Tumor cell $\alpha_3\beta_1$ integrin and vascular laminin-5 mediate pulmonary arrest and metastasis

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Arrest of circulating tumor cells in distant organs is required for hematogenous metastasis, but the tumor cell surface molecules responsible have not been identified. Here, we show that the tumor cell $\alpha_3\beta_1$ integrin makes an important contribution to arrest in the lung and to early colony formation. These analyses indicated that pulmonary arrest does not occur merely due to size restriction, and raised the question of how the tumor cell $\alpha_3\beta_1$ integrin contacts its best-defined ligand, laminin (LN)-5, a basement membrane (BM) component. Further analyses revealed that LN-5 is available to the tumor cell in preexisting patches of exposed BM in the pulmonary vasculature. The early arrest of tumor cells in the pulmonary vasculature through interaction of $\alpha_3\beta_1$ integrin with LN-5 in exposed BM provides both a molecular and a structural basis for cell arrest during pulmonary metastasis.

Introduction

The lung is a frequent site for metastasis of many different tumor types. Tumor cells enter the circulation from the primary tumor and then colonize distant organs. The interactions between the tumor cells and the vessels that allow the initial arrest of the tumor cell in the lung are poorly characterized, although in individual cases contributing molecules have been identified (Abdel-Ghany et al., 2001; Abdel-Ghany et al., 2003). Recently, we developed methods that allow the observation of the early events in metastasis after entry of the cells into the pulmonary circulation (Al-Mehdi et al., 2000). Fluorescent tumor cells were introduced i.v. into mice or rats, and their lungs were isolated and maintained and observed under physiological conditions. Using these methods, we showed that intravascular proliferation rather than early extravasation characterized the initial events in metastatic colony formation. Here, we have adapted these methods to study the initial arrest of tumor cells in the pulmonary vasculature and investigate the role of integrins in this process.

It has long been supposed that integrins play an important role in metastasis. This supposition is based in part on the role of integrins in motility, and in part on data showing that agents that interrupt integrin–ligand interaction also inhibit metastasis. For example, peptides containing the motif RGD (Arg-Gly-Asp) that compete for binding of integrins to fibronectin, or peptides that block binding of integrins to laminin (LN) can inhibit metastasis when cojected with tumor cells (Humphries et al., 1986; Saiki et al., 1989; Yamamura et al., 1993). Antibodies directed against surface integrins that affect LN binding also reduced lung metastasis (Vollmers et al., 1984). Each integrin is a heterodimer composed of both an $\alpha$ and a $\beta$ subunit. The ligand-binding domain of the integrin heterodimer is a globular region that requires both subunits to engage the ligand. Thus, antibodies specific for either the $\alpha$ or the $\beta$ chain can be blocking. Integrins bind to components of ECM such as collagens, fibronectin, and LNs, and can mediate adhesion, spreading, or migration on these substrates (Schwartz, 2001; van der Flier and Sonnenberg, 2001).

Using an attachment assay based on the observation of fluorescent tumor cells in isolated lungs, we have evaluated the involvement of integrins in the arrest of tumor cells in...
the pulmonary circulation. This report shows for the first time that the αvβ3 integrin is an important (but not exclusive) component in that process. The importance of αvβ3 integrin in pulmonary vascular attachment by tumor cells raised the question of access to its ligands. LN-5, -8, -10, and -11 are ligands for the αvβ3 integrin (Nissinen et al., 1997; Fukushima et al., 1998; Kikkawa et al., 2000; Fujiwara et al., 2001). Each is mainly found in ECM with LN-8/9 and LN-10/11 also present in the stroma of the bone marrow (Siler et al., 2000). How ECM or basement membrane (BM) components could be exposed to tumor cells in pulmonary vessels was not immediately apparent. Unexpectedly, examination of vessels at the sites of tumor cell attachment revealed exposed BM, enabling the binding of αvβ3 integrin.

## Results

### Assay for tumor cell arrest in the pulmonary vasculature

To assess the arrest of tumor cells in the lung, we injected different numbers of fluorescent metastatic HT1080 cells into the renal vein of rats. Immediately after the injection, the isolated, ventilated, and perfused lungs were examined. Fluorescent tumor cells were counted in 60 consecutive nonoverlapping images under low power (10×). The curve of arrested versus injected cells was effectively linear over a range up to 2 × 10⁶ cells. Therefore, injection of cell numbers within the linear range was used in the subsequent experiments to measure pulmonary arrest (Fig. 1a).

