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Integrin $\alpha_V\beta_3$ Promotes M21 Melanoma Growth in Human Skin by Regulating Tumor Cell Survival[1]

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ABSTRACT

Growth and dissemination of malignant melanoma has a profound impact on our population, and little is known concerning the mechanisms controlling this disease in humans. Evidence is provided that integrin $\alpha_V\beta_3$ plays a critical role in M21 melanoma tumor survival within human skin by a mechanism independent of its known role in angiogenesis. Antagonists of $\alpha_V\beta_3$ blocked melanoma growth by inducing tumor apoptosis. Moreover, M21 melanoma cell interactions with denatured collagen, a known ligand for $\alpha_V\beta_3$, caused a 5-fold increase in the relative Bcl-2:Bax ratio, an event thought to promote cell survival. Importantly, denatured collagen colocalized with $\alpha_V\beta_3$-expressing melanoma cells in human tumor biopsies, suggesting that $\alpha_V\beta_3$ interaction with denatured collagen may play a critical role in melanoma tumor survival *in vivo*.

INTRODUCTION

In recent years, the incidence of malignant melanoma has continued to rise (1). Thus, understanding the molecular and biochemical mechanisms regulating melanoma growth is of paramount importance. Cutaneous melanoma is thought to originate from a series of mutational events within melanocytes present in the basal layers of the epidermis (1, 2). These genetic alterations allow the melanocytes to escape the tightly regulated growth control mechanisms leading to malignant transformation (3). Therefore, the microenvironment in which human melanoma cells typically grow may profoundly influence the course of the disease. To this end, studies have identified families of molecules that potentiate malignant melanoma (4, 5). Interestingly, integrin receptors known to mediate cell-ECM[4] interactions have been suggested to promote this process (6).

The integrin family of cell adhesion receptors is known to mediate cellular interactions with the ECM (7). Interestingly, ligation of α_v and β_1 integrins may promote cell survival *in vitro* (8, 9). Moreover, reports indicate that expression of $\alpha_V\beta_3$ correlates with the vertical growth phase of human melanoma, suggesting that this integrin plays an important role in melanoma progression (10). However, little information is available concerning the potential mechanisms mediating these processes.

In this report, we provide evidence that $\alpha_V\beta_3$ plays a critical role in melanoma cell survival within true human skin, where cutaneous melanomas typically arise. Furthermore, we have identified denatured collagen as a physiologically relevant $\alpha_V\beta_3$ ligand present in human melanoma biopsies. Finally, we provide evidence for a mechanism by which $\alpha_V\beta_3$ may regulate human melanoma growth *in vivo* by interaction with denatured collagen.

MATERIALS AND METHODS

Antibodies, Reagents, and Chemicals. Mab LM609 directed to integrin $\alpha_V\beta_3$, Mabs 17E6 and ID7 directed to α_v and β_3, and Mab AP3 directed to β_3 have been described previously (11–13). Mab W6/32 (anti-MHC class I) was obtained from the American Type Culture Collection (Rockville, MD). Mab 5G3 directed to LI-CAM has been described previously (14). Mab HUI77 directed to denatured collagens was developed by subtractive immunization.[5] Polyclonal antibodies Ab-1 and Ab-2 directed to Bcl-2 and Bax, respectively, were obtained from Calbiochem (La Jolla, CA). Mab 4062 directed to Ki67 was obtained from Chemicon (Temecula, CA). Goat antimouse and goat antirabbit FITC and rhodamine-conjugated IgGs were from BioSource International (Camarillo, CA). Bacterial collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). OCT embedding compound was obtained from VWR Scientific Products (San Franscisco, CA). The ApopTag Apoptosis Detection Kit was obtained from Oncor (Gaithersburg, MD).

Cells and Cell Culture. Human melanoma cell line M21 was obtained from D. L. Morton (University of California, Los Angeles, CA). M21 variants M21L (α_v negative), M21L4 (α_v positive), and M21L12 (α_v-negative transfection control) were described previously (15). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and Pen-Strep.

Tumor Growth Assays. Tumor growth assays were performed essentially as described previously (16). Briefly, subconfluent cultures of M21 melanoma cell variants were harvested and washed three times with serum-free RPMI 1640. Melanoma cells (1×10^6) were injected s.c. into the flanks of 6-week-old SCID or nude mice. In the human/SCID mouse chimeric model, fresh human neonatal foreskins were obtained from the Cooperative Human Tissue Network (Cleveland, OH). SCID mice were prepared for surgical transplantation of human skin as described previously (17). Tumors were allowed to propagate for up to 37 days. Tumor growth was monitored by measuring the dimensions (length and width) of the growing tumors with calipers. Mabs were injected i.p. at a concentration of 250 μg/mouse. Antibody treatments were given three times per week. Mice were sacrificed, and tumors were resected and snap-frozen in liquid nitrogen for further analysis.

