Integrin Agonists as Adjuvants in Chemotherapy for Melanoma

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Abstract

Purpose: Metastatic melanomas are generally resistant to chemotherapy and radiation, even when wild-type for p53. These tumors often grow in small nests where many of the cells have little contact with extracellular matrix (ECM). Previous work showed that M21 melanomas undergo apoptosis in response to chemotherapy when cells are adherent to ECM but not in suspension. Thus, reduced integrin-dependent adhesion to ECM could mediate therapy resistance. The goal of this study was to test whether stimulation of integrin signaling could increase chemotherapeutic efficacy.

Experimental Design: Colony forming assays and survival assays were used to test the responses of melanoma lines *in vitro*. Severe combined immunodeficient mice with subcutaneous human melanomas received chemotherapy with or without reagents that stimulate integrin signaling; tumor volume was then monitored over time.

Results: Clonal growth assays confirmed that M21 cells showed reduced sensitivity to the chemotherapeutic drug 1-β-D-arabinofuranosylcytosine (araC). When five additional primary melanoma lines were screened, 80% showed higher sensitivity when adherent compared with suspended. Subcutaneous M21 tumors *in vivo* showed minimal ECM between tumor cells. To evaluate the importance of integrin signaling in chemoresistance in this model, mice were treated with araC, with or without the multivalent snake venom disintegrin contortrostatin or the activating anti-β1 integrin antibody TS2/16. Although araC, TS2/16, or contortrostatin alone had little effect on M21 tumor growth, combining araC with either integrin signaling reagents strongly reduced growth ($P = 0001$).

Conclusions: Loss of integrin-mediated adhesion is rate limiting for therapeutic response in this model. Combining chemotherapy with reagents that stimulate integrin signaling may therefore provide a new approach to therapy.

Melanomas are usually resistant to chemotherapy and radiation even at early stages (1). Surgery is therefore the major mode of treatment, which, if unsuccessful, leaves few options. As a consequence, 5-year survival rates for patients with unresectable or metastatic disease are <10% (2). Because melanomas often have wild-type p53 genes and lack known defects in other DNA damage and apoptosis pathways, the reasons for chemotherapy resistance are poorly understood.

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Integrin-mediated adhesion promotes the transmission of many signaling pathways initiated by growth factor receptors, including Erk mitogen-activated protein kinase, phosphatidylinositol-3-OH-kinase, and Rho family GTPases (3). As a result of these synergies, many cell types require integrin-mediated adhesion to extracellular matrix (ECM) for survival (4). This mechanism, however, is generally lost in metastatic cancers. Among cells that survive well in suspension, a subset exhibits a synergy between adhesion and DNA damage pathways (5, 6). These include mouse embryo fibroblasts, human fibrosarcoma, and human melanoma. In these systems, including melanomas, loss of adhesion results in decreased p53 levels, and reduced activation of c-Abl and stabilization of the p53 homologue p73. These cell types consequently show little apoptosis in response to DNA damage by radiation and chemical agents. These results suggest that cancer cells may become resistant to therapy through such a mechanism. Low integrin occupancy could occur within solid tumors due to matrix destruction, whereas cells that have extravasated into the bloodstream would be completely nonadherent. This hypothesis has not, however, been tested *in vivo*.

Invasive and metastatic melanomas typically grow in nests, surrounded by interstitial matrix and various types of stromal and immune cells. Although the surrounding tissue is typically matrix rich, the nests are almost entirely cellular with only

Translational Relevance

This work tests whether loss of integrin-mediated adhesion is a significant factor in chemoresistance in melanoma. Patients with melanomas that cannot be removed surgically show very poor survival even when tumors are wild-type for p53. We report that 4 of 5 melanomas examined showed lower responses to chemotherapy when cells were nonadherent. When grown as a subcutaneous xenograft in mice, a human melanoma cell line showed minimal staining for extracellular matrix proteins, indicating that integrin engagement is low. When tumor-bearing mice were treated with the chemotherapeutic agent araC, responses were minimal; however, two different reagents that stimulate integrin signaling significantly improved the response to chemotherapy. We conclude that integrin adjuvant therapy may improve outcomes in the treatment of metastatic melanoma.

small fragments of basement membrane proteins and fibronectin, and overall very low ECM compared with normal melanocytes or hyperplastic nevi (7, 8). The high production of active proteases, both metalloproteinases and components of the plasmin system, probably contributes to the minimal ECM present within these tumors (9, 10). The control of DNA damage pathways by cell adhesion may therefore be relevant to melanomas.

