Human Cancer Biology

Clinical Cancer Research

Increased Circulation of Galectin-3 in Cancer Induces Secretion of Metastasis-Promoting Cytokines from Blood Vascular Endothelium

Chen Chen, Carrie A. Duckworth, Qicheng Zhao, David Mark Pritchard, Jonathan M. Rhodes, and Lu-Gang Yu

Abstract

Purpose: Cytokines such as interleukin (IL)-6 and granulocyte colony-stimulating factor (G-CSF) are important metastasis promoters. This study has investigated the functional significance of the increased circulation of galectin-3, a common feature in patients with cancer and in particular those with metastasis, on cytokine secretion from the blood vascular endothelium in cancer.

Experimental Design: The effects of galectin-3 on secretion of cytokines from human microvascular lung endothelial cells were assessed *in vitro* by cytokine array and *in vivo* in mice. The consequences of galectin-3–induced cytokine secretion on endothelial cell behaviors were determined, and the relationship between the levels of circulating galectin-3 and cytokines in patients with colorectal cancer with and without metastasis was investigated.

Results: Galectin-3 at pathologic concentrations found in patients with cancer induces secretion of IL-6, G-CSF, sICAM-1, and granulocyte macrophage colony-stimulating factor from blood vascular endothelial cells *in vitro* and in mice. These cytokines autocrinely/paracrinely interact with the vascular endothelium to increase the expressions of endothelial cell surface adhesion molecules integrin$\alpha_v\beta_1$, E-selectin, ICAM-1, and VCAM-1, resulting in increased cancer cell–endothelial adhesion and increased endothelial cell migration and tubule formation. In patients with metastatic colon cancer, higher serum galectin-3 levels correlated significantly with increased serum G-CSF, IL-6, and sICAM1 concentrations.

Conclusion: The increased circulation of galectin-3 in patients with cancer induces secretion of several metastasis-promoting cytokines from the blood vascular endothelium that enhances endothelial cell activities in metastasis. Targeting the actions of circulating galectin-3 in patients with cancer therefore represents a promising therapeutic strategy to reduce metastasis and improve survival. *Clin Cancer Res; 19(7); 1693–704. ©2013 AACR.*

Introduction

Adhesion of disseminating tumor cells to the blood vascular endothelium and endothelial cell migration and tubule formation are critical steps in the cancer metastasis cascade.

Galectin-3 is a galactoside-binding protein that is expressed by many types of human cells and is found intracellularly, on the cell surface, as well as in the circulation. Intracellular galectin-3 is an apoptosis inhibitor and mRNA splicing promoter (1), whereas cell surface-associated extracellular galectin-3 acts as an adhesion molecule in cell–cell and cell–matrix interactions and facilitates cancer progression and metastasis (2). Recent studies have revealed that the concentration of circulating galectin-3 is increased up to 31-fold in the bloodstream of patients with various cancers including breast, colorectal (3), lung (4), bladder (5), head and neck (6), and melanoma (7). Patients with metastatic disease have higher concentrations of circulating galectin-3 than those with only localized tumors.

Recently, we have shown that the increased circulation of galectin-3 in cancer promotes cancer metastasis in an animal model (8). We showed that this effect of galectin-3 is partly attributed to its interaction with the oncofetal Thomsen-Friedenreich carbohydrate (Galβ1,3GalNAcα-, TF) antigen on the transmembrane mucin protein MUC1 expressed by cancer cells (9). The galectin-3-TF/MUC1 interaction induces MUC1 cell surface polarization leading to exposure of underlying adhesion molecules, thus resulting in increased tumor cell heterotypic adhesion to blood

Authors' Affiliation: Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom

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Corresponding Author: Lu-Gang Yu, Department of Gastroenterology, University of Liverpool, Liverpool L69 3GE, United Kingdom. Phone: 44-151-794-6820; Fax: 44-151-794-6825; E-mail: lgyu@liv.ac.uk

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Translational Relevance

Disseminating tumor cell adhesion to blood vascular endothelium and endothelial cell migration and tubule formation are important steps in the metastasis cascade. This study shows that circulating galectin-3, whose concentration is increased up to 31-fold in patients with cancer and in particular those with metastasis, induces the secretion of several well-known [e.g., interleukin (IL)-6 and granulocyte colony-stimulating factor (G-CSF)] metastasis-promoting cytokines from the blood vascular endothelium *in vitro* and *in vivo*. These cytokines autocrinely/paracrinely interact with the vascular endothelium to enhance the expression of endothelial cell surface adhesion molecules, resulting in increased cancer–endothelial adhesion and increased endothelial migration and tubule formation. This indicates that the increased circulation of galectin-3 commonly seen in various types of cancers can have profound influence on metastasis through induction of metastasis-promoting cytokines from the blood vascular endothelium. Targeting the actions of circulating galectin-3 therefore represents a promising therapeutic strategy to reduce metastasis and improve survival.

vascular endothelium and increased tumor cell homotypic aggregation in the circulation (10). Our studies also showed that, in addition to interaction with cancer-associated MUC1, circulating galectin-3 has other as yet unidentified MUC1-independent actions that contribute considerably to its effect on metastasis promotion (8).

We reveal in this study that the increased circulation of galectin-3 in cancer induces secretion of several metastasis-promoting cytokines from the blood vascular endothelium that enhances endothelial cell activities in metastasis.

Materials and Methods

Materials

Recombinant human galectin-3, interleukin (IL)-6, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), ICAM-1 and human cytokine ELISA kits, mouse sICAM-1 ELISA kit, and human Cytokine Protein Array were purchased from R&D Systems. Calcein AM Cell Labeling Solution was from Invitrogen. Mouse recombinant IL-6, G-CSF, GM-CSF, ICAM-1, and mouse cytokine ELISA kits were from PeproTech. *In Vitro* Angiogenesis Assay Endothelial Cell Invasion kits and *In Vitro* Angiogenesis Tube Formation kits were from AMS Biotechnology Ltd. Non-Enzymatic Cell Dissociation Solution (NECDS) and all other chemicals were from Sigma.

Cell lines

The MUC1-negative HCT116 human colon cancer cells (11) were obtained from the European Cell Culture Collections via the Public Health Laboratory Services (Porton Down, Wiltshire, UK) and cultured in McCoy's5a medium. The MUC1-negative ACA19$^-$ cells selected from human melanoma A375 cells (12) were kindly provided by Dr. John Hilkens (The Netherland Cancer Institute) and cultured in Dulbecco's Modified Eagles Medium (DMEM). (No authentication of the cell lines was done by the authors.) Human microvascular lung endothelial cells (HMVEC) and human umbilical vein endothelial cells (HUVEC) were obtained from Lonza and cultured in EGM-2 endothelial growth media and supplements (EGM-2 Bulletkits, Lonza). Less than 5 passages of the endothelial cells were used in all experiments.

