Inhibition of phosphatidylinositol-3-kinase and mitogen-activated protein kinase kinase 1/2 prevents melanoma development and promotes melanoma regression in the transgenic TPRas mouse model

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Abstract

A number of human melanomas show hyperactivation of the Ras pathway due to mutations of the molecule or alteration of upstream or downstream effectors. In this study, we evaluated the effect of blocking the two Ras downstream pathways phosphatidylinositol-3-kinase/Akt and Raf/mitogen-activated protein kinase kinase/extracellular signal–regulated kinase on melanoma development and regression in the TPRas mouse model. The inhibition of these two signaling cascades by topically applied Ly294002 and U0126 significantly delayed melanoma development and significantly decreased the tumor incidence, particularly when the drugs were applied in combination. Treatment with the inhibitors of established melanomas resulted in complete remission in 33% of mice and partial regression in 46% of mice when drugs were delivered in combination. These responses correlated with increased apoptosis and decreased proliferation both *in vitro* and *in vivo* and reduced tumor angiogenesis. In conclusion, this study strongly supports the role of the phosphatidylinositol-3-kinase/Akt and Raf/mitogen-activated protein kinase kinase/extracellular signal–regulated kinase pathways in the development and maintenance of Ras-dependent melanomas and supports the notion that specific inhibition of these effectors may represent a very promising avenue for the treatment and prevention of the disease. [Mol Cancer Ther 2006;5(12):3071–7]

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Introduction

Malignant melanoma is a very aggressive cancer with a high propensity to metastasize and with resistance to most current therapeutic regimens. Therefore, melanoma represents a challenging disease for the development of new effective chemopreventive and therapeutic approaches (1, 2).

Common genetic alterations in human melanomas include loss of the tumor suppressor locus *CDKN2a*, known as a melanoma susceptibility locus (3), and acquisition of activating mutations or hyperactivation of the Ras pathway.

NRas is mutated in ~30% to 50% of human melanomas and dysplastic nevi (4, 5). However, activation of the Ras pathway could also result from aberrant signaling upstream of Ras (6) or from deregulation of Ras downstream targets such as BRaf and Akt3 (7, 8).

Confirmation of the pivotal role played by Ras in melanomagenesis comes from the development of transgenic mice bearing an active mutant of the molecule. These TPRas mice express the human activated Ha-Ras gene under the mouse tyrosinase promoter, which restricts the expression of the molecule mainly in melanocytes. In the model developed by Powell et al. (9), mice develop cutaneous melanoma only after topical application of the carcinogen dimethylbenz(*a*)anthracene (DMBA) or after exposure of neonates to UV light (10, 11). In the model by Chin et al. (12), melanomas originate spontaneously only when the TPRas mouse is crossed with a *CDKN2a* knockout animal. Interestingly, in the DMBA-induced melanoma model, tumors often present loss of the INK4a-ARF proteins, which are codified by the *CDKN2a* locus (13). Both models, therefore, show genetic characteristics that are commonly observed in the human disease, thus representing a suitable system for testing chemopreventive and therapeutic agents for melanoma.

Inhibition of the Ras pathway represents a promising avenue in the treatment of melanoma. Indeed, inhibitors such as perillyl alcohol have shown some efficacy in reducing the incidence of melanoma in TPRas mice (14). However, Ras functions could also be affected by targeting downstream Ras effectors through which survival, proliferation, angiogenesis, and invasion are regulated (15).

We have previously shown that disruption of the phosphatidylinositol-3-kinase (PI3K)/Akt and Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal–regulated kinase (Erk) pathways by the use of topically applied specific inhibitors leads to the reduced development of a Ras-dependent melanoma cell line in

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severe combined immunodeficient mice (SCID; ref. 16). Tumor xenografts are used as models for preclinical anticancer drug development because they are predictive indicators of the probable clinical activity of new therapeutic drugs (17). However, these models do not recapitulate the stages of tumor development and primarily allow the study of drug response from previously established tumors, but not the outcome of tumor-preventive approaches. On the other hand, the TPRas mouse represents a model for melanoma where the tumors show similar characteristics to the human disease and present a moderate latency period and a high incidence of tumor development, providing an ideal system for evaluating both chemopreventive and therapeutic agents.