The goal of these studies was to ascertain whether low levels of integrin-mediated adhesion contribute to chemotherapy resistance in melanoma *in vivo*. We used a severe combined immunodeficient mouse xenograft model to study human M21 melanoma cells injected s.c. We found that triggering integrins substantially enhances the response of M21 cells to chemotherapy *in vivo*. Four of five primary melanoma lines also showed reduced sensitivity to chemotherapy *in vitro*. These data show that low integrin signaling can be a significant factor mediating therapy resistance in melanoma.

Materials and Methods

Materials. 1-β-D-arabinofuranosylcytosine (araC) and other reagents were obtained from Sigma. The TS2/16 hybridoma was obtained from American Type Culture Collection, and purified IgG was prepared in the University of Virginia hybridoma facility. Contortrostatin was prepared as described (11).

Cell culture. Human M21 cells and primary melanomas (12) were cultured in DMEM supplemented with penicillin-streptomycin and 10% fetal bovine serum (Life Technologies-Bethesda Research Laboratories). For survival assays, cells that were adherent on tissue culture plastic or suspended in 2% methyl cellulose were treated with 5 μm araC for 72 h. Cells were then replated on fibronectin-coated tissue culture plastic for 4 h and counted. Survival was calculated relative to untreated cells from the same cohort. For clonogenicity assays, $\sim 10^5$ cells were either left on tissue culture plastic in DMEM plus 0.5% fetal bovine serum (adherent) or trypsinized and suspended in the same medium with 1% methyl cellulose (suspended). Cells were treated with the indicated concentration of araC for 24 h, then harvested and plated in 10-cm dishes. They were maintained in growth medium for 10 d then fixed and stained with Coomassie blue. Colonies per plate were scored for three plates per condition.

Immunohistochemistry. Excised tumor tissue was fixed in zinc formalin and embedded in paraffin, and 5-μm sections were cut. ECM proteins were stained as previously described (13). For fibronectin and collagen, sections were subject to heat-induced antigen retrieval tissue using Antigen Unmasking Solution (Vector Laboratories). For laminin, sections were treated with trypsin. Sections were either incubated with rabbit anti-fibronectin (Sigma-Aldrich) or rabbit IgG control serum (Santa Cruz Biotechnology) at 1:400 overnight at 4°C; or monoclonal antibodies to human laminin (clone LAM-89; Novocastra Laboratories; 1:50 dilution) and to human collage IV (clone CIV 22; Dako; 1:50 dilution) were applied for 1 h at room temperature. Antibodies were detected using the avidin-biotin complex method (Vector Labortories). Antibodies were visualized using diaminobenzidine (DAKO Corp). Sections were then counter stained with hematoxylin. Slides were analyzed by light microscopy using an Olympus BX51 microscope and Image Pro Plus 3.0 software (Media Cybernectics).

To detect collagen fibrils, sections were stained using the Picrosirius red method (14).

Mouse tumor growth. Severe combined immunodeficient/bg mice (Jackson Laboratories) were injected s.c. with 100,000 M21 cells suspended in 100 μL cold PBS. Ten mice were used for each condition. Injection sites were clipped and prepped with iodine before injection. Starting at week 4 when tumors were first palpable, mice were injected i.p. with araC at 100 mg/kg IP, TS2/16 (100 μL at 1 mg/mL) or both. Alternatively, they received the same concentration of araC with or without injections of contortrostatin at 50 μg per mouse (i.p. at 1 mg/mL). Animal weights were measured weekly and tumors were measured. Individual mice were euthanized according to our institutional Animal Care and Use Committee guidelines or when largest tumor diameter was >1 cm or mice lost >20% of their body weight. In general, experiments spanned 12 to 14 wk after inoculation.