Human serum samples

Fifty serum samples from patients with colorectal cancer, 39 without clinically detectable metastasis and 11 with liver metastasis were obtained from CTBRC cancer tissue bank (Liverpool, UK; Supplementary Table S1). Serum samples had been obtained from patients at the time of primary tumor resection at the Royal Liverpool University Hospital.

Human cytokine array

HMVECs (1×10^5 cells/well) were cultured in 6-well plates, or ACA19$^-$ cells (5×10^5 cells/ml) were cultured in 6-well plates precoated with poly-HEMA (10) for 24 hours before introduction of galectin-3 or control bovine serum albumin (BSA) for 24 hours at 37°C. The culture media were collected and cytokine concentrations were analyzed using Human Cytokine Protein Arrays as per the manufacturer's instructions. These arrays assayed 36 cytokines (C5/C5a, CD40 Ligand, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α, sTREM-1), each in duplicate. The arrays were quantified with BioRad Image Lab software.

Serum galectin-3 assay

Serum galectin-3 concentrations in patients with colorectal cancer were determined by galectin-3 ELISA as described in our previous study (3).

Assessment of cancer cell–endothelial adhesion

HMVEC monolayer was treated with galectin-3 or control BSA for 24 hours. The monolayer was washed and used for subsequent assessment of cancer cell adhesion. In other experiments, after treatment of HMVECs with galectin-3 or BSA, the culture media were collected and used for assessment of cancer cell adhesion to fresh HMVEC monolayer.

ACA19$^-$ and HCT116 cancer cells were detached from culture plates with NECDS, washed, and resuspended at 5×10^6cells/mL in DMEM. The cells were labeled with 10 μL/mL Calcein AM at 37°C for 30 minutes, washed, and resuspended at 1×10^5/mL with serum-free DMEM containing 0.5 mg/mL BSA before application (1×10^4cells/well) to HMVEC monolayer for 1 or 24 hours at 37°C. After 2 washes with PBS, the endothelial cell–associated

fluorescence was measured using a fluorescence microplate reader at 485-nm excitation/535-nm emission.

Analysis of cell surface adhesion molecules by flow cytometry

HMVECs were treated with or without galectin-3 for 24 hours before the cells were released with NECDS. The cells were washed with PBS, fixed with 2% paraformaldehyde, and incubated with 5% goat serum/PBS for 30 minutes before application of antibodies against CD44, integrin $\alpha_v\beta_1$ or $\alpha_v\beta_3$, E-selectin, VCAM-1, or ICAM-1 (all at 1:400 dilution) for 1 hour. After wash with PBS and incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:400 in 1% BSA in PBS) for 1 hour, the cells were analyzed by flow cytometry.

In vitro measurement of angiogenesis: (i) endothelial cell invasion and (ii) endothelial tubule formation

HMVECs (1×10^5 cells/well) were cultured in 24-well plates for 24 hours before treatment with galectin-3 for 24 hours. The culture media were collected and used, with or without subsequent introduction of a combination of recombinant cytokines or a combination of neutralizing anti-cytokine antibodies, to assess migration of fresh HMVECs through basement matrix proteins using the *In Vitro* Angiogenesis Assay Endothelial Cell Invasion Kit or to assess HUVECs tubule formation using the *In Vitro* Angiogenesis Assay Endothelial Cell Tube Formation Kit. The length and branch points of tubules formed were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

In vivo measurement of the effect of galectin-3 on cytokine secretion in mice

Nine 6- to 8-week-old female Balb/c athymic mice, obtained from Charles River Laboratories and maintained and used in accordance with the animal care protocol approved by University of Liverpool, were randomly divided into 3 equal groups, and 5 μg/mouse recombinant human galectin-3 (2.5 μg/mL, assuming a 2-mL blood volume) was introduced by intravenous tail vein injection. Blood was obtained by cardiac puncture at 0, 24, and 48 hours, and the serum concentrations of G-CSF, GM-CSF, IL-6, and sICAM-1 were determined by ELISA.

Statistical analysis

Unpaired *t* test was used for single comparison, one-way ANOVA followed by Bonferroni for multiple comparisons, and Spearman rho correlation analysis were used where appropriate. Differences were considered significant when 2-tailed, $P < 0.05$.

Results

Galectin-3 induces secretion of soluble molecules from endothelial cells, but not from cancer cells, that enhance cancer cell–endothelial adhesion

Our previous studies have shown that galectin-3–MUC1 interaction–associated cancer cell adhesion occurs rapidly, within 1 hour (8–10). In searching for the MUC1-independent action of circulating galectin-3, we first investigated whether the presence of galectin-3 for a longer time has any influence on the behavior of MUC1-negative cells. Addition of galectin-3 for 24 hours (Fig. 1A), but not 1 hour (Fig. 1B), at a concentration (1 μg/mL) similar to that in patients with cancer caused a significant increase ($48.2\% \pm 8.5\%$, $P < 0.05$ and $22.9\% \pm 4.4\%$, $P < 0.05$, respectively) in adhesion of MUC1-negative ACA19$^-$ and HCT116 cells to HMVECs. The culture medium obtained from HMVECs treated with galectin-3 for 24 hours also induced a similar increase in adhesion of fresh ACA19$^-$ ($43.0\% \pm 14.3\%$, $P < 0.05$) and HCT116 ($74.4\% \pm 21.2\%$, $P < 0.05$) cells to fresh HMVECs (Fig. 1C), whereas the culture medium from ACA19$^-$ or HCT116 cells treated with galectin-3 showed no effect on subsequent adhesion of fresh ACA19$^-$ or HCT116 cells to fresh HMVECs (Fig. 1D). These results indicate that the presence of galectin-3 for a longer period induced the release of soluble molecules from HMVECs, but not from cancer cells, and that these soluble molecule(s) are largely responsible for the galectin-3–mediated adhesion of MUC1-negative cells to endothelial cells.

Galectin-3 induces endothelial secretion of cytokines that increase cancer cell–endothelial adhesion

As many cytokines, in particular proinflammatory cytokines, such as TNFα, IL-1, and IL-6, are well-known for their prometastatic promotion of cancer cell–endothelial adhesion (13, 14), we investigated whether the soluble molecules secreted by HMVECs in response to galectin-3 and responsible for galectin-3–mediated adhesion of MUC1-negative cells were cytokines. Treatment of HMVECs with galectin-3 (1 μg/mL) for 24 hours resulted in increased concentrations of 4 cytokines: IL-6 (2.1-fold), G-CSF (2.2-fold), GM-CSF (3.2-fold), and sICAM-1 (2.3-fold) in the culture medium (Fig. 2A), whereas treatment of ACA19$^-$ cells with galectin-3 had no significant effect on cytokine abundances in the culture medium when the cytokine profiles were analyzed using a human cytokine assay array (Fig. 2B). This suggests that galectin-3 enhances cytokine secretion from HMVECs but not ACA19$^-$ cells.

