The α6β3 Integrin Maintains the Survival of Human Breast Carcinoma Cells In vivo


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Abstract

The α6β3 integrin has been widely implicated in carcinoma function in vitro; however, in vivo data are scarce. To determine the importance of α6β3 in tumor progression, a SUM-159 breast carcinoma cell line that is essentially devoid of α6β3 expression was generated using an RNA interference strategy. Loss of α6β3 expression inhibits colony formation in soft agar assays, suggesting a vital role for α6β3 in survival signaling and anchorage-independent growth. Orthotopic injection of the β3-deficient cell line into the mammary fat pad of immunocompromised mice yielded significantly fewer and smaller tumors than the control cell line, revealing a role for the α6β3 integrin in tumor formation. Under conditions that mimicked the in vivo environment, decreased expression of the α6β3 integrin led to enhanced apoptosis as determined by the percentage of Annexin V-FITC+, PI− cells and the presence of caspase-3 cleavage products. Recombinant vascular endothelial growth factor (VEGF) significantly inhibited the cell death observed in the β3-deficient cell line, demonstrating the importance of VEGF expression in this survival pathway. Furthermore, loss of α6β3 expression leads to enhanced apoptosis and reduced expression of VEGF in breast carcinoma cells in vivo. Importantly, the specificity of α6β3 in both the in vitro and in vivo assays showed that reexpression of the β3 subunit into the β3-deficient cell line could rescue the functional phenotype. Taken together, these data implicate the α6β3 integrin in tumor formation by regulating tumor cell survival in a VEGF-dependent manner.

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Introduction

Although the α6β4 integrin provides a well-characterized adhesive function in normal epithelial cells by anchoring the epithelium to its underlying basement membrane (1, 2), the tumor-associated functions of this integrin are becoming increasingly recognized. Importantly, the expression of this integrin is maintained as epithelial structures dissociate during the initiation and progression of carcinomas and, moreover, several types of carcinomas express significant amounts of α6β4 (3, 4). Numerous studies by our group and others on carcinoma-derived cell lines have revealed that α6β4 facilitates their ability to migrate, invade, and resist apoptotic stimuli (3–6). The ability of α6β4 to affect these diverse functions results largely from its effects on multiple signaling pathways, a process that may result from its association with specific growth factor receptors, tetraspanins and possibly other molecules (7). The dichotomy of α6β4 function is summarized best by the hypothesis that α6β4 switches from a mechanical adhesive device into a signaling competent receptor during the progression from normal epithelium to invasive carcinoma (8).

Despite this considerable data implicating α6β4 in tumor-associated functions in vitro, there is a paucity of data on its contribution to tumor behavior in vivo. The most compelling in vitro data that exist are from studies on squamous carcinoma in which α6β4 has been implicated in the formation of these tumors (9, 10). For other types of carcinomas including breast carcinoma, that have been studied extensively in vitro with respect to α6β4 function, limited in vivo data exists. Moreover, the available data were obtained from model systems in which exogenous α6β4 was expressed in cells that lack expression of this integrin (11, 12). To obtain more insight into the contribution of α6β4 in carcinoma progression, we introduced short hairpin RNA (shRNA) oligonucleotides using RNA interference to deplete expression of this integrin in human breast carcinoma cells. Following orthotopic injection of these cell lines, we assessed the effect of the loss of α6β4 expression on the behavior of these cells. These experiments reveal that α6β4 is necessary for the ability of these cells to survive in vivo, a conclusion substantiated by their behavior in three-dimensional cultures. Interestingly, breast carcinoma cells deficient in α6β4 expression produce significantly less vascular endothelial growth factors (VEGF) in vivo, a finding that relates to previously published data on the ability of α6β4 to regulate VEGF expression in vitro as well as the importance of VEGF for the survival of breast carcinoma cells (13–15).

Materials and Methods

Cell lines. The SUM-159 scrambled (Scr)-shRNA and β4-shRNA human breast carcinoma cell lines were generated and maintained as described previously (16). The β3 subunit was reexpressed into the β4-shRNA cell line by infecting these cells with a human β3 retrovirus (obtained from Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) to generate the β4-shRNA + β3 cell line.

