Increased circulation of galectin-3 in cancer induces secretion of metastasis-promoting cytokines from blood vascular endothelium

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Abstract

Purpose: Cytokines such as IL-6 and G-CSF are important metastasis promoters. This study has investigated the functional significance of the increased circulation of galectin-3, a common feature in cancer patients 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 colorectal cancer patients with and without metastasis was investigated.

Results: Galectin-3 at pathological concentrations found in cancer patients induces secretion of IL-6, G-CSF, sICAM-1 and GM-CSF 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 integrinαvβ1, 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 cancer patients 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 cancer patients therefore represents a promising therapeutic strategy to reduce metastasis and
improve survival.
**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 cancer patients and in particular those with metastasis, induces the secretion of several well-known (e.g. IL-6 and 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 represent a promising therapeutic strategy to reduce metastasis and improve survival.
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) whilst 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 demonstrated 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 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, IL-6, G-CSF, 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 (Abingdon, UK). Calcein AM Cell Labeling Solution was from Invitrogen (Paisley, UK). Mouse recombinant IL-6, G-CSF, GM-CSF, ICAM-1 and mouse cytokine ELISA kits were from PeproTech (London, UK). In Vitro Angiogenesis Assay Endothelial Cell Invasion kits and In Vitro Angiogenesis Tube Formation kits were from AMS Biotechnology Ltd (Abingdon, UK). Non-Enzymatic Cell Dissociation Solution (NECDS) and all other chemicals were from Sigma (Dorset, UK).

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 (No authentication of the cell lines was done by the authors). Human micro-vascular lung endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Wokingham, UK) 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 colorectal cancer patients, 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 (1x10^5 cells/well) were cultured in 6-well plates, or ACA19- cells (5x10^5 cells/ml) were cultured in 6-well plates pre-coated with poly-HEMA(10) for 24 hr before introduction of galectin-3 or control BSA for 24 hr 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 colorectal cancer patients 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 hr. 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.

ACA197 and HCT116 cancer cells were detached from culture plates with NECDS, washed and resuspended at 5x10^6 cells/ml in DMEM. The cells were labeled with 10μl/ml Calcein AM at 37°C for 30 min, washed and re-suspended at 1x10^5/ml with serum-free DMEM containing 0.5mg/ml BSA before application (1x10^4 cells/well) to HMVEC monolayer for 1 or 24hr at 37°C. After two washes with PBS, the endothelial cell-associated fluorescence was measured using a fluorescence microplate reader at 485nm excitation/535nm emission.

Analysis of cell surface adhesion molecules by flow cytometry

HMVECs were treated with or without galectin-3 for 24 hr 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 αvβ3, E-selectin, VCAM-1 or ICAM-1 (all at 1:400 dilution) for 1hr. After wash with PBS and incubation with FITC-conjugated secondary antibodies (1:400 in 1% BSA in PBS) for 1 hr, the cells were analyzed by flow cytometry.

In vitro measurement of angiogenesis: (i) endothelial cell invasion and (ii) endothelial tubule formation
HMVECs (1×10⁵ cells/well) were cultured in 24-well plates for 24 hr before treatment with galectin-3 for 24 hr. The culture media was 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 (Margate, Kent, UK) and maintained and used in accordance with the animal care protocol approved by University of Liverpool, were randomly divided into three equal groups and 5µg/mouse recombinant human galectin-3 (2.5µg/ml, assuming a 2ml blood volume) was introduced by intravenous tail vein injection. Blood was obtained by cardiac puncture at 0, 24 and 48 hr and the serum concentrations of G-CSF, GM-CSF, IL-6 and sICAM-1 were determined by ELISAs.