The galectin-3–mediated cytokine secretion from HMVECs was both dose-dependent, occurring at galectin-3 concentrations similar to those in the serum of patients with cancer (Fig. 2C), and time-dependent, occurring significantly only after treatment with galectin-3 for more than 20 hours (Fig. 2D). This effect of recombinant galectin-3 was not related to contamination by endotoxin as a 100-fold higher endotoxin concentration (100 EU) than that in the recombinant galectin-3 (<1.0 EU) did not show any effect on secretion of these cytokines (data not shown).

The galectin-3–mediated cytokine secretion was completely inhibited by the presence of the galectin-3 inhibitor lactose (Fig. 3A), whose presence also effectively inhibited galectin-3–mediated ACA19$^-$ cell adhesion to HMVECs (Fig. 3B). To determine whether the galectin-3–induced secretion of these cytokines from HMVECs was responsible for galectin-3–mediated adhesion of

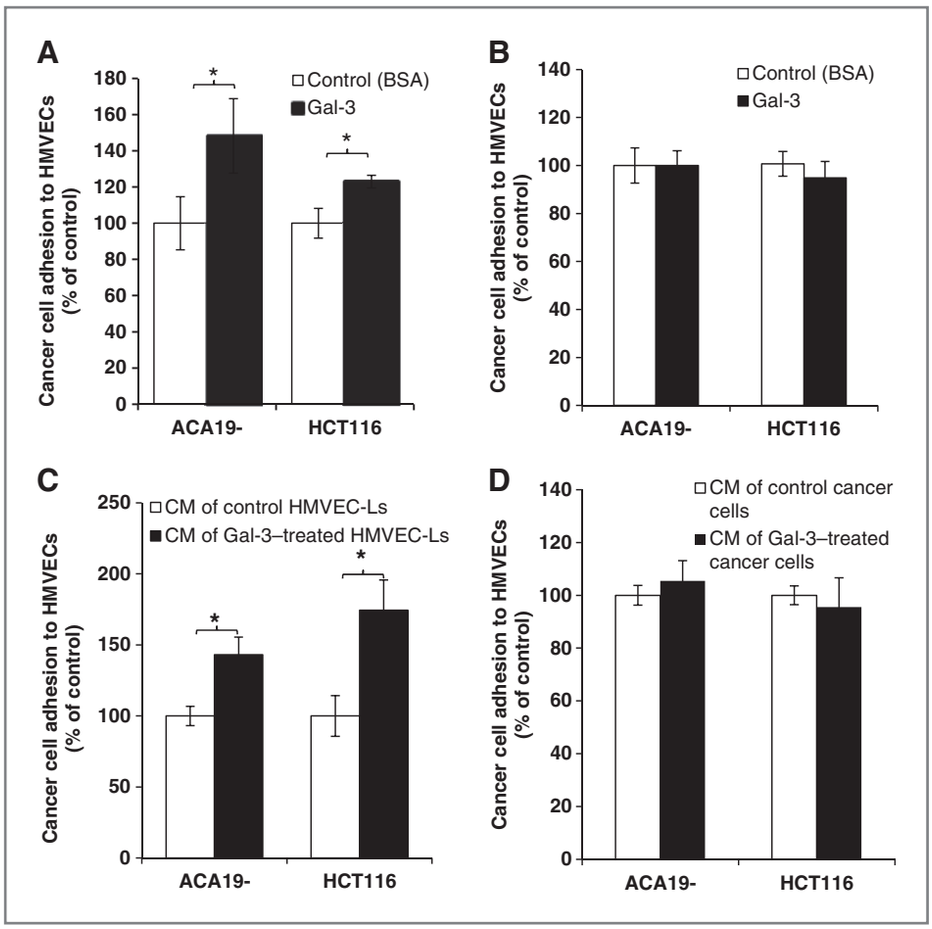

Figure 1. Galectin-3 induces endothelial secretion of soluble molecules that increase cancer cell–endothelial adhesion. Lengthy (24 hours, A) but not short (1 hour, B) presence of 1 μg/mL galectin-3 increases ACA19$^-$ and HCT116 cell adhesion to HMVECs. Galectin-3 induces secretion of soluble molecules from endothelial (C), but not cancer (D), cells that cause cancer cell–endothelial adhesion. The 24-hour culture media (CM) from HMVECs (C), ACA19$^-$ or HCT116 (D) cells treated with or without 1 μg/mL galectin-3 under suspension were used as culture medium to assess adhesion of fresh ACA19$^-$ or HCT116 to fresh HMVEC monolayer. Data are expressed as percentage compared with BSA-treated controls (mean ± SD) from 3 independent experiments, each in triplicate. *, $P < 0.05$.

MUC1-negative cells, we assessed the effect of neutralizing antibodies against these cytokines on galectin-3–mediated ACA19$^-$ cell adhesion. The presence of a combination of anti-G-CSF, GM-CSF, IL-6, and sICAM-1 antibodies completely prevented ACA19$^-$ cell adhesion induced by conditioned medium from galectin-3–treated HMVECs (Fig. 3C). Furthermore, the presence of a combination of recombinant IL-6, G-CSF, GM-CSF, and sICAM-1 in concentrations similar to those in the conditioned medium from 1 μg/mL galectin-3–treated HMVECs (Fig. 2) induced a similar increase of ACA19$^-$ and HCT116 cell adhesion (Fig. 3D) as the conditioned medium from galectin-3–treated HMVECs. These results indicate that galectin-3–induced secretion of IL-6, G-CSF, GM-CSF, and sICAM-1 by HMVECs is essential for galectin-3–induced adhesion of MUC1-negative cancer cells to endothelial cells.

Galectin-3–induced endothelial secretion of cytokines increases expression of endothelial cell surface adhesion molecules that promote cancer cell–endothelial adhesion

To gain insight into the mechanism of the galectin-3–induced, cytokine-mediated cell adhesion, we analyzed the expression of several common cell surface adhesion molecules on HMVECs in response to galectin-3. HMVECs treated with galectin-3 for 24 hours increased the expression of cell surface integrin$\alpha_v\beta_1$ (43%), E-selectin (19%), VCAM-1 (17%), and ICAM-1 (33%), whereas the expression of cell surface CD44 and integrin$\alpha_v\beta_3$ was not affected (Fig. 4A).

To determine whether the increased expression of these endothelial cell surface adhesion molecules by galectin-3 was linked to galectin-3–induced cytokine secretion, the expression of integrin$\alpha_v\beta_1$, the adhesion molecule that showed the most increase in response to galectin-3, was analyzed further. A combination of neutralizing antibodies against G-CSF, GM-CSF, IL-6, and sICAM-1 in the culture medium resulted in 30% reduction of the galectin-3–mediated increase of integrin$\alpha_v\beta_1$ (Fig. 4B). Furthermore, as had been seen with recombinant galectin-3, incubation of HMVECs with a combination of recombinant G-CSF, GM-CSF, IL-6, and sICAM-1 at concentrations similar to those in the conditioned medium of galectin-3–treated HMVECs caused a 40% increase in cell surface integrin$\alpha_v\beta_1$ expression (Fig. 4B). This indicates that the increased expression of endothelial cell surface adhesion molecules by galectin-3 is associated with autocrine/paracrine actions of galectin-3–induced secretion of cytokines on endothelial cells.