Biochemical analysis. For semiquantitative PCR, RNA isolation was done with the RNeasy mini kit (Qiagen, Valencia, CA) and 1 μg of total RNA was used with the Qiagen one-step reverse transcription-PCR kit as described by the manufacturer. The following primer sets were used: β4-FWD, 5′ GCATCGTGGTCATGAGAGCG 3′; β4-REV, 5′ CAACTGTCCCTCGTGCAACAGC 3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-FWD, 5′ CCTGCGCAAGGTGATCATGAC 3′, GAPDH-REV, 5′ TGTCTACACGGAAATGACCTG 3′.
For immunoblotting, cell lysates (30 µg) and membranes were prepared as described previously (17). The blots were incubated in a 1:5,000 dilution of rabbit polyclonal anti-β4-integrin (505; ref. 18) or 0.5 µg/mL anti-β-actin (Sigma, St. Louis, MO) followed by 0.04 µg/mL of peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). All membranes were visualized by SuperSignal West Pico chemiluminescence (Pierce, Rockford, IL).

To evaluate cell surface expression, cells were incubated with 5 µg/mL of the following primary antibodies for 30 minutes at 4°C: 3E1, mouse anti-β4 integrin monoclonal antibody (mAb; obtained from Rita Falconi, Regina Elena Cancer Institute, Rome, Italy); GOH3, rat anti-66 integrin mAb (Immunotech, Westbrook, ME); MC13, mouse anti-β1 integrin mAb (obtained from Steve Akiyama, NIH, Research Triangle Park, NC); P1B5, mouse anti-α3 integrin mAb (Life Technologies, Gaithersburg, MD); rat and mouse control IgG (Sigma). Cells were then conjugated with either antimouse phycoerythrin (β4, β1, and α3) or antirat CY2 (α6; both from Jackson ImmunoResearch) at 200 µg/mL for 30 minutes at 4°C and analyzed by flow cytometry.

Matrigel cultures. In duplicate wells of a 24-well dish, a base layer of Matrigel (BD Biosciences, Bedford, MA; 200 µL/well) was overlaid with 1 × 10⁵ cells suspended in 300 µL of a 2:1 mixture of PBS and Matrigel. Complete serum-containing medium (0.5 mL/well) covered the cell-containing layer and was replaced every 3 days. For the experiments with recombinant growth factors, either human VEGF165 or epidermal growth factor (EGF; R&D Systems, Minneapolis, MN), both at a final concentration of 100 ng/mL, were used in the medium prior to use. Binding of recombinant VEGF was inhibited by the presence of 10 ng/mL heparin (Sigma). Images were captured with IP Lab Spectrum software (Webster, NY). Single-cell suspensions of the Matrigel cultures were yielded by dispase treatment (BD Biosciences) for 2 hours at 37°C followed by trypsin (Life Technologies) digestion for 10 minutes at 37°C. Survival assays were done on the recovered cells as outlined below.

Survival assays. The Matrigel-recovered cells were labeled with either Annexin V-FITC and propidium iodide (PI; Biosource, Camarillo, CA) or cleaved caspase-3 (Asp175) antibody (Cell Signaling, Beverly, MA). For Annexin V-FITC, PI analysis, cells were incubated for 15 minutes with 5 µg/mL Annexin V-FITC in Annexin buffer (10 mmol/L Hepes-NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂). Cells were then resuspended in Annexin buffer containing 5 µg/mL PI. For cleaved caspase-3 detection, cells were prepared as outlined by the manufacturer and stained with a 1:25 dilution of rabbit anti-cleaved caspase-3 or 5 µg/mL rabbit IgG as a control (Sigma) for 30 minutes. Cells were then labeled with 200 µg/mL peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch). All labeled cells were analyzed by flow cytometry.

