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Cancer Cell Glycocalyx Mediates Mechanostransduction and Flow-Regulated Invasion

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Abstract

Mammalian cells are covered by a surface proteoglycan (glycocalyx) layer, and it is known that blood vessel-lining endothelial cells use the glycocalyx to sense and transduce the shearing forces of blood flow into intracellular signals. Tumor cells *in vivo* are exposed to forces from interstitial fluid flow that may affect metastatic potential but are not reproduced by most *in vitro* cell motility assays. We hypothesized that glycocalyx-mediated mechanotransduction of interstitial flow shear stress is an un-recognized factor that can significantly enhance metastatic cell motility and play a role in augmentation of invasion. Involvement of MMP levels, cell adhesion molecules (CD44, α3 integrin), and glycocalyx components (heparan sulfate and hyaluronan) were investigated in a cell/collagen gel suspension model designed to mimic the interstitial flow microenvironment. Physiologic levels of flow upregulated MMP levels and enhanced the motility of metastatic cells. Blocking the flow-enhanced expression of MMP actvity or adhesion molecules (CD44 and integrins) resulted in blocking the flow-enhanced migratory activity. The presence of a glycocalyx-like layer was verified around tumor cells, and the degradation of this layer by hyaluronidase and heparinase blocked the flow-regulated invasion. This study shows for the first time that interstitial flow enhancement of metastatic cell motility can be mediated by the cell surface glycocalyx – a potential target for therapeutics.

INTRODUCTION

Mammalian cells are covered by a surface glycocalyx layer that serves many cellular functions (1, 2). It has been demonstrated that endothelial cells that line blood vessels use the glycocalyx to sense and transduce the mechanical shearing forces of blood flow into intracellular signals (3–5). In a recent study we demonstrated that interstitial flow in a model of tumor microenvironment suppressed the invasion of non-metastatic glioma cells replicating cell behavior observed in orthotopically-implanted glioma tumors (6, 7). This

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suppression of migration in non-metastatic cells had not been observed in other *in vitro* models, likely due to a lack of interstitial flow and associated cell surface shear stress that our study captured (6). Metastatic cells on the other hand have been hypothesized to have enhanced migration rates in response to flow (8–11). In related work with vascular smooth muscle cells and fibroblasts, interstitial flow enhanced migration (12). Building upon these studies, the present investigation hypothesized that interstitial flow and related shear stress can also enhance the invasive potential of metastatic cells.

The cell lines selected for this study were the SN12C and SN12L1 renal carcinoma cells. The SN12L1 cell line was previously isolated from tumors that led to frequent and distant metastases (13, 14). The SN12C cell line, though isolated from the same tumor and established to be metastatic, did not lead to as many tumors at distant sites (13, 14). The SN12L1 has recently been confirmed as more invasive in orthotopically implanted tumors of the kidney when compared to the SN12C cell line (15).

In our earlier study, tumor cells were exposed to a maximum of 4 hours of flow (6). Though short, exposure to 4 hours of flow dramatically suppressed the migation of non-metastatic tumor cells (6). Several recent studies have examined flow effects on metastatic cells with flow periods of up to 24 hours (16–18). These studies determined that metastatic cells can utilize flow-induced chemokine gradients to direct migration either away from or towards the main tumor mass (16, 18). In the present study we examined the effects of both short-term (1 or 4 hours) and long-term (24 hours) of flow on the invasive potential of metastatic cells.

One major factor influencing cell invasion is the activity and regulation of matrix metalloproteinases (MMPs) (19–23). In the study of glioma cell lines, MMP-1 and MMP-2 were the primary MMPs that were modulated to decrease invasion potentials (6). Flow may regulate MMPs by mechanotransduction signals transmitted through surface glycocalyx proteoglycans (24, 25). Treating smooth muscle cells with heparinase to degrade heparan sulfate proteoglycans blocked the expression of MMP-13 and inhibited flow-induced migration - highly suggestive of glycocalyx-mediated mechanotransduction (24, 26). In this study we assess whether similar mechanisms can enhance invasion of metastatic tumor cells.

In a recent animal model CD44, α3 integrin, and caveolin were identified as genes regulated in metastasis rates for the SN12L1 and SN12C cells (15). We therefore examined the effect of flow on these genes as well. Because of the direct link between CD44 and hyaluronic acid (27–31), a constituent of the glycocalyx (32–34), hyaluronan-mediated flow-signaling was also investigated.

METHODS

Cell culture and chemoattractant

SN12L1 (High metastatic potential) and SN12C (Low metastatic potential) human renal carcinoma cell lines (courtesy of Dr. Isaiah Fidler, MD Anderson Cancer Center) were investigated. Cells were cultured following MD Anderson protocols. MDA-MB-435S cells were obtained from ATCC (HTB-129) and cultured according to ATCC protocols. 1 nM

TGF-α (transforming growth factor - alpha; Sigma), the optimal chemoattractant concentration in MEM culture media without serum, was added to the companion wells for all invasion assays.

Three-dimensional (3D) interstitial flow model

The three-dimensional model simulated the interstitial flow forces that tumor cells encounter within the interstitium. Cells were suspended in type I collagen and exposed to fluid flow forces via a Darcy flow experimental apparatus (Supplement Figure 1). Suspension, incubation, and flow protocols were followed as previously established (6). Both time of exposure to flow (1, 4, and 24 hours) and shear stress levels were varied by application of 1 or 3 cm H_2O hydrostatic pressure differentials. Shear stress (τ) transmitted to the cell surface through the glycocalyx layer (see Figure 4) was calculated knowing the permeability coefficient (K_p) of the gel suspension, the superficial fluid velocity (U_∞) and the estimated glycocalyx thickness (H) using the previously described formula (25):

$$\tau = \mu U_\infty H / K_p \quad [1]$$

Fluid filtrate effluent was quantified after initiation of flow to determine the volumetric flow rate, J_v. For the longer term 24-hour flow experiments a fixed flow velocity of 1 μm/sec was utilized.