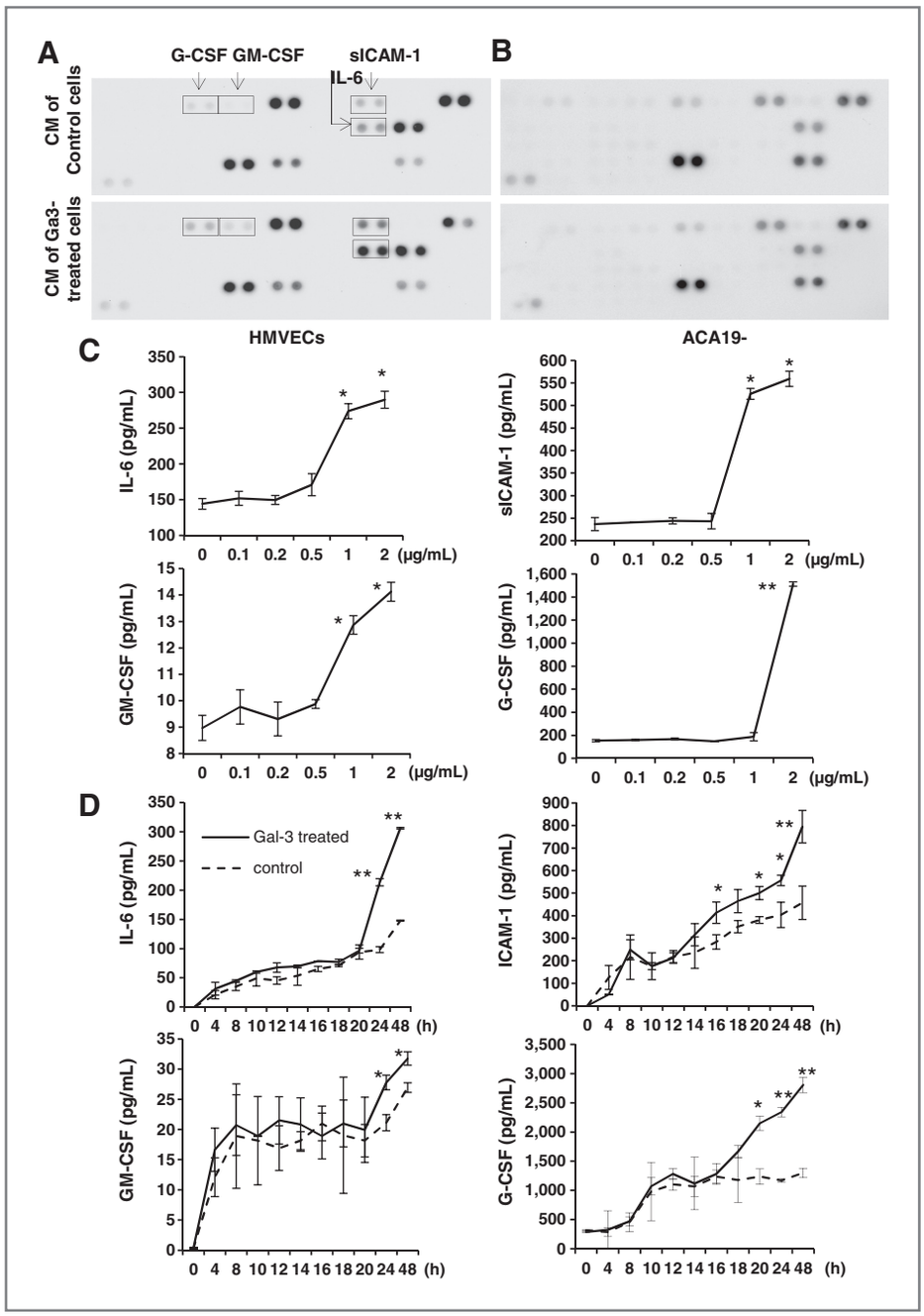

Figure 2. Galectin-3 induces secretion of IL-6, G-CSF, GM-CSF, and sICAM-1 from endothelial but not cancer cells. Cytokine profile in the conditioned medium (CM) of 24-hour 1 μg/mL galectin-3 or BSA-treated HMVECs (A) or ACA19$^-$ cells (B). Galectin-3 (1.5 μg/mL) induces dose- (C) and time- (D) dependent secretion of IL-6, G-CSF, GM-CSF, and sICAM-1 from HMVECs. Data are expressed as mean ± SD of triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The presence of each of these cytokines increased integrin$\alpha_v\beta_1$ expression on HMVECs, albeit to different extents. A 34% increase was observed by G-CSF, 32% by GM-CSF, 17% by IL-6, and 36% by ICAM-1 (Fig. 4C). This suggests that the galectin-3–induced cytokines likely all make a contribution to the galectin-3–mediated increase in expression of endothelial cell surface adhesion molecules

The presence of a combination of neutralizing antibodies against integrin $\alpha_v\beta_1$, E-selectin, VCAM-1, and ICAM-1 caused significant inhibition of galectin-3–associated ACA19$^-$ cell adhesion to HMVECs (Fig. 4D). Collectively, these results indicate that the cytokine-induced expression of endothelial cell surface adhesion molecules in response to galectin-3 is responsible for the increased adhesion of MUC1-negative cells induced by galectin-3.

Galectin-3 promotes endothelial cell migration and microvascular tube formation in angiogenesis

As proinflammatory cytokines, such as IL-6, have previously been shown to promote angiogenesis (15), and the