### Effects of integrins on pulmonary attachment in vivo

HT1080 cells are known to express β1, β3, β4, β5, and β6, and α1, α2, α3, α4, α5, α6, and αv integrin subunits (Petersmann et al., 1993). Before i.v. infusion, HT1080-GFP cells were incubated with blocking antibodies to the β1, β3, or β4 integrin subunits or to the αvβ3 integrin. Because blocking antibody to β3 integrin is not available, the effect of this integrin was evaluated with the blocking antibody to αv, the exclusive heterodimeric partner for the β3 integrin subunit (Nishimura et al., 1998). Of the anti-β subunit antibodies tested, only earlier treatment with anti-β1 blocking antibody resulted in a significant reduction in cell attachment (Fig. 1b). Treatment of HT1080-GFP cells with a nonblocking antibody to β1 integrin did not alter pulmonary arrest, nor did infusion of the blocking antibody into the lung before infusion of the tumor cells (Fig. 1c and d). These results demonstrated that integrins including the β3 subunit were involved in the arrest of HT1080 in the pulmonary vasculature.

HT1080-GFP cells were incubated with blocking antibodies to the α1, α2, α3, α4, or αv integrin subunits. As shown in Table I, blocking antibodies against the α1, α2, α4, or αv integrin subunits did not affect pulmonary attachment. Anti-α3 integrin subunit antibody had the greatest effect with 34% inhibition, whereas slight reductions of 15 and 16% were seen after the treatments with anti-α3 and α4 integrin subunits, respectively. The effect of blocking the α2 integrin subunit was statistically significant; the effect on the α4 integrin subunit was not. Combining these three antibodies increased the inhibition, but was not entirely additive (unpublished data). Blocking antibodies to α3 (but not αv) integrin subunits similarly reduced pulmonary arrest by two metastatic breast carcinoma cell lines, MDA-MB-231 and ASC-1. A summary of the results is shown in Table I.

### Table I. Effect of anti-α integrin subunit blocking antibodies on pulmonary arrest

| Antibody specificity | Control   | Antibody    | Inhibition |
|----------------------|-----------|-------------|------------|
|                      | cells/mm² | cells/mm²   | %          |
| α1                   | 6.00 ± 0.53 | 5.78 ± 0.65 | 4          |
| α2                   | 7.23 ± 0.29 | 6.10 ± 0.52 | 15         |
| α3                   | 7.42 ± 1.07 | 4.93 ± 1.14 | 34         |
| α4                   | 5.95 ± 0.64 | 5.02 ± 0.66 | 16         |
| αv                   | 4.65 ± 1.73 | 5.03 ± 2.57 | 8          |
| α3                  | 5.94 ± 1.87 | 6.20 ± 1.83 | 5          |
| α4                  | 6.20 ± 1.50 | 6.00 ± 2.07 | 3          |

HT1080-GFP cells were treated with the indicated blocking antibodies as described in the Materials and methods section. Each value is expressed as cells/mm² ± SD. The anti-αv subunit antibody used was P1B5. Similar results were obtained with ASC-1 (unpublished data). The comparison of means using t test was P = 0.03 for α3 integrin subunit blocking antibody and P = 0.05 for αv. The value for α4 was P = 0.15.
expression vector resulted in the recovery of pulmonary attachment (Fig. 2 a). Likewise, genetic elimination of the α3 integrin subunit in ESb cells compared with K562 parental cells enhanced cell attachment by 40%, but expression of the α1 integrin subunit expression had no effect (Fig. 2 c). These data further support the contention that the α3β1 integrin participates in tumor cell pulmonary attachment.

Anti-α3 or anti-β1 integrin subunit antibody treatment of HT1080-GFP cells also had an inhibitory effect on early colony formation 1 wk after injection. The cells were exposed to the indicated antibody before injection without any subsequent exposure to the antibody, as in the experiments shown in Fig. 1 b. This initial exposure was sufficient to reduce colony formation. The decrease in colony number exceeded the decrease in pulmonary attachment (Table III). Initial pulmonary arrest using the α3β1 integrin appears to play a critical role in subsequent colony formation.

