Survivin promotion of melanoma metastasis requires upregulation of α5 integrin

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Survivin is an apoptotic and mitotic regulator that is overexpressed in melanoma and a poor prognostic marker in patients with metastatic disease. We recently showed that Survivin enhances melanoma cell motility through Akt-dependent upregulation of α5 integrin. However, the functional role of Survivin in melanoma metastasis is not clearly understood. We found that overexpression of Survivin in LOX and YUSAC2 human melanoma cells increased colony formation in soft agar, and this effect was abrogated by knockdown of α5 integrin by RNA interference. We employed melanoma cell xenografts to determine the in vivo effect of Survivin overexpression on melanoma metastasis. Although Survivin overexpression did not affect primary tumor growth of YUSAC2 or LOX subcutaneous tumors, or indices of proliferation or apoptosis, it significantly increased expression of α5 integrin in the primary tumors and formation of metastatic colonies in the lungs. Additionally, Survivin overexpression resulted in enhanced lung colony formation following intravenous (i.v.) injection of tumor cells in vivo and increased adherence to fibronectin-coated plastic in vitro. Importantly, in vivo inhibition of α5 integrin via intraperitoneal injection of an αβ integrin-blocking antibody significantly slowed tumor growth and reduced Survivin-enhanced pulmonary metastasis. Knockdown of α5 integrin in cells prior to i.v. injection also blocked Survivin-enhanced lung colony formation. These findings support a direct role for Survivin in melanoma metastasis, which requires α5 integrin and suggest that inhibitors of α5 integrin may be useful in combating this process.

Introduction

Melanoma arises from epidermal melanocytes and is the deadliest type of skin cancer, due to its high propensity for metastasis (1). Metastatic spread of tumor cells from a primary tumor to secondary organs is a highly specialized program that involves tumor cell migration, survival in the circulation, extravasation and colonization of distant sites (2). Despite recent therapeutic breakthroughs for metastatic melanoma with the approval of the BRAF kinase inhibitor vemurafenib (3) and the anti-cytotoxic T-lymphocyte-associated antigen 4 monoclonal antibody ipilimumab (4), there still remains a dismal prognosis for patients with advanced disease as in the majority of patients their tumors develop mechanisms of resistance (5,6). Therefore, defining molecular mechanisms that govern melanoma progression may aid the development of more effective therapeutic strategies for combating melanoma metastasis.

Survivin, an inhibitor of apoptosis protein, is overexpressed in melanoma and has been identified as a biomarker of poor prognosis in metastatic disease (7,8). Survivin is expressed embryonically, silenced in most adult tissues and aberrantly reactivated in malignant cells (9). Both antiapoptotic and promitotic roles for Survivin have been described in malignancies including melanoma (10,11). However, the role of Survivin in melanoma metastasis has not been elucidated. We reported previously that Survivin is required for constitutive melanoma cell motility and its overexpression promotes motility through the Akt-dependent upregulation of α5 integrin (12). These studies suggested a potential alternate role for Survivin independent of apoptotic or mitotic regulation, in melanoma metastasis.

Integrins are a family of α-β heterodimeric transmembrane proteins, which act as receptors for cell adhesion molecules and mediate bidirectional signaling between cells and the extracellular matrix (13). The diverse pairings of α and β integrin subunits regulate signaling pathways that regulate proliferation, differentiation, apoptosis and motility (14), suggesting a role for integrin-mediated signaling in tumor cell development and metastasis. Indeed, integrin expression is associated with metastatic phenotype in melanoma (15), and signaling through α5β3 integrin can promote melanoma metastasis in mice (16). As noted above, we previously implicated α5 integrin in Survivin-enhanced melanoma cell motility in vitro (12), suggesting a potential role for this integrin in melanoma metastasis in vivo.

Here we demonstrate that Survivin promotes melanoma metastasis in vivo in an α5 integrin-dependent manner. In this study, we investigated the effect of Survivin on melanoma metastasis using an orthotopic mouse model with melanoma cells overexpressing Survivin. We found that Survivin overexpression resulted in upregulation of α5 integrin expression in vivo and enhanced colony formation and metastasis to the lungs following either subcutaneous (s.c.) or intravenous (i.v.) injection. These prometastatic effects of Survivin were dependent on tumor cell expression of α5 integrin.

Materials and methods

Cell culture

Human melanoma cell lines (LOX and YUSAC2) were maintained and passaged as described previously (12).

