stress-induced amplification of endothelial adherence by soluble IL-6 receptors has been implicated recently in metastatic initiation.[21] In a manner analogous to its inhibitory effect on tumor cell adhesion to endothelium, hypertonic saline also significantly impeded tumor cell interaction with both laminin and fibronectin (Fig 4).

**Effect of hypertonicity on endothelial laminin expression.** To evaluate the effects of extracellular hypertonicity/hyperosmolarity on ECM protein secretion, laminin expression by activated endothelial cells was determined by antibody binding. Incubation of vascular endothelium in a hypertonic

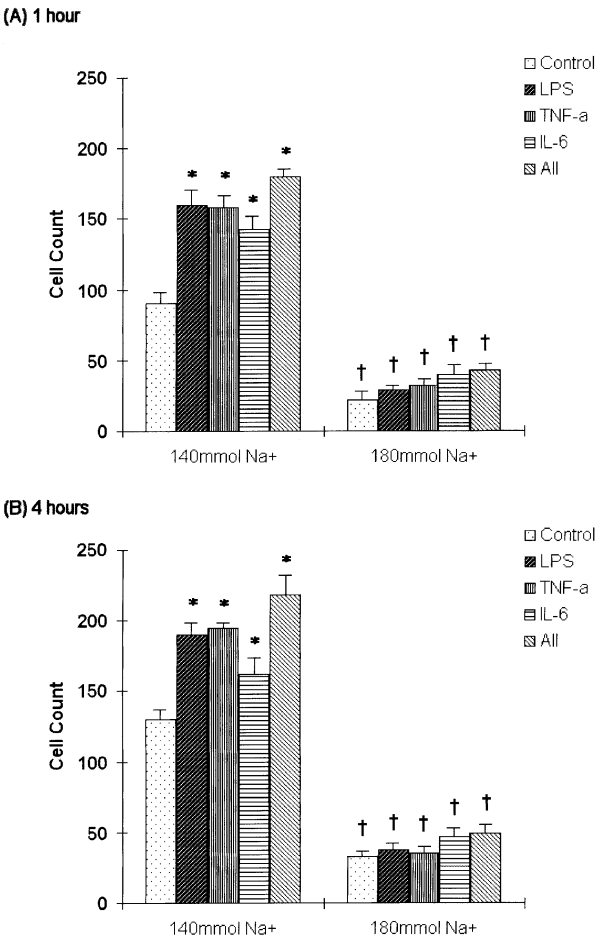


**Fig 3.** Effect of exposure of endothelial monolayer to perioperative cell stressors and hypertonicity on number of tumor cells adherent after 2 hours of incubation. Endothelium was exposed to one of LPS, TNF-α, and IL-6, or all 3 cytokines, under hypertonic or isotonic conditions. **A,** Treatment for 1 hour before quantitative determination of cell adhesion. **B,** Treatment for 4 hours before quantitative determination of cell adhesion. Data expressed as mean (SEM) and are representative of 5 separate experiments. *$P < .05$ vs control 140 mmol $Na^+$. †$P < .05$ vs corresponding 140 mmol $Na^+$ groups.