Darcy permeability and three-dimensional shear stress

The flow rates, flow velocities, Darcy permeabilities, hydrostatic pressure drops, and estimated shear stresses (0.84 dyn/cm^2 at ΔP = 1 cm H_20; 2.53 dyn/cm^2 at ΔP = 3 cm H_20) in the 4 hour flow experiment are listed in Supplemental Table 1. In the 24 hour flow experiment, flow velocity (1 μm/sec) and flow rate (6 μl/min) were held constant, permeability was approximated as 3.0×10^{-15} m^2 based on Supplemental Table 1, and shear stress was estimated to be 1.40 dynes/cm^2. All experimental values were similar to those used in other studies and were within the range of physiological values (6, 12, 16).

Compacting and flushing cell suspensions

Fluid flow permanently changes the thickness of gels by compaction of collagen and thereby alters the cell distribution, collagen density and permeability (6). Therefore, following the initial inoculation of the cell/gel suspension and following the 24-hour incubation of the suspension , no-flow control gels were subjected to a short (10 minutes) exposure to flow (1.5 cm ΔP) to compact all gels, flush out MMPs that accumulated during the incubation period, and to replenish nutrients (Supplement Figure 2).

Three-dimensional (3D) invasion assays

Following completion of the flow period (Supplement Figure 1-time 3), any cells on the underside of the inserts were mechanically removed to “zero” the initial migration count (6, 12). Suspensions were then incubated with 700 μl of 1 nM TGF-α in the companion well for either 24 or 48 hours of migration (without flow; Supplement Figure 1–4). The time of exposure to flow and these migration periods were selected for comparison to previous studies and to minimize adverse effects (contamination, increased cell population) of

lengthening the overall experiment (6, 12, 16, 17). At the end of the no-flow migration period, cells that had migrated to the underside of the inserts were quantified (6, 35). The flow and migration periods were separated so that flow effects could not be interpreted as resulting from the convection of signaling molecules produced by the suspended cells.

Collagen and gelatin zymography

To determine the effects of flow on MMP-1 and MMP-2 expression, collagen and gelatin zymography were performed (detailed by Shi et al; 12) on conditioned media collected at the end of the migration period. Representative zymography gels were quantified using the Quantity One software (Bio-Rad) and were presented as percentage of controls (12). During the investigation of the flushing effects of flow (Supplement Figure 2), conditioned media was collected directly from the gels after centrifugation to force the media out the gel.

Quantitative RT-PCR

Quantitative reverse transcription-polymerase chain reaction was performed to validate that interstitial flow was modulating the genes associated with key proteins involved in invasion. RNA was extracted from SN12L1 cells immediately following exposure to either 4 hours or 24 hours of flow (Supplement Figure 1–3), and also for cells at the end of either 24 hours or 48 hours of migration (Supplement Figure 1–4). Previously established RNA extraction, reverse transcription, and quantitative real-time PCR protocols were followed (6). CD44, α3 integrin, and caveolin gene expressions were analyzed since they have been shown to be regulators of metastasis in renal carcinoma cells (15). MMP-1 and MMP-2 were also analyzed in this study since they play a major role in invasion (6). RT-PCR was also performed for additional genes involved in matrix degradation. GAPDH served as an internal control for each sample. The forward and reverse primers utilized to amplify the gene products and product lengths along with GenBank Accession numbers have been listed (Supplement Table 2).

Invasion assays with protein inhibitors

Invasion assays were conducted with MMP, CD44, and α3 integrin inhibitors. The inhibitors and negative controls (MMP-NC and IgG1 isotype) utilized in this study along with concentrations and product information are listed in Supplement Table 3. Chemokine gradients have been shown to enhance migration by directing cell migration via an autologous chemotaxis mechanism (16). To confirm that autologous chemotaxis flow effects had been isolated and removed during the no-flow migration period, invasion assays were also carried out without the use of a chemoattractant. The potency of 1 nM TGF-α was also determined during the no-flow migration period for the SN12L1 cells.

Heparan sulfate proteoglycan immunofluorescence staining

SN12L1 cell suspensions were stained for heparan sulfate proteoglycans following previously established protocols (26). The gels were flushed with the fixative, primary antibody HepSS-1 (US Biological) and secondary antibody Alexa Fluor 350 goat anti-mouse IgM (Invitrogen) for 15 minutes. Lastly, the gels were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Confocal fluorescent microscopy (Zeiss

LSM 510, Plan-Neofluar 40x Oil DIC objective) was used to image isolated cells, estimate thickness of the pericellular heparan sulfate layer, and to image larger sections of the gels. This study also assessed the cleavage of heparan sulfate glycosaminoglycans by heparinase treatment (described below).

Hyaluronidase and heparinase treatment

To assess the potential role of glycocalyx components as flow sensors, flow and invasion experiments were conducted on gel-suspended cells with degraded hyaluronan or heparan sulfate. SN12L1 cells were suspended with media supplemented with either 6.7 IU/L heparinase III or 1.5 U/ml *Streptomyces hyalurolyticus* hyaluronidase (Supplement Table 3) (26, 36). Additionally, 1.5 U/ml hyaluronidase and 1 IU/L heparinase were added to the flow media ensuring that enzymatic activity was sustained during the flow period. For the 4-hour and 24- hour flow experiments total enzyme treatment time was 16 and 36 hours, respectively. Following standard experimental protocols, at the end of the migration period, the effect of hyaluronidase and heparinase on flow-induced MMP-1, MMP-2, CD44, α3 integrin, and caveolin gene expression were examined.

Statistical analysis

All data were normalized by their respective controls and are presented as mean ± standard error of the mean. Each 'N' sample size represents a set of 2 or more gels. The two-tailed Student’s t-test was utilized to determine statistical significance.