Statistical analysis. To compare the tumor growth curves, repeated measures models were used to estimate tumor growth as a function of experiment, treatment, and time. These models can account for (*a*) nonlinear growth in tumors, (*b*) correlations between tumor volume measurements within animals, (*c*) increasing variability in tumor volume over time, and (*c*) variability in tumor growth in animals in different experiments. Polynomials (three-knot restricted cubic splines) were used to model nonlinear tumor growth; random coefficient models were used to allow for increasing variability in tumor volumes over time and to account for within-animal correlations. Specific comparisons of tumor growth between groups, and for interactions between araC and contortrostatin, were carried out with F tests. The analyses were carried out in SAS 9.1 and GAUSS 8.0.

Results

Colony forming assays. Previous work showed that human M21 melanoma cells in suspension underwent less apoptosis after treatment with araC or ionizing radiation compared with adherent cells (5). However, radiation and chemotherapy can work through growth arrest or nonapoptotic cell death, so that apoptosis may not accurately predict the therapeutic response *in vivo* (15, 16). We therefore analyzed the role of cell adhesion to ECM in response to chemotherapy using a clonogenic growth assay. M21 cells were exposed to varying doses of araC for 24 h while adherent or in suspension in methyl cellulose. Cells were then rinsed, replated at low density in growth medium, and subsequent clonogenic growth was scored. We found that cells that were adherent during treatment were highly sensitive to araC, showing almost complete loss of colony formation at the lowest dose (Fig. 1A). By contrast,

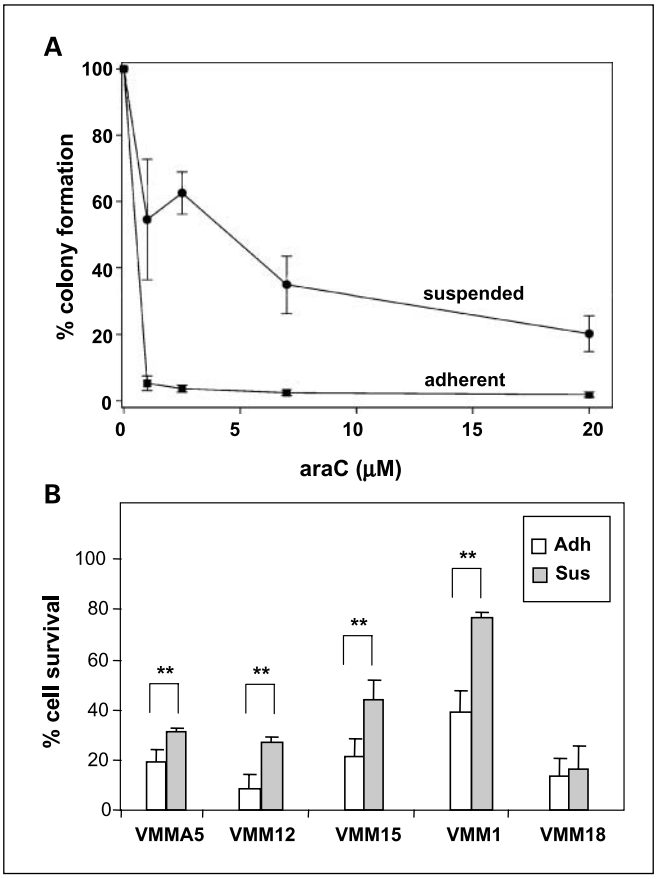

Fig. 1. Melanoma chemosensitivity *in vitro. A,* M21 melanoma cells were treated with the indicated concentration of araC for 24 h while adherent or in suspension, then washed and replated. The ability to form colonies was scored after 2 wk. Points, mean; bars, SD. $n = 3$ plates per condition. ■, adherent (*adh*) cells; ●, suspended (*sus*) cells. *B,* primary melanoma lines either adherent to tissue culture plastic or suspended on methyl cellulose were treated with 5 μm araC for 72 h while surviving cells were then counted. Columns, mean; bars, SD; $n = 3$-4. **, $P < 0.01$.

suspended cells were largely resistant, with only partial decreases in clonogenicity even at high doses. We conclude that the response of M21 cells to chemotherapy is strongly modulated by cell adhesion as suggested by apoptosis assays.