**Statistical analysis**

Unpaired t test was used for single comparison, one-way ANOVA followed by Bonferroni for multiple comparisons and Spearman’s rho correlation analysis was used where appropriate. Differences were considered significant when two-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 hr (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 hr (Fig 1A), but not 1 hr (Fig 1B) at a concentration (1μg/ml) similar to that in cancer patients caused a significant increase [48.2±8.5%, p<0.05 and 22.9±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 hr also induced a similar increase in adhesion of fresh ACA19- (43.0±14.3%, p<0.05) and HCT116 (74.4±21.2%, p<0.05) cells to fresh HMVECs (Fig 1C) whilst 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 pro-inflammatory cytokines such as TNFα, IL-1 and IL-6,
are well known for their pro-metastatic 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 (1µg/ml) for 24 hr resulted in increased concentrations of four 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) while 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 cancer patients (Fig 2C) and time-dependent, occurring significantly only after treatment with galectin-3 for more than 20 hr (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 (<1.0EU) 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 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 1µg/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 24hr increased the expression of cell surface integrinαvβ1 (43%), E-selectin (19%), VCAM-1 (17%) and ICAM-1 (33%) whilst the expression of cell surface CD44 and integrinαvβ3 were 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 $\alpha_\text{v} \beta_1$, 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 $\alpha_\text{v} \beta_1$ (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 $\alpha_\text{v} \beta_1$ 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.

The presence of each of these cytokines increased integrin $\alpha_\text{v} \beta_1$ 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 $\alpha_\beta_1$, 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
Galectin-3 promotes endothelial cell migration and micro-vascular tube formation in angiogenesis

As pro-inflammatory cytokines such as IL-6 have previously been shown to promote angiogenesis(15) and the galectin-3-induced secretion of cytokines from the vascular endothelium included the pro-inflammatory 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 micro-tubule formation, two important components of the angiogenesis process. The conditioned medium from 24 hr 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 hr 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 5µg/mouse galectin-3, equating approximately to a pathological circulating galectin-3 concentration seen in cancer patients 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±26.7% of IL-6 and 58.4±28.2% of sICAM-1 was observed after 48 hr (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 (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 colorectal cancer patients (Table 1). However, when patients with and without metastasis were considered separately, significant correlations of galectin-3 levels were 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 cancer patients. Neither the galectin-3 concentrations (p=0.16) nor the concentrations of the four 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, was a marker of metastasis in colorectal cancer but a combined analysis of galectin-3 and -4 concentrations detect metastasis in these patients(17).
Discussion

This study shows that galectin-3 at pathological 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 these cytokines in colorectal cancer patients 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 tumour cell metastatic spread to remote tumour 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-6Ra, causing activation of JAK/STAT, Ras/ERK or PI-3/Akt signaling pathways(18) 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(19) and stomach(20). IL-6 can stimulate the release of angiogenesis-promoting factors such as VEGF and bFGF(21) and
increase epithelial-mesenchymal transition(22). 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(23). The IL-6-mediated activation of Stat-3 signaling in inflammatory cells can lead to transcriptional activation of NF-kB 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(24). 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, 25, 26).

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(27). Serum G-CSF concentrations are raised in uroepithelial cancer and correlate with a poor prognosis(28). Circulating G-CSF can mobilize Ly6G+Ly6C+ granulocytes in pre-metastatic tissues at distant organs before arrival of tumor cells and facilitate subsequent tumor cell homing and promote tumor cell migration, angiogenesis and metastasis(29). 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(29).
GM-CSF, often used following chemotherapy in cancer patients, promotes the invasiveness and survival of cancer cells by activation of MEK/ERK and PI3K/Akt signaling (30). Serum GM-CSF concentrations are higher in breast cancer(31).

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(32) including breast, gastrointestinal, lung, stomach, melanoma, ovary and bladder(33) and in particular those with metastasis(34). High serum sICAM-1 concentrations correlate with TNM stage in colorectal cancer(35) and elevated pre-operative serum sICAM-1 level has been shown to be an independent prognostic marker for stage II colorectal cancer(36). The circulation of sICAM-1 inhibits T cell interaction with tumour cells(37), blocks NK cell –mediated toxicity of tumour cells(38) and promotes tumour cell escape from immune-surveillance.

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 cancer patients 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 (33, 39). Previous studies have shown that pro-inflammatory 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 (40-42).