RESULTS

Invasion enhanced by three-dimensional shear stress and flow

The migratory activity of the SN12L1 (higher metastatic) cell line was enhanced as the time of exposure to interstitial flow was increased, whereas the SN12C (lower metastatic) cell line was not affected by flow (Figure 1A). Preliminary data suggests that the migratory activity of a previously studied cell line MDA-MB-435S is comparably enhanced in this model (Supplemental Figure 3). Fluid flow enhanced SN12L1 migratory activity by up to 3-fold compared to normalized controls, a significantly greater invasive potential than the SN12C cells ($p < 0.005$). Increasing flow (by increasing ΔP) did not have a significant effect on the SN12L1 migration although migration trended higher. There was no difference in cell suspension density between the SN12C and SN12L1 cells. The gel permeabilities and fluid dynamics (flow rates, velocities, shear stresses) were essentially the same for the two cell lines (Supplemental Table 1) and consistent with previously-reported values (6, 12, 16–18).

Flow upregulates MMP-1 and MMP-2 expression

Zymography of media collected from the wells of suspensions exposed to 4 hours of flow demonstrated flow-induced upregulation of MMP-1 and MMP-2 expression (Figure 1B). The SN12L1 (higher metastatic potential) cells had a much greater increase in MMP-1 expression than the SN12C l cells. Both cell lines displayed modest increases in MMP-2 levels after flow exposure.

Flow affects key genes involved in motility

Alpha-3 integrin, CD44, and caveolin gene expression were previously examined by Bockhorn et al. in an animal model where interstitial flow was present and found to be essential in differentiating the metastatic potentials of renal carcinoma cell lines (15). The flow environment was not characterized in that study. When we exposed SN12L1 cells to 4 hours of flow and monitored their gene expression before the migration period, the expression of α3 integrin and CD44 was enhanced significantly (Figure 1C). Caveolin expression was also enhanced but to a lesser extent. It is noteworthy that flow did not affect the expression of these genes in the SN12C cells.

Blocking MMP, CD44 or α3 integrin inhibits flow-induced migration

Flow-induced enhancement of migration in the SN12L1 cells exposed to 4 hours of flow and 48 hours of migration towards TGF-α was successfully suppressed with the use of MMP-Inhibitor, Anti-CD44, and Anti-α3 integrin (Figure 2A). Although migration was significantly reduced with the use of anti-α3 integrin, migration was still slightly above baseline levels by 27% ($p < 0.05$). MMP inhibitor and Anti-CD44 were the most efficient in blocking the migration enhancing effect of flow as migratory activity remained at the no-flow control levels. The effect of these blockers on baseline migration was also determined and has been reported for comparison (Supplemental Figure 4).

Invasion is enhanced by longer-term exposure to flow

Migratory activity of the SN12L1 (highly metastatic) cell line was enhanced with exposure to 24 hours of flow (1.4 dynes/cm^2 shear stress) followed by 24 hours of migration towards TGF-α, when compared to no-flow controls (Figure 2B). In this case, there was no evidence of lasting enhanced invasion by flow-induced chemokine gradients during the 24 hour invasion experiment without chemoattractant (Figure 2B). It should be noted that there was negligible migration immediately following the 24 hour flow period, 15 ± 5 cells/field (compared to 220 ± 70 cells/field after the migration period), and all filters were "zeroed" before the migration period commenced (see methods).

Gene expression changes affected by flow and migration

After exposure to 4 hours of flow the SN12L1 cells had elevated MMP-1, MMP-2, CD44, α3 integrin, and caveolin gene expressions (Figure 3A). At the end of the migration period, MMP-1 gene expression decreased but was still elevated relative to the no-flow control while MMP-2 gene expression remained elevated during the migration period. CD44, α3 integrin, and caveolin gene expression, on the other hand, returned to baseline by the end of the migration period.

SN12L1 gene expression of MMP-1, MMP-2, CD44, α3 integrin, and caveolin varied with time of exposure to flow (Figure 3B). MMP-1 gene expression maintained a significantly elevated expression out to 24 hours of flow exposure. In response to the longer-term exposure to flow, MMP-2 gene expression decreased modestly to a point where it was no longer significantly elevated relative to control ($p = 0.23$). There was a notable drop in the gene expression of CD44, α3 integrin, and caveolin with the extended flow period as CD44

and α3 integrin gene expressions returned to baseline (no-flow) levels and caveolin became suppressed below baseline.

SN12L1 cells were evaluated for changes in gene expression after exposure to 24 hours of flow followed by a 24 hour migration period without flow (Figure 3C). MMP-1 gene expression decreased significantly during the migration period but remained elevated compared to no flow controls. At the end of the migration period most expression levels were near no-flow control levels.

Glycocalyx staining in three-dimensional suspensions

Because flow modulated gene expressions in SN12L1 cells, a mechanotransduction process was suspected to be the upstream event driving the underlying mechanism. The glycocalyx glycosaminoglycan component heparan sulfate was hypothesized to be a mechanosensor based on our previous studies with smooth muscle cells and endothelial cells (3–5, 26, 37). High magnification images of single cells suspended in collagen gels showed that heparan sulfate proteoglycans were concentrated around metastatic tumor cells in our 3D cell suspensions (Figure 4A). Heparan sulfate thickness was estimated to range between 0.2 to 3 μm. Thicker and more extended proteoglycans were found at sites where multiple cells overlapped (Figure 4B). For comparison, heparan sulfate staining is shown for aortic smooth muscle cells (SMC) in 3D gels (Supplemental Figure 5). Treatment with the specific enzyme heparinase removed heparan sulfate from the surface of SN12L1 cells (Figure 4C, D).

Heparinase and hyaluronidase block flow-enhanced invasion

We used heparinase to enzymatically degrade heparan sulfate and inhibit mechanotransduction. In addition, because the hyaluronan receptor CD44 was affected by flow (Figure 3) and hyaluronan has been shown to play a role in mechanotransduction in other cell types (4), we used hyaluronidase to enzymatically degrade hyaluronan. With the degradation of hyaluronan and heparan sulfate, flow-enhanced SN12L1 migration was inhibited (Figure 5A). Hyaluronidase inhibited migration induced by flow after 4 hours or 24 hours of flow exposure. Heparinase, on the other hand, inhibited flow-induced migration only after longer term flow exposure.