Survivin-expressing lentivirus and infection

The Survivin gene (BIRC5) was PCR amplified from human melanocyte complementary DNA. The PCR product was cloned into pS-C-B-AmplKan (Stratagene, Agilent Technologies, Santa Clara, CA), confirmed by sequencing and then subcloned into the modified pEi2 lentiviral expression vector obtained from Bryan Welm (Huntsman Cancer Institute, Salt Lake City, UT), which has been described previously (17). Validation of the lentiviral construct and production of the virus were carried out as described (18). For melanoma cell infection, 8 µg/ml polybrene (AB01643-00001, American Bioanalytical, Natick, MA) was added as a lipophilic reagent. Cells were maintained in culture as stable lines, which consisted of >95% green fluorescent protein (GFP)-positive cells. LOX and YUSAC2 cells stably expressing GFP (control) or GFP-tagged Survivin are referred to as LOX-GFP, LOX-Surv, YU2-GFP and YU2-Surv throughout the text.

Immunoblotting

Western blotting was performed as described previously (12).

Invasion and migration assays

Transwell migration assays were performed as described previously (12).

Colonies formation assay

The underlayer mix was created by mixing equal parts of a 1.6% SeaPlaque Genetic Technology Grade agarose (50111, Lonza, Allendale, NJ) solution with 2× Iscove’s medium (12200-036, Invitrogen Life Technologies, Grand Island, NY) containing 20% fetal bovine serum and 2% penicillin-streptomycin–glutamine (Invitrogen), to give a final underlayer mix of 0.8% Genetic Technology Grade agar with 10% fetal bovine serum and 1% penicillin-streptomycin–glutamine. Underlayer mix (2 ml) was poured into sterile 6 cm plates and allowed to solidify on a level surface at 4°C. The cell layer consisting of 5×10^4 (LOX) or 2.5×10^4 (YUSAC2) cells in Iscove’s

Abbreviations: GFP, green fluorescent protein; HPF, high-power field; i.v., intravenous; PBS, phosphate-buffered saline; pHH3, phospho-histone H3; s.c., subcutaneous; SEM, standard error of the mean; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
medium containing 0.4% Genetic Technology Grade agarose, 5% fetal bovine serum and 0.5% penicillin-streptomycin-glutamine in 5ml was gently poured onto the solidified underlayer in each plate. Plates were incubated at 37°C, monitored daily and medium was replenished as needed to prevent dehydration of the agarose. After 11 days, colonies were photographed and quantitated using ImageJ software. We found that 11 days was the optimal time for growth using these cell lines. For soft agar assays incorporating RNai, cells were seeded into agar 24 h after transfection with a scrambled or an α5 integrin (ITGA5)-specific small interfering RNA (siRNA). The oligonucleotides and RNAi transfection protocol have been reported previously (12).

Mice
NOD.CB17/SCID mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animal procedures were in accordance with national guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

Spontaneous metastasis assay
A total of 2 × 105 cells in 0.25 ml phosphate-buffered saline (PBS) were injected s.c. into the right flank. Once tumors could be palpated, the long and short dimensions (dL and dS, respectively) were measured using digital calipers twice weekly. Dimensions were later used to calculate tumor volume using the following equation: \( V = \frac{dL \times dS^2}{2} \). Mice were killed once tumors approached 2 cm3 or the experimental endpoint was reached, and then primary tumors and lungs were harvested. For paraffin sections, tissues were fixed in 10% neutral buffered formalin and later embedded in paraffin. For cryosections, lungs were fixed in 4% parafomaldehyde and later embedded in optimal cutting temperature compound (4583, Fisher Scientific, Pittsburgh, PA). Three sections were taken per lung, and two fields were examined per section. For assays involving blocking of α5 integrin, once tumor dimensions (dL and dS) were = 9.5 cm, mice were injected intraperitoneally twice weekly for 4 weeks with either M200 (Volociximab, 10 mg/kg) or the same dose of a control mouse immunoglobulin G antibody (GBW-275D92, GenWay Biotech, San Diego, CA). M200 is an α5(β3) integrin-blocking antibody, kindly provided by Abbott Laboratories (Abbott Park, IL).