Soft agar assay. Cells (50 × 10³ cells per well of a six-well plate) were suspended in 2 mL of serum-containing medium containing 0.3% agar (Life Technologies) and plated over a 1 mL base layer of 0.75% agar in triplicate. The cultures were overlaid with complete medium (0.5 mL/well) and replaced every 3 days. After 2 weeks, the total number of colonies was quantified by counting 50 fields per well using bright-field optics. Images were captured by IP Lab Spectrum software.

Mammary fat pad injections. Cells were trypsinized, washed thrice in PBS, and resuspended in 40 µL phenol-free Matrigel immediately prior to orthotopic injection. For each experiment, cell survival at the time of injection was assessed by measuring the percentage of Annexin V-phycoerythrin- (PharMingen, San Diego, CA) cells as described previously (17). Following cell preparation, female immunocompromised mice (National Cancer Institute, Frederick, MD) at 7 to 9 weeks of age were anesthetized and injected in the mammary fat pad (2 × 10⁵ cells per injection, one injection site per mouse). At 15 to 17 weeks of age, the animals were sacrificed and the tumors were harvested, weighed, and fixed overnight in 10% neutral-buffered formalin (Fisher, Pittsburgh, PA) for histologic processing (Rodent Histopathology Core, Dana-Farber/Harvard Cancer Center, Boston, MA). The average number of mitoses (per mm²) was determined by scoring H&E stained tumor sections for definite mitotic figures.

Terminal nucleotidyl transferase–mediated dUTP nick end labeling. Tumor tissues were analyzed for apoptosis as outlined by the manufacturer (Roche, Indianapolis, IN). Briefly, the paraffin-embedded tissues were dewaxed and rehydrated prior to treatment with 20 µg/mL proteinase K (Roche) for 30 minutes at 37°C. Tissues were incubated with or without fluorescein-labeled terminal transferase for 60 minutes at 37°C followed by incubation with a peroxidase-conjugated antifluorescein antibody for 30 minutes. The presence of peroxidase was detected by staining with 3,3′-diaminobenzidine + substrate-chromogen (DakoCytomation, Carpinteria, CA) for 10 minutes at room temperature. Tissues were counterstained in hematoxylin prior to image capture with Spot software (Diagnostic Instruments, Sterling Heights, MI).

Immunohistochemistry. Paraffin-embedded tumors were dewaxed and rehydrated, endogenous peroxidase activity was quenched for 30 minutes with 0.3% hydrogen peroxide, and trypsin pretreatment was done for 12 minutes with 0.1% trypsin (EMD Biosciences; La Jolla, CA) followed by a 5 minute incubation with 0.1 µg/mL soybean trypsin inhibitor (EMD Biosciences). To inhibit nonspecific staining, a biotin blocking system (Dako) was used as outlined in the manufacturer's protocol prior to the staining procedure. The tissues were blocked with goat serum for 60 minutes followed by overnight incubation at 4°C with 5 µg/mL antihuman VEGF antibody (Calbiochem, La Jolla, CA) or rabbit IgG (Sigma) as a negative control. Goat-anti-rabbit biotinylated secondary antibody solution and immunoperoxidase detection reagents were obtained from the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and incubations were done as outlined by the manufacturer. Peroxidase activity was detected with 3,3′-diaminobenzidine substrate-chromogen for 10 minutes prior to counterstaining with hematoxylin. Tissues were then mounted and images were captured as described above.

Results

Generation of a breast carcinoma cell line deficient in α6β3/4 integrin expression. The SUM-159 breast carcinoma cell line (19, 20) was transduced with retroviruses expressing either a β4-shRNA or a Scr-shRNA as a control. The β4-shRNA cell line was largely devoid of β4 expression compared with the Scr-shRNA cell line at both the mRNA (Fig. 1A) and protein (Fig. 1B) level. Moreover, cell surface expression of β4 was decreased by 62% on the β4-shRNA cell line compared with the Scr-shRNA cell line (Fig. 1C). The specificity of the β4 knock-down was confirmed by comparing the cell surface expression of other integrin subunits including α3, α6, and β1 in the β4-shRNA and Scr-shRNA cell lines. No significant difference in the cell surface expression of α3 or β1 subunits was observed between the two cell lines; however, the β4-shRNA cell line showed an ~40% decrease in expression of the α6 subunit (Fig. 1D). The α6 integrin heterodimerizes with both β1 and β4 subunits (21, 22); however, the majority of α6 associates with β4 in this cell line. Thus, the observed loss of α6 expression is a predictable consequence of reduced β4 expression on the cell surface.