Heparinase and hyaluronidase inhibit the flow-induced upregulation of MMPs

SN12L1 cells were evaluated at the end of the migration period to determine if gene expressions for MMP-1 and MMP-2 were altered after treatment with hyaluronidase or heparinase (Figure 5B, 5C). Hyaluronidase treatment effectively blocked the upregulation of MMP-1 in both the 4-hour and 24-hour flow experiments. Flow-induced MMP-2 expression was also blocked in the 4-hour experiment (Figure 5B), however, there was no effect of hyaluronidase on MMP-2 for the 24-hour flow case (Figure 5C). On the other hand, heparinase treatment effectively blocked the upregulation of MMP-2 in both 4-hour and 24-hour flow experiments. Heparinase failed to block MMP-1 expression in the shorter-term flow experiment (Figure 5B), but blocked the flow-induced MMP-1 gene expression in the 24-hour flow experiment (Figure 5C).

DISCUSSION

This study demonstrated, for the first time, that mechanotransduction of flow forces via glycocalyx components (hyaluronan and heparan sulfate glycosaminoglycans) can enhance the invasion potential of tumor cells. Fluid flow changed the invasive potential of metastatic cells through activation of MMPs, adhesion molecules, receptors, and integrins, acting through multiple signaling pathways. Physiologic flow velocities and shear stress levels applied to the SN12C and SN12L1 cells induced heterogeneous responses in the cells in a manner similar to *in vivo* experiments (14, 15). Exposure to physiologic levels of interstitial flow and shear stress increased the migration of the SN12L1 and the MDA-MB-435S cells, but not the SN12C cells (lower metastatic rates *in vivo*; Figure 1A). The flow-induced migration trends observed in this study are consistent with the reported dissemination of implanted renal cell carcinomas: SN12L1 cells are more metastatic *in vivo* (15), and the present study demonstrates that the glycocalyx mediates the flow - induced phenomenon.

In previous work with minimally invasive glioma cells we showed that interstitial flow downregulates MMP expression and suppresses migration (6). In contrast, the metastatic renal carcinoma cells in the present study responded to flow by increasing MMP activity and gene expression (Figures 1B, 3) which likely contributed to matrix degradation and higher invasion rates; blocking MMPs inhibited the enhanced migration (Figure 2). Flow-induced upregulation of MMP-13 and migration have been shown previously in rat smooth muscle cells (12). In U87 glioma cells flow suppressed MMP-1 expression, and in CNS-1 glioma cells flow downregulated MMP-2 expression (6, 12). Clearly, flow effects on migration are dependent on cell type (6, 12), but MMPs are key mediators of invasion in all cell types published to date.

Integrin (α3), CD44, and caveolin were also investigated since they were shown to be involved in differentiating the metastatic potential of SN12C and SN12L1 cells *in vivo* (15). α3 integrin and CD44 are adhesion-related receptors that interact with a variety of extracellular matrix proteins (15, 27). When exposed to flow, SN12L1 – but not SN12C – cells upregulated expression of α3 integrin and CD44 (Figure 1C), suggesting that cell/cell and cell/matrix interactions may be involved in the enhanced migration. Out of the four integrins (α3, α5, α6, β3) investigated by Bockhorn et al, only α3 integrin gene expressions differed between cells with altered metastatic potentials (15). Other studies have also suggested that α3 integrin can interact with collagens, laminins, MMPs, and other key proteins to enhance tumor invasion (38–40). Indeed, inhibitors of α3 integrin and CD44 suppressed flow-induced migration after four hours of flow (Figure 2A).

Interestingly, CD44, α3 integrin, and caveolin returned to baseline during the no-flow migration period, indicating that the activation of these genes required sustained exposure to flow (Figure 3A). Furthermore, SN12L1 cells appeared to become desensitized to flow exposure over time with respect to CD44, α3 integrin and caveolin expression: there was dramatic upregulation at 4 hours of flow exposure, but this returned to baseline or below at 24 hours (Figure 3B). In contrast, MMP-1 and MMP-2 gene expression were upregulated regardless of the time of exposure to flow, suggesting sustained augmentation of invasion rates. MMP-1 expression dropped significantly during the migration period following 24

hours of flow (Figure 3C), again indicating that flow is required to sustain upregulation of this gene. The dramatic drops in gene expression for CD44, α3 integrin, and caveolin observed in this study are consistent with the decrease in gene expression for the SN12L1 cells shed from a tumor compared to primary tumor cells (15).

The functional studies were consistent with the gene expression results. The most significant suppression in migration was brought about by inhibiting either MMPs or CD44 (Figure 2). CD44 is known to interact with the surrounding matrix, specifically hyaluronan, and CD44 isoforms interact with proteoglycans (27). Hyaluronan/CD44 interaction directly affects actin polymerization/cytoskeleton rearrangement, MMP signaling, and a number of processes affecting tumor progression (28, 32). Hyaluronan/CD44 interactions are also known to provide resistance against tumor therapeutics and if disrupted could result in better prognosis (26). The present work suggests that the hyaluronan/CD44 signaling pathway may also promote invasion through flow-sensory mechanisms.

This study suggests that the intrinsic ability of the cells to actively invade may be influenced by how cells detect the forces exerted by fluid flow. A potential mechanism is transmission of the force by the cell surface glycocalyx. We previously demonstrated that flow-induced migration could be knocked down in smooth muscle cells by degrading heparan sulfate proteoglycans (26). Syndecans and glypicans (cell-surface proteoglycans associated with heparan sulfate) can promote cell signaling, focal adhesion kinase phosphorylation, tumor growth, and migration (32). Hyaluronan binds to the transmembrane receptor CD44 and can initiate intracellular signaling (4). Therefore it is possible that hyaluronan and heparan sulfate glycosaminoglycans serve as mechanosensors for flow.

