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Integrin $\alpha 2$ on the Surface of Tumor Cells Alter Sites of Metastases

Kiyoshi Yoshimura

Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-855, Japan (Received June 14, 2010)

Abstract The finding that integrin $\alpha 2$ expression on tumor cells confers increased metastatic potential to certain tissue sites via direct physical adhesion, as well as the finding of the activation of an intracellular signaling cascade linked to integrin $\alpha 2$ that yields an invasion and growth phenotype is novel. These findings can potentially be exploited to increase our knowledge of which patients are at higher risk of forming (hepatic) metastases, thus aiding our ability to discriminate proper adjuvant therapy based on molecular phenotyping. Additionally, expression of this cell surface molecule may be blocked, or the internal signaling cascade that results from binding of this cell surface molecule may be blocked to potentially decrease the metastatic potential of cancers in a therapeutic fashion.

Key words: integrin, metastasis, cancer, liver

Introduction

Tumor progression and metastasis depend on factors intrinsic to the tumor cell such as growth factors/receptors, extracellular matrix proteins, proteases, chemokines, and cellular adhesion molecules, as well as host factors. Cellular adhesion molecules are important in various biologic processes, including organogenesis, tissue homeostasis, wound healing, and inflammatory/immune responses.¹⁾ Adhesion molecules are expressed on the cell surface and can bind to themselves or other surface molecules. They mediate adhesion between cells of the same type or of different types and support tumor cell survival during extravasation, dissemination, mechanical arrest in capillaries, and intravasation.

Among the most important adhesion molecules implicated in metastasis are the integrins, heterodimeric transmembrane receptors found throughout metazoan development.²⁾ Most integrins bind to components of the extracellular matrix (ECM) such as laminins,

collagens, and fibronectin. Integrins are involved in various aspects of cellular behavior including stable adhesion to basement membranes, formation of extracellular matrices and migration on such matrices, the formation of platelet aggregates, the establishment of intercellular junctions in the immune system, and the support of bacterial and viral entry during infectious diseases.³⁾

The initiation and progression of cancer is accompanied by alterations in the expression of adhesion molecules, including integrins. Integrin-mediated migration and interactions with other cells such as endothelial cells contributes to tumor cell invasion and metastasis. The integrin profile that promotes metastatic spread is often not the same as the profile that promotes the initial transformation and growth. During tumor progression tumor cells gain and lose expression of specific integrins depending on the environmental restraints encountered.

While molecular parameters of the general metastatic process continue to be elucidated, much less is known about those involved in

organ-specific metastases. The liver is one of the most important targets for organ specific metastasis in many cancer types and is commonly the sole site of metastasis for gastrointestinal cancers. While a few studies have identified potential molecular correlates of hepatic metastasis, none have directly demonstrated their role in selective metastasis to the liver. In order to define cell membrane molecules mediating liver metastasis, we employed the classic approach of repetitive *in vivo* metastasis selection to derive liver-metastasizing sublines of a tumor with normally low intrinsic potential for hepatic metastasis. Analysis of a broad array of cell membrane adhesion molecules and chemokine receptors revealed that the integrin α_2 (CD49b) was uniquely upregulated relative to the non-liver metastasizing parental line. Antibody blockade and forced expression of integrin α_2 demonstrated a direct role in liver metastasis, collagen type IV binding and collagen type IV-dependent activation of Focal adhesion kinase (FAK), a critical intracellular kinase involved in the metastatic process. Analysis of primary and metastatic colon tumors from patients provided evidence that integrin α_2 plays a role in liver metastasis in human cancer.

Results

In vivo selection of liver metastasizing subline of B16 melanoma

B16-F0 melanoma cells were subjected to 8 rounds of *in vivo* selection for liver metastasis using an intrasplenic injection route. This route favors hepatic metastasis since the splenic veins drain into the portal vein. Nonetheless, intrasplenic injection of B16-F0 generates very few hepatic metastases; instead demonstrating massive peritoneal dissemination. A rare liver metastasis was explanted and expanded *in vitro* followed by intrasplenic re-administration (Fig. 1). After four (B16-KY4) and eight rounds of selection (B16-KY8), tumor lines were established. B6 mice were challenged with four different tumor lines (B16-F0, B16-KY4, B16-KY8 and B16-F10, a subline selected for lung metastatic potential) in the hepatic metastasis model. Mice challenged with B16-F0 and B16-F10 had marked