To control for the specificity of the β4-shRNA in subsequent functional experiments, we reexpressed the human β4 integrin in the β4-shRNA cells to generate a β4-shRNA + β4 SUM-159 cell line (Fig. 1E). For this purpose, we took advantage of the observation that the RISC enzyme complex responsible for shRNA can be saturated (23) and we used a β4 retrovirus to infect the β4-shRNA cell line and consequently “swamp” the shRNA-controlling enzyme. This approach alleviated concerns that a nonhuman β4 subunit or a mutant form of β4 resistant to the designed shRNA sequence would not properly heterodimerize with endogenous human α6 subunits. Taken together, these data show the production of a SUM-159 breast carcinoma cell line that is specifically deficient in α6β3/4 integrin expression and the generation of a β4-shRNA + β4 cell line as a functional control.

1 S.O. Yoon and A.M. Mercurio, unpublished observation.
Loss of endogenous α6β4 integrin expression promotes cell death in vitro. To assess the contribution of α6β4 to the survival of breast carcinoma cells in a three-dimensional matrix, we embedded Scr-, β4-, and β4-shRNA + β4 cells in Matrigel. After 10 days, cells were isolated from the cultures and the percentage of Annexin V-FITC+, PI− cells or intracellular caspase-3 cleavage. A, columns, percentage of Annexin V-FITC+, PI− cells from three independent experiments done in duplicate; bars, ± SD. The level of cell death in the Scr-shRNA cell line was set at 20% in each experiment to permit comparisons between separate assays and cell lines. Loss of α6β4 integrin expression led to a significant increase in the level of apoptosis under these growth conditions (*, two-tailed t test; P = 0.012). This cell death could be reversed to the same level as observed in the Scr-shRNA cell line by reexpressing the β4 subunit into the β4-shRNA SUM-159 cell line. B, columns, level of caspase-3 cleavage was measured in two independent experiments done in triplicate; bars, ± SD. For these experiments, the Scr-shRNA cell line was incubated with rabbit IgG antibody as the isotype control and the resulting nonspecific staining was set at 5% and subtracted from all values. The percentage of cleaved caspase-3 was significantly increased following loss of α6β4 expression (*, one-tailed t test; P < 0.05) and this cell death could be rescued with the β4-shRNA + β4 cell line (*, one-tailed t test; P < 0.05). C, the morphology of these cell lines in Matrigel cultures illustrates the survival results shown in (A) and (B). A healthy stellate morphology characterized the Scr-shRNA cell line (a), whereas the β4-shRNA cell line appeared aggregated and devoid of cellular projections (Fig. 2C). Reexpression of β4 largely restored the stellate appearance in the β4-shRNA cells. Interestingly, the apoptosis observed in the β4-shRNA cell line was dependent on the three-dimensional architecture of the cell line and could not be induced by serum deprivation alone (data not shown). Thus, reduced α6β4 expression promotes apoptosis in SUM-159 breast carcinoma cells when grown in three-dimensional culture conditions and this cell death can be prevented by reexpression of β4 into the β4-deficient cell line.

Recombinant vascular endothelial growth factor rescues the β4-shRNA cell line from apoptosis. Given that α6β4 can regulate Scr-shRNA cell line (19), whereas the β4-shRNA cell line appeared aggregated and devoid of cellular projections (Fig. 2C). Reexpression of β4 largely restored the stellate appearance in the β4-shRNA cells. Interestingly, the apoptosis observed in the β4-shRNA cell line was dependent on the three-dimensional architecture of the cell line and could not be induced by serum deprivation alone (data not shown). Thus, reduced α6β4 expression promotes apoptosis in SUM-159 breast carcinoma cells when grown in three-dimensional culture conditions and this cell death can be prevented by reexpression of β4 into the β4-deficient cell line.