### α3β1 integrin ligands in vivo

LN-5 is the best-characterized ligand for the α3β1 integrin. Because it is mainly a BM component in the lung, we expected that it would be covered by endothelium and not available to circulating tumor cells in the pulmonary vessels (Mizushima et al., 1998; Coraux et al., 2002). To search for LN-5 in vascular channels, we infused two different fluorescently labeled antibodies to the α1 chain of LN (MIG-1 and CM6) sequentially after the injection of HT1080-GFP-vimentin cells (these mAbs react with rat, but not mouse LN-5). The α3 chain of LN is found in LN-5, -6, and -7 (Colognato and Yurchenco, 2000; Nguyen et al., 2000b). However, LN-5 is considered to be markedly more abundant than the others (Adair-Kirk et al., 2003). Although MIG-1 generally gave a stronger signal than CM6, both anti-LN-5 antibodies stained small areas surrounding the arrested tumor cells (Fig. 3, a and b). A similar signal was obtained after the infusion of antibody against the LN γ2 chain (Fig. 3, e and g). The LN γ2 chain is unique to LN-5 confirming its presence, but not excluding the presence of LN-6 or -7.

GFP-vimentin was used to label the cells because the signal from cytoplasmic GFP alone overwhelmed the signal. To test whether these patches existed before the infusion of tumor cells, fluorescently labeled antibodies to either the α3 chain (MIG-1) or the γ2 chain of LN were injected into naïve rats. Foci of antibody staining were found in lungs (Fig. 3, c, d, and f). They are infrequent, approximately only on 10–20 high power fields surveyed. Fluorescent antibodies to irrelevant molecules, the cartilage-specific collagen II, or to the platelet integrin αIIb failed to stain any vascular patches (Fig. 3 h). These results have led us to suggest that exposed BM preexists in the pulmonary vasculature.

To further test the hypothesis that LN-5 acts as a ligand for the α3β1 integrin on tumor cells, we infused antibodies to the α3 chain (CM6) before the injection of HT1080-GFP tumor cells. These antibodies reduced the extent of pulmonary attachment.

### Table II. Effect of α integrin subunit blocking antibodies on pulmonary attachment by breast cancer cell lines

| Antibody | MDA-MB-231 (Control) | Blocking antibody | Inhibition | MDA-MB-435s (Control) | Blocking antibody | Inhibition |
|----------|----------------------|------------------|------------|----------------------|------------------|------------|
| α3       | 6.38 ± 0.67          | 4.74 ± 0.42      | 26%        | 7.79 ± 1.36          | 5.19 ± 0.92      | 33%        |
| α6       | 6.45 ± 0.77          | 6.40 ± 0.61      | 1%         | 7.25 ± 1.28          | 7.22 ± 0.88      | 0%         |

The effect of anti-α3 or anti-α6 integrin subunit blocking antibodies on the breast carcinoma cell lines MDA-MB-231 and MDA-MB-435s was determined as described in the Materials and methods section. Each value is expressed as cells/mm² ± SD.
nary attachment compared with the pulmonary arrest seen when the antibodies were infused after injection of the tumor cells (Fig. 3i).

**Interaction between tumor cells and vascular BM**

We used EM as an alternative means to visualize tumor cell–pulmonary vessel interactions. HT1080-GFP-vimentin cells were labeled with ferritin before injection to allow their identification. 38 tumor cells were identified, all within the pulmonary vessels. Of these, nine cells showed distinct attachment to the vessel wall in EM images. In each of these cases, at the point of contact between the tumor cell and the vessel, the vessel was missing the expected endothelial covering of the basal lamina (Fig. 4, a–c). When the tumor cells were pretreated with a blocking anti-β1 integrin subunit antibody, no points of contact were identified (0 out of 33 tumor cells; P = 0.001). Rare patches of exposed BM could be found in the absence of tumor cells (Fig. 4d). These observations lead to the hypothesis that the foci of exposed BM between endothelial cells may be a prerequisite for tumor cell vascular attachment in the lung.