Tail vein metastasis assay
Mice were injected with 5 × 105 cells in 0.25 ml PBS i.v. through the lateral tail vein. After 24h or 14 days, mice were euthanized, their lungs resected and fixed in 4% paraformaldehyde and then embedded in optimal cutting temperature compound for cryosectioning. Two sections were taken per lung, and one field was examined per section. The 24h time point was used as an indicator of immediate extravasation, whereas 14 days served as a delayed time point as others have done (19). GFP-positive tumor cells were visualized by fluorescence microscopy.

Cell adhesion assay
Dishes (12-well, flat bottom) were coated with 0.5 ml recombinant human fibronectin (10 μg/ml, Invitrogen) in PBS overnight at 37°C. After removing fibronectin solution, cells (1–2.5 × 104 per well) in medium were added and incubated for 2h at 37°C. Using disposable pipettes, medium was gently removed and cells were washed three times with PBS. Adherent cells were fixed with 0.5 ml 4% paraformaldehyde in PBS for 30 min at room temperature. After three gentle washes with PBS, 0.5 ml 1% Toluidine Blue in PBS was added. After 1h, wells were washed extensively with water and then left on the bench to dry overnight. Cells were solubilized in 2% sodium dodecyl sulfate (1 ml/well), and absorbance at 620 nm was determined using a plate reader.

Immunohistochemistry
Sections were dewaxed by citrus clearing solvent (8301, Fisher Scientific) and rehydrated using a decreasing ethanol gradient from 100 to 70%. Antigen retrieval was performed by boiling for 20min in sodium citrate (pH 6.0). Immunohistochemistry was performed on 5μm tissue sections using an immunoperoxidase method (K4010, Dako, Agilent Technologies), with rabbit polyclonal antibodies against human α5 integrin (1:300, HPA002642, Sigma, St Louis, MO) or phospho-histone H3 (ppH3) (Ser10) (1:200, 9701, Cell Signaling Technology, Danvers, MA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay
Paraffin-embedded tumor sections (5 μm) were dewaxed and rehydrated as described above. Sections were then incubated with protease K (0111587901, Roche, Genetech, San Francisco, CA) at 10 μg/ml for 1h at room temperature. Slides were then stained using the In Situ Cell Death Detection Kit TMR Red (12156792910, Roche), according to the manufacturer’s protocol.
Survivin promotes melanoma metastasis

Given that Survivin promoted lung metastasis without affecting tumor growth, we next assessed the capacity of Survivin-overexpressing cells to establish lung metastases following injection directly into the tail vein. This approach bypasses the need to navigate from the primary tumor and measures the ability of tumor cells to survive in the bloodstream, extravasate and colonize the lung (20). We injected YU2-GFP or YU2-Surv cells into the tail vein, then killed mice and resected the lungs to measure extravasated cells 24 h postinjection (*P < 0.001, **P < 0.0001). Cells were seeded in soft agar for 11 days. Cell colonies were then photographed and quantified. LOX cell colonies are shown at right. *P = 0.007, **P = 0.005. YU2-GFP and YU2-Surv cells were transfected with scrambled (Scr) or α5 integrin-specific siRNA. After 24 h, cells were placed in soft agar assay as in (c). *P = 0.003. Western blot of α5 integrin knockdown is shown. All error bars represent SEM of triplicates.

Injection revealed a significant increase in lung metastases in mice injected with Survivin-overexpressing cells compared with control cells (Figure 3c). The lack of difference in pH3 staining (Figure 3d) confirmed that increased numbers of cells had become established in the lungs, rather than proliferating after reaching the lungs. Thus, although Survivin overexpression does not appear to affect primary tumor growth or immediate extravasation of cells into the lungs, there is enhanced capacity of these cells to establish metastatic colonies in the lung.

Survivin promotes cellular adherence

We considered the possibility that increased numbers of Survivin-overexpressing cells in the lungs may reflect enhanced cellular adhesion to extracellular matrix. We examined YU2-GFP and YU2-Surv cells in an in vitro adherence assay in dishes coated...
with fibronectin to mimic matrix. As shown in Figure 3e, Survivin overexpression was associated with increased adherence.