Figure 1. The generation of a SUM-159 breast carcinoma cell line deficient in α6β4 integrin expression. A, semiquantitative PCR was done with RNA isolated from Scr- and β4-shRNA cell lines using β4 and GAPDH primers. A sample without RNA (−) was done as a negative control. B, extracts were prepared from Scr- and β4-shRNA cell lines and equal amounts of total protein (30 μg) were resolved by SDS-PAGE and immunoblotted for β4 and β-actin. C, to evaluate the cell surface expression of α5β4, Scr-, and β4-shRNA cell lines were incubated with either mouse anti-β4 integrin antibody (filled histograms) or mouse IgG as a control (unfilled histograms) followed by antimouse phycoerythrin-conjugated secondary antibody and analyzed by flow cytometry. D, the cell surface expression of other integrin subunits including α5, β1, and α6 was compared in the Scr-shRNA (unfilled histograms, heavy outline) and β4-shRNA (unfilled histograms, light outline) cell lines. Filled histograms, analysis of the Scr-shRNA cells following incubation with either mouse IgG (α3 and β1) or rat IgG (αβ) as a control. E, to reexpress the β4 subunit into the β4-shRNA SUM-159 cell line, a β4 retrovirus was used to infect β4-shRNA cells (−) yielding a β4-shRNA + β4 cell line (+). Extracts (30 μg protein) were resolved by SDS-PAGE and immunoblotted for β4 and β-actin.

Figure 2. Reduced α6β4 expression enhances apoptosis. Scr-, β4-, and β4-shRNA + β4 cell lines were cultured in Matrigel for 10 days. Single-cell suspensions were isolated by dispase treatment and apoptosis was measured by flow cytometry as a percentage of Annexin V-FITC+, PI− cells or intracellular caspase-3 cleavage. A, columns, percentage of Annexin V-FITC+, PI− cells from three independent experiments done in duplicate; bars, ± SD. The level of cell death in the Scr-shRNA cell line was set at 20% in each experiment to permit comparisons between separate assays and cell lines. Loss of α6β4 integrin expression led to a significant increase in the level of apoptosis under these growth conditions (*, two-tailed t test; P = 0.012). This cell death could be reversed to the same level as observed in the Scr-shRNA cell line by reexpressing the β4 subunit into the β4-shRNA SUM-159 cell line. B, columns, level of caspase-3 cleavage was measured in two independent experiments done in triplicate; bars, ± SD. For these experiments, the Scr-shRNA cell line was incubated with rabbit IgG antibody as the isotype control and the resulting nonspecific staining was set at 5% and subtracted from all values. The percentage of cleaved caspase-3 was significantly increased following loss of α6β4 expression (*, one-tailed t test; P < 0.05) and this cell death could be rescued with the β4-shRNA + β4 cell line (*, one-tailed t test; P < 0.05). C, the morphology of these cell lines in Matrigel cultures illustrates the survival results shown in (A) and (B). A healthy stellate morphology characterized the Scr-shRNA cell line (a), whereas the β4-shRNA cell line (b) is aggregated and devoid of cellular projections. Reexpression of β4 expression in the β4-deficient cell line largely restores the stellate appearance observed in the Scr-shRNA cell line (c). Magnification, ×10.
Expression of the $\alpha 6\beta 4$ integrin is necessary for formation of orthotopic tumors. To investigate our hypothesis that $\alpha 6\beta 4$ is an important contributor to tumor formation in vivo, we injected Scr-, $\beta 4$, and $\beta 4$-shRNA + $\beta 4$ cells into the mammary fat pad of immunocompromised mice. Prior to injection, the percentage of apoptotic cells was assessed and no significant difference in survival was found between the cell lines for any experiment (data not shown). In mice injected with $\beta 4$-shRNA cells, the frequency of tumor formation was decreased by 48% compared with mice injected with Scr-shRNA cells (Table 1). The $\beta 4$-shRNA + $\beta 4$ cell line increased the percentage of tumor formation by 24% compared with the $\beta 4$-shRNA-injected mice, thereby demonstrating a partial rescue of the $\beta 4$-shRNA phenotype. The mean tumor weight of the mice bearing $\beta 4$-shRNA tumors was ~3-fold less than the average weight of the Scr-shRNA tumors, whereas the tumors that formed following injection of the $\beta 4$-shRNA + $\beta 4$ cells were relatively the same weight, or slightly heavier, than the Scr-shRNA tumors. Further analysis revealed that the number of mitotic figures in the Scr-shRNA (41.1 ± 20.6 mm$^2$) and $\beta 4$-shRNA (48.7 ± 16.1 mm$^2$) tumors was statistically equivalent and indicated that a role for $\alpha 6\beta 4$ in tumor cell proliferation was not responsible for the differences in tumor formation. 

**Tumors deficient in $\alpha 6\beta 4$ expression exhibit elevated levels of apoptosis and decreased vascular endothelial growth factor expression.** To determine whether the link between loss of $\alpha 6\beta 4$ expression and cell death extends in vivo, tumors formed following orthotopic injection of Scr-, $\beta 4$, and $\beta 4$-shRNA + $\beta 4$ cells were analyzed for apoptosis using in situ terminal nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technology. The nonnecrotic regions of tumors formed from $\beta 4$-shRNA injected cells showed dramatically increased levels of apoptosis compared with tumors resulting from either Scr-shRNA- or $\beta 4$-shRNA +
Table 1. Expression of the α6β4 integrin is necessary for tumor formation in vivo

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of mice</th>
<th>No. of tumors</th>
<th>Tumor formation (%)</th>
<th>Tumor weight (g ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scr-shRNA</td>
<td>24</td>
<td>19</td>
<td>79</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>β4-shRNA</td>
<td>13</td>
<td>4</td>
<td>31</td>
<td>0.18 ± 0.07</td>
</tr>
<tr>
<td>β4-shRNA + β4</td>
<td>11</td>
<td>6</td>
<td>55</td>
<td>0.55 ± 0.29</td>
</tr>
</tbody>
</table>

NOTE: Scr-, β4-shRNA, and β4-shRNA + β4 SUM-159 cells (2 × 10⁶ cells/injection) were suspended in Matrigel (40 μL) and injected into the mammary fat pad of immunocompromised mice. After 7 to 10 weeks, the frequency of tumor formation was reduced by 48% and 24% in mice injected with β4-shRNA cells compared with Scr-shRNA and β4-shRNA + β4 cells, respectively. The mean tumor weight in grams (g ± SE) of the four mice bearing β4-shRNA tumors was significantly less than the average weight of the Scr-shRNA tumors (two-tailed t test, P < 0.005), whereas the β4-shRNA + β4 tumors were comparable in weight to the Scr-shRNA tumors.

β4-injected cells (Fig. 5A-D). We speculated that this increased apoptosis might result from decreased VEGF expression in the absence of the α6β4 integrin (14). To compare the expression of VEGF in tumors formed from the Scr-, β4-, and β4-shRNA + β4 cells, we did immunohistochemistry using an antibody that recognizes human VEGF. This approach enabled us to distinguish VEGF produced by the human tumor cells from VEGF produced by host stromal cells. Tumors that arose from β4-shRNA cells displayed limited VEGF immunostaining (Fig. 5E-H). In comparison, high levels of VEGF expression were observed in the tumors isolated from Scr-shRNA- and β4-shRNA + β4-injected mice. Taken together, these data indicate that loss of α6β4 expression in breast carcinoma cells promotes apoptosis in vivo that is associated with decreased VEGF expression.

Discussion

We conclude from the data presented here that the α6β4 integrin contributes to the survival of human breast carcinoma cells injected into immunocompromised mice. Cells that are deficient in the expression of this integrin exhibit a significant decrease in their ability to form orthotopic tumors compared with control cells because of increased apoptosis. Interestingly, loss of α6β4 expression had no effect on the proliferative capacity of these cells in vivo as determined by the number of mitotic figures in the tumors. The ability of α6β4 to sustain the survival of breast carcinoma cells in vivo is linked to the expression of VEGF by these cells, an observation that substantiates an important role for VEGF signaling in tumor formation (24). Collectively, our data provide an important extension of the numerous in vitro studies that have highlighted the importance of α6β4 for the function of breast and other carcinoma cells (3–6), and they reinforce a limited number of studies using patient samples that have linked α6β4 to breast cancer progression (25, 26).