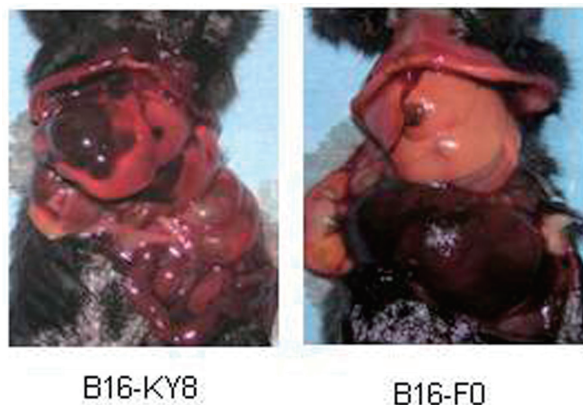


Fig. 1 Mice challenged with the parental B16-F0 had very few liver metastases with peritoneal dissemination. Mice challenged with B16-KY8 had mostly hepatic metastases.

peritoneal dissemination of their disease and relatively small disease burden in the liver. In striking contrast, mice challenged with B16-KY8 had significant disease burden in the liver with little peritoneal dissemination.

Assessment of cell surface molecule expression associated with liver metastasis

The four B16 cell lines were analyzed by flow cytometry to survey a broad panel of adhesion molecules potentially relevant to differential patterns of adhesion, attachment, implantation, and growth. Of over 30 molecules analyzed, including chemokine receptors previously implicated in hepatic homing, only integrin α_2 (also known as CD49b) was differentially expressed on B16-KY8 cells relative to the other three B16 cell lines. Because integrin α_2 combines in a heterodimeric complex with integrin β_1 (also known as CD29) to form VLA2, we also performed flow cytometric assays to test differences in expression of integrin β_1 . Consistent with the reported ubiquitous expression of integrin β_1 , the four B16 cell lines expressed similar amounts of integrin β_1 (data not shown) but only the B16-KY8 cell line expressed higher amounts of integrin α_2 (Fig. 2).

In order to further determine whether integrin α_2 was correlated with liver metastatic potential, B16-KY8 cells were sorted into two populations: integrin α_2 high and low expressing cells. These cells were then

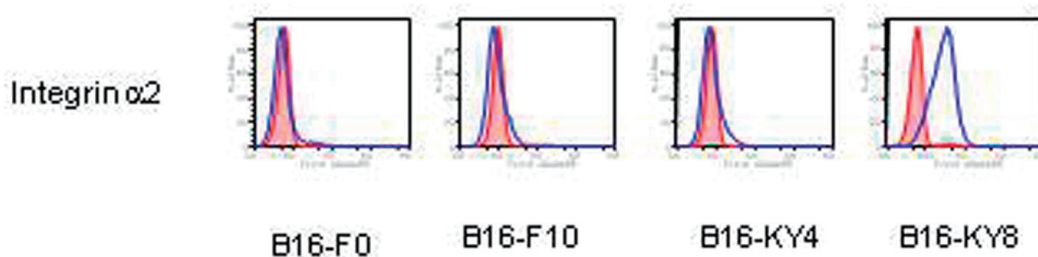


Fig. 2 Analysis of integrins α_2 reveals that integrin α_2 is selectively upregulated in B16-KY8.

used to challenge mice in the intrasplenic injection model and then sacrificed on day 21. When the integrin α_2 high cells were used, 30% of mice had limited peritoneal disease. In contrast, 100% of mice receiving intrasplenic injection of the integrin α_2 low cells developed peritoneal dissemination. While, 100% of mice in both groups developed hepatic metastases, the number of nodules in the liver was significantly higher with the integrin α_2 high than the one from mice injected integrin α_2 low B16-KY8 cells. At the same time, the number of peritoneal nodules in the integrin α_2 high injected mice was much less than the number in the integrin α_2 low. These results provided initial evidence that integrin α_2 expression might indeed enhance formation of liver metastasis.

Integrin α_2 blockade inhibits hepatic metastasis while forced expression promotes selective hepatic metastasis

In order to definitively determine whether integrin α_2 expression was functionally involved in hepatic metastasis, the effect of blocking B16-KY8 cells with anti-integrin α_2 antibody in the hepatic metastasis model was studied. While treatment of B16-KY8 cells by blocking with anti-integrin α_2 antibody did not completely eliminate hepatic metastasis, it was significantly reduced relative to treatment with the isotype control antibody. Importantly, anti-integrin α_2 antibody treatment concomitantly increased peritoneal dissemination of B16-KY8, demonstrating that integrin α_2 blockade did not reduce generic metastatic potential but rather shifted metastasis away from the liver. As a comparison group in this experiment, mice injected with B16-F0 cells, which express integrin α_2

on <3% of the cells, displayed peritoneal carcinomatosis with <30% showing evidence of (small volume) hepatic metastases. To further demonstrate that integrin α_2 can promote selective hepatic metastasis, we transfected B16-F0 with the integrin α_2 gene to generate the B16-SKY line and confirmed surface expression of integrin α_2 by flow cytometry. We used B16-SKY to challenge mice by both intrasplenic and intravenous routes. After intrasplenic injection, B16-SKY produced significantly more hepatic metastatic nodules than B16-F0. We also studied the effect of blocking integrin α_2 expression on B16-SKY on the pattern of metastases after intrasplenic and tail vein injection. The most notable effect of blocking integrin α_2 expression on B16-SKY in the hepatic metastasis model was to increase the rate of peritoneal metastases though there was additionally a decrease in liver metastases.