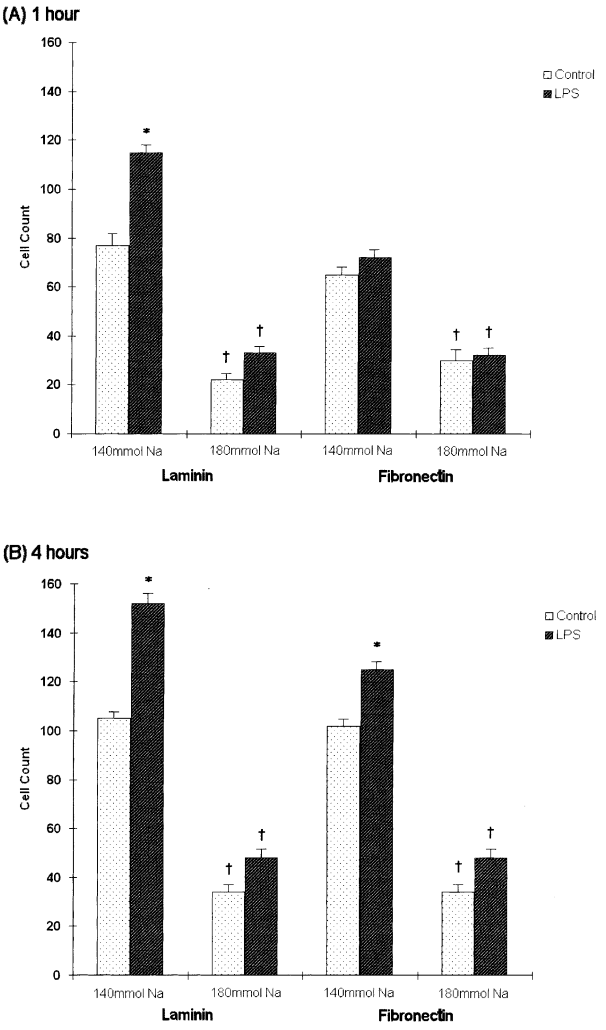


**Fig 4.** Effect of hypertonicity on number of tumor cells adherent to the ECM proteins laminin and fibronectin after 2 hours of incubation. Tumor cells were exposed to LPS under hypertonic or isotonic conditions 1 or 4 hours before quantitative determination of cell adhesion. Data expressed as mean (SEM) and are representative of 3 separate experiments. *$P < .05$ vs control 140 mmol $Na^+$. †$P < .05$ vs corresponding 140 mmol $Na^+$ groups.

environment for 60 minutes significantly reduced LPS-induced secretion of laminin (Table I).

**Effect of ionic hypertonicity on tumor cell β-1 integrin expression.** LPS-induced β-1 integrin expression was assessed at increments of osmolarity (140 mM, 160 mM, and 180 mM NaCl), and 180 mM NaCl caused a significant time-dependent decline in integrin expression on the tumor cell surface, compared to both control and LPS-stimulated cells in an isotonic environment. The response elicited by hypertonicity was apparent within 30 minutes of exposure to the saline solutions, with integrin expression reaching its nadir of 61% of control values at 6 hours with 180 mM NaCl. HTS significantly impaired upregulation of adhesion molecules on the cell surface despite chemokine activation; however, β-1 integrin expression also fell markedly below constitutive levels, suggesting shedding of receptors, a finding in keeping with previous studies.[17] Integrin expression recovered somewhat over successive time points, notwithstanding sustained incubation in hypertonic medium. While never exceeding 80% of control values, this implies a degree of time-dependent accommodation to a hypertonic environment (Fig 5).

**Effect of hypertonicity on tumor cell viability.** We have previously described induction of

**Table 1.** Cellular effects of increasing sodium levels

| | *Endothelial cell laminin expression* | *Tumor cell apoptosis (12 Hours)* | |
|---|---|---|---|
| | | *Control* | *LPS* |
| 140 mmol/L $Na^+$ | 33.74 (2.4) | 12.7 (0.2)% | 8.3 (0.5)% |
| 160 mmol/L $Na^+$ | 32.04 (1.8) | 13.2 (0.8)% | 8.7 (0.7)% |
| 180 mmol/L $Na^+$ | 22.78 (2.2)* | 18.0 (1.1)%* | 9.0 (0.4)% |
| 200 mmol/L $Na^+$ | 24.33 (1.7)* | 22.7 (1.9)%* | 12.4 (1.3)%* |

Data expressed as mean (SEM), and are representative of 3 separate experiments. Laminin expression represented as mean channel fluorescence. Apoptotic rates represented as cell counts.
*$P < .05$ vs 140 mmol/L $Na^+$.

apoptosis in activated neutrophils resulting from osmotic perturbation.[15] We postulated that exposure to a hypertonic environment may similarly induce tumor cells to enter the programmed cell death sequence, thereby curbing the tendency toward endothelial adhesion. However, only exposure to levels of hypertonicity above the clinical threshold ($\geq$ 200 mM NaCl) resulted in a significant induction of apoptosis in cells exposed to perioperative cell stressors. Osmotic perturbation of quiescent tumor cells did result in increased apoptosis at clinically relevant levels. Otherwise, apoptotic rates at all time points were equivalent between the experimental groups (Table I).

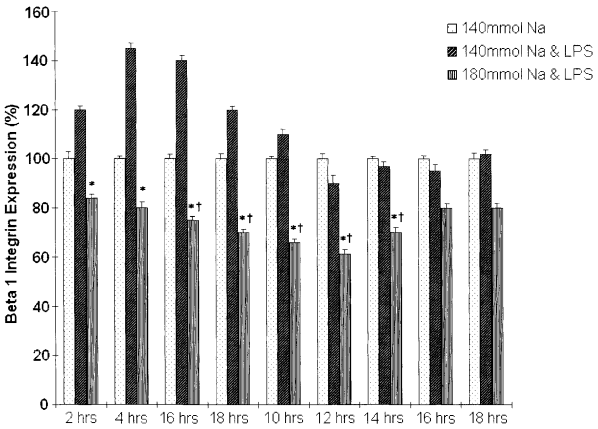


**Fig 5.** Effect of increased extracellular sodium concentration on tumor cell adhesion molecule expression. Tumor cells were exposed to LPS under hypertonic or isotonic conditions before quantitative determination of β-1 expression at 2 hour intervals. Data expressed as percentage of β-1 integrin at isotonicity (140 mmol/L $Na^+$) and are representative of 3 separate experiments. *$P < .05$ vs 140 mmol $Na^+$ and LPS. †$P < .05$ vs 140 mmol $Na^+$.