In this study, we show that inhibition of PI3K/Akt and Raf/MEK/Erk pathways by topical application of the specific inhibitors, Ly294002 and U0126, significantly reduces the incidence of melanoma and promotes tumor regression in DMBA-treated TPRas mice. These effects are associated with increased apoptosis and reduced proliferation both *in vivo* and *in vitro* and with reduced tumor angiogenesis.

Taken together, these findings show that topical treatment with inhibitors of the PI3K/Akt and Raf/MEK/Erk pathways can be an effective means for both the prevention and treatment of melanoma and shows that both signaling cascades are required in Ras-dependent melanoma development and maintenance.

Materials and Methods

Chemicals and Plasmids

DMBA was purchased from Sigma and dissolved in acetone (Sigma, St. Louis, MO). The PI3K inhibitor, Ly294002, and the MEK1/2 inhibitor, U0126, were obtained from LC Laboratories (Woburn, MA). The final concentrations used for Ly294002 and U0126 were 50 and 10 μmol/L, respectively, for both *in vitro* and *in vivo* studies. The PI3K and MEK1/2 dominant negative constructs have been previously described (16).

TPRas Mice

The TPRas transgenic mice have also been previously described (9). This mouse contains a mutated T24 Ha-Ras gene driven by a 2.5 kb fragment of the mouse tyrosinase promoter.

In vivo Experiments and Immunohistochemistry

Mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care–approved facility with 12 h light cycles. Food and water were provided *ad libitum*. In the first experiment, 68 mice were treated with DMBA (50 μg) once a week for 5 weeks to induce melanoma growth and randomly divided into four treatment groups: ethanol (control), Ly294002, U0126, and Ly294002 + U0126. Treatments with the inhibitors started a week before DMBA treatment and were done three times a week for the duration of the experiment (36 weeks). The primary outcome was time to first tumor. Tumor incidence was examined using nonparametric survival analysis (Kaplan-Meier). In the second experiment, 62 mice already bearing melanomas (size range, 150–400 mm^3), were divided into four treatment groups as above and followed for 35 weeks to see if tumors progressed, partially regressed, or completely regressed. These data were analyzed using Stata v9's ologit procedure, an ordinal logistic regression model to designate tumors that stopped growing or decreased in size.

For immunohistochemical studies, tumors were excised and frozen in O.C.T. Compound (Sakura, Tokyo, Japan). Five-micron section were cut and stained with antibodies according to the manufacturer's instructions. Briefly, primary antibodies were incubated overnight followed by incubation with biotinylated secondary antibody (1:200; The Jackson Laboratories, Bar Harbor, ME) and with either FITC-conjugated avidin D (1:500; Vector Labs) or streptavidin/horseradish peroxidase (The Jackson Laboratory). Antibodies used were: rat anti-CD31 (platelet/endothelial cell adhesion molecule 1, 1:50; BD PharMingen, Bedford, MA), rat anti-Ki67 (1:50; Dako Corporation, Santa Barbara, CA), rabbit anti-cleaved caspase 3 (1:100), rabbit anti-phosphor-Akt (1:50, immunohistochemistry specific), and rabbit anti-phosphor-MEK (1:50; Cell Signaling Technology, Danvers, MA).

Cell Line

The 1984-1 cell line, derived from a cutaneous melanoma on a TPras mouse, was described previously (14).

Cell Proliferation

Cells (10^5) were plated in triplicate in 6 cm plates and were counted every 3 to 4 days using an electronic particle counter (Beckman Coulter, Fullerton, CA). Ly294002 and U0126, alone or in combination, were added each time cells were reseeded.

Detection of Apoptosis

Flow cytometry analysis of propidium iodide–stained cells was used to quantify the percentage of apoptotic cells as the fraction of cells with a hypodiploid amount of DNA (sub-G_1) as previously described (18). Cleaved caspase 3, a marker of caspase-mediated cell death, was detected by immunoblot analysis.