Primary melanomas. Having validated the use of cell survival as an end point, we next addressed whether this behavior was common to melanoma lines. Five primary melanomas established from patient specimens were analyzed. Cells were treated with an intermediate dose of araC and surviving cells counted after 72 hours. Four of the five lines showed significantly higher survival when treated in suspension (Fig. 1B). The differences ranged from ~1.5- to 3-fold in this assay. Because relatively modest differences in chemosensitivity *in vitro* can have major effect on patient survival (17), these data support the idea that loss of chemosensitivity in nonadherent melanomas may be clinically relevant.

ECM in xenografts. To test whether ECM organization in this model system resembles that reported for human tumors, M21 cells were injected s.c. into severe combined immunodeficient mice and tumors analyzed at 4 weeks. Staining with a polyclonal antibody that recognizes both mouse and human fibronectin showed positive staining in strands around tumor nests (*arrows*) but little or no staining within the nests (Fig. 2A). No staining was observed in controls with nonimmune antibody (Fig. 2B). In many cases, examination of the entire section showed that these strands were blood vessel walls (data not shown). Laminin was also present in thin streaks around the tumor nests (*arrows*) but was absent within the clusters of melanoma cells (Fig. 2C). An antibody that is specific for human collagen IV, a constituent of normal melanocyte basement membranes, showed no detectable staining (Fig. 2D), although a positive control with sections from human kidney done at the same time showed strong basement membrane staining (data not shown). Thus, human collagen IV is not present within tumors. When cells were stained with picrosirius red, which binds specifically to fibrillar collagens, staining of what seemed to be blood vessels was evident but no staining within the tumor nests was detected (Fig. 2E and F). H&E staining also suggested that tumors grew as tightly packed cells without detectable matrix (Fig. 2A-D). We conclude that melanoma xenografts in this model grow with only a minority of the tumor cells in contact with ECM, similar to many human melanomas.

Contortrostatin. To test whether stimulating integrin signaling could enhance the response to chemotherapy *in vivo*, we