Labeling cells for surface heparan sulfate or hyaluronan is a standard technique to visualize specific glycosaminoglycans which compose the overall glycocalyx (41). Hyaluronan is known to be present around cells in the tumor microenvironment (27, 42). Furthermore, we found that heparan sulfate, a key component of the cellular glycocalyx, surrounds individual SN12L1 tumor cells *in vitro* (Figure 4A) similar to that seen associated with smooth muscle cells (Supplemental Figure 5) (26). Degrading the cellular glycocalyx using hyaluronidase or heparinase blocked the flow-enhanced invasion of SN12L1 cells (Figure 5A). These findings strongly suggest that heparan sulfate and hyaluronan play essential roles in the mechanotransduction pathways involved in tumor invasion. It is not yet clear if each drives a distinct downstream signaling pathway since degradation of heparan sulfate or hyaluronic acid is expected to leave other glycocalyx components intact (43). It has been shown in endothelial cells that degradation of either heparan sulfate or hyaluronic acid with specific enzymes can block shear-induced nitric oxide (4). And in this case both hyaluronidase and heparinase blocked the flow-enhanced upregulation of MMPs after flow exposure in a mixed manner (Figure 5). In glioma cells MMP-1 and MMP-2 were independently regulated by shear; U87 cell migration was modulated by MMP-1 and CNS-1 cell migration by MMP-2 (6). In this study the combined response of MMP-1 and MMP-2 gene expressions suggests a possibility of an integrated mechanism in some tumor cell lines - an important topic for continued research.

Other evidence exists to support our conclusion that glycocalyx components transmit mechanical signals. It has been demonstrated recently through a mathematical model that the glycocalyx can sense interstitial flow and transmit solid stresses to the cell membrane that are one to two orders of magnitude higher than fluid shear stress (25). The transmission of these forces to the cell membrane and cytoskeleton can induce mechanotransduction regulating cell function. The breakdown of heparan sulfate has been previously shown to inhibit flow-induced upregulation of MMP-13 in rat smooth muscle cells (26). Additionally, in glioma invasion, hyaluronan has been linked to MMP secretion, matrix degradation, and cell motility (44). Therefore the role of flow-induced cell signaling in glioma cell migratory activity may also be explained by mechanotransduction through glycocalyx components (6).

This study suggests that in addition to biochemical factors known to promote invasion, exposure to enhanced flow in the tumor microenvironment can also facilitate metastasis. There are additional factors *in vivo* that could affect invasion with flow such as regions of hypoxia and recruitment of stromal cells which have been shown to augment MMP-1 and MMP-2 activities, similar to the effects reported here (8–10, 17, 23, 45–50). Furthermore, it remains unclear how antiangiogenic therapy and vasculature normalization, which alter the interstitial fluid flow forces (10, 51), would affect the varied invasive potential and growth of tumors. It will be vital to incorporate the findings of the present study in *in vivo* models to test how flow and flow-altering therapies affect invasion. The findings discussed herein will be valuable for future studies of interstitial flow effects on the migratory activity of tumor cells that may be utilized in the identification of novel therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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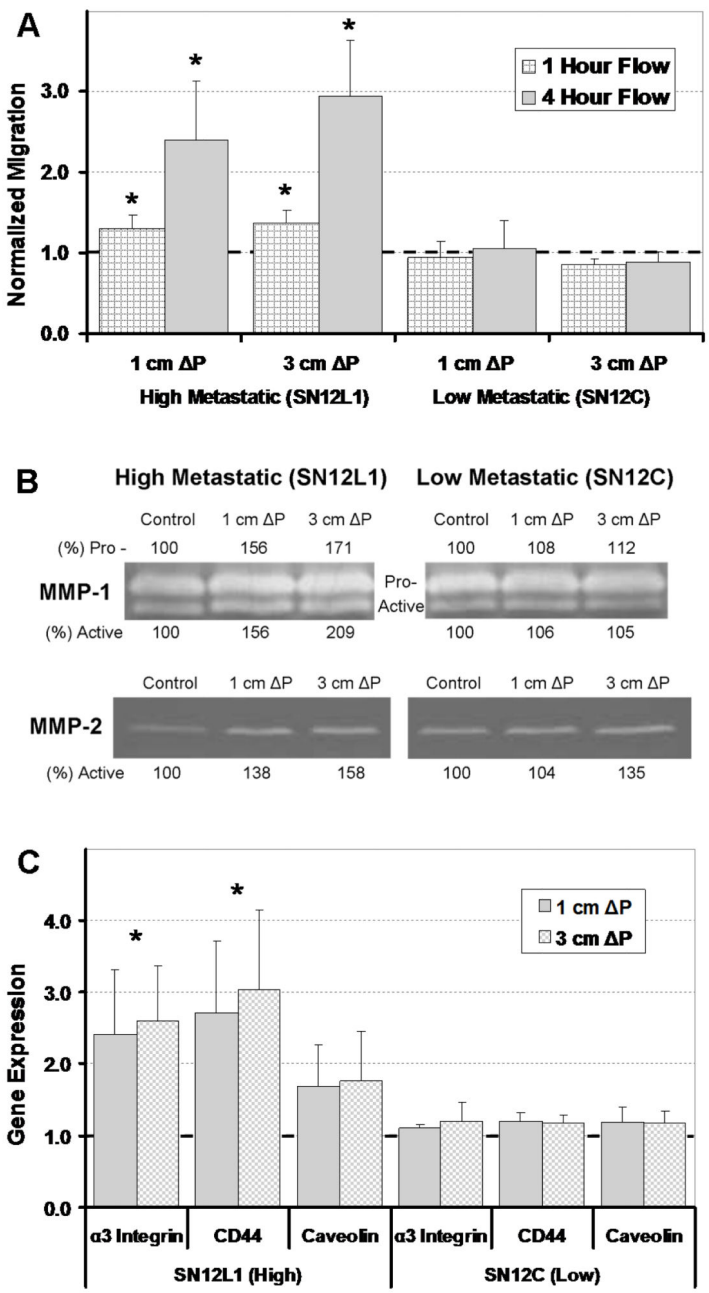

Figure 1.