**Discussion**

Our analyses have suggested that tumor cells arrest in the pulmonary vasculature through interaction of their α3β1 integrin with exposed BM. We have shown that the α3β1 integrin contributes to metastasis through mediation of early adhesion to the vasculature using a variety of cells, HT1080, MDA-MB-231 and -435s, ESB cells, K562 cells, and immortalized keratinocytes. Interestingly, the α3β1 integrin is commonly expressed by most tumor cells. In some reports, higher expression of the α3β1 integrin correlated with increased metastasis (Morini et al., 2000). Involvement of the β1 integrin subunit in attachment is consistent with data showing reduced metastasis by a lung carcinoma cell line after treatment with blocking antibodies to the β1 integrin subunit (Takenaka et al., 2000). The α3β1 integrin associates with the urokinase receptor and with the tetraspanin CD151, both implicated in metastasis (Scherberich et al., 1998; Testa et al., 1999; Wei et al., 2001; Zhang et al., 2001). In keratinocytes, secretion of matrix metalloproteinase-9, a molecule known to influence metastasis, is dependent on the α3β1 integrin (DiPersio et al., 2000). Our data

**Table III. Inhibition of early colony formation by pre-treatment with anti-integrin subunit antibodies**

| Treatment                  | Colonies/lung ± SD | Range (of colonies) | # of mice | t test against no antibody |
|----------------------------|--------------------|---------------------|-----------|---------------------------|
| No antibody                | 2.25 ± 1.71        | 0–5                 | 12        |                           |
| Anti-β1 blocking           | 0                  | 0                   | 7         | 0.001                     |
| Anti-β1 nonblocking        | 2.43 ± 1.72        | 1–6                 | 7         |                           |
| Anti-α3 (P1B5)            | 0.71 ± 0.76        | 0–2                 | 7         | 0.016                     |
| Anti-α3 (ASC-1)           | 0.71 ± 0.95        | 0–2                 | 7         | 0.022                     |
| Anti-α6                   | 1.86 ± 2.27        | 0–5                 | 7         |                           |

HT1080-GFP cells were treated with the indicated antibodies for 0.5 h before injection as described in the Materials and methods section. After washing, 5 × 10⁵ cells were injected into the tail vein of nu/nu mice. The numbers of colonies were counted after scanning the entire surface of the left lobe of the isolated mouse lung. A two-tailed t test is shown only for those with a significant difference as compared to the no antibody control.

Figure 3. **LN-5 can act as a ligand in the pulmonary vasculature.** Immediately after infusion of HT1080-GFP-vimentin cells into rats, the labeled antibodies to LN-5 were infused. 10 min later, the lungs were isolated and perfused. Panels show representative images obtained with labeled (a) anti-LN α1 chain antibody (MIG-1), or (b) CM6. (c and d) Typical images seen after infusion of the Alexa Fluor® 647-labeled MIG-1 antibody in naive rats. (e) An image after Alexa Fluor®-labeled anti-LN γ2 chain antibody staining. The image is a rotation of a three-dimensional reconstruction showing the direct proximity of the γ2 staining to the cell. (f) LN γ2 chain antibody staining in naive rats. (g) An HT1080-GFP-vimentin cell in a vessel filled with tetramethylrhodamine dextran. LN γ2 staining is seen in blue on the vessel wall. (h) Alexa Fluor® control image. (i) Anti-LN α3 chain antibody (CM6) was infused either before or after infusion of HT1080-GFP cells. When antibody infused before, cell attachment was less, P = 0.019. Bars, 20 μm.
expected platelet aggregation. However, the exposure of the surface of the basal lamina or the BM might not expose collagen, or more specifically, their helical domains. In some BMs, the collagens appear to be beneath the basal lamina (Nguyen et al., 2000a). Consistent with this possibility, anti-collagen IV antibody only stained poorly in our hands (unpublished data). This would also explain why the exposed BM regions do not have associated platelet aggregation. Metastasis may favor the lung because of this unique feature of its vessels. Whether arrest in the bone marrow is influenced by the α3β1 integrin binding with LN-8/9 or LN-10/11 remains to be studied. Other organs may also use different adhesion factors. For example, expression of the α5 integrin subunit enhances the arrest of tumor cells in the kidney glomeruli, but does not affect cell attachment in the lung or the liver (Tani et al., 2003).

