Inhibition of Experimental Metastasis by Targeting the HUIV26 Cryptic Epitope in Collagen

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Metastasis from the primary tumor to distant sites involves an array of molecules that function in an integrated manner. Proteolytic remodeling and subsequent tumor cell interactions with the extracellular matrix regulate tumor invasion. In previous studies, we have identified a cryptic epitope (HUIV26) that is specifically exposed after alterations in the triple helical structure of type IV collagen. Exposure of this cryptic epitope plays a fundamental role in the regulation of angiogenesis *in vivo*. However, little is known concerning the ability of tumor cells to interact with this cryptic site or whether this site regulates tumor cell metastasis *in vivo*. In this regard, many of the same cellular processes that regulate angiogenesis also contribute to tumor metastasis. Here we provide evidence that tumor cells such as B16F10 melanoma interact with denatured collagen type IV in part by recognizing the HUIV26 cryptic site. Systemic administration of a HUIV26 monoclonal antibody inhibited experimental metastasis of B16F10 melanoma *in vivo*. Taken together, our findings suggest that tumor cell interactions with the HUIV26 cryptic epitope play an important role in regulating experimental metastasis and that this cryptic element may represent a therapeutic target for controlling the spread of tumor cells to distant sites. (Am J Pathol 2006, 168:1576–1586; DOI: 10.2353/ajpath.2006.050176)

Metastasis or the spread of malignant tumor cells from the primary tumor mass to distant sites involves a complex series of interconnected events.^{1–6} Understanding the biochemical, molecular, and cellular processes that regulate tumor metastasis is of great importance. The metastatic cascade is thought to be initiated by a series of genetic alterations leading to changes in cell-cell in-

teractions allowing disassociation of cells from the primary tumor mass. These events are followed by local invasion and migration through proteolytically modified extracellular matrix (ECM). To establish secondary metastatic deposits, the malignant cells evade the host immune surveillance, arrest in the microvasculature, and extravasate out of the circulation. Finally, tumor cells can invade the local ECM, proliferate, recruit new blood vessels by induction of angiogenesis, and expand to form secondary metastatic foci.^{1–6} Although it is well accepted that tumor cell interactions with the ECM play an important role in metastasis, relatively little is known concerning the ability of tumor cells to interact with proteolytically remodeled ECM or whether these interactions contribute functionally to the spread of tumor cells to distant sites.

Because tumor metastasis involves a complex cascade of interdependent events, studying this process is a difficult task. In this regard, a number of *in vitro* assays have been developed to study individual cellular processes in metastasis including adhesion, invasion, migration, and proliferation.^{7–10} *In vitro* assays, although valuable, do not completely recapitulate the physiological events that facilitate tumor dissemination. Therefore, investigators have developed several *in vivo* systems, including a number of murine and rat metastasis models.^{11–13} Depending on the particular questions to be addressed, these models can provide distinct insight into the metastatic cascade.

A major impediment to effective treatment of malignant tumors involves the development of resistance to standard therapeutic modalities as well as metastatic dissemination of tumor cells. Thus, the identification of novel therapeutic targets and treatment strategies are of paramount importance. Although many studies have confirmed the importance of targeting specific secreted growth factors, proteases, cell surface adhesion receptors, and many intracellular regulatory molecules, these

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approaches have met with only limited success due in part to the genetic instability of tumor cells.^{14–17} Therefore identifying new functional targets within the noncellular compartment may provide an effective new clinical strategy. To this end, our previous studies have identified a unique cryptic site (HUIV26) within collagen that regulates angiogenesis and endothelial cell behavior.^{18–21} This functional cryptic site has been shown to be highly expressed within the ECM of malignant tumors and within the subendothelial basement membrane of tumor-associated blood vessels.^{18–21} However, little is known concerning the capacity of tumor cells to interact with this cryptic site or whether interactions with this site alter tumor cell behavior *in vitro* and metastasis *in vivo*. Here we provide evidence for the first time that malignant tumor cells can interact with the HUIV26 cryptic epitope and that blocking these interactions inhibits tumor cell adhesion and migration *in vitro* and experimental metastasis *in vivo*.