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 (43) while IL-6 can induce complex secretion of IL-8, GM-CSF, VEGF and MCP-1 from tumor cells (44). 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 non-endothelial 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 supra-pathological concentrations, has been reported previously to induce endothelial cell morphogenesis(45) and enhance VEGF- and bFGF-mediated angiogenesis(46). As clustering by galectin-3 of its ligands can markedly enhance galectin-3 binding affinity (47), the effect of galectin-3 on VEGF- and bFGF-mediated angiogenesis demonstrated in vitro in these earlier studies with higher than pathological galectin-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 cancer patients 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 cancer patients therefore represents a promising therapeutic strategy to reduce metastasis and improve cancer survival.

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References


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

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<th>Serum concentrations</th>
<th>Spearman’s rho correlation coefficient</th>
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<td>Median (range, ng/ml)</td>
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<td><strong>All patients (n=50)</strong></td>
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<td>Gal-3</td>
<td>106.8 (7.5-5106.3)</td>
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<td>IL-6</td>
<td>5.0 (2.1-287.8)</td>
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<td>GM-CSF</td>
<td>15.8 (5.4-833.3)</td>
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<td>G-CSF</td>
<td>43.0 (11.0-13266.0)</td>
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<td>sICAM1</td>
<td>14442.0 (7593.5-30235.9)</td>
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<td><strong>Patients without metastasis (n=39)</strong></td>
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<td>Gal-3</td>
<td>82.0 (7.5-1603.9)</td>
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<td>IL-6</td>
<td>5.2 (2.7-72.1)</td>
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<td>GM-CSF</td>
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<td>G-CSF</td>
<td>33.1 (11.1-3973.0)</td>
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<td>sICAM1</td>
<td>15148.1 (8414.1-30235.9)</td>
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<td><strong>Patients with metastasis (n=11)</strong></td>
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<td>Gal-3</td>
<td>215.0 (30.9-5106.3)</td>
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<tr>
<td>IL-6</td>
<td>3.5 (2.1-287.8)</td>
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<tr>
<td>GM-CSF</td>
<td>15.8 (12.0-833.3)</td>
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<tr>
<td>G-CSF</td>
<td>29.0 (15.0-13266.0)</td>
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<tr>
<td>sICAM1</td>
<td>12516.7 (7593.5-21621.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>All patients</strong></td>
<td></td>
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<tr>
<td>Gal-3 and IL-6</td>
<td>0.17</td>
<td>0.12</td>
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<tr>
<td>Gal-3 and GM-CSF</td>
<td>0.24</td>
<td>0.045</td>
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<td>Gal-3 and G-CSF</td>
<td>-0.11</td>
<td>0.22</td>
<td></td>
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<tr>
<td><strong>Patients without metastasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-3 and IL-6</td>
<td>0.12</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Gal-3 and GM-CSF</td>
<td>0.11</td>
<td>0.26</td>
<td></td>
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<td>Gal-3 and G-CSF</td>
<td>0.19</td>
<td>0.13</td>
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<td>Gal-3 and sICAM-1</td>
<td>-0.056</td>
<td>0.37</td>
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<td><strong>Patients with metastasis</strong></td>
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<tr>
<td>Gal-3 and IL-6</td>
<td>0.51</td>
<td>0.05</td>
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<tr>
<td>Gal-3 and GM-CSF</td>
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<td>0.26</td>
<td></td>
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<tr>
<td>Gal-3 and G-CSF</td>
<td>0.55</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Gal-3 and sICAM-1</td>
<td>0.73</td>
<td>0.005</td>
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Figure legends

Fig 1: Galectin-3 induces endothelial secretion of soluble molecules that increase cancer cell-endothelial adhesion.

Lengthy (24 hr, A) but not short (1 hr, B) presence of 1µg/ml galectin-3 increases ACA19̣ 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 hr-culture media (CM) from HMVEC (C), ACA19- or HCT116 (D) cells treated with or without 1µg/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 to BSA-treated controls (mean±SD) from 3 independent experiments, each in triplicate. *P<0.05.

Fig 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 CM of 24 hr-1µg/ml galectin-3, or BSA-treated HMVECs (A) or ACA19- cells (B). Galectin-3 (1.5µg/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.

Fig 3: Galectin-3-mediated cytokine secretion increases cancer cell-endothelial adhesion.

A and B: Galectin-3(1µg/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 anti-cytokine neutralizing antibodies. HMVECs were treated with 1µg/ml galectin-3 or BSA for 24 hr, the culture media was harvested and used for assessing ACA19- and HCT116 cell adhesion to fresh HMVECs with or without a combination of neutralizing antibodies against G-CSF (25ng/ml), GM-CSF (300pg/ml), IL-6 (2ng/ml) and sICAM-1 (5ng/ml). D: A combination of recombinant G-CSF (2.5ng/ml), GM-CSF (30pg/ml), IL-6 (200pg/ml) and sICAM-1 (500pg/ml) increases ACA19- and HCT116 cell adhesion to HMVECs. The data are expressed as percentage compared to BSA-treated controls from 3 independent experiments, each in triplicate. P*<0.05, **p<0.01, ***p<0.001.

**Fig 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 1.5µg/ml galectin-3 (green) for 24 hr induces expressions of cell surface integrinαvβ1, E-selectin, VCAM-1 and ICAM-1 but not CD44 nor integrin αvβ3 compared with control 1.5µg/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 1.5µg/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 24hr before the expression of integrin αvβ1 on HMVECs were analyzed. D: The presence of neutralizing antibodies against
integrinαvβ1 (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.

Fig 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 hr. The culture media was collected and used for subsequent assessment of fresh HMVECs 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.
Chen et al, Fig 1

A

Cancer cell adhesion to HMVECs (% of control)

ACA19- HCT116

B

Cancer cell adhesion to HMVECs (% of control)

ACA19- HCT116

C

Cancer cell adhesion to HMVECs (% of control)

ACA19- HCT116

D

Cancer cell adhesion to HMVECs (% of control)

ACA19- HCT116
Chen et al, Fig 2

A  

CM of Control cells

CM of Ga3- treated cells

B  

G-CSF GM-CSF sICAM-1 IL-6

C

IL-6 (pg/ml)

GM-CSF (pg/ml)

sICAM-1 (pg/ml)

D

Gal-3 treated  

control

IL-6 (pg/ml)

sICAM-1 (pg/ml)

GM-CSF (pg/ml)

G-CSF (μg/ml)

SF (pg/ml)
Chen et al, Fig 3

A

Cytokine secretion (%)

Control
Gal3
Gal3+Lac
Lac

IL-6
sICAM-1
GM-CSF
G-CSF

B

A19- adhesion to HMVECs (% of control)

Control
Gal-3
Gal-3 + Lactose

C

ACA19- cell adhesion to HMVECs (% of control)

CM of control HMVECs
+ - - +
CM of Gal-3-treated HMVECs
- + + -
Anti-cytokine mAbs
- - + +

D

Cell adhesion to HMVECs (% of control)

ACA19-
HCT116
Chen et al, Fig 4

A. Flow cytometry analysis of integrin αvβ1, E-Selectin, VCAM-1, ICAM-1, CD44, and integrin αvβ3.

B. Cytokine analysis with rGM-CSF, rICAM-1, rG-CSF, and rIL-6.

C. Bar graph showing ACA19 adhesion to HMVECs from various conditions with CM from control HMVECs, CM from Gal-3-treated HMVECs, and Anti-CAM mAbs.
Chen et al, Fig 5

A

**HMVECs migration (\% of control)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>BSA CM</th>
<th>Gal3 CM</th>
<th>Gal3/Lac CM</th>
<th>Gal3 CM + Cytokine mAbs</th>
<th>BSA CM + rCytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM from BSA-treated HMVECs</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CM from Gal-3-treated HMVECs</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CM from Gal-3/Lac-treated HMVECs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytokine neutralizing antibodies</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Recombinant cytokines</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

B

**Tubule length (\%)**

C

**Branch points**

D

BSA CM | Gal3 CM | Gal3/Lac CM | Gal3 CM + Cytokine mAbs | BSA CM + rCytokines

E

**Cytokines (ng/ml)**

- **ICAM-1**
- **G-CSF**
- **GM-CSF**
- **IL-6**

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, et al.

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