Integrin α_2 expression enhances binding to collagen type IV and mediates FAK activation upon collagen type IV binding

Integrins are known to mediate collagen binding and the liver is particularly rich in type IV collagen. We therefore asked whether integrin α_2 up-regulation might enhance collagen type IV binding and whether there is binding mediated activation of kinases known to be involved in metastasis. To study the intensity of attachment of the B16 cell lines to collagen type IV coated plates, which is a known ligand of integrin α_2 , an attachment assay was performed using B16-F0 and B16-KY8. The B16-F0 group displayed low numbers of attached tumor cells on the collagen type IV coated plate. In contrast, the B16-KY8 group displayed a >6 fold increase

in attachment of tumor cells on the collagen type IV coated plates.

We next determined whether integrin α_2 dependent binding to collagen type IV mediated activation of FAK in tumor cells. As described above, FAK, which plays a major role in cell motility, has been shown to be activated by engagement of a number of integrins, including integrin α_2 , and is thought to be an important factor in the metastatic process. Indeed, attachment of B16-KY8 and B16-SKY to collagen type IV coated plates resulted in significant FAK activation, peaking at 90 minutes, whereas B16-F0 cells plated on collagen IV failed to activate FAK. FAK activation was collagen IV dependent and specific, since another kinase involved in metabolic regulation of tumor cells, AKT, was equally activated in B16-F0, KY8 or SKY and was not affected by binding to collagen IV. Taken together, these studies demonstrate that induction of integrin α_2 results in collagen IV dependent FAK activation and provide a potential mechanistic basis for its role in mediating selective hepatic metastasis.

Correlation between integrin α_2 expression and hepatic metastasis in human colorectal cancer

We also analyzed colorectal tumors from patients with simultaneous liver and lung metastases. Metastases are significantly less infiltrated with leukocytes than primary colorectal tumors, allowing direct analysis of Integrin α_2 on tumor cells by immunohistochemical staining. Sections of concurrent liver and lung metastases available from seven colorectal cancer patients were stained with anti-integrin α_2 antibody and processed for immunohistochemistry as described. In six of seven cases, the liver metastases had higher levels of staining than the pulmonary metastases. In roughly half the cases, hepatic metastases showed extremely strong staining for Integrin α_2 , whereas the corresponding lung metastases had extremely low staining. These results in both primary and metastatic human colorectal cancers show a correlation between integrin α_2 expression and propensity to metastasize to the liver.

Discussion

The formation of metastatic disease from a tumor primary requires multiple complex steps including invasion through the normal tissue architecture, intravasation, arrest, extravasation, and growth. Many of these processes have been associated with the up-regulation of various cell surface molecules, and in particular integrins.⁴⁾

We used *in vivo* selection to derive a B16 cell line (B16-KY8) that has a higher propensity to form hepatic metastases and lower propensity to form peritoneal metastases than the parental cell line (B16-F0) in both a hepatic metastasis (splenic injection) and a pulmonary (tail vein injection) model. We hypothesized that differential expression of one or more cell surface molecules would be responsible for this difference in metastatic pattern. Remarkably, analysis of a broad panel of integrins, chemokine receptors previously implicated in liver homing and cellular adhesion molecules revealed a single molecule, integrin α_2 , that was differentially expressed between the non-liver metastasizing parental B16-F0 tumor and the liver metastasizing B16-KY8. Multiple complimentary approaches, including sorting of high vs. low expressers, antibody blockade and forced expression via transfection, demonstrated that integrin α_2 plays a causal role in liver metastasis. Analysis of both primary and metastatic human colorectal cancers demonstrated an association between integrin α_2 expression and liver metastasis, suggesting that integrin α_2 is relevant to the liver-specificity of metastasis in human cancer. Mechanistically, *in vitro* studies suggested that integrin α_2 may facilitate hepatic metastasis via binding to collagen type IV and consequent activation of FAK. With this information in our murine system, we studied integrin α_2 expression in two different sets of patient samples. In the first set, primary colorectal cancers were selected and divided into patients with and without hepatic metastases. We found that the patients with hepatic metastases had higher expression of integrin α_2 mRNA in the microdissected tumor cells compared to those patients without hepatic metastases. In the second set, we studied matched hepatic and

pulmonary metastases from patients with colorectal cancer. In two cases we also had the matched primary specimens. We found increased cell surface expression of the integrin α_2 protein on the cell surface of tumor cells in hepatic metastases compared to pulmonary metastases.