The α5β1 integrin also binds to LN-5, yet anti-α6 integrin subunit antibody failed to alter attachment in vivo in our experiments. There are other situations in which the α5β1 integrin and the α3β1 integrin function differently. Antibodies to the α3 integrin subunit blocked migration of pancreatic carcinoma cell lines and inhibited adhesion of keratinocytes to LN-5, whereas antibodies to the α6 subunit failed to have these effects (Tani et al., 1997; Hintermann et al., 2001). The signaling pathways through these two integrins are distinct in keratinocytes (Hintermann et al., 2001; Mercuro et al., 2001b). The α5β1 integrin is critical for the formation of hemidesmosomes, whereas the α3β1 integrin regulates adhesion, spreading, and migration in association with the ECM (Borradori and Sonnenberg, 1999). Nonetheless, the α5β1 integrin clearly plays a role in metastasis, if not a demonstrable role in the early arrest of tumor cells in the lung (Mercuro et al., 2001a; Jauliac et al., 2002). Metastasis to the mouse lung is inhibited both by pretreatment of the lung with anti-α6 integrin subunit antibody and treatment of the tumor cells with the antibody (Ruiz et al., 1993). Furthermore, α6 integrin was shown to contribute to survival of breast carcinoma cells as they formed metastatic colonies (Wewer et al., 1997). The α5β1 can also bind LN-5. Some of the actions attributed to the α6 subunit may involve this integrin. Thus, the α6 integrin subunit may augment metastasis, not through enhancing pulmonary arrest, but by facilitating survival or proliferation. Pauli’s group has shown that the β1 integrin subunit on tumor cells can ligate to endothelial-bound CLCA1, mediating a signaling cascade that includes FAK activation (Abdel-Ghany et al., 2002). Although they have postulated that this interaction initiates vascular arrest, their data are equally comparable with a model synthesizing our data in which the tumor cell α3β1 integrin interacts with exposed LN-5, allowing subsequent interactions that then signal for intravascular survival and proliferation. CLCA1 may be one of the factors that contribute to pulmonary arrest in addition to the α5β1 integrin.

This model is consistent with the observation that pulmonary metastasis is enhanced by endothelial damage. Both hypoxia and bleomycin or other chemotherapeutic drugs induce endothelial injury and lead to exposure of the BM (Nicolson and Custead, 1985; Orr et al., 1986; Lichtner and Nicolson, 1987). Orr et al. (1986) demonstrated that endothelial damage with bleomycin promoted pulmonary me-

Figure 4. EM shows tumor cells attached to exposed BM. HT1080-GFP-vimentin cells were exposed to ferritin before injection into rats. Lungs were isolated and fixed with glutaraldehyde by perfusion. (a) A representative image from EM with a tumor cell with internalized ferritin within a small vessel and two contact points seen between the tumor cell and the vessel wall. (b) An enlargement of a contact point. At the contact point, the expected overlying endothelium is absent, showing the tumor cell attached to the BM. (c) A tracing of the structures in b. (d) An incidental portion of exposed BM found in lung not involved by tumor.
tastasis, and most of the arrested cells were found attached to the endothelial BM. It has been proposed that tumor cells induce the retraction of endothelial cells, enhancing metastasis (Honn et al., 1989, 1994). Our results suggest that LN-5 is available before interaction with tumor cells, but they do not preclude further retraction after arrest.