Migration response of highly metastatic SN12L1 cells and lower metastatic SN12C cells, representative zymography gels, and expression of key genes involved in motility and migration. All results were normalized to no-flow controls (1.0 or 100%). ΔP = 1 cm corresponds to a shear stress of 0.84 dynes/cm^2; ΔP = 3 cm corresponds to 2.53 dynes/cm^2. (A) Exposure to 4 hours of interstitial flow elicited a heterogeneous response - SN12L1 migration was enhanced (increased invasion potential) while the SN12C cell line was not responsive. (B) Representative collagen and gelatin zymography gels are presented with

quantified MMP expression. Flow caused a marked increase in both pro- and active MMP-1 expression in the SN12L1 cells, and little effect on SN12C cells. (C) Exposure to 4 hours of flow (without additional migration) enhanced α3 integrin and CD44 gene expression in SN12L1 cells, but not in SN12C cells. Note: * $p < 0.05$; N = 3–10.

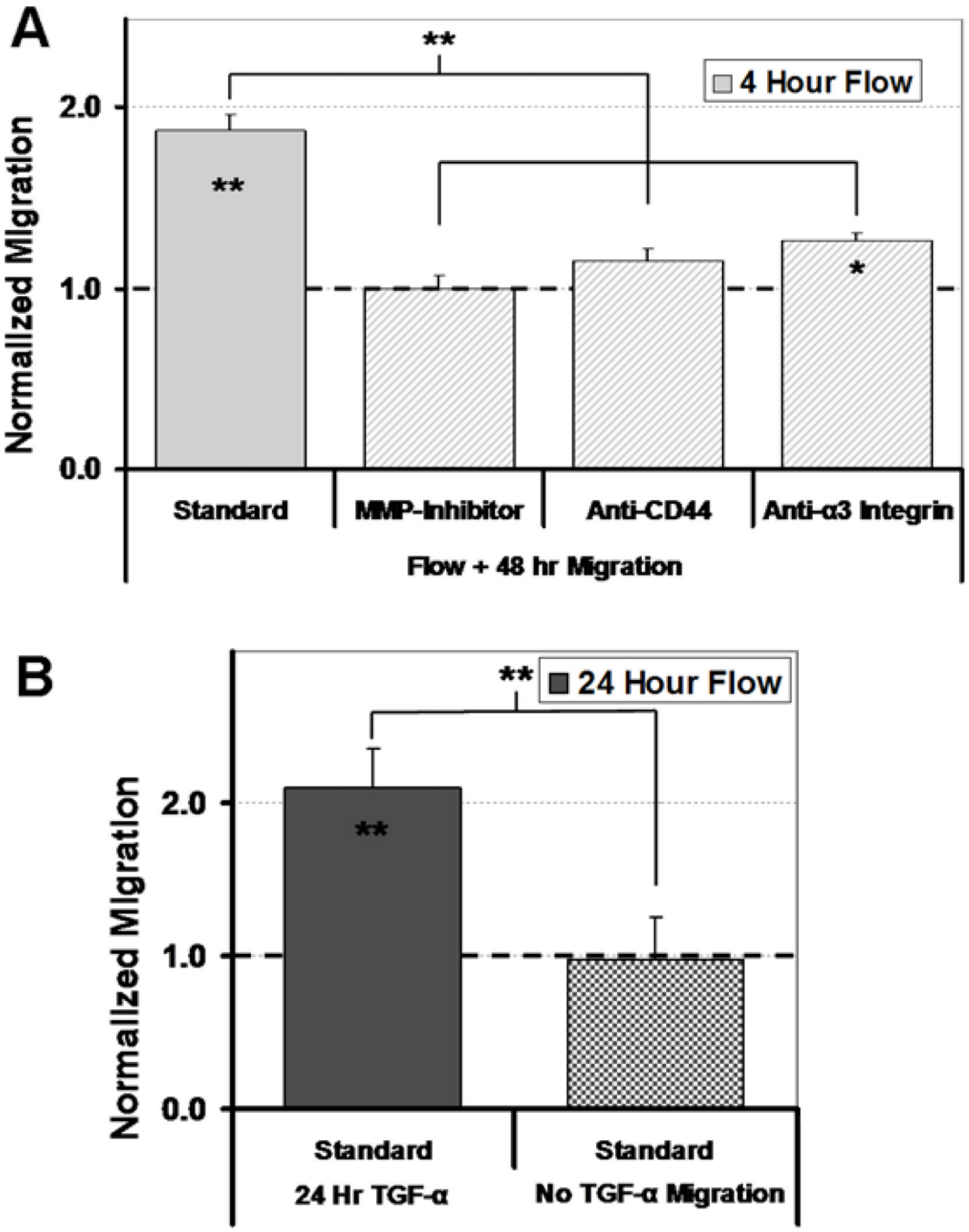

Figure 2.

Migration response of the SN12L1cell line after exposure to flow in the presence of MMP, CD44, or α3 integrin inhibitors. All results were normalized to their respective no-flow plus treatment controls (1.0) and compared to the "Standard" – no inhibitor or antibody case. (A) 4 hours of flow (short term treatment) enhanced SN12L1 invasion rates and inhibiting MMPs, CD44, or α3 integrin reduced the flow-induced migration response to near baseline levels. (B) 24 hours of exposure to flow enhanced invasoin rates as quantified after the no-

flow migration period. Cell migration without the chemoattractant TGF-α in the companion well yielded no enhancement of migration. Note: * $p < 0.05$, ** $p < 0.005$; N = 3–10.