Immunofluorescence Analysis of Tumor Tissue. Tissue sections (4 μm) were fixed in acetone for 30 s and then stored at -70°C until use. For apoptosis analysis, sections were washed in 70% ethanol, followed by three washes with PBS, and blocked with 2.5% BSA. Tissues were then incubated with Mabs 5G3 anti-L1 (50 μg/ml) and HUI77 (100 μg/ml). Tissue sections were washed five times with PBS, followed by incubation with rhodamine and FITC-conjugated IgGs at a dilution of 1:400. ApopTag staining was performed according to the manufacturer's instructions. As a second method for the detection of apoptosis, Tdt-mediated nick end labeling staining was performed with the Promega In Situ Cell Death Detection Kit according to the manufacturer's instructions.

Flow Cytometric Analysis. Single cell suspensions were prepared by bacterial collagenase digestion as described previously (16). The cells were washed three times with serum-free RPMI 1640 containing 1% BSA and fixed

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[4] The abbreviations used are: ECM, extracellular matrix; Mab, monoclonal antibody; Tdt, terminal deoxynucleotidyl transferase; SCID, severe combined immunodeficient.

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in 1% paraformaldehyde. The fixed cells were washed three times with PBS and stained with the ApopTag reagent according to the manufacturer's instructions. Cell fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Negative control gates were set by using cell suspensions incubated in the absence of Tdt enzyme.

Western Blot Analysis. M21 and M21L tumors were washed three times with PBS and minced. The tumor mince was resuspended in lysis buffer containing 300 mM NaCl, 50 mM Tris, and 1.0% Triton X-100 (pH 7.5) and homogenized. Tumor lysates (100 μg) were separated by 15% SDS-PAGE. Proteins were transfered to nitrocellulose and probed with polyclonal antibodies Ab-1 (anti-Bcl-2) or Ab-2 (anti-Bax), followed by incubation with goat antirabbit peroxidase-labeled secondary antibody. Western blots were visualized by an enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham Life Sciences, Arlington Heights, IL).

Cell-Ligand ELISA. ELISA plates were coated with 50 μl of human native collagen type I, denatured collagen type I, fibronectin, laminin, or vitronectin at a concentration of 25 μg/ml for 18 h at 4°C. Wells were blocked with 1% BSA. Cultures of M21 or M21L cells were washed three times and incubated in serum-free RPMI 1640 for 24 h. Serum-starved cells were harvested and resuspended in modified adhesion buffer containing 0.5% BSA, 1.0 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.2 mM $MnCl_2$ in PBS (pH 7.5) at a concentration of 1×10^6 cells/ml. Fifty μl of cell suspensions were added to either BSA-coated or matrix-coated wells. Cells were allowed to attach for 5 h at 37°C in a humidified CO_2 incubator. Lids were then removed, and the plates were placed in a 37°C dry incubator overnight. This procedure resulted in lysis of the cells and drying the cell lysates to the bottom of the wells. Plates were then washed, and ELISA was performed by standard procedures as described previously (18).

Statistical Analysis. Statistical analysis was performed with the Stat works program for Macintosh computers. Data were analyzed for statistical significance using Student's *t* test.

RESULTS

$\alpha_V\beta_3$ Antagonists Inhibit Human M21 Melanoma Growth *in Vivo*. Studies have demonstrated that increased expression of α_v integrins correlates with enhanced melanoma growth (19). Thus, $\alpha_V\beta_3$ expressed in human melanoma may contribute to tumor growth independently of its role in angiogenesis. To test this possibility, SCID mice were injected with $\alpha_V\beta_3$-expressing M21 human melanoma cells, followed by injections with either function-blocking $\alpha_V\beta_3$-specific Mab LM609 or AP3, a control Mab directed to β_3. As shown in Fig. 1*A*, Mab LM609 inhibited M21 tumor growth, whereas control AP3 had little, if any, effect. To confirm these results, a second $\alpha_V\beta_3$ blocking Mab (17E6) was evaluated in an independent model of tumor growth. As shown in Fig. 1*B*, Mab 17E6 dramatically inhibited M21 tumor growth in nude mice, whereas control anti-β_3 Mab ID7 had no effect. Moreover, M21 tumor growth was not inhibited by systemic administration of the $\alpha_V\beta_5$ function-blocking Mab P1F6 in similar experiments (data not shown). Importantly, the inhibition of tumor growth observed in SCID mice was not due to LM609 blocking angiogenesis, as has been reported in earlier studies, because LM609 does not react with murine $\alpha_V\beta_3$ (16, 20).

Growth of Human M21 Melanoma within Full-thickness Human Skin. Cutaneous melanoma is thought to originate from a series of mutational events within melanocytes present in the basal layers of the epidermis (2, 21). To determine whether $\alpha_V\beta_3$ expression contributes to human M21 melanoma growth within human skin, we evaluated the growth of M21 melanoma cells in the human/SCID mouse chimeric model. As shown in Fig. 2*A*, $\alpha_V\beta_3$-expressing M21L4 cells formed large tumors. In contrast, $\alpha_V\beta_3$-negative M21L12 cells showed minimal tumor growth. In fact, by 34 days, M21L4 tumors were, on average, 6-fold larger than M21L12 tumors ($P < 0.009$). After 20 days of incubation, $\alpha_V\beta_3$-negative M21L12 cells began to establish measurable tumors. These findings suggest that whereas $\alpha_V\beta_3$ plays a role in M21 melanoma tumor development,

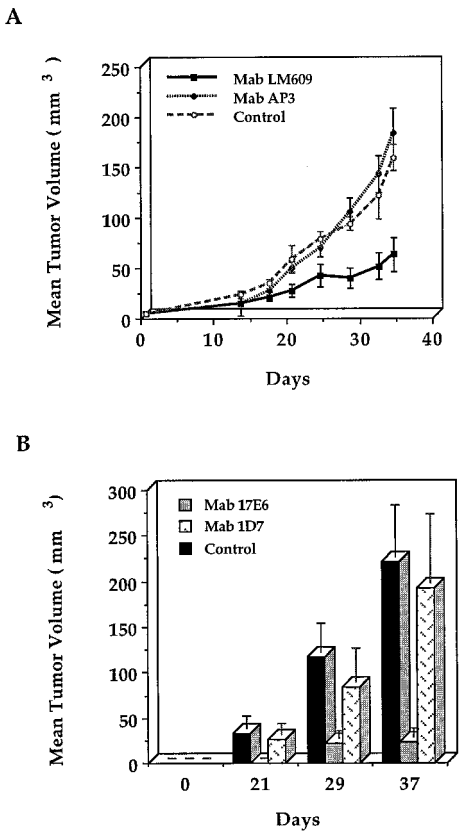

Fig. 1. $\alpha_V\beta_3$ antagonists inhibit melanoma growth *in vivo*. M21 human melanoma cells were injected s.c. into either (*A*) SCID mice or (*B*) nude mice, followed by treatment with the indicated Mabs or control PBS. Data points indicate the mean ± SE of tumor volumes from 5–6 mice/condition. Mab LM609, function-blocking antibody directed to $\alpha_V\beta_3$ integrin. Mab AP3, non-function-blocking antibody directed to the β_3 integrin subunit. Mab 17E6, function-blocking antibody directed to the α_v integrin subunit. Mab ID7, non-function-blocking antibody directed to the β_3 integrin subunit.

other integrins besides $\alpha_V\beta_3$ are also likely contribute to this process. Previous studies suggest that once a critical mass of M21L melanoma cells was established in mice, the tumors grew at similar rates (19). Consistent with these results, once M21L12 cells establish tumor foci in the human skin, no significant difference in tumor cell proliferation could be detected by staining with a Mab directed to nuclear proliferation antigen Ki67 (data not shown). These findings suggest that the differences in tumor size may not be directly associated with proliferation rates, but rather with the early establishment of initial tumor foci.

Expression of $\alpha_V\beta_3$ Protects Human M21 Melanoma from Apoptosis within Full-thickness Human Skin. Studies suggest that the growth of solid tumors depends on the balance between cell growth and cell death (22, 23). Interestingly, ligation of $\alpha_V\beta_3$ has been shown to regulate cell survival *in vitro* (8, 15). However, no information is known concerning whether this mechanism functions in tumors growing in human skin, where melanoma is typically found. Moreover, our results suggest that $\alpha_V\beta_3$ expression may be a requirement for the establishment of early tumor foci. To examine this possibility, M21L4 and M21L12 cells were grown for 5 days within the human skin. These tissues were analyzed for apoptosis by costaining with ApoTag reagent and Mab 5G3 directed to human L1, which is highly expressed in M21 cells (data not shown). As shown in Fig. 2*B*, M21L4 cells were detected within the human skin (*top left panel*) and exhibited little apoptosis (*bottom left panel*). In contrast, $\alpha_V\beta_3$-negative M21L12 cells showed extensive cell death throughout the tumor foci (*bottom right panel*). To quantitate the extent of cell death,

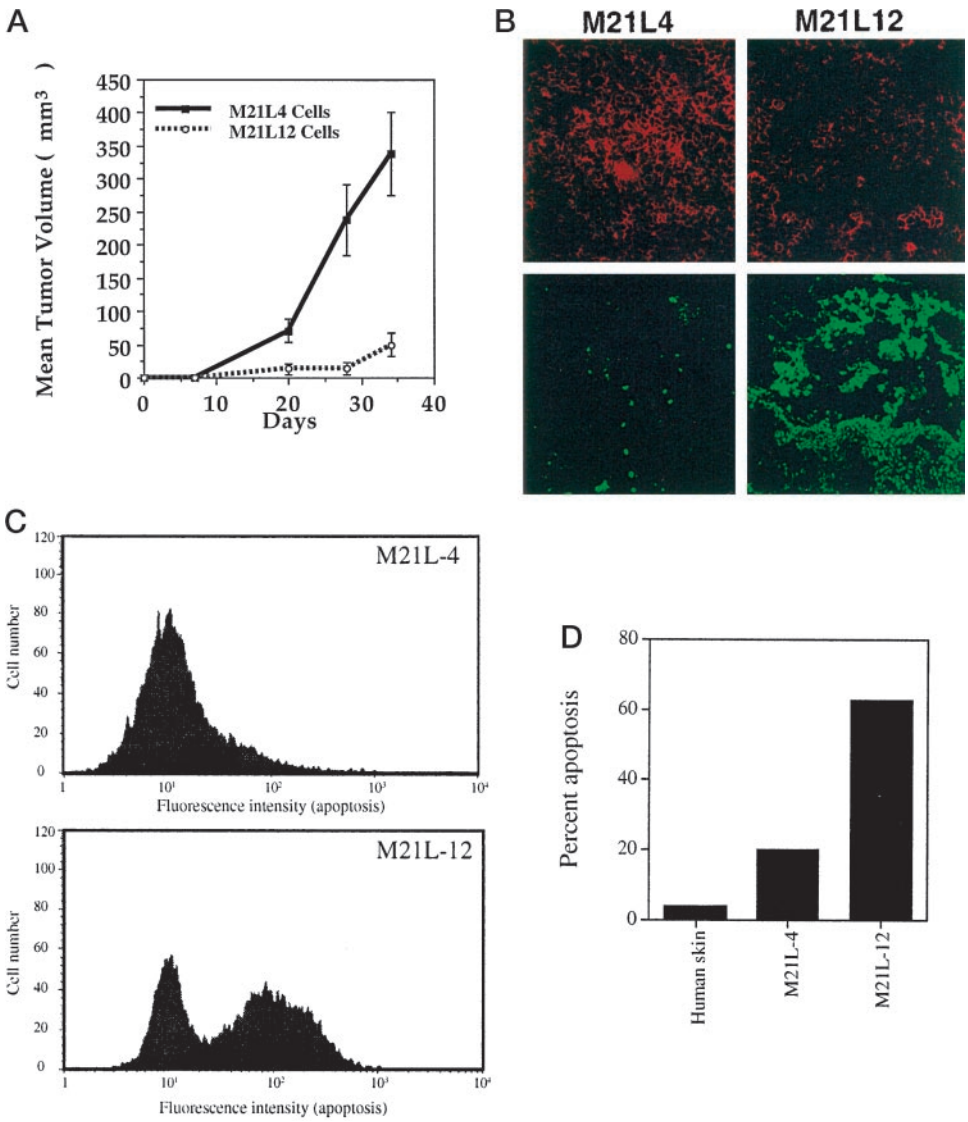

Fig. 2. Growth of human melanoma cells within full-thickness human skin. Melanoma cells expressing $\alpha_V\beta_3$ (M21L4) or lacking $\alpha_V\beta_3$ (M21L12) were injected intradermally into full-thickness human skin. *A*, comparison of M21L4 and M21L12 melanoma tumor growth in full-thickness human skin. *B*, immunofluoresence analysis of M21L4 and M21L12 melanoma tumors grown in full-thickness human skin for 5 days. *Top panels* indicate LI-CAM expression (Mab 5G3) within melanoma cells (*red*). *Bottom panels* indicate tumor apoptosis (ApopTag; *green*). *C*, fluorescence-activated cell-sorting analysis of single cell suspensions from M21L4 tumors (*top panel*) and M21L12 tumors (*bottom panel*) stained with ApopTag. *D*, quantification of apoptosis from single cell suspensions of human skin in the presence or absence of M21L4 and M21L12 cells. The percentage of apoptosis indicates the percentage of cells that stained positive for apoptosis as compared to the negative control (no Tdt enzyme).

cell suspensions derived from these tumors were analyzed for apoptosis by flow cytometry. As shown in Fig. 2*C*, cells from M21L12 tumors showed a dramatic increase in apoptosis as compared to cells from M21L4 tumors. In fact, M21L12 tumors showed a 3-fold increase in apoptosis, whereas cells from normal human skin in the absence of tumor cells showed little, if any, evidence of cell death (Fig. 2*D*.).

Antagonists of $\alpha_V\beta_3$ Induce Apoptosis of M21 Melanoma Tumors *in Vivo*. Our findings indicate that a specific antagonist of $\alpha_V\beta_3$ can block melanoma growth *in vivo* and, furthermore, that $\alpha_V\beta_3$ plays an important role in melanoma survival within human skin. To confirm these observations, SCID mice were injected with M21L4 melanoma cells, followed by treatments with either control Mab W6/32 or Mab LM609. Tissues from control- and LM609-treated tumors were analyzed for apoptosis with the ApopTag reagent. As shown in Fig. 3*A*, tumors from mice treated with LM609 exhibited extensive apoptosis as compared to tumors from W6/32-treated mice. Importantly, whereas some apoptotic cells were detected within the centers of both control- and LM609-treated tumors, only the LM609-treated tumors showed extensive apoptosis throughout the tumor, including the margins near the host-tumor interface. Similar results were obtained with function-blocking Mab 17E6 in the human/mouse chimeric model using an independent Tdt-mediated nick end labeling staining assay (data not shown).