These analyses put forward a new model for pulmonary metastasis in which tumor cells use the α5β1 integrin to bind to LN-5 in exposed BM. Colony formation was decreased point out that we did not see cells in aggregates in the lung after injection. Single-cell suspension before injection. As additional confirmation, we treatment with anti-integrin antibodies (10 ng/ml) in a ratio of 10:1 to the same medium. Although some reports have used as follows: anti-β1, clone 6SA (Shang et al., 2001); anti-β3, clone B3A; anti-β3, clone CD104; clone ASC-3; anti-αvβ3, clone P1E6; anti-αv, clone AV1; anti-α6, clone DB1B2; anti-α6, clone P1E6; anti-αv, clone P1B5 and anti-α5, clone ASC (Wayner et al., 1988; Wagner et al., 1991); anti-αv, clone P1H4; anti-α6, clone P1D6; and anti-αv, clone NK1-Gof3. The anti-β integrin nonblocking stimulating antibody is clone 21C8. MIG-1 and CM6 are mouse mAbs raised against different domains of the αv chain of LN-5. Both antibodies react with rat, but not murine LN-5 (Plopper et al., 1998; Shang et al., 2001). Anti-LN γ1 chain antibody was purchased from CHEMICON International. Antibodies against LNs, or collagen II, were freshly labeled with Zenon mouse IgG labeling kits; Alexa Fluor® 647 ( Molecular Probes, Inc.) in a ratio of 10 μg antibody:50 μl labeling reagent:50 μl blocking reagent.

Treatments of cells with antibodies Cells were harvested, washed with serum-free medium, and resuspended in the same medium containing 0.1% BSA. The suspensions were incubated with antibodies (10 μg/ml) at 4°C for 30 min on a rotator, followed by dilution with equal volume of the same medium. Although some reports have indicated that treatment with anti-αv, integrin subunit antibody can lead to aggregation of keratinocytes (Nguyen et al., 2001), to ensure that aggregation was not occurring in our experiments, we verified that the cells were in single-cell suspension before injection. As additional confirmation, we point out that we did not see cells in aggregates in the lung after injection.

Intravital attachment test Tumor cells were labeled by either stable GFP expression (HT1080-GFP and HT1080-GFP-vimentin) or vital fluorescence dyes. ESB cells and derivatives were incubated in 100 μM CellTracker™ Green CMFDA ( Molecular Probes, Inc.) in a medium containing 10% FBS at 37°C for 30 min. K562 cells, MK cells, and MDA-MB-231 or -435s cells were labeled with the vital dye Mitotracker® Red CMXROS (200 nM; Molecular Probes, Inc.) by 5 min exposure in culture medium. Experiments using CMFDA or Mitotracker® Red on HT1080-GFP cells gave the same results as monitoring GFP. Male rats (~250 g; Sprague-Dawley) were injected into the renal vein with 3 × 10^6 cells unless otherwise indicated. For colony assays, female mice (CD-1 nude: Charles River Laboratories) received 5 × 10^3 cells in the lateral tail vein. 30 min after injection for the attachment assays or 1 wk after injection for the colony assays, the lungs were ventilated, perfused, and isolated under physiological pressures as described previously (Al-Mehdi et al., 1998, 2000). Fluorescent tumor cells were observed in the isolated lungs using an inverted fluorescent research microscope (model DMIRB; Leica), and images were recorded with a camera (Orcia; Hamamatsu Photonics) with OpenLab software (Improvision). The quantitative assays were based on counting cells in 60 consecutive and nonoverlapping fields with a 10×/1× lens (1.1 mm² field/picture). Rats and mice were matched by weight, and each antibody was tested in matched pairs of animals. The results were obtained from at least three independent experiments in all cases.

Confocal scanning laser microscopy 5 min after injection of HT1080-GFP-vimentin cells (10^6) into the left renal vein, antibody–Alexa Fluor® complexes were introduced through the inferior vena cava. Lungs were isolated as described above. The pulmonary vasculature was labeled by perfusion with tetracythylrhodamine dextran ( Molecular Probes, Inc.). The images were captured using a laser scanning system (Radiance 2000; Bio-Rad Laboratories) and a microscope (Eclipse TE300; Nikon). The 2-line Argon/krypton laser (wavelength 488 nm/568 nm) and the red laser diode (wavelength 638 nm) were used for observation.

Transmission EM observation HT1080-GFP-vimentin cells were labeled with the electron-dense marker ferritin at 4°C for 10 min. 5 or 30 min after i.v. injection (2 × 10^6 cells) into rats, the lung was dissected as described above. Afterwards, the lung was washed with PBS, followed by perfusion with 5% glutaraldehyde. Samples were collected from the upper region of the left lung and fixed in 2.5% glutaraldehyde at 4°C. The images were photographed with a transmission electron microscope, 80 kV (JEM-1010; JEOL USA, Inc.)

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