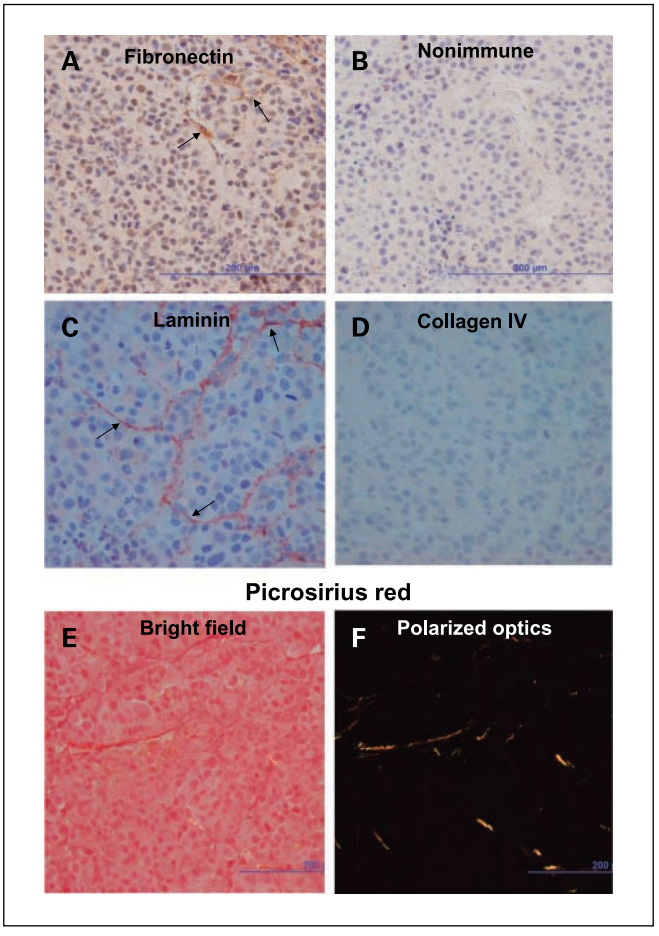

Fig. 2. ECM in M21 xenografts. Subcutaneous M21 tumors from mice were removed, fixed, and processed for immunohistochemistry as described in Materials and Methods. Sections were stained for fibronectin (*A*), with nonimmune IgG (*B*), for laminin (*C*), or for type IV collagen (*D*). *E* and *F*, the same field of a section stained with picrosirius red viewed in bright field (*E*) or polarization optics (*F*). Similar results were observed in tumors from three mice.

used the snake venom disintegrin contortrostatin (11). Unlike most disintegrins, contortrostatin is a homodimer and at nanomolar concentrations efficiently cross-links integrins to potently promote signaling, increasing tyrosine phosphorylation of integrin targets such as focal adhesion kinase and $p130^{cas}$. Contortrostatin binds to both αv integrins and α5β1 (18), which are both generally expressed on melanoma cells (19). We therefore examined effects of contortrostatin on melanoma responses to chemotherapy in the M21 mouse model.

Mice bearing subcutaneous tumors were treated with araC alone, contortrostatin alone, or both araC and contortrostatin. Mice were treated starting at week 4, when tumors were first palpable. They received two treatments, ~1 week apart, and tumor size was monitored as before (Fig. 3). We found that araC alone had no effect (araC versus control, $P = 0.83$), and contortrostatin alone seemed to have a slight but still insignificant effect ($P = 0.20$). The combined treatment, however, led to a dramatic and highly significant decrease in tumor size ($P < 0.0001$). When compared with sum of the individual effects of araC and contortrostatin, the combined treatment was clearly synergistic ($P = 0.023$).

Antibody TS2/16. Contortrostatin can bind integrins on mouse cells as well as the human melanoma cells. Although the synergy between contortrostatin and araC suggest that melanoma was the target, effects on the host cannot be completely excluded, even in immunodeficient mice. To address this issue, we examined the monoclonal antibody TS2/16, which is specific for the human β1 integrin (20). This divalent IgG can trigger integrin signaling directly by cross-linking integrins on the cell surface (21). Additionally, TS2/16 binding increases the affinity of integrins affinity for ligands (20), thus, may promote integrin binding to the sparse ECM within the nests. Severe combined immunodeficient mice were injected s.c. with M21 cells; again, starting at 4 weeks, mice were treated with araC alone, with TS2/16 anti-IgG alone, or with both simultaneously. Mice were subjected to three rounds of treatment and tumor growth followed (Fig. 4). Growth curves showed that araC alone had no effect on tumor growth ($P = 0.99$). TS2/16 alone seemed to have an effect but it did not reach statistical significance ($P = 0.33$). By contrast, combining araC with TS2/16 significantly inhibited tumor growth ($P = 0.002$).

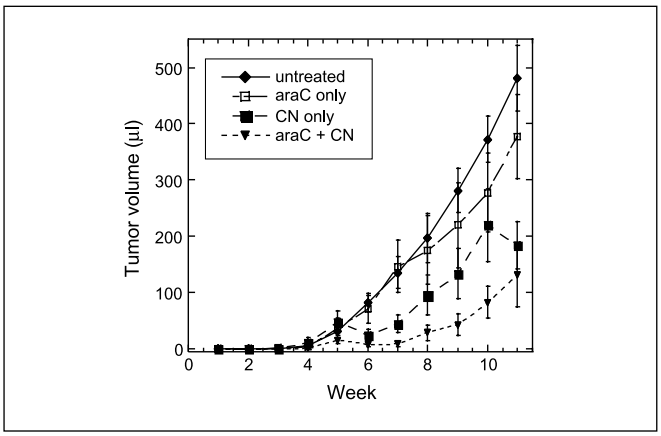

Fig. 3. Chemotherapy plus contortrostatin. Mice with subcutaneous M21 tumors were treated twice beginning at week 4 with araC alone, contortrostatin (*CN*) alone, or araC plus contortrostatin. Tumor volume was then followed. Points, mean; bars, SE. The decrease in tumor size for contortrostatin only mice at 11 wk was due to death of the mice with larger tumors. Qualitatively similar results were obtained in three experiments.