## DISCUSSION

The present study describes the novel finding that exposure to hypertonic saline impedes tumor cell attachment to vascular endothelium via a decline in adhesion molecule and laminin expression. Assessment of adhesion processes has previously been conducted by labeling cells with radioactive chromium ($^{51}C$), followed by quantification of lysate radioactivity by gamma spectrometry.[22] Transfection of cells to express green fluorescent protein facilitates the direct visual assessment of adherent cells.

Tumor cell migration beyond the original location is predicated upon the tight integration of disparate processes, such as biophysical forces concerned with the generation of cell locomotion,[23] expression of adhesion molecules,[24] and the underlying adhesion-mediated bidirectional signaling responses, resulting in firm attachment of tumor cells to the microvasculature, facilitating transmural penetration.[25] In this study, exposure of tumor cells to inflammatory mediators led to an upregulation of β-1 integrin expression and enhanced binding avidity. Synergistic adhesive responses were observed when the cytokines were coadministered. This may result from distinct sites of action within the cell, despite the fact that there is undoubted interrelation at the signaling and target levels.

Hypertonic saline has been noted to exert a myriad of effects on neutrophils, serving to inhibit function in a reversible and transient manner, by inducing a decline in adhesion molecule expression. Therefore, although the immunomodulatory effects of HTS provide potential strategies for attenuating inappropriate neutrophil activation, the same facility may diminish the postoperative acceleration observed in tumor growth. Recent studies have proposed that the timing of the osmotic shock to the neutrophil may be crucial in determining the eventual cellular response.[26,27] Murao et al have suggested that HTS reduces neutrophil potency if administered early in states of immunologic disarray.[26] Similarly, infusion of HTS before and during oncologic surgery may

abrogate the priming effect of inflammatory mediators on shed tumor cells.

Plasma NaCl concentration is tightly regulated, being a major determinant of plasma osmolality. Previous studies have demonstrated that infusion of 7.5% NaCl results in a rapid, though transient, rise in plasma $Na^+$ levels to 40 mM above isotonic levels,[28] which remain elevated for up to 4 hours.[27] To evaluate whether a universal and indiscriminate increase in extracellular tonic and osmotic strength exerts an inhibitory effect on integrin-mediated adhesion and signaling, tumor cells were exposed to media of increased osmolarity, and quantitative analysis of integrin expression was performed. We observed impaired adhesion molecule expression by both tumor and endothelial cells exposed to a hypertonic environment. The correlation with decreased adhesive potential provides further confirmation of the importance of integrin-mediated processes in metastatic initiation. Cells exposed to a hypertonic environment for 1 hour exhibited a reduction in adhesive ability of more than 50%; however, this was accompanied by a more tempered decline in β-1 integrin expression. This apparent discrepancy may be attributed to the influence of hypertonic shock on expression of other cell surface adhesion molecules.[5,11,12,27] Previous studies have shown that physicochemical alterations in the microvascular barrier are mediated by the local release of vasoactive substances in response to cytokine stimulation.[29] The transient increase in interstitial osmolarity that results from the infusion of hypertonic saline may impair this proinflammatory response of the immune-competent endothelial cells.

Apoptosis and phagocytosis of circulating tumor cells serves to limit tumor–endothelial cell interaction. Dysfunction of this regulatory mechanism is a pivotal component in the enhancement of adhesive potential evident post surgery. However, we have demonstrated in our model system that, within clinical limits, HTS does not amplify stimulated tumor cell apoptotic rates, implying that the beneficial effects of hypertonicity are not mediated through a pro-apoptotic response.

Integrins offer a promising pharmacologic target for suppressing unchecked and unrestrained tumor dissemination and drug resistance. Exposure of tumor cells to a hypertonic environment reveals reversible and transient attenuation of integrin-dependent adhesion. In the clinical environment, HTS infusion profoundly suppresses neutrophil-endothelial interaction by reducing adhesion molecule expression. An analogous effect on tumor cells would potentially provide protection from the deleterious effects of the tumor-permissive environment engendered by cytoreductive surgery.

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