**α5 Integrin is required for Survivin-enhanced pulmonary metastasis**

This increased adherence to fibronectin is consistent with our finding that overexpression of Survivin in primary tumors correlated with higher expression levels of α5 integrin (Figure 2d) and our previous finding that Survivin overexpression results in upregulation of α5 integrin in melanoma cells (12). To determine if increased α5 integrin expression is critical for Survivin-enhanced lung metastasis, we repeated the spontaneous metastasis assay using s.c. injected cells and incorporated an α5β1 integrin-blocking antibody (M200). We first confirmed that M200 effectively blocked Survivin-enhanced melanoma cell motility in vitro, whereas a control immunoglobulin G antibody did not (data not shown). Mice with Survivin-overexpressing tumors (YU2-Surv) were injected intraperitoneally with M200 or the control antibody twice weekly for 4 weeks and compared with mice with YU2-GFP tumors injected with the control antibody. We found that tumor growth was similar between YU2-GFP and YU2-Surv tumors in mice treated with the control antibody (Figure 4a) and Supplementary Figure 3, available at Carcinogenesis Online. However, there was a significant reduction in the growth of YU2-Surv tumors in mice treated with M200 (Figure 4a and Supplementary Figure 3, available at Carcinogenesis Online). TUNEL analysis revealed that although the level of apoptosis was similar between the control antibody-treated groups, the Survivin-overexpressing tumors in mice treated with M200 contained significantly more apoptotic cells (Figure 4b). Similarly, M200 treatment of YU2-Surv tumors was associated with decreased tumor cell proliferation (Figure 4c). Consistent with our findings in Figure 2d, YU2-Surv tumors exhibited higher levels of α5 integrin compared with control tumors in animals treated with the control antibody (Figure 4d). However, expression of α5 integrin was noticeably reduced in YU2-Surv tumors from animals treated with M200 (Figure 4d). It is possible that M200 binding to the α5β1 receptor results in a conformational change affecting the α5 integrin epitope or its detection (21). Consistent with our findings in Figure 2e, we found that among mice treated with the control antibody that those with YU2-Surv tumors had more lung metastases than mice with YU2-GFP tumors (Figure 4e). Importantly, examination of the lungs from mice-bearing YU2-Surv tumors treated with M200 revealed fewer metastatic lung colonies (Figure 4e). Finally, we investigated whether the mitigating effect of α5 integrin inhibition on Survivin-enhanced metastasis was related to, or independent of, decreased growth of the primary tumor. To address this question, we examined the effect of knocking down enhanced α5 integrin expression in YU2-Surv cells on their capacity to form lung metastases following i.v. delivery. Since there was no precedent for administering M200 i.v., and we had shown that RNAi-mediated depletion of α5 integrin was effectively sustained for up to 11 days (Figure 1d), we examined the metastatic capability of these cells. As shown in Figure 4f, RNAi-mediated knockdown of α5 integrin abrogated the Survivin-enhanced pulmonary metastasis. These in vivo results support our hypothesis that Survivin promotes melanoma cell metastasis in an α5 integrin-dependent manner.

**Discussion**

Survivin was originally identified as an inhibitor of apoptosis (22) although subsequent studies revealed it to be a bifunctional protein that regulates both apoptosis and mitosis (23). Survivin has been widely implicated in metastasis and chemoresistance in a number of malignancies including melanoma (7,8), breast cancer (24) and
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colorectal cancer (25). We recently reported that Survivin is required for constitutive motility of melanoma cells and that Survivin overexpression increases melanoma cell motility in vitro via upregulation of $\alpha_5$ integrin (12). Here we demonstrate that Survivin can promote melanoma cell colony formation and metastasis to the lungs in an $\alpha_5$ integrin-dependent manner.

The antiapoptotic and mitotic regulatory functions of Survivin depend on cellular context and compartmentalization of Survivin signaling networks (26). In our earlier work, we showed that Survivin depletion in melanoma cells reduced their motility without inducing apoptosis and that promotion of motility in Survivin-overexpressing cells was evident in the presence of a mitosis inhibitor (12). In our experimental in vivo system, enhancement of melanoma metastasis by Survivin also appeared to be independent of any potential apoptotic or mitotic effects. Although Survivin-overexpressing tumors were more metastatic, we did not observe significant differences in growth rates compared with control tumors. The lack of effect of Survivin overexpression on tumor growth, consistent with our finding of no alterations in either apoptotic or mitotic indices in the tumors, suggests that the prometastatic effect of Survivin could not be attributed to an effect on primary tumor growth. When tumor cells were injected i.v., there was no immediate (at 24h) effect of Survivin.

Fig. 3. Survivin overexpression enhances melanoma cell colonization of the lungs. (a) YU2-GFP or YU2-Surv cells were injected into the tail vein of immunodeficient mice ($n = 5$ per group). Mice were killed 24h later, and the lungs resected and cryosectioned. Number of GFP-positive cells per HPF was counted in lung sections. ns, not significant. (b) Proliferating tumor cells in the lungs from mice in (a) were assessed by pH3 staining. (c) Cells were injected as in (a), then mice were killed 14 days later. GFP-positive foci were then quantified. *$P < 0.001$. (d) pH3 staining of lungs from mice in (c). (e) Cells (150 or $250 \times 10^3$ indicated) were plated on fibronectin-coated wells, then after washing, adherent cells were quantitated by staining with Toluidine Blue. *$P < 0.001$. All error bars indicate SEM. Representative images show GFP-positive (a, c) and pH3-staining (b, d) cells in lungs.
overexpression on lung seeding, suggesting that the effect of Survivin was not simply to aid immediate extravasation or increase cell survival in the bloodstream. However, examination of the lungs 14 days following i.v. injection revealed higher numbers of lung colonies in mice injected with Survivin-overexpressing versus control cells. We chose the time points of 24 h and 14 days to represent early and late experimental windows, based on prior publications using this assay. It is possible that some extravasation occurred beyond the 24 h time point, with continued seeding of the lung parenchyma. Although we did not examine any time points between 24 h and 14 days, the lack of difference in proliferation at either the earlier or later time point suggests that the greater number of Survivin-overexpressing cells in the lungs at 14 days was not a result of the cells proliferating after reaching the lungs. We suspect that Survivin-overexpressing cells reaching the lungs had higher rates of survival but were not able to demonstrate this directly since TUNEL staining of tumor cells in the lungs was technically problematic (data not shown). Given our finding that Survivin increases cell adhesion to fibronectin-coated dishes, a more likely explanation is that Survivin promotes adhesion to the extracellular matrix in the lungs.

We observed that the prometastatic effect of Survivin overexpression was associated with increased tumor expression of α5 integrin. We noted previously that α5 integrin expression was higher in metastatic compared with primary human melanoma cell lines and is a critical regulator of cell motility downstream of Survivin (12). Others have shown that α5 integrin increases ovarian cancer metastasis and its expression is predictive of metastasis and poor prognosis in ovarian cancer patients (27,28). In melanoma, α5 integrin promotes in vivo metastasis of murine B16F10 melanoma cells (29). Integrins mediate outside-in and inside-out signaling, which may impact cell growth and death, adhesion and motility (30). Integrin-based interactions are also known to be involved in matrix degradation and remodeling, which can aid in tumor cell dissemination from the primary tumor (31).

We considered the possibility that increased α5 integrin expression promoted dissemination of cells from the tumor into the bloodstream, thereby facilitating seeding of tumor cells in the lungs. To address this issue, we attempted to quantify circulating tumor cells in blood from mice with Survivin-overexpressing tumors by quantitative real-time polymerase chain reaction for the human melanoma marker melan-A. However, we were unable to detect such circulating cells by this method in any of the mice over the 6 week period of tumor growth with our limit of detection being 250 melanoma cells in 0.25 ml blood (J.A.M., T.L. and D.G., data not shown).

Nevertheless, our results suggest that Survivin-mediated upregulation of α5 integrin in tumors promotes subsequent colonization of tumor cells in the lungs. We found that upregulation of α5 integrin is critical for Survivin-enhanced lung metastasis, as evidenced by the reduction in pulmonary metastasis seen when α5 integrin was targeted with the antibody M200 (Volociximab). M200 treatment had the concomitant effect of reducing tumor growth, associated with higher levels of apoptosis and lower levels of proliferation in tumor cells. These findings are consistent with those of others.
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**References**


**Supplementary material**

**Supplementary Figures 1–3** can be found at http://carcin.oxfordjournals.org

**Funding**

Med-into-Grad Program funded by the Howard Hughes Medical Institute to J.A.M.; National Institutes of Health (T35 grant, HL007744 to B.B.J.); National Institutes of Health (R01 grant, AR050102 to D.G.); the Department of Dermatology and the Huntsman Cancer Foundation to D.G.

**Acknowledgements**

We thank Bryan Welm for providing the pEi2 plasmid and Debra Chao at Abbott Laboratories for helping us acquire the M200 antibody. We also acknowledge the Dermatopathology Laboratory at the University of Utah, and Anneli Bowen for photographing some of the immunostained slides.

**Conflict of Interest Statement:** None declared.

Received January 17, 2013; revised April 22, 2013; accepted April 25, 2013