Our data support the hypothesis that the most critical function for α6β4 in cancer is to impede apoptosis and sustain tumor survival. Initially, our group showed that α6β4 expression is necessary to prevent the apoptosis of breast carcinoma cells in response to serum and matrix deprivation by activating the phosphoinositide-3-kinase/Akt pathway (27). The ability of α6β4 to promote survival in these studies was specific to carcinoma cells that expressed mutant forms of p53 (28). These events were not observed in carcinoma cells that expressed wild-type p53 because α6β4 stimulated p53-dependent caspase-3 activity that led to

Figure 5. Increased apoptosis and decreased VEGF in mammary tumors formed in β4-shRNA–injected mice. Assessment of the level of cell death (A–D) occurring in the mammary tumors that formed following orthotopic injection of the Scr-shRNA (B), β4-shRNA (C), and β4-shRNA + β4 (D) cell lines was determined by TUNEL labeling. The red-brown staining is indicative of apoptosis and is dramatically enhanced in the tumors formed following injection of the β4-shRNA cell line (C) compared with either the Scr-shRNA (B) or β4-shRNA + β4 cell lines (D). A, β4-shRNA tumor incubated in the absence of terminal transferase enzyme as a negative (−) control. VEGF immunohistochemistry (E–H) done on tumors resulting from injection of Scr-shRNA (F), β4-shRNA (G), and β4-shRNA + β4 (H) cell lines revealed a much higher level of VEGF expression in the Scr-shRNA (F) and β4-shRNA + β4 (H) tumors than in the β4-shRNA (G) tumors. E, an isotype or negative (−) control-labeled sample obtained from a Scr-shRNA–injected mouse. Representative of data observed in three independent experiments that were done on a minimum of four separate mammary tumors obtained from each cell line. Bar, 50 μ.
inactivation of Akt (27). Subsequent work indicated that one mechanism by which α6β4 sustains survival is to regulate the translation of VEGF and consequent autocrine VEGF survival signaling (14). Specifically, α6β4 regulates 4E-BP1 phosphorylation, a translational repressor that inhibits the function of eukaryotic translation initiation factor 4E, in a phosphoinositide-3-kinase- and mTOR-dependent pathway. Moreover, α6β4 lost its ability to prevent the cell death of carcinoma cells maintained under "stress" conditions in which VEGF expression had been reduced, thus, directly implicating VEGF as a downstream effector of α6β4-mediated cell survival (14).

More recently, studies employing three-dimensional cultures have substantiated the importance of α6β4 for the survival of breast carcinoma cells. Initially, it was reported that activation of α6β4 signaling in three-dimensional mammary epithelial cells conferred resistance to apoptosis by maintaining a polarized tissue architecture (29). Additional studies have suggested that α6β4 can mediate the anchorage-independent survival of malignant mammary epithelial cells by a mechanism that involves secretion of laminin-5 followed by ligation of α6β4 and subsequent activation of a Rac GTPase/nuclear factor κB signaling pathway (30). Although the involvement of VEGF was not examined in these studies, it is worth noting that VEGF can activate nuclear factor κB (31), suggesting that the mechanisms that have been proposed for α6β4-mediated survival signaling are not mutually exclusive. Taken together, the existing in vitro data support our in vivo results. The alternative hypothesis, however, that could have been predicted from the existing literature is that loss of α6β4 expression would affect tumor invasion and metastasis and not the formation of the primary tumor. As such, our observation that loss of α6β4 expression significantly diminished primary tumor formation is significant.

A role for α6β4 in carcinoma formation is suggested by other recent studies on the genesis of squamous cell carcinomas. Using a model system that involves the retroviral expression of specific genes in primary, human keratinocytes, and the subsequent use of these cells for grafting on immune-deficient mice, Khavarri’s group reported that coexpression of oncogenic Ras with molecules that impede Ras-induced growth arrest results in invasive epidermal carcinoma resembling squamous cell carcinomas (9). Of interest, keratinocytes deficient in the expression of either the β4 subunit or laminin-5 (isolated from patients with blistering skin disease) were unable to form tumors, but reexpression of the respective genes restored their ability to form invasive tumors (9). In a related study using a transgenic model that targeted α6β4 expression in the suprabasal levels of the mouse epidermis, an increased frequency of papillomas, carcinomas, and metastases was observed following induction with a chemical carcinogen (10). The mechanism seems to involve the suppression of transforming growth factor-β signaling by α6β4 and the resulting inhibition of transforming growth factor-β from suppressing the clonal expansion of initiated cells in the epidermal basal layer (10). Although survival was not assessed directly in these models, a link between α6β4 and the survival of squamous carcinoma cells is a reasonable hypothesis based on our data. Given these tantalizing reports, it will be informative to assess the contribution of α6β4 to survival in other models of carcinoma formation.

The data presented in this report strengthens the hypothesis that autocrine VEGF signaling is an important component of tumor progression. More specifically, we showed that VEGF_165 functions as a survival factor in breast carcinoma cells by signaling through the neuropilin-1 receptor (13), a finding recently confirmed by others (32). In vivo, enhanced tumor growth in polyoma virus middle T-antigen mice was observed in a transgenic model targeting overexpression of VEGF to mammary epithelial cells (24). This increased tumor growth was attributed, in part, to inhibition of apoptosis by autocrine VEGF signaling. A role for autocrine VEGF in tumor formation and growth has also been observed in xenografted human leukemias and lymphomas (33, 34). Thus, the ability of exogenous VEGF to rescue the β4-deficient breast carcinoma cells from apoptosis and the striking decrease in VEGF expression in the orthotopic tumors reported here adds to the increasing body of evidence supporting the importance of autocrine VEGF pathways in tumor formation and survival.

An issue that arises from the foregoing discussion is whether α6β4-induced VEGF expression in breast carcinoma cells stimulates tumor angiogenesis. Indeed, VEGF produced by both tumor and stromal cells could affect angiogenesis (35). Although we did not observe gross differences in the vasculature between control and β4-deficient tumors, we cannot exclude some effect of α6β4 expression on angiogenesis in these experiments. With this in mind, recent studies have examined the expression of α6β4 on endothelial cells and its possible role in angiogenesis. Based on the analysis of α6β4 expression in vascular endothelial cells during the development of the mouse whisker pad, it was inferred that this integrin actually inhibits the angiogenic switch (36). In contrast, another study argued that α6β4 promotes the migration and invasion of endothelial cells during the invasive phase of tumor angiogenesis (37). This conclusion was supported by the observation that mice carrying a targeted deletion of the COOH-terminal portion of the β4-tail formed smaller and less vascularized tumors than wild-type mice following s.c. injection of cancer cells (37). Interestingly, however, loss of α6β4 signaling in the latter study did not affect tumor angiogenesis in an orthotopic model of mammary carcinogenesis, suggesting that the role of α6β4 in tumor angiogenesis may not be universal (37). Clearly, more studies are warranted to assess the contribution of α6β4 to angiogenesis in breast tumors. Nonetheless, the collective data argue strongly that α6β4 influences the survival of breast tumor cells and that one mechanism involves its ability to regulate autocrine VEGF signaling.

In summary, our data reveal a contribution of the α6β4 integrin to tumor formation, and they substantiate the hypothesis that α6β4 plays an important role in regulating the expression of VEGF in carcinoma cells and that VEGF functions as a survival factor in nascent tumors. We emphasize, however, that a role for α6β4 in the survival of nascent tumors does not preclude its involvement in later stages of invasion and metastasis. With this in mind, the use of a conditional system to express β4-shRNA at specific stages of tumor formation and progression will be valuable.

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