Integrin $\alpha_2\beta_1$ also known as very late antigen 2 (VLA2) is primarily found on activated T cells, platelets, and have been described on epithelial cells such as keratinocytes.⁵⁾ Interestingly on keratinocytes, VLA2 is considered a differentiation marker. Upon terminal differentiation, keratinocytes not only lose their contacts to the basement membrane, but also decrease their expression of VLA2 drastically.⁶⁾ The expression of the other two collagen-binding integrins, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, is more restricted to chondrocytes and fibroblasts, respectively.⁷⁾⁸⁾

Integrin $\alpha_2\beta_1$ can bind with collagen type I, II, and IV.⁹⁾¹⁰⁾ Collagen type I is found primarily in bone, dermis, tendon, ligaments, and cornea. Collagen type II is found primarily in cartilage, vitreous body, and nucleus pulposus. Collagen type IV is found primarily in basement membranes.¹¹⁾ In our murine models of hepatic, pulmonary, and peritoneal metastases, collagen type IV, which is highly enriched in the liver, is the most relevant collagen subtype to implicate in an integrin-collagen interaction.

Although a number of studies have implicated integrin expression in the metastatic process, including liver metastases, the findings presented here are the first to directly demonstrate a role for integrin α_2 in selective liver metastasis. A few reports have implicated α_6 integrins (associated with various β subunits), which bind laminin, in liver metastases.¹²⁾¹³⁾¹⁴⁾ The most convincing report demonstrated that both components of a complex of integrin $\alpha_6\beta_2$ and the D6.1A tetraspanin were necessary for liver metastasis in a rat pancreatic adenocarcinoma model.¹⁵⁾ Other adhesion molecules, including integrin $\alpha_v\beta_3$, osteopontin (a ligand for integrin $\alpha_v\beta_3$), integrin $\alpha_v\beta_5$, integrin $\alpha_4\beta_1$ and VCAM-1 have been associated with liver metastasis in various models,¹⁶⁻²⁰⁾ but their direct role has not been comprehensively confirmed by blockade and overexpression analyses, nor compara-

tive expression analysis in human tumors as we have done for integrin α_2 in the current report.

While this is the first report to implicate integrin α_2 in hepatic metastasis, 2 earlier studies have shown that integrin α_2 plays a role in the intrahepatic migration of tumor cells. One report showed that chemotactic and haptotactic migration of hepatocellular carcinoma cells in response to growth factors produced in a fibrotic microenvironment involves integrin α_2 .²¹⁾ A second report showed that while integrin α_2 did not affect extravasation of rhabdomyosarcoma cells in the liver, it facilitated their intrahepatic migration to the subcapsular space once in the liver parenchyma. It remains to be determined whether the collagen IV-dependent, FAK-mediated role of integrin α_2 in hepatic metastasis suggested in the current report also applies to its capacity to enhance intrahepatic migration of tumor cells.

The link between integrins and intracellular signaling molecules involved in cell migration, such as FAK, is an emerging theme in metastasis research.²²⁾ Of note, a protein tyrosine phosphatase that affects cell motility, PRL-3, has been implicated in enhanced metastatic potential of colon cancer.²³⁾²⁴⁾ Whether PRL-3 specifically enhances liver metastasis is unclear, since it was equivalently up regulated in metastases from multiple organs.²⁵⁾ This contrasts with our findings in animal models that integrin α_2 expression did not significantly enhance lung metastasis after intravenous injection of tumors and was expressed at lower levels in lung vs. liver metastasis of human colon cancer. Whether there is a direct link between integrin α_2 and PRL-3 remains to be determined.

In summary, we have provided direct evidence in murine models and correlative evidence in human tumors that integrin $\alpha_2\beta_1$ is one of the determinants conferring potential for selective hepatic metastasis. We hypothesize that integrin α_2 expression may be particularly important for liver metastases in tumors whose primary venous drainage flows through the liver, such as gastrointestinal cancers. In this circumstance, integrin α_2 would putatively engage collagen type IV in the liver, facilitating arrest of tumor cells

and FAK activation, which in turn effects cell motility changes that would facilitate metastatic distribution within the liver parenchyma.

This finding has both prognostic and therapeutic significance. Analysis of integrin α_2 in primary tumors may help determine which patients are at higher risk for forming hepatic metastases. Additionally, there is potential to block the formation of hepatic metastases in patients who are discovered to have colorectal cancer and who have expression of integrin α_2 in their resected primaries. This therapy could potentially be used from the time of diagnosis immediately after colonoscopic biopsy, through the period of surgical therapy in which the primary was removed, and immediately after in the adjuvant setting. The possibility of decreasing the hepatic recurrence rate of patients who have undergone curative resection of hepatic colorectal metastases would need to be explored.

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