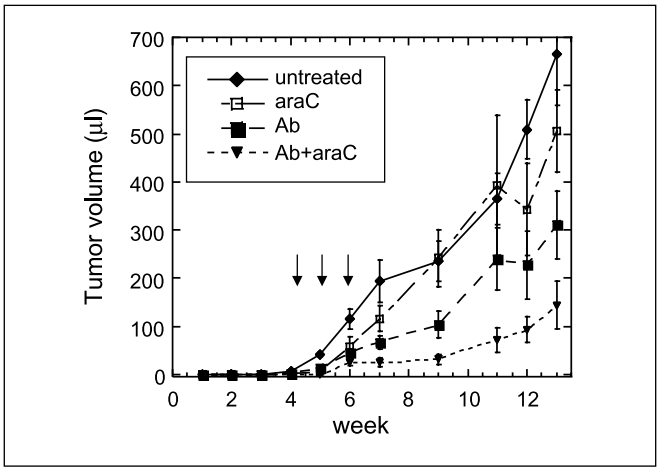

Fig. 4. Chemotherapy plus integrin antibody. Mice with subcutaneous M21 tumors were treated thrice beginning at week 4 with araC alone, antibody TS2/16 alone, or araC plus TS2/16. Tumor volume was then followed. Points, mean; bars, SE. Qualitatively similar results were obtained in two experiments.

Discussion

In this study, we addressed the mechanism of therapy resistance in melanoma. Previous reports showed that loss of integrin-mediated adhesion in melanoma and some other cell types resulted in decreased sensitivity to DNA damage from chemotherapy or ionizing radiation (5, 6). We show here (Fig. 1B) that this behavior, although not universal, is common among melanomas. It has also been shown that established melanomas grow in nests that contact stromal matrix at the edges but have very little matrix between the cells. Thus, a sizable fraction of the tumor cells would have only minimal integrin occupancy and therefore low activity of integrin-dependent signals.

To test whether loss of integrin-mediated signals contribute to therapy resistance, mice with subcutaneous tumors were treated with araC and contortrostatin, a snake venom disintegrin that is highly effective at stimulating signaling through several integrins that are prominently expressed on melanoma cells. In these experiments, contortrostatin alone or araC alone had little effect, whereas combining araC and contortrostatin caused substantial and highly significant decreases in tumor size. To test our hypothesis without interference from possible effects on the murine host, we also examined an anti-human β1 antibody that can stimulate signaling both directly by cross-linking the receptors and indirectly by increasing affinity for ECM ligands. This antibody had only a slight and insignificant effect on its own but substantially increased the response to chemotherapy. These data therefore strongly argue that loss of integrin-mediated adhesion is an important causative factor in therapy resistance of melanoma.

These experiments used only 2 to 3 treatments, compared with regimens of many months for chemotherapy in patients. In our experiments, the tumors seemed to recover and resume growth ~1 month after treatment ended. However, the substantial effects seen with this limited treatment regimen

suggest that stronger responses may be obtained using clinically relevant protocols. On the other hand, whether the reagents tested in this study are suitable for use in patients is unclear. The use of TS2/16 may be limited by its ability to activate the widely expressed β1 integrins on nearly every cell in the body, which is likely to cause some side effects. Contortrostatin seems more promising, although its likely immunogenicity may limit its use in standard chemotherapy. However, recent efforts to deliver contortrostatin encapsulated in liposomes may circumvent this issue (18). Although much more work remains to be done to develop and test clinically viable approaches, these results show that combining chemotherapy or radiation with reagents that stimulate integrin signaling may be an important new direction for improving therapeutic outcomes in the treatment of patients with metastatic melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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