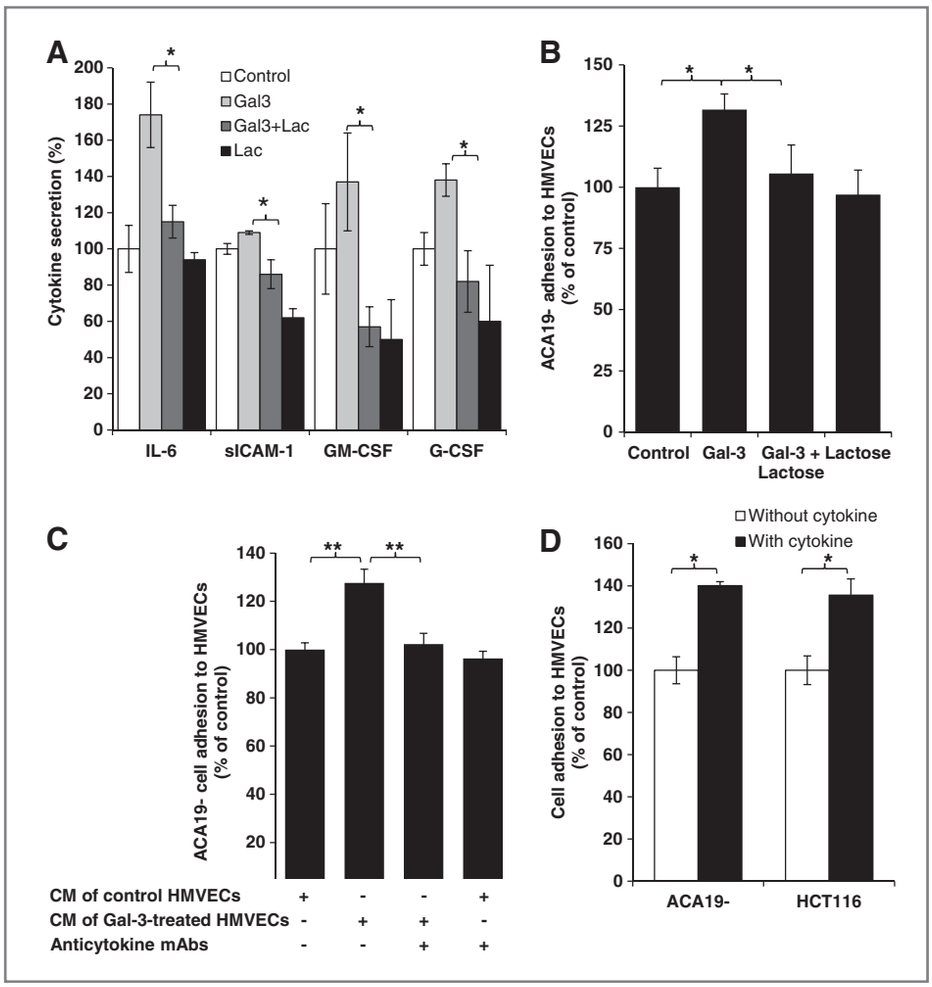

Figure 3. Galectin-3–mediated cytokine secretion increases cancer cell–endothelial adhesion. A and B, galectin-3(1 μg/mL)-mediated cytokine secretion (A) and cancer cell adhesion to HUVECs (B) is inhibited by lactose. C, galectin-3–mediated cancer cell–endothelial adhesion is inhibited by anticytokine neutralizing antibodies. HMVECs were treated with 1 μg/mL galectin-3 or BSA for 24 hours; the culture media were harvested and used for assessing ACA19$^-$ and HCT116 cell adhesion to fresh HMVECs with or without a combination of neutralizing antibodies against G-CSF (25 ng/mL), GM-CSF (300 pg/mL), IL-6 (2 ng/mL), and sICAM-1 (5 ng/mL). D, a combination of recombinant G-CSF (2.5 ng/mL), GM-CSF (30 pg/mL), IL-6 (200 pg/mL), and sICAM-1 (500 pg/mL) increases ACA19$^-$ and HCT116 cell adhesion to HMVECs. The data are expressed as percentage compared with BSA-treated controls from 3 independent experiments, each in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

galectin-3–induced secretion of cytokines from the vascular endothelium included the proinflammatory IL-6 and G-CSF, we further assessed the effect of galectin-3–induced cytokine secretion on endothelial cell migration through basement matrix proteins as well as on endothelial microtubule formation, 2 important components of the angiogenesis process. The conditioned medium from 24-hour galectin-3–treated HMVECs caused a 48.8% ± 2.5% ($P < 0.001$) increase in migration of fresh HMVECs compared with that of BSA-treated control (Fig. 5A). A combination of neutralizing antibodies against G-CSF, GM-CSF, IL-6, and sICAM-1 significantly reduced galectin-3–associated HMVEC cell migration, suggesting that the galectin-3–induced secretion of these cytokines is responsible for the observed increase in HMVEC migration. This was further supported by a similar increase in HMVEC cell migration (63.1% ± 10.6%, $P < 0.001$) when a combination of recombinant G-CSF, GM-CSF, IL-6, and sICAM-1 at similar concentrations as in the conditioned medium from galectin-treated HMVECs was added to the culture.

HUVECs (the most commonly used endothelial cells for investigating *in vitro* tubule formation) cultured in the conditioned medium obtained from 24-hour galectin-3–treated HMVECs showed significant increase in tubule length and branch points compared with HUVECs cultured in the conditioned medium from BSA-treated HMVECs (Fig. 5B–D). These effects of galectin-3 were prevented when lactose was introduced at the same time as galectin-3 or a combination of cytokine neutralizing antibodies was added to the conditioned medium. Moreover, introduction of a combination of recombinant G-CSF, GM-CSF, IL-6, and sICAM-1 to BSA-treated control medium resulted in similar increases in HUVEC tubule length and formation of branch points as that induced by the galectin-3 conditioned medium. Together, these results suggest that galectin-3–induced secretion of cytokines from vascular endothelium also promotes endothelial angiogenesis.

Galectin-3 induces cytokine secretion *in vivo*

When 5 μg/mouse galectin-3, equating approximately to a pathologic circulating galectin-3 concentration seen in patients with cancer with metastasis (3), was injected intravenously into the tail vein, a 45.1% ± 14.4% increase of serum G-CSF, 293.3% ± 93.7% of GM-CSF, 111.1% ±

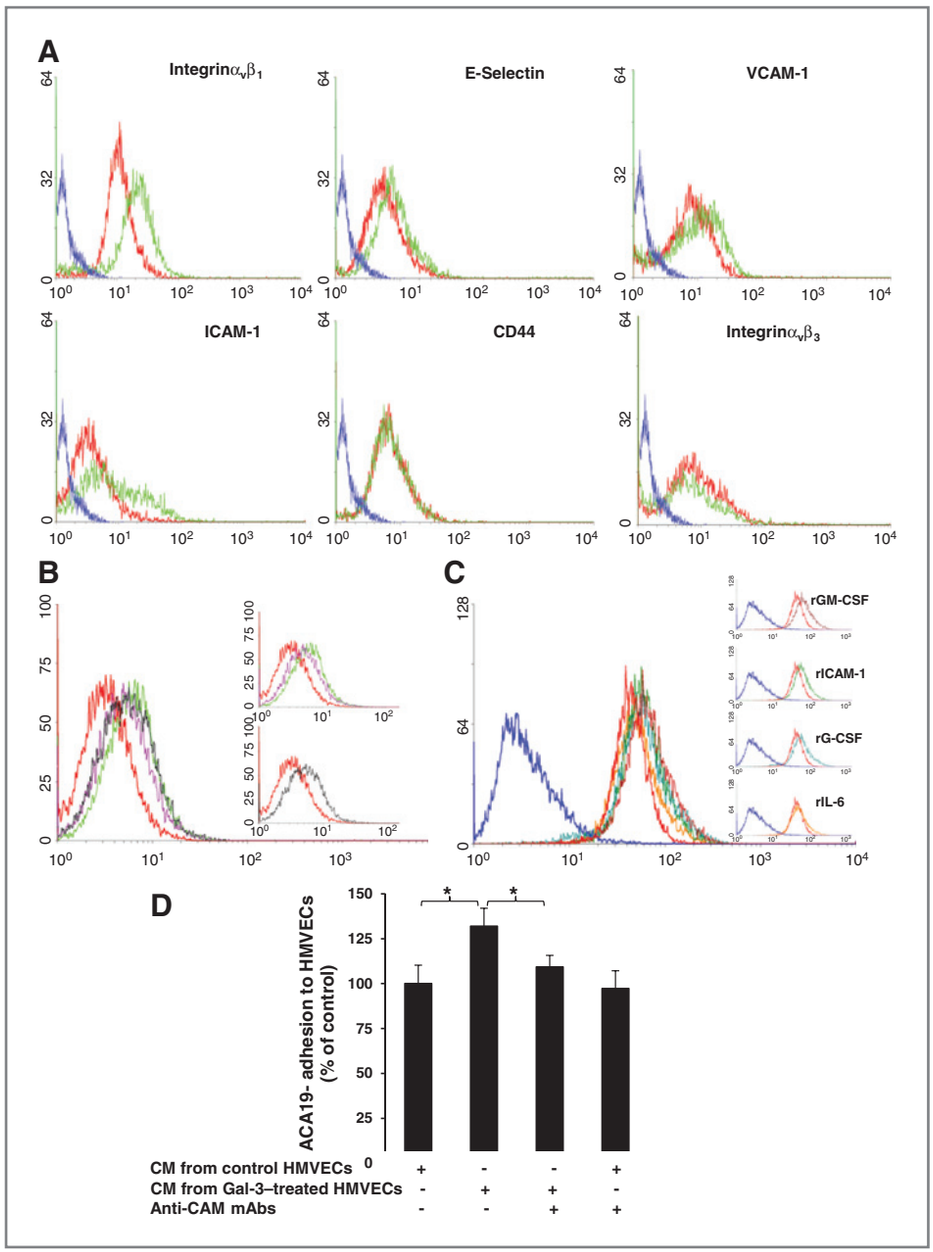

Figure 4. Galectin-3–induced cytokine secretion enhances expressions of endothelial cell surface adhesion molecules which are responsible for galectin-3–mediated cancer cell–endothelial adhesion. A, the presence of 1.5 μg/mL galectin-3 (green) for 24 hours induces expressions of cell surface integrin$\alpha_v\beta_1$, E-selectin, VCAM-1, and ICAM-1 but not CD44 nor integrin $\alpha_v\beta_3$ compared with control 1.5 μg/mL BSA-treated cells (red). B and C, galectin-3–mediated increase of endothelial cell adhesion molecules is the consequence of galectin-3–induced cytokine secretion. HMVECs were treated without (red) or with 1.5 μg/mL galectin-3 in the absence (green) or presence of a combination of neutralizing antibodies against IL-6, G-CSF, GM-CSF, and ICAM-1 (purple), a combination of recombinant IL-6, G-CSF, GM-CSF, or ICAM-1 (black; B) or in the presence of each individual recombinant GM-CSF (dark red), ICAM-1 (green), G-CSF (light blue), or IL-6 (orange; C) for 24 hours before the expression of integrin $\alpha_v\beta_1$ on HMVECs were analyzed. D, the presence of neutralizing antibodies against integrin$\alpha_v\beta_1$ (10 μg/mL), E-selectin (10 μg/mL), VCAM-1 (10 μg/mL), and ICAM-1 (10 μg/mL) inhibits galectin-3 (1.5 μg/mL)–mediated ACA19$^-$ cell adhesion to HMVECs. IgG control shown in blue.

26.7% of IL-6, and 58.4% ± 28.2% of sICAM-1 was observed after 48 hours (Fig. 5E). This provides strong evidence of a direct impact of circulating galectin-3 on secretion of these cytokines *in vivo*.

As circulating galectin-3 might be in complex with serum glycoproteins (16), we also tested whether serum galectin-3 affects endothelial secretion of cytokines. Treatment of HMVECs with high galectin-3–containing human serum caused significantly more IL-6, G-CSF, and GM-CSF secretion than with low galectin-3–containing serum (Supplementary Fig. S1), an effect that was markedly prevented by the presence of lactose. This indicates that circulating galectin-3, although it may sometime be in complex with serum glycoproteins, is still a functionally active molecule in the circulation. This is in keeping with the well-known nature of the weak and reversible binding of galectins to their glycans.

Relationship between circulating galectin-3 and cytokine secretion in colon cancer patients

A significant correlation between circulating galectin-3 concentration was observed with serum G-CSF concentration ($P < 0.05$) but not with the other 3 cytokines when all 50 patients with colorectal cancer (Table 1). However, when patients with and without metastasis were considered separately, significant correlations of galectin-3 levels were

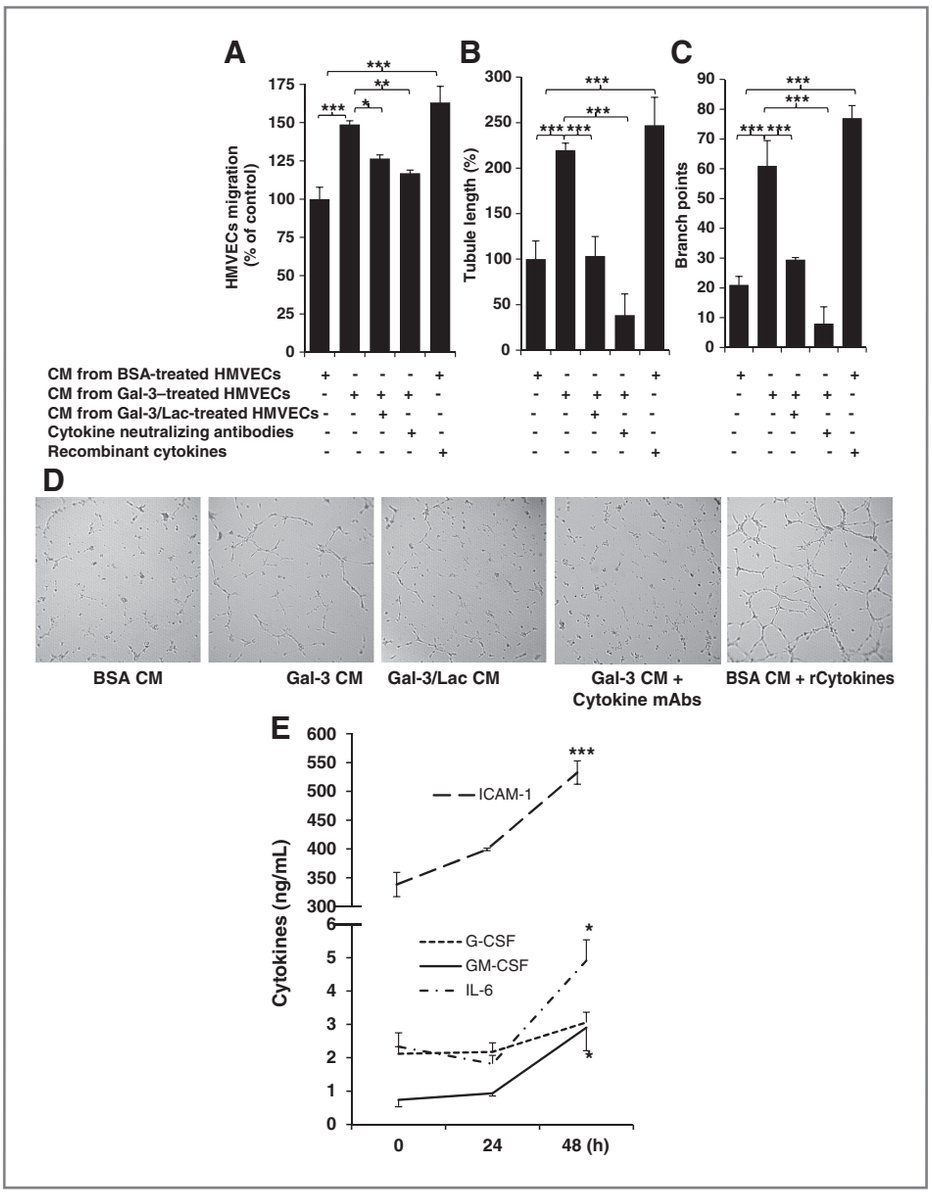

Figure 5. Galectin-3 induces secretion of cytokines *in vivo* and their secretion promotes angiogenesis. Galectin-3–induced cytokine secretion promotes endothelial cell migration (A) and tubule formation (B–D). HMVECs were treated with 1.5 μg/mL BSA or galectin-3 with or without lactose for 24 hours. The culture media were collected and used for subsequent assessment of fresh HMVEC migration through matrix proteins or HUVEC tubule formation, with or without the presence of a combination of antibodies against G-CSF, GM-CSF, IL-6, and sICAM-1 or a combination of recombinant G-CSF, GM-CSF, IL-6, and sICAM-1. Tubule length (B) and branch points (C) were quantified. Data are expressed as percentage compared with BSA-treated controls from 3 independent experiments, each in triplicate. Representative images are shown in D. E, intravenous injection of galectin-3 increases serum concentrations of sICAM-1, G-CSF, GM-CSF, and IL-6 cytokine in mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

observed with G-CSF ($P = 0.04$), IL-6 ($P = 0.05$), and sICAM-1 ($P = 0.005$) only in patients with metastasis. This further supports a role of galectin-3–induced cytokine secretion in metastasis promotion in patients with cancer. Neither the galectin-3 concentrations ($P = 0.16$) nor the concentrations of the 4 cytokines showed statistically significant correlation with the presence of metastasis in these patients (G-CSF, $P = 0.74$; IL-6, $P = 0.06$, sICAM-1, $P = 0.64$; GM-CSF, $P = 0.74$). This is in keeping with a recent report showing that neither galectin-3 nor -4 levels, when analyzed individually, were a marker of metastasis in colorectal cancer but a combined analysis of galectin-3 and -4 concentrations detects metastasis in these patients (17).

Discussion

This study shows that galectin-3 at pathologic concentrations induces secretion of IL-6, sICAM-1, G-CSF, and GM-CSF from blood vascular endothelium *in vitro* and *in vivo*. These cytokines autocrinely/paracrinely interact with the endothelium to enhance expression of endothelial cell surface adhesion molecules, resulting in increased cancer cell–endothelial adhesion and increased endothelial cell migration and tubule formation, all important steps of the metastasis cascade. This likely represents a very important mechanism for the MUC1-independent action of circulating galectin-3 on metastasis promotion (8). Such a conclusion is supported by the observed correlations between levels of circulating galectin-3 and

Table 1. Relationships between serum galectin-3 and IL-6, GM-CSF, G-CSF, and sICAM-1 in patients with colon cancer with and without metastasis

	Serum concentrations, median (range, ng/mL)	Spearman' rho correlation coefficient	*P*
All patients ($N = 50$)			
Gal-3	106.8 (7.5–5,106.3)		
IL-6	5.0 (2.1–287.8)		
GM-CSF	15.8 (5.4–833.3)		
G-CSF	43.0 (11.0–13,266.0)		
sICAM1	14,442.0 (7593.5–30,235.9)		
Patients without metastasis ($n = 39$)			
Gal-3	82.0 (7.5–1,603.9)		
IL-6	5.2 (2.7–72.1)		
GM-CSF	14.8 (5.4–1,57.1)		
G-CSF	33.1 (11.1–3,973.0)		
sICAM1	15,148.1 (8,414.1–30,235.9)		
Patients with metastasis ($n = 11$)			
Gal-3	215.0 (30.9–5,106.3)		
IL-6	3.5(2.1–287.8)		
GM-CSF	15.8 (12.0–833.3)		
G-CSF	29.0 (15.0–13,266.0)		
sICAM1	12,516.7 (75,93.5–21,621.2)		
All patients			
Gal-3 and IL-6			
Gal-3 and GM-CSF		0.17	0.12
Gal-3 and G-CSF		0.24	0.045
Gal-3 and sICAM-1		−0.11	0.22
Patients without metastasis			
Gal-3 and IL-6		0.12	0.23
Gal-3 and GM-CSF		0.11	0.26
Gal-3 and G-CSF		0.19	0.13
Gal-3 and sICAM-1		−0.056	0.37
Patients with metastasis			
Gal-3 and IL-6		0.51	0.05
Gal-3 and GM-CSF		0.22	0.26
Gal-3 and G-CSF		0.55	0.04
Gal-3 and sICAM-1		0.73	0.005

these cytokines in patients with colorectal cancer with metastasis. As increased circulation of galectin-3 is commonly seen in many types of cancers and as several of these cytokines (e.g., IL-6 and G-CSF) are well-known metastasis promoters, the galectin-3–induced, cytokine-mediated metastasis promotion likely also represents a general mechanism in disseminating tumor cell metastatic spread to remote tumor sites.

IL-6 is a pleiotropic cytokine that plays diverse roles as a regulator of the acute inflammatory response as well as a growth and survival factor. IL-6 binds to its cell surface receptor IL-6Rα, causing activation of JAK/STAT, Ras/ERK, or PI3/Akt signaling pathways (15) leading to the expression of a large variety of gene products that are involved in cell proliferation and growth. High serum concentrations of IL-6 correlate with presence of metastasis and poor prognosis in many types of cancers including colorectal (18) and stomach (19). IL-6 can stimulate the release of angiogenesis-promoting factors such as VEGF and bFGF (20) and increase epithelial–mesenchymal transition (21). IL-6 produced in a primary tumor can promote the recruitment of circulating tumor cells back into their primary tumor, creating a process called tumor self-seeding that accelerates tumor growth, angiogenesis, and stromal cell recruitment (22). The IL-6–mediated activation of Stat-3 signaling in inflammatory cells can lead to transcriptional activation of NF-κB with consequential promotion of additional IL-6 and IL-8 secretion, thus generating a positive feedback loop between immune cells and tumor cells that further stimulates tumor progression and metastasis (23). As a result of such divergent influences of IL-6 on

tumor progression and metastasis, inhibition of IL-6–mediated cell signaling has been the subject of intense investigation as a possible cancer treatment and several phase I and II clinical trials using either anti-IL-6 antibodies or IL-6 inhibitors are currently underway (15, 24, 25).

G-CSF and GM-CSF both stimulate the bone marrow to produce granulocytes. G-CSF binds to its cell surface receptor G-CSFR, resulting in activation of intracellular signaling pathways including JAK/STAT, Ras/ERK, and PI3K/Akt (26). Serum G-CSF concentrations are increased in uroepithelial cancer and correlate with a poor prognosis (27). Circulating G-CSF can mobilize Ly6G + Ly6C + granulocytes in premetastatic tissues at distant organs before arrival of tumor cells and facilitate subsequent tumor cell homing and promote tumor cell migration, angiogenesis, and metastasis (28). Direct injection of recombinant G-CSF into the tail vein of nude mice before and after tumor cell injection increases lung metastasis in animals injected with human breast cancer cells (28).

GM-CSF, often used following chemotherapy in patients with cancer, promotes the invasiveness and survival of cancer cells by activation of MEK/ERK and PI3K/Akt signaling (29). Serum GM-CSF concentrations are higher in breast cancer (30).

sICAM-1 is a soluble form of the transmembrane cell adhesion molecule ICAM-1. ICAM-1 binds to Mac-1 and integrin LFA-1 and promotes cell–cell interactions. Higher serum sICAM-1 concentrations are seen in various cancers (31) including breast, gastrointestinal, lung, stomach, melanoma, ovary, and bladder (32) and in particular those with metastasis (33). High serum sICAM-1 concentrations correlate with tumor–node–metastasis (TNM) stage in colorectal cancer (34), and elevated pre-operative serum sICAM-1 level has been shown to be an independent prognostic marker for stage II colorectal cancer (35). The circulation of sICAM-1 inhibits T-cell interaction with tumor cells (36), blocks natural killer (NK) cell-mediated toxicity of tumor cells (37) and promotes tumor cell escape from immunosurveillance.

Thus, IL-6, G-CSF, GM-CSF, and sICAM-1 each can have, via different mechanisms, a very harmful influence on cancer progression and metastasis. The increased secretion of these cytokines into the blood circulation by the vascular endothelium in response to increased circulation of galectin-3 in patients with cancer is therefore likely to have a profound influence on cancer metastasis and prognosis locally, remotely, and systematically.

The galectin-3–mediated endothelial secretion of IL-6, G-CSF, GM-CSF, and sICAM-1 is shown in this study to increase the expression of endothelial cell surface integrin$\alpha_1\beta_v$, E-selectin, VCAM-1, and ICAM-1. Many of these cell surface adhesion molecules are responsible for recruiting leukocytes onto the vascular endothelium in inflammation and are believed to be also crucial in adhesion of disseminating tumor cells to the blood vascular endothelium in metastasis (32, 38). Previous studies have shown that proinflammatory cytokines, such as TNFα and IL-1, can induce endothelial expression of cell surface adhesion molecules that increase adhesion of circulating tumor cells to the capillary bed both *in vitro* and *in vivo* (39–41).

It is not yet known whether the increased secretions of IL-6, G-CSF, GM-CSF, and sICAM-1 by galectin-3 are all a direct consequence of the galectin-3 action or whether one or more of these could be triggered by the secretion of the others. Some cytokines are certainly capable of inducing secretion of other cytokines autocrinely or paracrinely. IL-1, for example, can induce the production of GM-CSF and G-CSF from endothelial cells (42), whereas IL-6 can induce complex secretion of IL-8, GM-CSF, VEGF, and MCP-1 from tumor cells (43). The observation that circulating galectin-3 concentrations correlate with G-CSF, IL-6, and sICAM-1 but not with GM-CSF in patients with metastasis implies that the increase of some cytokines (e.g., GM-CSF) in endothelial response to galectin-3 might likely be the consequence of an increase of the others (e.g., IL-6).

We cannot rule out the possibility that some of the cytokine increase observed in mice after galectin-3 injection might be the result of galectin-3 interaction with nonendothelial cells. The identity of the galectin-3–binding receptor responsible for galectin-3–induced endothelial cytokine secretion is not yet known, and it is unclear whether the expression of this receptor and its glycosylation status are the same between HMVECs and native human endothelial cells.

The presence of exogenous galectin-3 in the culture medium, albeit at what are probably suprapathologic concentrations, has been reported previously to induce endothelial cell morphogenesis (44) and enhance VEGF- and basic fibroblast growth factor (bFGF)-mediated angiogenesis (45). As clustering by galectin-3 of its ligands can markedly enhance galectin-3–binding affinity (46), the effect of galectin-3 on VEGF- and bFGF-mediated angiogenesis showed *in vitro* in these earlier studies with higher than pathologic glectin-3 concentrations may also be functionally relevant in the circulation and contribute to metastasis.

Thus, the increased circulation of galectin-3 in the bloodstream of patients with cancer has several important and distinctive influences on metastasis. It can interact directly with disseminating tumor cells through TF/MUC1, causing increased cancer cell heterotypic adhesion (8) and homotypic aggregation (10). It can also interact with the blood vascular endothelium and induce endothelial secretion of metastasis-promoting cytokines and thus indirectly promote metastasis. Targeting the actions of circulating galectin-3 in patients with cancer therefore represents a promising therapeutic strategy to reduce metastasis and improve cancer survival.

Disclosure of Potential Conflicts of Interest

J.M. Rhodes has received commercial research support [linked research grant part funded by industry (Provexis Ltd) for investigation of soluble plantain fiber in prevention of diarrheal diseases], has honoraria from speakers bureau (various in relation to treatment of inflammatory bowel

disease but none in relation to the topic of this article), and ownership interest in patents held/applied for in conjunction with University of Liverpool: (i) for treatment of diarrheal diseases with soluble plantain fiber and (ii) for treatment of cancer with modified heparins. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.M. Rhodes, L.-G. Yu
Development of methodology: C. Chen, Q. Zhao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Chen, C.A. Duckworth, D.M. Pritchard
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Chen, L.-G. Yu
Writing, review, and/or revision of the manuscript: C. Chen, C.A. Duckworth, D.M. Pritchard, J.M. Rhodes, L.-G. Yu
Study supervision: J.M. Rhodes, L.-G. Yu

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