Northern Blotting

Total RNA from 1984-1 cells treated for 24 h with inhibitors or from cells overexpressing the PI3K or MEK1/2 dominant negative constructs were harvested using TRIzol Reagent (Life Technologies, Inc., Carlsbad, CA) as per the manufacturer's instructions. RNA samples were run on a 1% formaldehyde gel and transferred to Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ). Blots were hybridized with ^{32}P-labeled human vascular endothelial growth factor (VEGF) cDNA and imaged with a Storm phosphor-screen (Amersham Biosciences).

Immunoblot Analyses

1984-1 cells were plated in RPMI 1640 plus 10% fetal bovine serum, allowed to adhere, then treated with the inhibitors in low serum media (0.5% fetal bovine serum). Cells expressing the dominant negative constructs were placed in low serum media and protein lysates were harvested 24 h later. Protein lysates were made using 9 mol/L

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of urea, 0.075 mol/L of Tris (7.6) buffer, quantified with the Bradford assay, and run on SDS-PAGE using standard methods. Antibodies used were: anti-phospho-p44/42 mitogen-activated protein kinase (Thr^{202}/Tyr^{204}, 1:1,000), anti-phospho-Akt (Ser^{473}, 1:1,000), anti-cyclin D1 (1:1,000), anti-cleaved caspase 3 (1:1,000; Cell Signaling), anti-cyclin E (1:1,000, Upstate Biotechnology, Lake Placid, NY), anti–basic fibroblast growth factor (bFGF; 1:500; Transduction Laboratories, Lexington, NY), anti–hypoxia-inducible factor 1α (HIF1α; 1:1,000; Bethyl Laboratories, Montgomery, TX), and anti-β-actin (1:200, Santa Cruz Biotechnology, Santa Cruz, CA).

Results

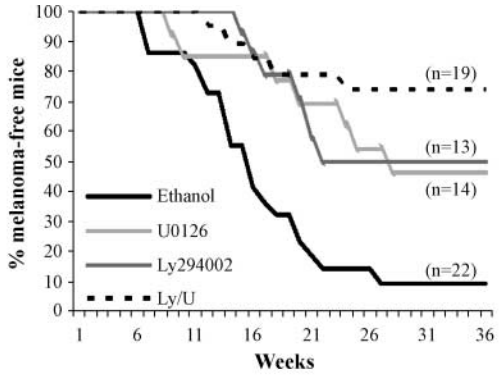
PI3K and MEK1/2 Inhibition Reduces Melanoma Incidence

To test whether selective inhibition of the Ras downstream pathways PI3K/Akt and Raf/MEK/Erk had an effect on melanoma development, we treated TPRas mice with the specific inhibitors Ly294002 and U0126. The treatment was started when mice were free of tumor, a week before five weekly treatments with the carcinogen DMBA. Control mice received DMBA in combination with ethanol, used as a vehicle for the inhibitors. At the end of the study, only 9% of mice were melanoma-free in the control (vehicle) group. In the group of animals treated with U0126, mice showed melanoma development starting at week 10, whereas in Ly294002-treated mice, tumor development was delayed by about 8 weeks with respect to controls. Both inhibitors, when applied alone, reduced the overall melanoma incidence by ~40%. When the agents were delivered in combination, 70% to 75% of mice did not develop melanoma (Fig. 1A). All treatment groups showed significantly lower tumor incidences than the control group, with the combined agents presenting a higher survival rate ($P < 0.0001$). Importantly, during the prolonged treatment, no systemic toxicities or skin irritations were observed.

PI3K and MEK1/2 Inhibition Promotes Melanoma Regression in TPRas Mice

To further investigate the efficacy of blocking these two pathways on melanoma growth, TPRas mice bearing 150 to 400 mm^3 cutaneous tumors (Fig. 2A, *a*) were treated with Ly294002 and U0126, alone or in combination, or with ethanol. Melanomas in 93% of the control animals progressed, measured as an increase in size, and in some cases, gained an invasive and metastatic behavior, with the presence of tumor cells in peripheral lymph nodes (Fig. 2A, *b* and *c*). For all treatment groups, the regression of tumors was significant with respect to the control group ($P < 0.0001$). The combination of the two drugs was most effective in promoting tumor regression compared with either agent alone (Ly294002 versus Ly294002 + U0126, $P = 0.005$; U0126 versus Ly294002 + U0126, $P = 0.008$; Fig. 1B). As shown in Fig. 2B, tumors treated with the drugs showed reduced Akt and MEK activity.

A



B

treatment group		regressed	partially regressed	progressed	total
ethanol	# of mice	0	1	13	14
	% mice	0	7.1	92.9	100
Ly294002	# of mice	2	8	2	12
	% mice	16.7	66.7	16.7	100
U0126	# of mice	3	5	4	12
	% mice	25	41.7	33.3	100
Ly + U	# of mice	8	11	5	24
	% mice	33.3	45.8	20.8	100

Figure 1. Ly294002 and U0126 reduce the incidence of melanoma and improve melanoma regression in TPRas mice. **A,** Kaplan-Meier representation of melanoma incidence in TPRas mice. Mice (5–6 weeks old) were treated with Ly294002 (50 μmol/L) and U0126 (10 μmol/L) 1 wk prior to the five weekly treatments with DMBA. Drugs were applied topically three times a week for 36 wks. **B,** DMBA-induced melanomas (150–400 mm^3) were treated with Ly294002 and U0126 three times a week for 36 wks. Data were analyzed using an ordinal logistic regression model. The *P* values indicating significance of treatment groups compared with control group were: Ly294002, 0.002; U0126, 0.004; Ly294002 + U0126, 0.0004.

Interestingly, of all treatment groups, the U0126-treated mice showed a higher percentage of progression, although this difference was not statistically significant, perhaps due to the small sample sizes.

Inhibition of PI3K and MEK1/2 Correlates with Reduced Cell Proliferation and Increased Apoptosis

Treatment with Ly294002 and U0126 reduces cell proliferation *in vivo*. The average number of Ki67-positive cells was significantly reduced in tumors treated with the combined agents (Fig. 3A). Treatments with Ly294002 or U0126 alone resulted in reduced proliferation overall, but with intermediate effects with respect to the inhibitors applied in combination (data not shown). These *in vivo* data correlate with the results *in vitro*, in which treatment of the TPRas mouse melanoma cell line 1984-1 with Ly294002 and U0126 significantly reduced cell proliferation (Fig. 4A and B). Also in this case, the effect of the inhibitors delivered in combination was more pronounced than the drugs applied singularly. Even though proliferation was significantly reduced, cells continued to grow, although slower than in control cells. This partial effect of the drugs is likely due, at least in part, to the instability of the compounds in culture.

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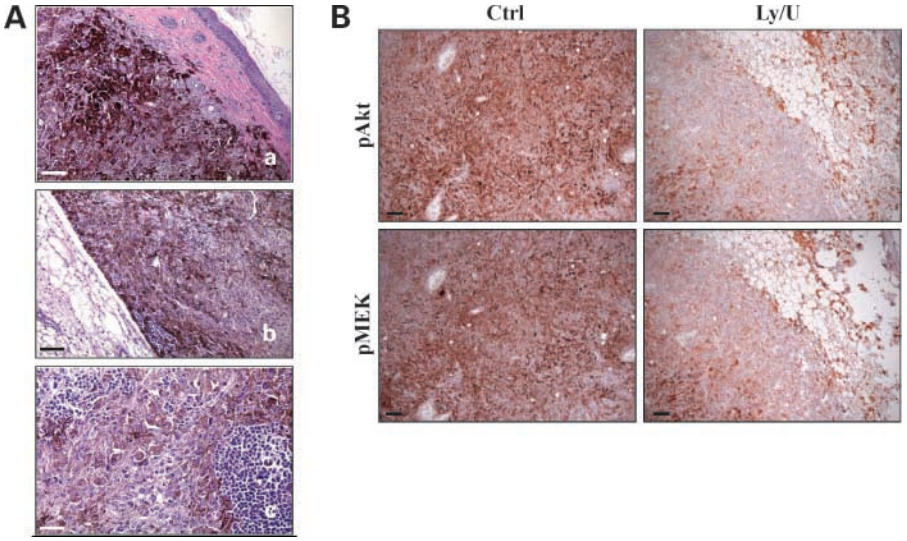

Figure 2. TPRas melanoma and effect of drugs *in vivo*. **A,** H&E staining of primary tumors (**a**) or metastasis in lymph nodes (**b** and **c**). **B,** phospho-Akt and phospho-MEK staining of control and Ly294002/U0126-treated tumors. Bars: **A, a**) and **b**): 50 μm; **c**): 20 μm; **B**, 50 μm.

Ras can induce cyclin D1 through both the Raf/MEK/Erk and the PI3K/Akt pathways (19, 20). Cyclin D1 contributes to Ras-dependent tumor growth by regulating the G_1-S cell cycle transition (19). Blocking the two signaling cascades downstream of Ras results in strong reduction of cyclin D1 expression associated with decreased growth rate, with the highest inhibition observed when both drugs were delivered together (Fig. 4C). A slight decrease in cyclin E was also seen in cells treated with both Ly294002 and U0126.

Inhibition of both PI3K/Akt and Raf/MEK/Erk pathways increases tumor apoptosis (Fig. 3B). In fact, the number of cells showing positive staining for cleaved caspase 3 was significantly higher in Ly294002/U0126-treated tumors than in controls. This result correlates with the data obtained *in vitro* on 1984-1 melanoma cells. Acute treatment with Ly294002 induces cell death in ~30% of the cells, whereas U0126 does not seem to exert a significant apoptotic effect. The drugs in combination induced cell death in ~40% of the treated cells (Fig. 5A), only slightly higher than the effect produced by Ly294002 alone, suggesting that cell death was mainly due to PI3K inhibition. These data correlate with an increased amount of cleaved caspase 3 in cells treated with Ly294002 and Ly294002/U0126 (Fig. 5B).

Inhibition of PI3K and MEK1/2 Decreases Tumor Angiogenesis

Tumor angiogenesis is a complex phenomenon and it is regulated by various pathways. Oncogenes such as Ras and its downstream pathways, PI3K/Akt and Raf/MEK/Erk, play an important role in the process (21). Melanomas arising in the DMBA-treated TPRas mice show high levels of vascularization. By blocking PI3K/Akt and Raf/MEK/Erk pathways with Ly294002 and U0126, we observed a significant decrease in tumor vessels (Fig. 3C). The number of blood vessels in the tumors, stained with the endothelial marker CD31, was in fact significantly reduced in Ly294002/U0126-treated mice. Treatments with Ly294002 and U0126 alone produced a less pronounced although significant effect (data not shown).

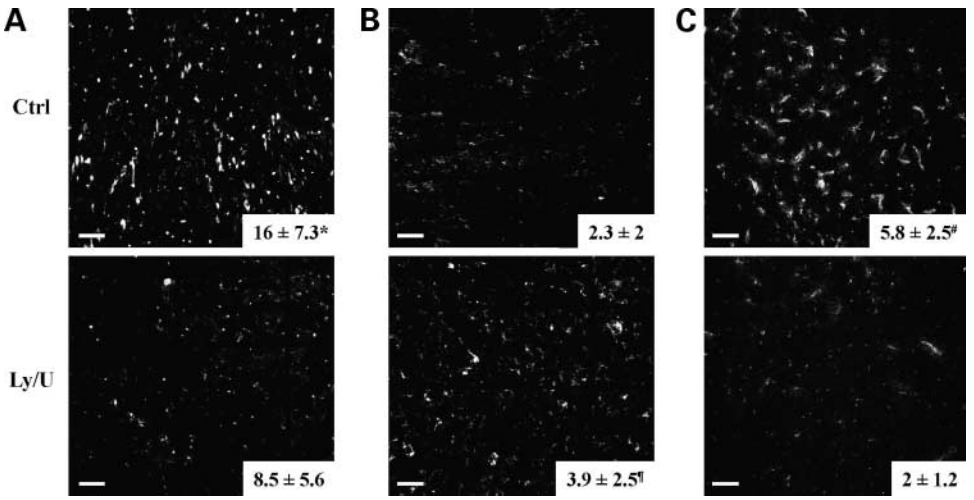

Figure 3. