Intravascular Metastatic Cancer Cell Homotypic Aggregation at the Sites of Primary Attachment to the Endothelium


ABSTRACT

The two major theories of cancer metastasis, the seed and soil hypothesis and the mechanical trapping hypothesis, view tumor cell adherence to blood vessel endothelium and cancer cell aggregation as corresponding key components of the metastatic process. Here, we demonstrate in vitro, ex vivo, and in vivo that metastatic breast and prostate carcinoma cells form multicellular homotypic aggregates at the sites of their primary attachment to the endothelium. Our results suggest that metastatic cell heterotypic adhesion to the microvascular endothelium and homotypic aggregation represent two coordinated subsequent steps of the metastatic cascade mediated largely by similar molecular mechanisms, specifically by interactions of tumor-associated Thomsen-Friedenreich glycoantigen with the β-galactoside-binding protein, galectin-3. In addition to inhibiting neoplastic cell adhesion to the endothelium and homotypic aggregation, disrupting this line of intercellular communication using synthetic Thomsen-Friedenreich antigen masking and Thomsen-Friedenreich antigen mimicking compounds greatly affects cancer cell clonogenic survival and growth as well. Thus, β-galactoside-mediated intravascular heterotypic and homotypic tumor cell adhesive interactions at the sites of a primary attachment to the microvascular endothelium could play an important role during early stages of hematogenous cancer metastasis.

INTRODUCTION

Aberrations in cancer cell adhesion play an important role in tumor progression, invasiveness, and metastasis (1, 2). Initially, a decrease in homotypic and heterotypic cancer cell adhesion, caused by the reduced expression of E-cadherins and elevated activity of proteolytic enzymes, including numerous metalloproteinases, promotes their escape from a primary tumor and intravasation (3, 4). During postintravasation stages, however, the increased ability of blood-borne metastatic cells to engage in new heterotypic and homotypic adhesive interactions could be critically important for establishing secondary lesions in distant tissues and organs (5, 6). Two major theories describing cancer metastasis, the seed and soil hypothesis and the mechanical trapping theory, emphasize heterotypic tumor cell adhesion to blood vessel endothelium and homotypic cancer cell aggregation as corresponding key components of the metastatic cascade (reviewed in Ref. 7).

Neoplastic cell heterotypic adhesion to the microvascular endothelium, resulting in tumor cell arrest in the vasculature of a target organ, is an essential early step in hematogenous cancer spread (5). Recent observations, suggesting that only endothelium-associated malignant cells are capable of giving rise to hematogenous cancer metastases, strongly support this idea (8). Moreover, a possible role for tumor-cell interactions in defining tissue specificity of breast and prostate cancer metastasis was recently revealed (9, 10).

Homotypic tumor cell adhesion (aggregation) leads to the formation of multicellular malignant cell aggregates. Because multicellular clumps could be trapped in small capillaries, the role for homotypic aggregation was postulated within the mechanical trapping theory of cancer metastasis (11). Early pioneering works from the groups of Drs. Isaiah Fidler, Reuben Lotan, Garth Nicolson, and Avraham Raz (12–14), as well as our previous results (6, 15), demonstrated that cancer cells with high metastatic potential exhibit a superior ability to form homotypic aggregates compared with their low metastatic counterparts. In several different types of cancer, the in vivo selection of tumor cells for high metastatic potential resulted in a selection of malignant cells exhibiting increased homotypic aggregation properties (6, 12). In addition, cancer cell lines selected in vitro for enhanced homotypic aggregation kinetics demonstrated significantly higher in vivo experimental metastatic potential than parental cells (14). To date, metastatic cancer cell heterotypic and homotypic adhesion have been largely studied as two independent unrelated processes. Here, we demonstrate in vitro, ex vivo, and in vivo experiments that these two processes appear to be linked together. Furthermore, it appears that in both breast and prostate cancer, heterotypic and homotypic adhesion mediated largely by similar molecular mechanisms, specifically by interactions between tumor-associated T3 glycoantigen (3) and galectin family proteins. The results presented in this article suggest that hematogenous cancer metastases could originate from multicellular aggregates formed at the sites of primary tumor cell attachment to the vascular wall. We identify interactions between T antigen and galectin-3 as one of the major pathways for mediating heterotypic and homotypic cancer cell adhesion and promoting metastatic cell clonogenic survival and growth.

MATERIALS AND METHODS

Antibodies, Chemicals, and Reagents. The TIB-166 hybridoma, producing rat monoclonal anti-galectin-3 antibody, was from American Type Culture Collection. T-HSA was from Dextra Laboratories (Reading, United Kingdom). T antigen-binding peptide P-30 (GRFILPWYASPS) was chemically synthesized using N-(9-fluorenylmethoxycarbonyl)-based chemistry. All other chemicals and reagents, unless otherwise specified, were from Sigma (St. Louis, MO).

Cell Lines and Cultures. The MDA-MB-435 human breast carcinoma cell line was provided by Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX). The DU-145 human prostate carcinoma cells were from American Type Culture Collection (Manassas, VA). The RPMI 1640 supplemented with l-glutamine, 10% FBS, sodium pyruvate, and nonessential amino acids was used for tumor cell lines. Immediately before adhesion experiments, cancer cells were prelabeled for 5 min with 3 µg/ml solution of acridine orange in
RESULTS

Although, many important facts regarding tumor-endothelial cell adhesion have been revealed using static adhesion assays, intravascularly they interact under dynamic flow conditions. Under these conditions, physiological shear force dramatically affects the adhesive behavior of both endothelial and neoplastic cells (19). Therefore, we attempted to dissect adhesive mechanisms relevant to cancer metastasis by investigating how metastatic cancer cells interact with endothelium derived from different anatomical sites in flow using parallel flow chamber techniques. Recently, we reported that under conditions of physiological shear force (0.8–4.0 dyn/cm²) upon adhesion to HLMVEC monolayer, highly metastatic MDA-MB-435, but not nonmetastatic MDA-MB-468, breast carcinoma cells acquired enhanced homotypic aggregation properties. Within minutes of adhesive interactions of a single cell suspension of MDA-MB-435 cells with HLMVEC monolayer, we frequently observed that adherent tumor cells captured other rolling and floating neoplastic cells and initiated a rapid formation of multicellular cancer cell aggregates at the sites of their primary attachment to the endothelium (18). Importantly, this adhesion pattern is not unique to breast carcinoma cell interactions with HLMVECs. Both metastatic breast (MDA-MB-435) and prostate (DU-145) cells exhibit similar adhesion behavior in flow (Fig. 1, A and B) while interacting with HBMECs representing a tissue most frequently targeted by breast and prostate cancer metastasis. This is an important observation because, although both MDA-MB-435 and DU-145 metastatic human cancer cells also adhere to endothelial cells from the tissues not targeted by tumor metastasis such as HUVECs (9, 10, 17), we previously demonstrated that multiple human prostate carcinoma cell lines, including DU-145 adhere 3–5-fold greater to HBMECs than to HUVECs, aortic endothelial cells, or even HLMVECs (9). We next investigated whether such homotypic cancer cell aggregation occurs intravascularly.

To address these questions, we have used an ex vivo experimental system for studying cancer cell adhesive interactions with perfused porcine dura mater microvessels recently developed in our laboratory.
These results demonstrate that homotypic aggregation by capturing other circulating tumor cells (Fig. 1, E). In perfused dura mater experiments, we often identified multiple homotypic tumor cell aggregates, formed in different microvessels, within the same observation field (Fig. 1F), implying that this is not a rare event but rather a common trend. Importantly, highly metastatic MDA-MB-435 breast carcinoma cells exhibited similar adhesion behavior in vivo. Injected i.v. as a single cell suspension into SCID mice, blood-borne MDA-MB-435 cells formed multiple intravascular homotypic aggregates stably adhered within mice lung microvessels (Fig. 1G).

Both ex vivo (Fig. 1, E and F) and in vivo (Fig. 1G) homotypic cancer cell aggregates reside frequently in microvessels exceeding significantly the size of the aggregates. This suggests that heterotypic adhesion to the endothelium rather than mechanical trapping is responsible for their arrest in the microcirculation. However, in some instances, it is possible that aggregates may detach and become blood borne once again, in which case one can reasonably envision their additional entrapment in small capillaries. In addition, we cannot completely rule out a multicellular neoplastic cell aggregation resulting from homotypic interactions between two or more floating tumor cells, which could lead, presumably, to mechanical lodging of such aggregates in blood microvessels as well. However, we demonstrate here (Fig. 2) that multiple circulating cancer cells, even while in a very close proximity to each other, can pass freely through a very narrow capillary network without forming multicellular clumps and being trapped (Fig. 2A). These same cells do form homotypic aggregates and become permanently lodged when halted by another single cancer cell stably adhered to the microvessel wall (Fig. 2, B–E). Once again, in this example, stable heterotypic tumor-endothelial cell adhesion precedes homotypic aggregate formation and permanent neoplastic cell arrest in the microcirculation.

Additional evidence suggests that heterotypic adhesion is not simply a result of mechanical trapping. When heterotypic cancer cell adhesion with endothelia does not take place, tumor cells demonstrate striking ability to avoid mechanical trapping by adjusting their shape and passing through the narrow microvessel sections (Fig. 3). In this particular experiment, prostate cancer cells avoided mechanical en-
trapment at the site of a microvascular spasm but became permanently attached to the vascular wall ~50 μm downstream of the constricted site in a wide part of the microvessel far exceeding the malignant cell size (Fig. 3). This observation highlights once again the role of heterotypic cancer-endothelial cell adhesion as an essential initial step in circulating metastatic cell permanent arrest within a microvasculature of a target organ.

Collectively, our in vitro, ex vivo, and in vivo results demonstrate that either within relatively large vessels, far exceeding tumor cells in diameter or within narrow capillaries, homotypic tumor cell aggregation is often initiated by preceding heterotypic adhesion between cancer cells and microvascular endothelium. These observations strongly suggest that intravascular heterotypic and homotypic metastatic cell adhesive interactions represent two coordinated subsequent steps of a metastatic cascade.

We next investigated how these processes may be mediated at the molecular level. We previously have reported that heterotypic and homotypic cancer cell adhesion appear to be mediated at least, in part, by similar molecular mechanisms, specifically by interactions between cancer-associated carbohydrate T antigen and β-galactoside-binding galectin family proteins (9, 10, 17, 18). The immunodominant portion of the T antigen, a simple mucin-type core 1 disaccharide Galβ1-3GalNAc, which is masked covalently or structurally on most normal cells, is exposed and immunoreactive on the outer cell surfaces of most human carcinomas and T-cell lymphomas (21, 22). This carbohydrate structure plays an important role in breast and prostate cancer cell adhesion to the endothelium and homotypic aggregation (10, 17, 18, 23–26). Here, we demonstrate that lactulosyl-L-leucine (23, 26), which mimic T antigen (10) but not lactitol-L-leucine (10), is capable of disrupting almost entirely the DU-145 human prostate carcinoma cell homotypic aggregation (Fig. 4A). This result indicates that, similarly to breast cancer, prostatic malignant cell homotypic aggregation appears to be largely T antigen mediated.

Of particular significance, in addition to promoting cancer cell homotypic aggregation, this line (T antigen mediated) of intercellular communication is also important in neoplastic cell clonogenic survival (Fig. 4B) and clonogenic growth (Fig. 4C). In clonogenic survival experiments, we plated breast (MDA-MB-435) and prostate (DU-145) carcinoma cells at the low density (100 or 200 cell/well in 24-well plates) in a presence of various concentration of either T antigen-mimicking lactulosyl-L-leucine (0–1.0 mM) or T antigen-masking P-30 peptide (0–50 μM). Although neither one of these two compounds causes any detectable cytotoxic or growth inhibitory effects in regular monolayer cultures, both lactulosyl-L-leucine (Fig.

Fig. 2. The group of 12 DU 145 prostate carcinoma cells, lined up in a long narrow capillary, slowly approaches stably adhered neoplastic cell (arrow) without interacting with each other or being arrested in circulation. Subsequent frames, illustrating dynamics of tumor cell transition through the microvessel, are represented by green, red, and yellow colors. B–E, cancer cells shown in A become permanently lodged and form multicellular aggregate when halted by the stably adhered tumor cell. Scale bar, 100 μm.

Fig. 3. Rolling tumor cell approaches the site of a microvascular spasm indicated with red arrow. By adjusting its shape and passing through the narrow microvessel segment, neoplastic cell avoids mechanical entrapment. However, it becomes permanently adhered ~50 μm downstream in a wide part of a microvessel. Scale bar, 50 μm.
Indeed, lactulosyl-L-leucine, which blocks galectin-3 specifically by cells clonogenic survival and growth. Antigen-mediated tumor cell adhesive interactions, could be essential during early stages of metastasis formation by supporting malignant cells clonogenic survival and growth.

This idea is consistent with T antigen interacting with galectin-3. Indeed, lactulosyl-L-leucine, which blocks galectin-3 specifically by mimicking T antigen (10), is capable of inhibiting significantly both incidence and number of spontaneous MDA-MB-435 breast cancer metastasis in vivo (Ref. 26 and Fig. 4D) in nude mice experiments alone without any cytotoxic drugs. The lactulosyl-L-leucine effects on cancer clonogenic survival and growth could be explained at least, in part, through an inhibition by this carbohydrate-amino acid conjugate of β-galactoside-binding lectin galectin-3. Several different groups demonstrated previously the role for this intriguing multifunctional protein in prostate cancer cell adhesion to bone marrow endothelium (9, 10, 17), homotypic cell adhesion (27), protection from apoptosis (28, 29), and MDA-MB-435 breast carcinoma cell anchorage-independent clonogenic growth (30). Recently, we have identified galectin-3 as a binding partner for the T antigen (10, 17). The lactulosyl-L-leucine interacts specifically with galectin-3 by mimicking T antigen (10), thus affecting the functions of this β-galactoside-binding lectin in tumor-endothelial cell heterotypic adhesion, breast and prostate cancer cell homotypic aggregation, and metastatic cell clonogenic survival and growth. Of note, another carbohydrate-based compound also targeting specifically galectin-3, modified citrus pectin developed by the group of Dr. A. Raz, was recently shown to inhibit tumor-endothelial cell adhesion and MDA-MB-435 breast cancer metastasis in vivo as well (31).

Consistent with its multiple functions, galectin-3 exhibits multidirectional intracellular trafficking upon various cellular processes. Previously, we reported that during cancer cell adhesion to the endothelia, the endothelium-expressed galectin-3 is clustering toward the sites of heterotypic contacts between endothelial and breast or prostate carcinoma cells (Refs. 10, 17 and Fig. 5, C–E, J, and L, red arrows). Recently, Yu et al. (32) documented the galectin-3 translocation to the perinuclear membranes in response to various apoptotic stimuli. Here, we demonstrate that in homotypic tumor cell aggregates, galectin-3 is clustering also toward the sites of cancer cell adhesion to each other (Fig. 5B, C, F, H, and K, yellow arrows). In addition and perhaps most intriguing, within minutes of breast and prostate tumor cell interactions with endothelial monolayer, a massive nuclear trafficking of this carbohydrate binding protein occurs in cancer cells (Fig. 5, C–F, and I–L, white arrows).

The galectin-3 clustering toward the sites of heterotypic adhesion on endothelial cells or to the homotypic cell-cell interfaces on tumor cells is consistent with its interactions with T antigen presented on neoplastic cell surfaces (10, 17). Furthermore, the translocation of the galectin-3 to the cell surfaces on both endothelial and cancer cells is also at least, in part, mediated by T antigen (Ref. 10 and Fig. 5, M and N). We demonstrated previously that T antigen not only acts as the galectin-3 binding partner but also causes rapid galectin-3 mobilization to the endothelial cell outer surfaces (10). Indeed, treating endothelial cells with T antigen conjugated to T-HSA causes >3-fold increase in endothelial cell surface area positively expressing galectin-3 (Fig. 5M). Similarly, ASF, a glycoprotein bearing multiple T antigen moieties, but not fetuin, on which T antigen epitopes are covalently masked with sialic (neuraminic) acid, induces rapid increase in galectin-3 surface expression on breast and prostate cancer cells (Fig. 5N). In contrast, fetuin was reported by Zhu and Ochieng (33) to induce rapid release of intracellular galectin-3 from tumor cells, suggesting that glycoprotein effect upon cancer cells may depend dramatically on carbohydrate moieties sialylation.

However, what factors cause galectin-3 nuclear translocation remains unknown. Interestingly, this process appears to be specific to neoplastic cell heterotypic adhesion. Galectin-3 nuclear trafficking does not occur when cancer cell interacts with each other (Fig. 5, B and H). Although, it is also not clear to which of the multiple functions of this intriguing carbohydrate-binding protein this phenomenon is related, it certainly reflects complex signaling pathways triggered by tumor-endothelial cell interactions, which could be critically important in determining the fate of metastatic cancer cells in circulation.

**DISCUSSION**

Our results suggest that β-galactoside-mediated intravascular heterotypic and homotypic tumor cell adhesive interactions at the sites of a primary attachment to the microvascular endothelium could play an important role in metastatic cancer spread. Interestingly, a similar adhesive behavior was previously reported for selectin-mediated leukocyte adhesion during inflammatory responses (34). Specifically, P-selectin-mediated leukocyte adhesion to the endothelium was shown to trigger a L-selectin-dependent homotypic leukocyte aggregation promoting their cooperative function at the site of inflammation. It appears that similarly heterotypic tumor cell adhesion to the endothelium induces homotypic cancer cell aggregation supporting metastatic cell arrest in microcirculation, clonogenic survival, and growth. However, in leukocytes, this sequence of adhesive events occurs via a coordinated action of P- and L-selectins interacting with their cognate carbohydrate ligands, mostly sialylated Lewis A and Lewis X core 2 oligosaccharides expressed on glycoproteins and
galectin-3 was directly implicated in malignant cell protection from apoptosis and clonogenic growth (28–30). Thus, the galectin-3 involvement in T antigen-mediated cell-to-cell interactions provides a rationale for explaining the role for this line of intercellular communication in metastatic cell clonogenic survival and growth. Indeed, in several experimental systems, the expression of galectin-3 in cancer cells was associated with increased malignant and metastatic phenotype (37, 38). However, this β-galactoside-binding lectin is not always abundantly expressed in tumor cells. For example, some prostate carcinoma cells such as LNCaP fail to express detectable galectin-3 levels (39, 40). This suggests that other than galectin-3 proteins, perhaps other galectin family members could potentially be involved in neoplastic cell β-galactoside-mediated homotypic interactions.

The signaling pathways triggered by a T antigen engagement most likely depend on which glycoprotein or glycolipid this carbohydrate structure is expressed. For example, the hyaluronan receptor CD44 was identified recently as a major T antigen carrier in colon carcinoma cells (41). The role for CD44 in tumor metastasis, specifically in homing of several different types of cancer to bone marrow as well as in mediating numerous major signaling pathways regulating cell growth, motility, and invasion, is well documented (42–44). The function of this glycoprotein is most studied in a context of its interactions with hyaluronan, a ubiquitous component of the extracellular matrix, which also occurs transiently intracellularly. However, it would be of paramount interest to investigate if CD44-associated signaling pathways in metastatic cells could be activated by galectins through interactions with T antigen. In addition, several other mucin family members implicated in tumor cell adhesion and metastasis such as MUC1, CD146/MUC18/MCAM, or GPI-linked surface mucin CD24 could also be potentially involved in T antigen-mediated adhesion and signaling by expressing this mucin-type disaccharide (45).

In summary, our results suggest that hematogenous cancer metastases could originate from multicellular aggregates formed at the sites of primary tumor cell attachment to the vascular wall resulting at least, in part, from β-galactoside, specifically T antigen-mediated heterotypic and homotypic metastatic cell adhesive interactions. In addition to promoting cancer cell adhesion (10, 17, 18, 26), galectin-3 was directly implicated in malignant cell protection from apoptosis and clonogenic growth (28–30). Thus, the galectin-3 involvement in T antigen-mediated cell-to-cell interactions provides a rationale for explaining the role for this line of intercellular communication in metastatic cell clonogenic survival and growth. Indeed, in several experimental systems, the expression of galectin-3 in cancer cells was associated with increased malignant and metastatic phenotype (37, 38). However, this β-galactoside-binding lectin is not always abundantly expressed in tumor cells. For example, some prostate carcinoma cells such as LNCaP fail to express detectable galectin-3 levels (39, 40). This suggests that other than galectin-3 proteins, perhaps other galectin family members could potentially be involved in neoplastic cell β-galactoside-mediated homotypic interactions.

REFERENCES


Intravascular Metastatic Cancer Cell Homotypic Aggregation at the Sites of Primary Attachment to the Endothelium

Vladislav V. Glinsky, Gennadi V. Glinsky, Olga V. Glinskii, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/13/3805

Cited articles
This article cites 44 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/13/3805.full.html#ref-list-1

Citing articles
This article has been cited by 35 HighWire-hosted articles. Access the articles at:
/content/63/13/3805.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.