Materials and Methods

Reagents, Chemicals, and Antibodies

Ethanol, methanol, acetone, and phosphate-buffered saline (PBS) were all obtained from Sigma (St. Louis, MO). OTC embedding compound was obtained from VWR International (Bridgeport, NJ). The monoclonal antibody HUIV26, which specifically reacts with denatured collagen type IV and does not react with the cell surface, has been previously described¹⁸ and was kindly provided by Cell Matrix Inc. a subsidiary of CancerVax Corp. (Carlsbad, CA). Hematoxylin, eosin-Y, xylene, and Permount were obtained from Fischer Scientific (Pittsburgh, PA). Monoclonal antibody (mAb) A103 directed to the melanoma-associated antigen MART-1 was obtained from Oncogene Research Products (San Diego, CA). Nonspecific normal mouse IgM control antibody was obtained from Pierce (Rockford, IL). Rhodamine-labeled goat anti-mouse secondary antibody was obtained from BioSource International (Camarillo, CA). Fluorescein isothiocyanate-labeled *Lycopersicon esculentum* lectin was obtained from Vector Laboratories (Burlingame, CA). WST-1 proliferation kits were obtained from Chemicon International (Temecula, CA). Purified collagen type I and IV were obtained from Sigma. Thermally denatured collagen was prepared by resuspending the collagen at a concentration 1.0 mg/ml in PBS and boiling the sample for 12 minutes.

Cells and Cell Culture

Murine B16F10 melanoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Tumor cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1.0% sodium pyruvate, glutamate, and Pen-Strep (Life Technologies, Inc.). Cells were maintained as subconfluent cultures before use and harvested with trypsin-

ethylenediaminetetraacetic acid (Life Technologies, Inc.).

Cell Adhesion and Proliferation Assays

Cell adhesion assays were performed as described previously with some modifications.²² Briefly, 48-well nontissue culture plates were coated with native triple helical or thermally denatured collagen types I and IV (10.0 μ g/ml) for 12 hours at 4°C. The plates were next washed with PBS and nonspecific binding sites were blocked by incubation with 1.0% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Tumor cells (B16F10) from subconfluent cultures were harvested, washed, and resuspended in adhesion buffer containing RPMI 1640, 1 mmol/L $MgCl_2$, 0.2 mmol/L $MnCl_2$, and 0.5% BSA in the presence or absence of function-blocking antibodies (0 to 100 μ g/ml) or an isotype-matched control antibody. Tumor cells were added to the coated plates in a total volume of 200 μ l and allowed to attach for 15 to 30 minutes. Nonattached cells were removed by washing and attached cells were stained with crystal violet as described previously.²² Cell adhesion was quantified by measuring the optical density of eluted crystal violet from attached cells at a wavelength of 600 nm.²² In cell proliferation assays, microtiter wells were coated with either native or denatured collagen type I or IV (10 μ g/ml). Tumor cells (B16F10) were resuspended in proliferation buffer containing 1.0% serum in the presence or absence of mAb HUIV26 or an isotype-matched control antibody (0 to 100 μ g/ml) and allowed to proliferate throughout a 3-day time course. Cellular proliferation was measured with a WST-1 tetrazolium salt cleavage assay kit (Chemicon International) according to the manufacturer's instructions. Cell proliferation was monitored using a microplate reader at a wavelength of 490 nm. Experiments were performed in triplicates and repeated twice with similar results.

Cell Migration Assay

Cell migration assays were performed as described previously with some modifications.²² Briefly, membranes (8.0- μ m pore size) from transwell migration chambers were coated with native triple helical or thermally denatured collagen type I or IV (10.0 μ g/ml) for 12 hours at 4°C. The transwells were next washed with PBS and nonspecific binding sites were blocked by incubation with 1.0% BSA in PBS for 1 hour at 37°C. Tumor cells from subconfluent cultures were harvested, washed, and resuspended in migration buffer containing RPMI 1640, 1 mmol/L $MgCl_2$, 0.2 mmol/L $MnCl_2$, and 0.5% BSA in the presence or absence of function-blocking antibody (0 to 100 μ g/ml) or an isotype-matched control. Tumor cells were allowed to migrate to the underside of the coated transwell membranes for 2 to 4 hours. Tumor cells remaining on the top-side of the membrane were removed and cells that had migrated to the under side were stained with crystal violet as described previously.²² Cell

migration was quantified by direct cell counts per microscopic field.

Chick Embryo Experimental Metastasis Assay

Twelve-day-old fertilized chick eggs were obtained from SPAFAS (North Franklin, CT) and maintained in a 48-place tabletop egg incubator (Lyon Electric, Chula Vista, CA) as described previously.^{23,24} Prominent blood vessels were visualized through the eggshell of the 12-day-old chick embryos with the aid of an egg candle.^{23,24} The area of the outer egg shell where prominent blood vessels are located close to the inner shell surface was swabbed with 70% ethanol and a small window was cut through the egg shell with a hobby grinding wheel (Dremel Emerson Electric Co., Racine, WI). The embryos were returned to the incubator until tumor cells were prepared for injection. Subconfluent cultures of B16F10 melanoma cells were washed with sterile PBS and harvested with trypsin ethylenediaminetetraacetic acid. Tumor cells were washed with serum containing Dulbecco's modified Eagle's medium and resuspended in sterile PBS at concentrations ranging from 0.5 to 5.0×10^6 per ml. Next, the small windows cut through the egg shell were carefully removed and a drop of mineral oil was added to the shell membrane to enhance visualization of the underlying blood vessels.^{23,24} Tumor cell suspensions were injected intravenously in a total volume of 100 μ l per embryo. The embryos were allowed to incubate undisturbed for 24 hours or a total of 7 days. To quantify experimental B16F10 lung metastasis, embryos were sacrificed at day 19 and both lobes of the chick lungs were dissected. The lungs were analyzed with the aid of a stereo microscope set at a defined magnification. The total number of isolated and discrete pigmented lung surface lesions was carefully counted on each side of each lobe for each embryo. A typical experiment included at least six embryos per condition. Experimental metastasis was described as the mean number of surface B16F10 melanoma lesions per lung per experimental condition.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of B16F10 Lung Tumor Lesions

To detect the presence of B16F10 melanoma cells at early time points (24 hours) semiquantitative RT-PCR was performed using primers specific for B16 M562 melanoma antigen and control primers for chick 18s ribosome as previously described.^{25,26} Briefly, chick lungs from each experimental condition were harvested at the end of a 24-hour incubation period after tumor cell inoculation and mRNA prepared as previously described.^{25,26} To examine the relative levels of B16F10 melanoma cells in the lungs of chick embryos at early time points, we used PCR primers directed to the B16-specific M562 melanoma antigen (forward) 5'-TGGGCTTGTAAGTACTG-GACTC-3' and (reverse) 5'-TTCACGTGATTTACAATC-

TCCTATTG-3'.^{25,26} In control experiments, PCR primers specific for chick 18S ribosome were used that included (forward) 5'-TTCGTATTGTGCCGCTAGAG-3' and (reverse) 5'-GCATCGTTAATGGTCGGAAC-3'.

Murine Experimental Metastasis Assay

The experimental metastasis assay was performed essentially as described with some modifications.^{24,27} Briefly, subconfluent cultures of B16F10 melanoma cells were harvested, washed, and resuspended in sterile PBS in the presence or absence of mAb HUIV26 or an isotype-matched control antibody (100.0 μ g/ml). Female BALB/c mice were injected intravenously (100 μ l) with tumor cells (2×10^5). After injection of the tumor cells, the mice were untreated or treated daily (7 days) (intraperitoneally) or treated with a single injection of mAb HUIV26 or normal mouse IgM control antibody (100 μ g) in a total volume of 100 μ l of sterile PBS. At the end of the treatment periods, the mice were sacrificed and the lungs were removed for analysis. To quantify experimental B16F10 lung metastasis, lungs were dissected and placed in 35-mm culture plates. The lungs were analyzed with the aid of a stereomicroscope set at a defined magnification ($\times 30$). The total number of isolated and discrete pigmented lung surface lesions was carefully counted on each lobe for each specimen. Experimental metastasis is described as the mean number of surface tumor lesions per lung per experimental condition. Presence of tumor lesions within the lungs was confirmed by histological analysis.

Immunohistochemical and Immunofluorescence Analysis

Lungs from chick embryos or mice were dissected and embedded in OTC, snap-frozen, and 4.0- μ m sections were cut with a cryostat as described previously.^{28,29} For histological analysis, frozen sections of lung tissue were fixed in 10% formalin and stained with hematoxylin and eosin. Immunohistochemical analysis was performed as previously described with some modifications.³⁰⁻³² Briefly, lung sections (4.0 μ m) were fixed for 30 seconds in 50% methanol and 50% acetone. The tissue sections were washed three times and incubated with 2.5% BSA in PBS to block nonspecific binding sites. mAb HUIV26 was diluted in 2.5% BSA in PBS to a final concentration of 10 μ g/ml and 100 μ l was added to the tissue sections. Tissues were incubated for a total of 2 hours at 37°C. The tissues were next washed five times with PBS for 5 minutes each followed by incubation with horseradish peroxidase-labeled goat anti-mouse secondary antibody (1:300) for 1 hour. The tissue sections were washed as before and photographed at a magnification of $\times 200$. For immunofluorescence co-staining, tissue sections were prepared as described above and incubated with goat anti-mouse rhodamine-labeled secondary antibody (1:400) and fluorescein isothiocyanate-labeled *L. esculentum* lectin (10 μ g/ml) for detection of blood vessels. The tissue sections were washed as before and a drop of

anti-fade mounting medium added. The tissue sections were then photographed at a magnification of $\times 630$ under oil emersion.

