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 α5β3 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 alternative 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 via 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/Amp/Kan (Stratagene, Agilent Technologies, Santa Clara, CA), confirmed by sequencing and then subcloned into the modified pE12 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 (2ml) was poured into sterile 6 cm plates and allowed to solidify on a level surface at 4°C. The cell layer consisting of 5 × 104 (LOX) or 2.5 × 104 (YUSAC2) cells in Iscove’s...
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 24h 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 × 106 cells in 0.25ml phosphate-buffered saline (PBS) were injected s.c. into the right flank. Once tumors could be palpated, the long and short dimensions (d1 and d2, respectively) were measured using digital calipers twice weekly. Dimensions were later used to calculate tumor volume using the following equation: 

\[ V = \frac{d_1 \times d_2 \times \sqrt{d_1^2 + d_2^2}}{2} \]

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% paraformaldehyde 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 (d1 and d2) were ~9-5cm, mice were injected intraperitoneally twice weekly for 4 weeks with either M200 (Voxoliximab, 10 mg/kg) or the same dose of a control mouse immunoglobulin G antibody (GBW-275D92, GenWay Biotech, San Diego, CA). M200 is an α5β1 integrin-blocking antibody, kindly provided by Abbott Laboratories (Abbott Park, IL).

Tail vein metastasis assay

Mice were injected with 5 × 104 cells in 0.25ml 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 0.5ml 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 0.5ml 4% paraformaldehyde in PBS for 30min at room temperature. After three gentle washes with PBS, 0.5ml 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 (1ml/well), and absorbance at 620nm 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 (pHH3) (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 (01115879001, 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.

Results

Survivin overexpression enhances melanoma cell metastatic potential

To examine the functional role of Survivin in melanoma metastasis, we utilized two human metastatic melanoma cell lines LOX and YUSAC2 (YU2) to generate in vivo models. LOX and YU2 were stably transfected with a control lentivirus-expressing GFP or a lentivirus-expressing GFP-tagged Survivin. Western blotting confirmed Survivin overexpression and upregulation of α5 integrin in both lines (Figure 1a). We then verified their increased migratory capacity in Transwell Migration Boyden chambers. Indeed, both LOX and YU2 lines transfected with GFP-Survivin demonstrated a >2-fold increase in the number of migratory cells compared with their GFP-transfected counterparts (Figure 1b).

Next, we examined the effect of Survivin overexpression on the capacity of melanoma cells to form anchorage-independent colonies in soft agar. As shown in Figure 1c, LOX and YU2 cells overexpressing GFP-Survivin yielded more numerous colonies than GFP-transfected control cells. To determine if α5 integrin is required for Survivin-enhanced colony formation, control and Survivin-overexpressing YU2 cells were transfected with a control siRNA or siRNA-targeting α5 integrin prior to seeding in soft agar. Knockdown of α5 integrin effectively negated Survivin-enhanced colony formation (Figure 1d).

Thus these in vitro studies indicate that Survivin enhances prometa-static activities in melanoma cells.

Survivin overexpression increases melanoma pulmonary metastasis in vivo

To determine whether Survivin overexpression in melanoma cells could enhance metastasis in vivo, we performed a spontaneous metastasis assay in which control or Survivin-overexpressing cells were injected s.c. into the flank of immunodeficient mice. Tumor size was monitored twice weekly for 6 weeks, then primary tumors were harvested and lungs were resected for analysis. Tumors grew rapidly (Supplementary Figure 1, available at Carcinogenesis Online), but tumor growth was not significantly affected by Survivin overexpression in YU2 cells (Figure 2a). To further investigate the mechanics of tumor growth, we performed histological analyses to determine the proliferative and apoptotic indices of these tumors. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining of tumor sections indicated no significant difference in apoptotic rate in Survivin-overexpressing versus control YU2 cells (Figure 2b). Similarly, no significant difference was seen in proliferation as measured by pHH3 staining in Survivin-overexpressing versus control cells (Figure 2c).