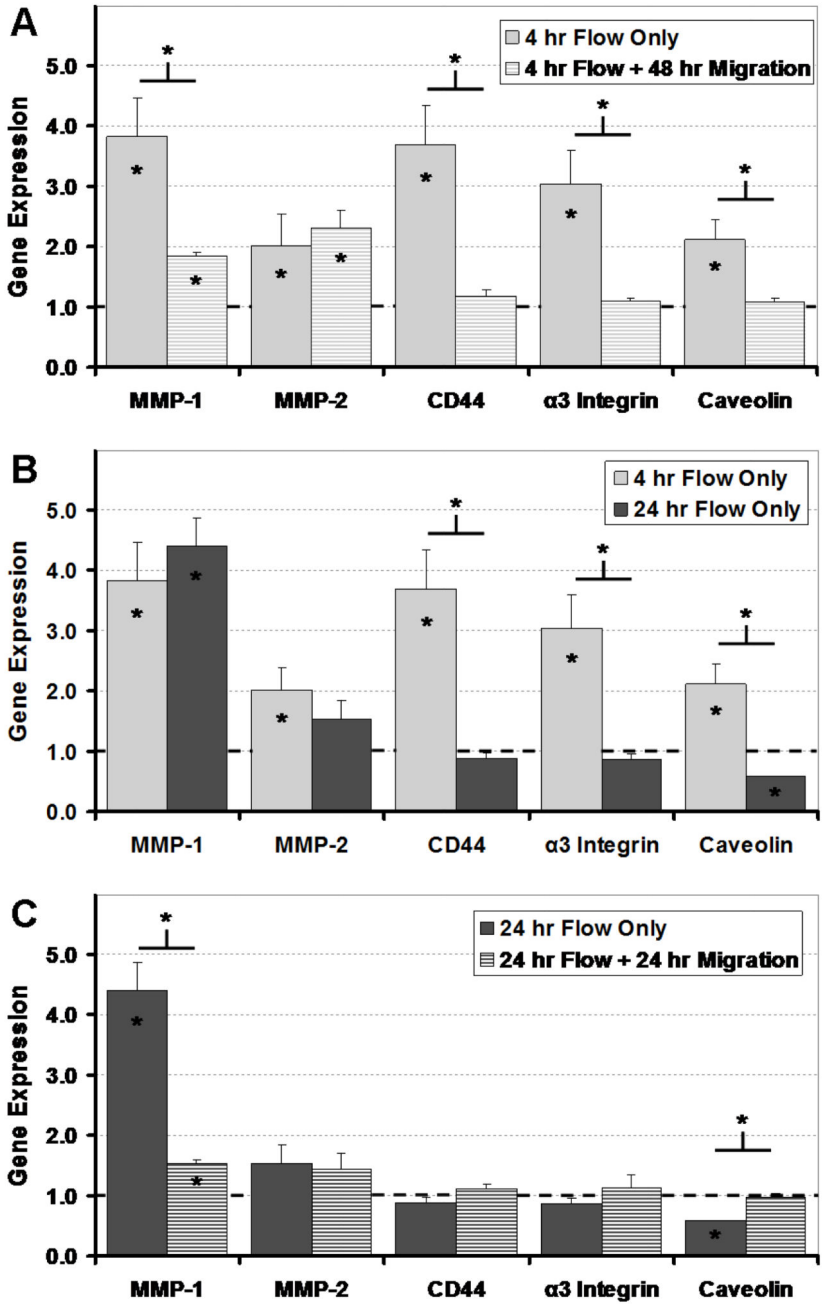

Figure 3.

Modification of SN12L1 MMP-1, MMP-2, CD44, α3 integrin, and caveolin gene expression after exposure to and removal of flow (0.84 dynes/cm^2 shear stress). All results were normalized to gene expression in cells from no-flow control gels (1.0). (A) With exposure to 4 hours of flow, all genes were elevated significantly; only MMP-1 and MMP-2 gene expression remained elevated after the 48 hour migration period (no flow). (B) Gene expression varied with time of exposure to flow. MMP-1 expression remained elevated in response to 24 hour exposure to flow. However, MMP-2, CD44 and α3 integrin gene

expression returned to near baseline levels as the flow period was lengthened. Caveolin gene was suppressed below baseline with longer term exposure to flow. (C) MMP-1 expression decreased significantly during the migration period but was still elevated above baseline. Note: * $p < 0.05$; N = 3–10.