Statistical Analysis

Statistical analyses of experimental data were analyzed using unpaired Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

mAb HUIV26 Inhibits Tumor Cell Interactions with Denatured Collagen Type IV

Our previous studies suggested that endothelial cell adhesion to proteolyzed and denatured type IV collagen depends in part on integrin-mediated cellular interactions with the HUIV26 cryptic epitope.¹⁹ Importantly, cellular interactions with this epitope were shown to be mediated by integrin $\alpha v \beta 3$.¹⁹ To examine whether malignant tumor cells also use the HUIV26 cryptic epitope in attaching to denatured collagen type IV, *in vitro* cell adhesion assays were performed as described in the Materials and Methods section. Highly metastatic B16F10 melanoma cells were resuspended in adhesion buffer in the presence or absence of mAb HUIV26 or an isotype-matched control antibody. Tumor cell suspensions were added to the plates and allowed to adhere to either native or denatured collagen-coated wells. Cell adhesion was quantified by measuring optical density of cell-associated crystal violet eluted from the attached cells. As shown in Figure 1, A and B, B16F10 melanoma cells attached to both native and denatured collagen type IV. Interestingly, mAb HUIV26 dose dependently inhibited adhesion of B16F10 cells to denatured collagen type IV by ~60% (maximum) as compared to either no treatment or treatment with an isotype-matched control antibody (Figure 1A). In similar studies, mAb HUIV26 had no effects on tumor cell adhesion to either native or denatured collagen type I (Figure 1, C and D). In addition, mAb HUIV26 had no effect on tumor cell adhesion to other ECM proteins such as fibronectin in either its intact or denatured forms (data not shown). Similar results were obtained with the metastatic breast carcinoma cell line 4T1, suggesting that the effects of mAb HUIV26 on adhesion are not restricted to a single tumor cell type (data not shown). Taken together, these findings suggest that tumor cell interactions with denatured collagen type IV are dependent, in part, on cellular recognition of the HUIV26 cryptic site. Cellular interactions with ECM components are also thought to contribute to the regulation of proliferation. Surprisingly, incubation of B16F10 melanoma cells with various concentrations (10 to 100 $\mu\text{g/ml}$) of mAb HUIV26 had little direct effect on tumor cell proliferation throughout a 3-day time course (data not shown).

mAb HUIV26 Inhibits Tumor Cell Migration on Denatured Collagen Type IV

Studies have suggested proteolytic remodeling of ECM components such as collagen type IV can regulate cellular invasion and migration. Thus, we examined the effects of mAb HUIV26 on malignant tumor cell migration *in vitro*. Membranes from transwell migration chambers were coated with native or denatured collagen type I or IV and B16F10 tumor cells were resuspended in migration buffer in the presence or absence of mAb HUIV26 or an isotype-matched control antibody. Tumor cell suspensions were added to the upper chambers of the migration wells and allowed to migrate to the under side of the coated membranes for 4 hours. Migration was measured by counting the number of tumor cells that migrated to the underside of the wells as described in the Materials and Methods section. As shown in Figure 2, A and B, B16F10 melanoma cells migrated on both denatured collagen type I and IV. Importantly, migration of B16F10 tumor cells was inhibited dose dependently as compared to an isotype-matched control antibody with maximal inhibition (50%) observed at 100 $\mu\text{g/ml}$. Moreover, mAb HUIV26 had little effect on tumor cell migration on either native or denatured collagen type I (Figure 2, C and D). These data are consistent with our previous studies and suggest that cellular interactions with the HUIV26 cryptic site play a role in regulating tumor cell migration on structurally altered collagen type IV.

B16F10 Melanoma Cells Rapidly Disseminate to the Lungs of Chick Embryos

Our previous studies have shown that cryptic epitopes within collagen are specifically exposed within basement membrane collagen type IV of malignant human and murine tumors as well as the subendothelial basement membrane of angiogenic blood vessels.^{18–21} Importantly, our current studies suggest that malignant B16F10 melanoma cells can interact with the HUIV26 cryptic site and function-blocking antibody directed to this site inhibits cellular adhesion and migration. Given these findings, the possibility exists that inhibiting cellular interactions with the HUIV26 site may impact tumor cell metastasis. Therefore, we established a rapid experimental metastasis assay to study the potential role of the HUIV26 epitope in this process. To facilitate these studies, we used the chick embryo model in conjunction with metastatic B16F10 melanoma cells. Subconfluent B16F10 melanoma cells were resuspended in sterile PBS. Twelve-day-old chick embryos were injected intravenously with 100 μl of B16F10 cell suspension, and the embryos were allowed to incubate for a total of 7 days. At the end of the 7-day incubation period, the embryos were sacrificed and the lungs were resected, washed, and placed in OTC embedding compound and snap-frozen as described in the Materials and Methods section. Frozen sections (4.0 μm) were cut and nonspecific binding sites were

Figure 1. Effects of mAb HUIV26 on tumor cell adhesion. To examine the effects that a function-blocking mAb directed to a cryptic collagen site has on tumor cell adhesion, *in vitro* adhesion assays were performed. Nontissue culture 48-well plates were coated (10.0 μ g/ml) with either native triple helical or denatured collagen types I and IV. Tumor cells (B16F10 melanoma) were resuspended in adhesion buffer in the presence (0 to 100 μ g/ml) or absence of mAb HUIV26 or an isotype-matched control antibody and cell adhesion was quantified. **A:** Quantification of B16F10 cell adhesion on denatured collagen type IV. **B:** Quantification of B16F10 cell adhesion on native collagen type IV. **C:** Quantification of B16F10 cell adhesion on denatured collagen type I. **D:** Quantification of B16F10 cell adhesion on native collagen type I. Data bars represent mean tumor cell adhesion as represented by the optical density of cell-associated crystal violet $\pm$ SDs from triplicate wells.

blocked with BSA. The tissue sections from each experimental condition were next incubated with control buffer or mAb HUIV26 and immunoreactivity was visualized by incubation with horseradish peroxidase-labeled goat anti-mouse secondary antibody. As shown in Figure 3A, left, no specific exposure of the HUIV26 cryptic epitope was detected within control lungs

whereas lungs from embryos injected with B16F10 melanoma cells stained positive (brown) for the HUIV26 cryptic epitope (Figure 3A, right), indicating the exposure of this cryptic collagen epitope (brown). To further examine the distribution of this cryptic epitope we performed immunofluorescence co-localization analysis. Frozen tissue sections from lungs from

Figure 2. Effects of mAb HUIV26 on tumor cell migration. To examine the effects that a function-blocking mAb directed to a cryptic collagen site has on tumor cell migration, *in vitro* migration assays were performed. Membranes from 24-well transwell migration chambers were coated (10.0 μ g/ml) with either native or denatured collagen types I and IV. Tumor cells (B16F10 melanoma) were resuspended in migration buffer in the presence (0 to 100 μ g/ml) or absence of mAb HUIV26 or an isotype-matched control antibody and seeded onto the upper wells of the migration chamber, and migration was quantified after a 4-hour incubation period by counting the number of tumor cells that migrated to the underside of the coated membrane. **A:** Quantification of B16F10 cell migration on denatured collagen type IV. **B:** Quantification of B16F10 cell migration on native collagen type IV. **C:** Quantification of B16F10 cell migration on denatured collagen type I. **D:** Quantification of B16F10 cell migration on native collagen type I. Data bars represent mean tumor cell migration as represented by the mean number of cells present on the underside of the coated membrane per experimental condition $\pm$ SEs from triplicate wells.

chick embryos previously injected with B15F10 tumor cells were stained with mAb HUIV26 followed by incubation with fluorescein isothiocyanate-labeled *L. esculentum* lectin, which has previously been shown to mark blood vessels.²⁰ As shown in Figure 3B, the HUIV26 cryptic epitope (red) was detected in close association

with blood vessels (green) at both early (24 hours) and late (7 days) time points after tumor cell inoculation. These findings are in agreement with previously published reports and indicate that the HUIV26 cryptic epitope is generated within B16F10 lung lesions in the chick embryo. Taken together, these findings confirm

Figure 3. Expression of the HUIV26 cryptic epitope in lungs of chick embryos. Chick embryos were either not injected or injected intravenously with B16F10 melanoma cells. The lungs were resected at either 24 hours or 7 days after tumor cell injection. **A:** Representative examples of age-matched chick lungs from either untreated (**left**) or B16F10-injected embryos (**right**). Exposure of the HUIV26 cryptic epitope in the lungs of chick embryos is indicated in brown. **B:** Co-localization of the HUIV26 cryptic epitope in association with blood vessels from the lungs of chick embryos. Representative examples of lungs from chick embryos taken either 24 hours (**top**) or 7 days (**bottom**) after tumor cell injection. Green color indicates blood vessels and red color indicates presence of the HUIV26 cryptic epitope. Yellow indicates co-localization (overlap). Photographs were taken under oil emersion. Original magnifications: $\times 200$ (**A**); $\times 630$ (**B**).

the suitability of this model to assess the potential anti-metastatic effects of mAb HUIV26.

mAb HUIV26 Dose Dependently Inhibits B16F10 Experimental Metastasis in the Chick Embryo Model

To examine the effects of mAb HUIV26 on B16F10 experimental metastasis, subconfluent B16F10 cells were harvested, washed, and resuspended in sterile PBS in the presence or absence of mAb HUIV26 or an isotype-matched control antibody. The B16F10 cell suspensions were injected intravenously ($100\ \mu\text{l}$ per embryo) and 7 days later the embryos were sacrificed and the lungs removed for analysis. As shown in Figure 4A, injection of untreated B16F10 melanoma cells resulted in the formation of numerous B16F10 lung tumor foci (black lesions). In contrast, lungs from chick embryos treated with mAb HUIV26 exhibited a reduction in B16F10 lung surface lesions. Histological examination of the lungs confirmed a reduction in infiltration of B16F10 melanoma cells into the

lung tissue. To quantify the effects of mAb HUIV26 on B16F10 experimental metastasis, the number of B16F10 surface lesions was counted on each side of the lung for each lung. As shown in Figure 4B, in the presence of mAb HUIV26 ($100\ \mu\text{g}$), the mean number of B16F10 lung foci was significantly ($P < 0.001$) reduced by $\sim 65\%$ as compared to no treatment or control antibody, whereas $1.0\ \mu\text{g}$ of mAb HUIV26 per embryo exhibited little effect. To confirm the specificity of these findings and exclude any nonspecific effects of the buffer, the antibody preparation was immune-depleted with denatured collagen type IV (depleted) or the control protein denatured collagen type I (cont-depleted). As shown in Figure 5C, the denatured collagen type IV-depleted antibody preparation failed to inhibit B16F10 experimental metastasis ($P > 0.200$). In contrast, the control depleted antibody preparation significantly ($P < 0.005$) inhibited experimental metastasis by $\sim 60\%$ as compared to controls providing additional evidence for specific effects of mAb HUIV26. To further examine the temporal effects of mAb HUIV26 on B16F10 cell metastasis in this model, we harvested chick lungs 24 hours after tumor cell injection and analyzed them for the presence of B16F10 tumor cells by semiquantitative RT-PCR using primers specific for B16 M562 melanoma antigen as has been previously described.^{25,26} As shown in Figure 4D, B16F10 melanoma cells could be detected within the lungs of untreated (Figure 4D, lanes 5 to 8) or control antibody-treated (Figure 4D, lanes 9 to 12) chick embryos 24 hours after tumor cells injection. In contrast, the relative levels of B16F10 tumor cells as measured by the B16 melanoma antigen RNA was reduced (Figure 4D, lanes 1 to 4) as compared to controls. Importantly, although the semiquantitative RT-PCR failed to detect signals from two of the lungs shown after a fixed number of cycles, lower intensity signals (Figure 4D, lanes 3 and 4) as compared to controls were detected in lung samples under identical conditions. These findings are consistent with the reduction in metastatic lung lesions observed at later time points. In addition, these results suggest an early reduction of experimental metastasis when angiogenesis is unlikely to play a significant role. Taken together, these findings suggest that mAb HUIV26 inhibits experimental metastasis of B16F10 to the lungs of chick embryos and that this inhibitory effect can occur early in the metastatic cascade.

mAb HUIV26 Inhibits B16F10 Experimental Metastasis in Mice

To confirm the anti-metastatic activity of mAb HUIV26 and to examine its effects in a second experimental system, we used a murine model. Subconfluent B16F10 cells were harvested, washed, and resuspended in sterile PBS in the presence or absence of mAb HUIV26 or an isotype-matched control antibody ($100\ \mu\text{g}$). Mice were treated (intraperitoneally) with either mAb HUIV26 or isotype-matched control antibody for a total of 7 days. At the end of the treatment period the mice were sacrificed and the lungs removed, washed, and analyzed with the aid of a

stereomicroscope. B16F10 melanoma lesions (Figure 5A, arrows) could be detected on the surface of the murine lungs whereas a reduction in lung tumor lesions was observed on lungs from mice treated with mAb HUIV26. To quantify the anti-metastatic effects of mAb HUIV26, the number of lung surface lesions were counted, and the mean number of lung lesions per lung per experimental condition was determined. As shown in Figure 5B, mAb HUIV26 significantly ($P < 0.05$) inhibited B16F10 experimental metastasis by ~50% as compared to either no treatment or treatment with an isotype-matched control antibody. To examine whether continuous treatment of mice with mAb HUIV26 was required for the anti-metastatic activity observed, mice were injected with B16F10 melanoma cells in the presence or absence of mAb HUIV26 or isotype-matched control and mice received no additional treatment. As shown in Figure 5C, a single injection of mAb HUIV26 at the time of tumor cell inoculation resulted in ~38% inhibition ($P < 0.05$) of experimental metastasis as compared to controls. These findings suggest that although a single injection of mAb HUIV26 can significantly inhibit experimental metastasis, continued treatment may be required for optimal effects. Collectively these data suggest that tumor cell interactions with the HUIV26 cryptic site may contribute to the regulation of metastasis and that blocking cellular interactions with the HUIV26 epitope may represent a novel approach to control the spread of malignant tumor cells to distant sites.

Discussion

The dissemination of malignant tumor cells to distant sites is a major contributing factor to the mortality associated with human cancer. Due in large part to the complexity of the interconnected processes required for tumor cells to metastasize, progress in understanding of the metastatic cascade has been slow. Interestingly, one of the most well studied integrin receptors thought to play an important role in malignant tumor growth and metastasis is integrin $\alpha v \beta 3$.³¹⁻³⁵ In particular, elevated expression of $\alpha v \beta 3$ integrin has been strongly correlated with the vertical growth phase of metastatic melanoma and a poor

Figure 4. mAb HUIV26 inhibits B16F10 experimental metastasis in the chick embryo. To evaluate whether mAb HUIV26 impacts tumor cell metastasis, B16F10 experimental metastasis was examined in the chick embryo model. Chick embryos were injected with B16F10 cells in the presence or absence of mAb HUIV26 or an isotype-matched control antibody (1.0 to 100.0 $\mu\text{g}/\text{embryo}$). Embryos were allowed to incubate for 7 days at which time they were sacrificed, lungs removed, and the number of pigmented lung tumor lesions quantified. **A:** Representative examples of age-matched chick lungs from each experimental condition. **B:** Quantification of effects of mAb HUIV26 on B16F10 experimental metastasis. Data bars represent the mean number of tumor lesions per lung per experimental condition $\pm$ SE. Experiments were completed three times with similar results. **C:** Quantification of the effects of mAb HUIV26 preparation before and after immune depletion with either denatured collagen type IV (depleted) or control denatured collagen type I (cont-depleted). **D:** Detection of B16F10 melanoma cells in the lungs of chick embryos 24 hours after injection of tumor cells by RT-PCR using primers specific for either mouse B16 M562 melanoma antigen (**top**) or control chick 18S ribosome (**bottom**). **Lanes 1 to 4,** examples from embryos injected with B16F10 cells and mAb HUIV26. **Lanes 5 to 8,** examples from embryos injected with B16F10 cells only. **Lanes 9 to 12,** examples from embryos injected with B16F10 cells and control antibody.

clinical prognosis in metastatic breast carcinoma.^{31–35} Previous studies have demonstrated that antagonists of $\alpha\beta 3$ integrin can inhibit metastasis in animal models.^{36,37} Although many ECM ligands for $\alpha\beta 3$ have been identified, including vitronectin and denatured collagen, little is known concerning their functional relevance *in vivo* in controlling invasive cellular processes such as metastasis.

Numerous studies have suggested that proteolytic remodeling of the ECM and subsequent cellular proliferation, invasion, and migration play important roles in tumor metastasis.^{38–40} In fact, inhibition of matrix metalloproteinases have been shown to inhibit metastasis in many models.^{38–40} Although mechanisms have been suggested to account for the anti-metastatic and anti-tumor activity associated with inhibition of proteolytic enzymes, their contribution to the metastatic cascade is still not completely understood. Proteolytic enzymes such as matrix metalloproteinases may function to release matrix-immobilized growth factors needed for tumor cell

Figure 5. mAb HUIV26 inhibits B16F10 experimental metastasis in a murine model. To evaluate whether mAb HUIV26 impacts tumor cell metastasis in a murine model, B16F10 experimental lung metastasis was examined. Mice were injected with B16F10 cells in the presence or absence of mAb HUIV26 or an isotype-matched control antibody. Mice were treated intraperitoneally (100 μ g antibody/injection/day) for 7 days (**A** and **B**) at which time they were sacrificed, lungs removed, and the number of lung tumor lesions quantified. **A:** Representative examples of lungs from each experimental condition after treatment for 7 days. **Arrows** indicate examples of B16F10 lung tumor lesions. **B:** Quantification of effects of daily (7 days) mAb HUIV26 treatment on B16F10 experimental metastasis. **C:** Quantification of effects of a single injection of mAb HUIV26 on B16F10 experimental metastasis. Data bars represent the mean number of tumor lesions per lung per experimental condition $\pm$ SE.