Ligation of $\alpha_V\beta_3$ has been reported to regulate the expression of Bcl-2 and Bax, proteins thought to modulate apoptosis (15). Moreover, studies have reported high levels of Bcl-2 expression within both human melanoma biopsies and cell lines (15, 24). Therefore, we compared the expression of both Bcl-2 and Bax within M21 melanomas grown in SCID mice by Western blotting. As shown in Fig. 3*B* (*top panel*), Bcl-2 was detected in $\alpha_V\beta_3$-expressing M21 tumors, whereas little was found in M21L tumors. Conversely, Bax was detected in $\alpha_V\beta_3$-negative M21L tumors, whereas M21 tumors failed to express detectable levels (Fig. 3*B, bottom panel*). These findings demonstrate a high Bcl-2:Bax ratio in melanomas expressing $\alpha_V\beta_3$, which is consistent with the possibility that ligation of $\alpha_V\beta_3$ in these tumors may regulate Bcl-2 and Bax expression, thereby contributing to cell survival (15, 25–28). Importantly, some $\alpha_V\beta_3$-negative melanoma cells eventually established small tumors, suggesting that other mechanisms in addition to the Bcl-2:Bax ratio are likely to contribute to melanoma survival.

ECM-mediated Regulation of Bcl-2 and Bax within $\alpha_V\beta_3$-expressing Melanoma Cells. Expression of Bcl-2 and Bax may be regulated by integrin-mediated interactions with the ECM (15, 29).

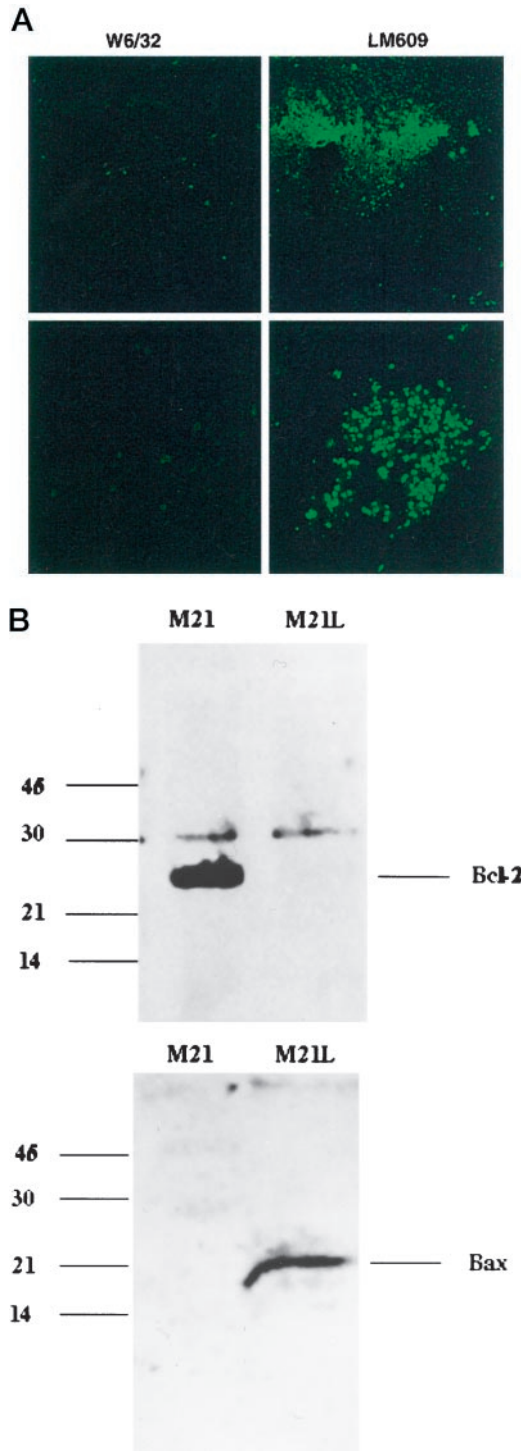

Fig. 3. Antagonists of $\alpha_V\beta_3$ induce apoptosis of M21 melanoma tumors *in vivo*. M21 melanoma cells were injected s.c. in SCID mice followed by treatments with or without Mabs LM609 or W6/32. Tissue sections were stained with the ApopTag apoptosis detection kit. *A*, *green staining* indicates apoptosis (fragmented DNA). Photographs of M21 tumors were taken near the host-tumor interface at ×100 (*top panels*) and ×200 (*bottom panels*). Mab W6/32, anti-human MHC class I. Mab LM609, anti-$\alpha_V\beta_3$ integrin. *B*, Western blot analysis of 100 μg of lysates from M21 and M21L melanoma tumors grown in SCID mice and probed with antibodies Ab-1 directed to Bcl-2 or Ab-2 directed to Bax.

Therefore, we examined the expression of Bcl-2 and Bax in M21 and M21L melanoma cells after interactions with $\alpha_V\beta_3$-specific ECM ligands or controls. As shown in Fig. 4*A*, little, if any, change in Bcl-2 expression was detected in M21 or M21L cells attached to either the $\alpha_V\beta_3$-dependent or control ECM ligands. In contrast, Bax expression was reduced 2- and 4-fold in M21 cells attached to $\alpha_V\beta_3$ -directed ligands vitronectin and denatured collagen, respectively (Fig. 4*B*). Interestingly, these results are consistent with the reduction in Bax expression observed in $\alpha_V\beta_3$-expressing M21 tumors *in vivo*. In addition, M21 cell attachment to native collagen also reduced Bax expression, suggesting a role for β_1 integrins in this response. Importantly, no change in Bax expression could be detected in $\alpha_V\beta_3$-negative M21L cells when attached to any of the ligands tested. These results implicate ligation of $\alpha_V\beta_3$ in the regulation of Bax expression because M21 cells predominately use $\alpha_V\beta_3$ in binding to vitronectin and denatured collagen (data not shown). Previous studies have suggested that a high Bcl-2:Bax expression ratio is associated with increased cell survival (15, 25–27). As shown in Fig. 4*C*, M21 cell attachment to vitronectin and denatured collagen induced a 3- and 5-fold increase in the mean Bcl-2:Bax ratio, respectively, whereas attachment to other ligands showed only a minimal change, if any. These results suggest that melanoma cell interaction with $\alpha_V\beta_3$-directed ligands may promote a high Bcl-2:Bax ratio that is thought to contribute to cell survival.

Generation of Denatured Collagen within Human Melanoma Tumors *in Vivo*. Whereas the $\alpha_V\beta_3$-directed ligand vitronectin has been detected in association with some tumors (29), collagen represents the majority of the ECM components within the dermal regions of human skin, where cutaneous melanomas typically arise. Moreover, M21 melanoma cell ligation of denatured collagen showed a 5-fold increase in the Bcl-2:Bax ratio compared to a 3-fold increase for interactions with vitronectin. Because denatured collagen is a ligand for $\alpha_V\beta_3$ and because $\alpha_V\beta_3$ ligation may regulate melanoma cell survival, we examined the possibility that denatured collagen was generated during human melanoma tumor growth *in vivo*. Tissue sections from both human melanoma biopsies and M21 tumors grown within full-thickness human skin were costained with anti-α_v polyclonal antibody and HUI77, a Mab specifically directed to denatured collagen but not to native collagen. As shown in Fig. 5, denatured collagen (*green*) was detected and shown to colocalize (*yellow*) with $\alpha_V\beta_3$-expressing melanoma cells (*red*) within human tumor biopsies (*left panels*). Moreover, similar results were observed with M21 melanoma cells grown within full-thickness human skin (*right panels*). Importantly, few if any melanoma cells observed within these tumors showed evidence of apoptosis when associated with denatured collagen after costaining experiments with ApopTag and Mab HUI77 (data not shown). Taken together, these findings suggest that the triple helical collagen within these human tumors is being selectively denatured, providing a physiologically relevant ligand for $\alpha_V\beta_3$ during melanoma growth *in vivo*.

DISCUSSION

The ability of malignant melanoma cells to establish a proliferating tumor depends on many factors (30, 31). Because collagen comprises the majority of the proteins present within the dermal ECM, where cutaneous human melanomas typically arise, it is likely to play an important role in regulating melanoma growth. Accordingly, integrin-ECM interactions can facilitate signal transduction events leading to the regulation of cell adhesion, migration, and survival, all factors that could facilitate melanoma progression (31–33). However, a major limitation in the study of melanoma arises from the fact that sites of tumor growth in many models often do not directly correspond to the tissue microenvironment found in the human disease. To address this issue, we evaluated the growth of human M21 melanoma tumors within full-thickness human skin, where cutaneous melanomas typically arise (2, 21). Our results suggest that $\alpha_V\beta_3$ plays an important role in the survival of human M21 melanomas within full-thickness

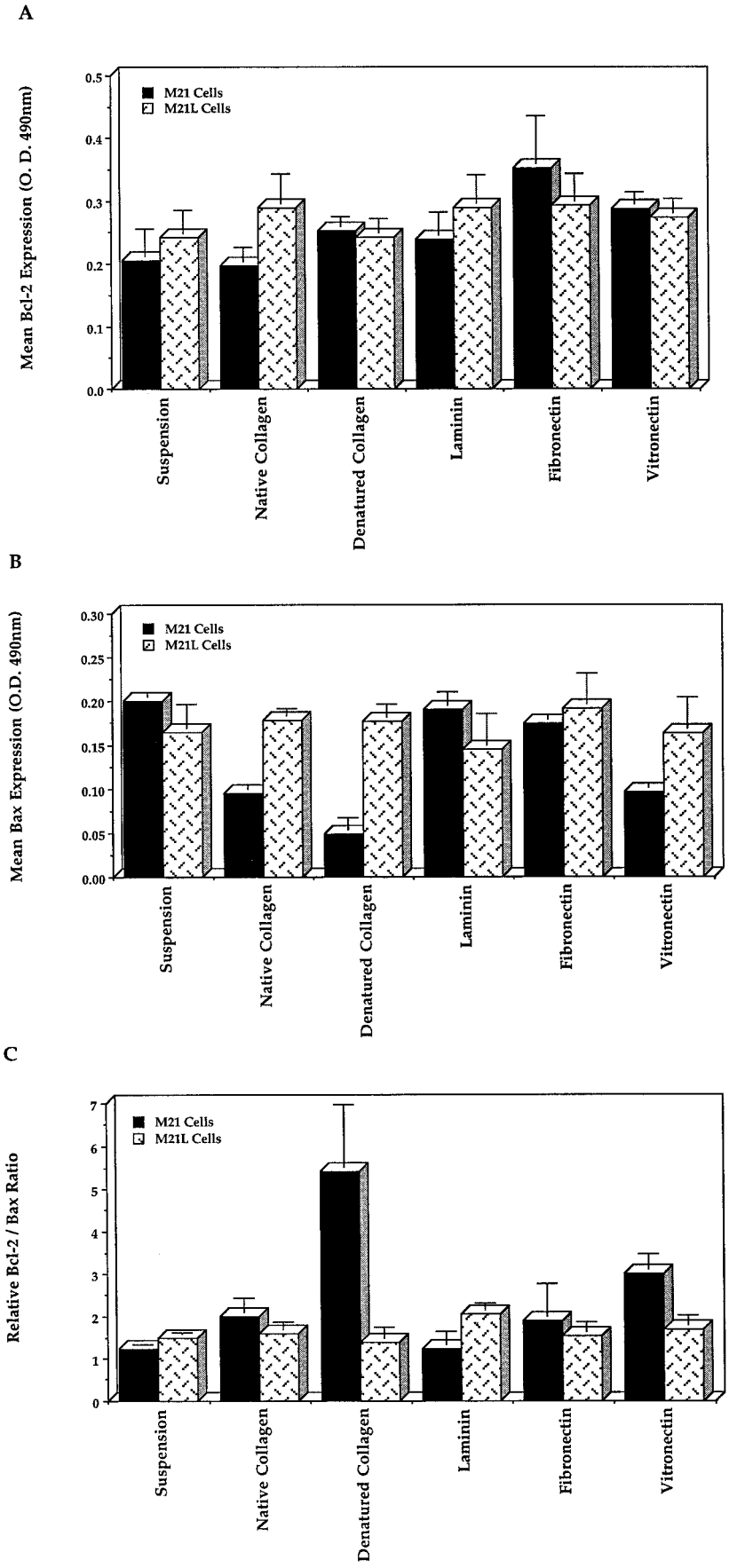

Fig. 4. Analysis of Bcl-2 and Bax expression in M21 and M21L cells attached to specific ECM proteins. M21 and M21L cells attached to specific ECM proteins were analyzed by ELISA for expression of Bcl-2 and Bax. *A*, relative expression of Bcl-2. *B*, relative expression of Bax. *C*, mean relative Bcl-2:Bax expression ratio. Data bars indicate mean absorbance ± SD from duplicate experiments derived from triplicate wells.

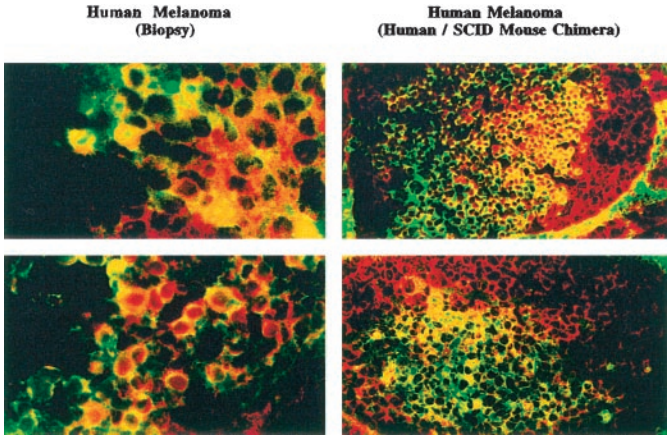

Fig. 5. Colocalization of denatured collagen and $\alpha v\beta 3$-expressing melanoma cells *in vivo*. Analysis of the generation of denatured collagen within human melanoma tumor biopsies and M21L4 tumors grown within full-thickness human skin. *Left panels*, costain analysis of human melanoma tumor biopsy (×630). *Right panels*, costain analysis of M21 tumors grown in human skin (×200). *Red* indicates $\alpha_V\beta_3$ integrin expression, and *green* indicates denatured collagen. *Yellow* indicates colocalization.

human skin. This contention is supported by the fact that melanoma cells lacking $\alpha_V\beta_3$ exhibited extensive apoptosis within human skin. Furthermore, $\alpha_V\beta_3$-negative M21L12 cells failed to establish measurable tumors for 20 days, as compared to 7 days for $\alpha v\beta 3$-expressing melanomas. These findings suggest a critical role for $\alpha_V\beta_3$ in the formation of melanoma foci within human skin.

Previous reports have correlated the expression of $\alpha_V\beta_3$ with the vertical growth phase of melanoma progression (10). However, little information is available concerning the mechanisms by which $\alpha_V\beta_3$ contributes to melanoma tumorgenicity. Here, we provide evidence for the first time that $\alpha_V\beta_3$ can facilitate human M21 melanoma growth in true human skin by regulating melanoma cell survival. Our findings suggest that the reduction in tumorgenicity observed in $\alpha_V\beta_3$-negative M21L tumors was likely associated with the inability of these tumor cells to interact with the $\alpha_V\beta_3$-directed ligands necessary for survival and was not due to alterations in tumor proliferation. This notion is supported by the fact that no change in proliferation was detected between M21 and M21L tumors stained for the Ki67 proliferation antigen. In addition, no difference in proliferation rates could be detected between $\alpha_V\beta_3$-negative or -positive M21 cells grown in culture (19). Finally, earlier studies indicate that once $\alpha_V\beta_3$-positive or -negative M21 cells established tumor foci in a murine model, their proliferation rates were similar (19).

The formation and expansion of solid tumors likely depend on a balance between cell growth and cell death (23, 27). Consistent with this hypothesis, our results indicate that melanoma cells lacking $\alpha_V\beta_3$ begin to undergo programmed cell death, whereas $\alpha_V\beta_3$-expressing cells exhibited little, if any, apoptosis. Moreover, systemic administration of functional blocking antibodies directed to $\alpha_V\beta_3$ but not $\alpha_V\beta_5$ blocked melanoma growth by inducing tumor apoptosis. Importantly, this inhibition of tumor growth was not a result of blocking angiogenesis because this antagonist does not react with $\alpha_V\beta_3$ expressed on murine blood vessels (16, 20). These findings are in agreement with $\alpha_V\beta_3$ playing a critical role in melanoma cell survival *in vivo*. In this regard, cell survival has been suggested to be regulated in part by the expression of Bcl-2 and Bax (9, 15, 16, 29). In fact, Yin *et al.* (28) recently demonstrated that overexpression of Bax increased tumor cell apoptosis, thereby reducing tumorgenicity. These findings are consistent with the expression of Bax that we observed in M21L tumors grown *in vivo* that exhibited similar phenotypic characteristics. In addition, our results also indicate that $\alpha_V\beta_3$-expressing M21 tumors exhibited a high relative Bcl-2:Bax ratio as compared to $\alpha_V\beta_3$-negative M21L tumors. These data are in agreement with the ability of the $\alpha v\beta 3$-expressing melanoma cells to interact with ECM components necessary for survival and the establishment of tumor foci. Thus, the possibility exists that melanoma cells lacking $\alpha_V\beta_3$ fail to interact with $\alpha_V\beta_3$-dependent ECM components and therefore begin to under go apoptosis. To this end, collagen is a major component of the dermal ECM of human skin (8). Moreover, many melanoma tumors have the capacity to proteolytically remodel their collagenous mircoenvironment by secreting collagen-degrading proteases (5, 8, 35). Importantly, denatured collagen has been shown to be recognized by integrin $\alpha_V\beta_3$ (8, 36). Thus, denatured collagen may provide a physiologically important ligand for $\alpha_V\beta_3$ to facilitate melanoma cell survival. Here, we provide evidence for the first time that denatured collagen is generated *in vivo* and, more importantly, colocalizes with $\alpha_V\beta_3$-expressing melanoma cells within human melanoma tumor biopsies. Significantly, few if any melanoma cells that were surrounded by denatured collagen exhibited any evidence of apoptosis. Furthermore, we demonstrate that $\alpha_V\beta_3$-mediated melanoma cell interactions with denatured collagen *in vitro* caused a 5-fold increase in the Bcl-2:Bax ratio. Whereas it is likely that other mechanisms besides $\alpha_V\beta_3$ and the relative expression of Bcl-2 and Bax also contribute to melanoma cell survival, it appears that $\alpha v\beta 3$ is a critical receptor in this process. Taken together, these findings suggest a mechanism by which $\alpha_V\beta_3$ expressed in melanoma cells may interact with denatured collagen, regulating the relative Bcl-2:Bax ratio, thereby contributing to melanoma cell survival.

In conclusion, our studies suggest that $\alpha_V\beta_3$ plays a critical role in melanoma cell survival in human skin and, furthermore, that $\alpha_V\beta_3$ antagonists represent a powerful new class of antitumor agents by virtue of their ability to disrupt tumor growth by inducing tumor cell apoptosis.

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