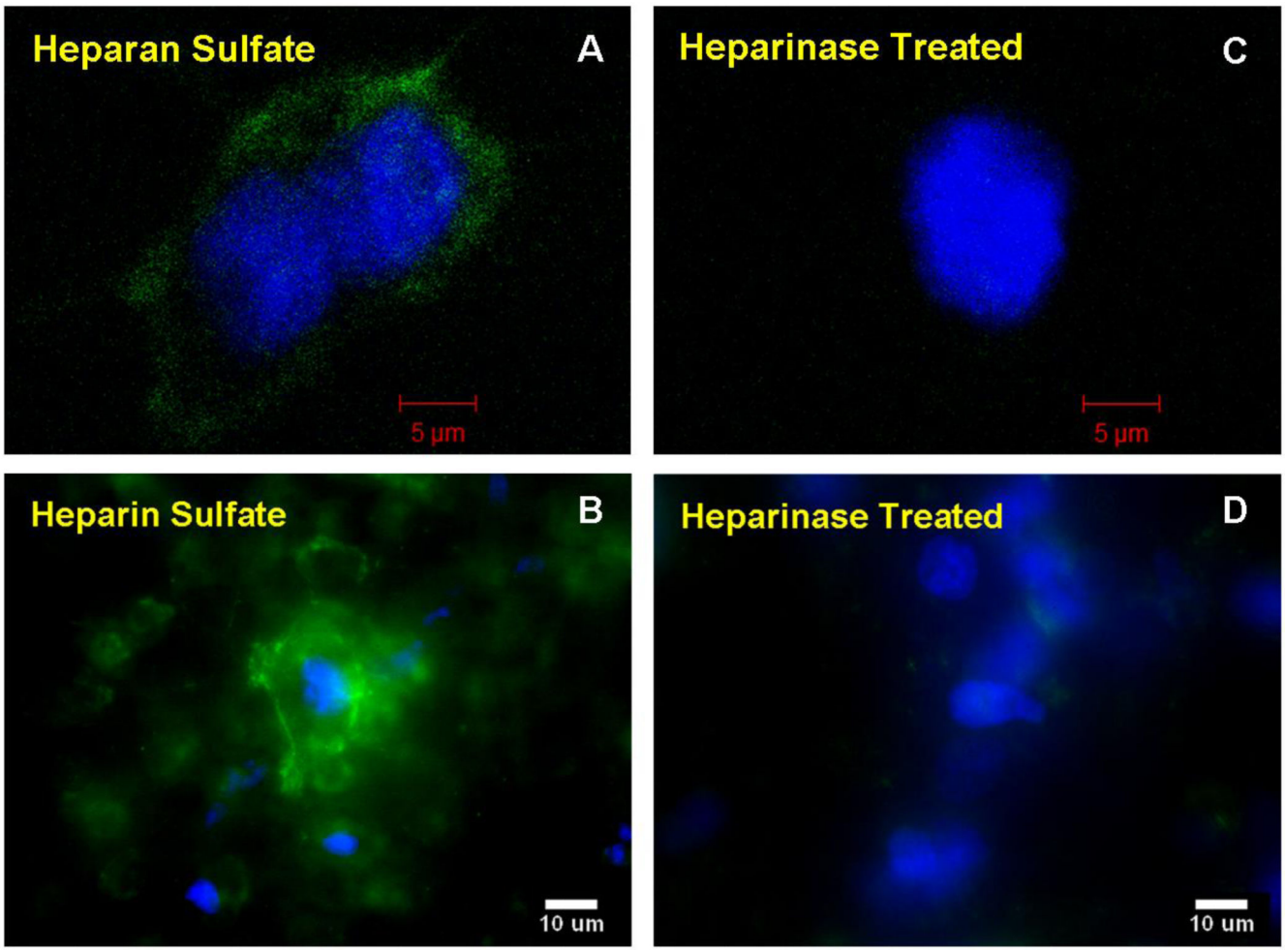

Figure 4.

Heparan sulfate staining of SN12L1 renal carcinoma cells in 3D collagen gels. (A-B) Immunostaining of SN12L1 cells shows that heparan sulfate proteoglycans are present on metastatic tumor cells. (A) Staining of single tumor cells shows that a layer of proteoglycans surrounds the cell periphery. (B) Staining of a group of cells. (C-D) Treatment with 16 hours of heparinase III degraded heparan sulfate. Note: heparan sulfate stained green; nuclei stained blue.

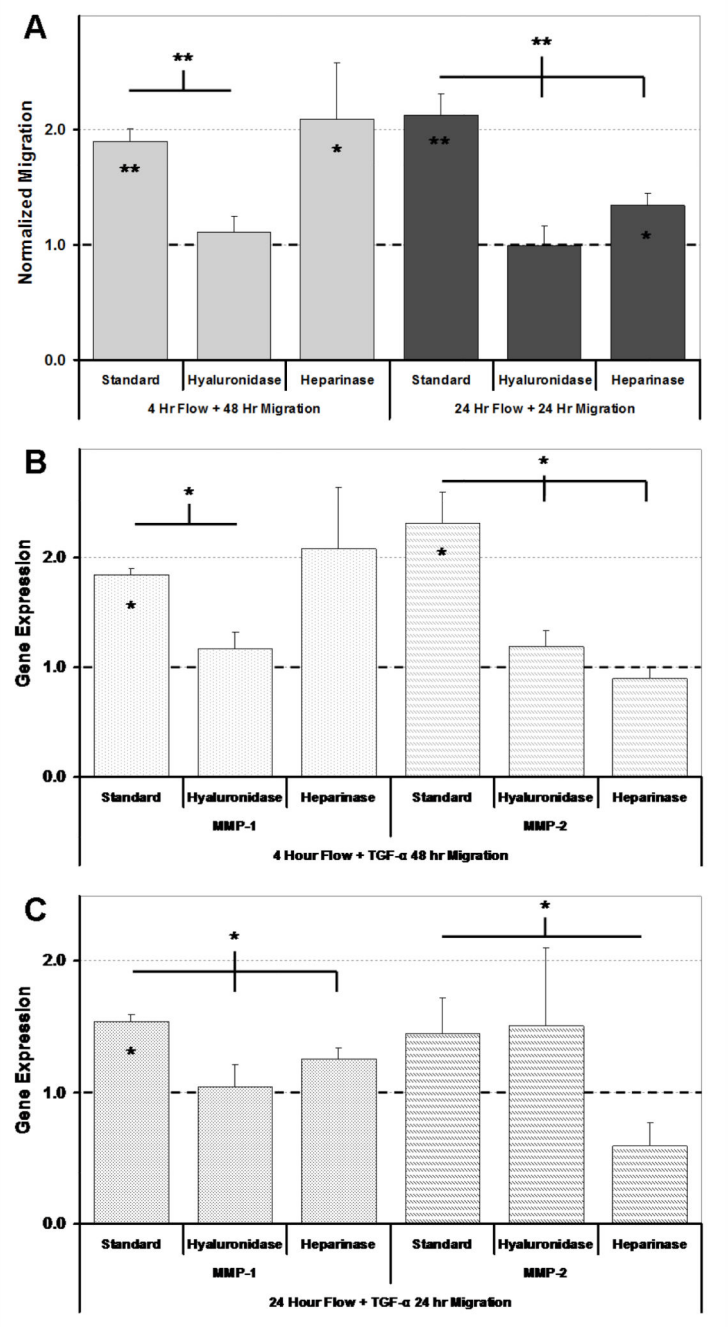

Figure 5.

Hyaluronidase and heparinase inhibit the flow-enhanced invasive potential of the SN12L1 metastatic renal carcinoma cells (0.84 dynes/cm^2 shear stress). All results were normalized to respective no-flow controls (1.0). “Standard” – no enzyme. (A) For the short-term 4 hour flow experiment, hyaluronidase inhibited the flow-induced migration and maintained migration at baseline levels. For the longer-term 24 hour flow experiments, both hyaluronidase and heparinase inhibited the flow-induced migration to near baseline levels. (B-C) Hyaluronidase blocked the 4 hour flow-induced upregulation of MMP-1 and MMP-2

whereas heparinase only blocked MMP-2 upregulation. MMP-1 gene expression correlated with the migration rate after the short time exposure to flow. Both hyaluronidase and heparinase inhibited the flow-induced upregulation of MMP-1 after 24 hours of flow, again consistent with the suppression of migration. Note: * $p < 0.05$, ** $p < 0.005$; $N = 3–9$.