Immunohistochemistry for α5 integrin confirmed higher levels of expression in Survivin-overexpressing versus control YU2 tumors (Figure 2d). Pulmonary metastases were quantified by counting GFP-positive foci in serial lung sections. Notwithstanding the similarity in tumor growth, a significant increase in the number of lung metastases was seen in mice bearing Survivin-overexpressing versus control YU2 tumors (Figure 2c). We could not identify apoptotic tumor cells in the lungs by TUNEL staining (data not shown). Importantly, there was no difference in proliferative activity of tumor cells in the lungs (Figure 2f), suggesting that the greater number of Survivin-overexpressing cells in the lungs was a result of metastatic seeding rather than subsequent expansion. Similar findings were observed in companion experiments with Survivin-overexpressing and control LOX tumors (Supplementary Figure 2, available at Carcinogenesis Online). These results indicate that Survivin overexpression in melanoma cells leads to increased expression of α5 integrin and promotes lung metastasis.
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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) or metastatic colonies (14 day postinjection). Both control and Survivin-overexpressing cells showed similar number of extravasated cells in the lungs 24 h postinjection (Figure 3a), suggesting that Survivin overexpression does not affect melanoma cell survival in the circulation or capacity to extravasate in the lungs. No difference in tumor cell proliferation in the lungs was observed either (Figure 3b). However, examination of the lungs 14 days following i.v. 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 pHH3 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

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**Fig. 1.** Survivin promotes melanoma cell migration and colony formation. (a) LOX-GFP, LOX-Surv, YU2-GFP and YU2-Surv cells were analyzed by western blotting for Survivin, α5 integrin and actin. (b) Cells listed in (a) were added to fibronectin-coated transwells (5 × 10⁴ cells per transwell). Migratory cells (per high-power field, HPF) were quantified 24 h later. *P < 0.001, **P < 0.0001. (c) 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. (d) 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.
Supporting Figure 4. "Survivin promotes metastasis of YUSAC2 melanoma cells in vivo. (a) YU2-GFP and YU2-Surv cells were injected s.c. into the flank of immunodeficient mice (n = 4–5 per group). Once palpable, tumors were measured twice weekly for 6 weeks. ns, not significant. (b) TUNEL staining was performed, and apoptotic cells per HPF are indicated. Representative images of GFP-positive tumor cells (green) and TUNEL-positive apoptotic cells (red) are shown. (c) Proliferating cells were quantified by staining tumor sections for pHH3. Representative images are shown. (d) Tumor sections were immunostained for α5 integrin. (f) pHH3 immunostaining of lung sections was quantified. Representative images are shown. All images were taken at ×200. All error bars indicate SEM. ns, not significant."
Survivin promotes melanoma metastasis. 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.

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**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 pHH3 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) pHH3 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 pHH3-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 (19). 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 indicated survival 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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showing that inhibitory antibodies against α5 integrin can decrease tumor growth (27, 32), possibly by inducing apoptosis in tumor-associated endothelial cells (33). Roman et al. (34) demonstrated that knockdown of α5 integrin by shRNA in Lewis lung carcinoma cells reduced primary tumor growth and metastasis. In addition, Sawada et al. (27) showed that inhibition of α5 integrin using the murine IIA1 α(β5) integrin-blocking antibody (M200 is a humanized version of IIA1) decreased metastasis of SKOV-3ip1 ovarian cancer cells in a mouse model. In that study, the reduced metastasis was also accompanied by a decrease in primary tumor growth of IIA1-treated animals (27). We believe that this diminished tumor growth, however, is unlikely to represent the basis for the decreased metastasis resulting from α5 integrin inhibition. To address this issue, we employed the modified metastasis assay in which tumor cells are injected i.v. rather than into the skin, which bypasses the effect of the primary tumor on the establishment of lung metastases. We found that knockdown of α5 integrin in tumor cells prior to injection decreased Survivin-enhanced lung colonization, suggesting that the reduced metastasis observed in the spontaneous metastasis assay was not solely due to the inhibitory effect of M200 on primary tumor growth.

In summary, the results of this study support a direct role for Survivin and α5 integrin in promoting melanoma metastasis and suggest that inhibitors of α5 integrin-like M200 may be useful in combating tumor progression and melanoma metastasis. Indeed, several clinical trials have been conducted examining the efficacy of M200 in metastatic melanoma and other cancers (35–37). A phase II trial of M200 in patients with metastatic melanoma found that when combined with dacarbazine, about half of the participants exhibited stable disease at 8 weeks, whereas the remaining patients suffered progressive disease (37). It is not clear that dosing of M200 was fully optimized to block α5 integrin in metastatic tumors, and perhaps future trials combining agents that target α5 integrin and oncogenic kinases (such as BRAF) or immunomodulatory molecules (such as cytotoxic T-lymphocyte-associated antigen 4) may prove more fruitful.

Supplementary material

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

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References


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