proliferation, migration, and survival.^{41,42} Moreover, proteolytic remodeling of ECM components may also remove restrictive physical barriers, allowing tumor cells to invade and migrate.^{38–40} Interestingly, recent evidence has suggested that proteolytic remodeling of ECM components may not simply destroy ECM molecules but cause structural alterations thereby exposing cryptic integrin-binding sites.^{18–21,43} Cellular interactions with these cryptic sites may facilitate unique signaling cascades regulating proliferation, migration, and survival.^{44,45} In this regard, we provide evidence for the first time that tumor cells including B16F10 melanoma can interact with the HUIV26 cryptic epitope recognized by $\alpha\beta 3$ because a mAb directed to this site inhibits adhesion and migration on denatured collagen type IV yet had little effect on triple helical collagen. These findings suggest that recognition of this functional ECM cryptic site in collagen is not restricted only to endothelial cells.

Interestingly, studies have indicated that cellular interactions with a cryptic epitope in laminin can regulate

breast carcinoma cell migration suggesting that cryptic ECM sites may play important roles in regulating invasive cellular processes.^{42,43} Our findings suggest that the HUIV26 cryptic site in collagen type IV plays a functional role in regulating tumor cell adhesion and migration *in vitro*. Surprisingly, mAb HUIV26 had little effect on tumor cell proliferation on denatured or native collagen under the conditions tested. Although these results are not completely understood, the lack of direct anti-proliferative activity may be associated with reduced exposure or modification of the HUIV26 cryptic epitope throughout time in culture because high levels of fibronectin within the serum used in the proliferation assays has been suggested to bind to denatured collagen/gelatin. Alternatively, matrix metalloproteinases present in the serum may modify the HUIV26 cryptic epitope within collagen type IV. Additional studies are now underway to examine this possibility. Our findings indicate that systemic administration of mAb HUIV26 directed to a unique $\alpha\nu\beta 3$ -binding cryptic collagen site inhibits experimental lung metastasis of B16F10 melanoma cells by ~50 to 65% in two independent animal models. Given previously published evidence indicating integrin-mediated regulation of p53 activity, it would be interesting to speculate that inhibiting $\alpha\nu\beta 3$ -mediated interactions with the HUIV26 cryptic collagen epitope may result in activation of p53, which in turn, may up-regulate expression of p21^{CIP1} thus impacting cell-cycle control and metastasis. Alternatively, modulating integrin-dependent ERK1/2 signaling may also contribute to the anti-metastatic activity observed. Our novel findings are consistent with the possibility that the HUIV26 cryptic epitope may represent a functionally important ligand for $\alpha\nu\beta 3$ that contributes to the regulation of tumor cell behavior. Although it cannot be completely excluded that the known anti-angiogenic effects of mAb HUIV26 contributes to the anti-metastatic effects observed, our data do suggest that mAb HUIV26 can directly inhibit tumor cell adhesion and migration on denatured collagen type IV, thus directly impacting tumor cell behavior. Moreover, our studies indicate that mAb HUIV26 can inhibit the spread of B16F10 melanoma cells to the lungs of chick embryos within 24 hours suggesting that HUIV26 can impact early events in the metastatic cascade when angiogenesis is likely to play less of a role. Taken together, a therapeutic strategy whereby an antagonist could impact both the endothelial cell compartment as well as the tumor cell compartment would likely be of great therapeutic benefit.

Given our current experimental findings, it is possible that mAb HUIV26 may reduce tumor cell metastasis in part by inhibiting tumor cell interactions with the HUIV26 cryptic epitope exposed with the basement membranes of epithelial sheets and blood vessels because functional exposure of this cryptic epitope was observed in B16F10 melanoma lung lesions and has been observed in several other tumor tissues.^{18–21} Further mechanistic studies are currently under way to examine the impact of disrupting tumor cell interactions with the HUIV26 epitope has on signaling pathways involved in melanoma cell-cycle control, proliferation, and survival. Collectively, our findings suggest that the HUIV26 cryptic epitope may represent

an effective and highly selective new therapeutic target for the treatment of metastatic disease.

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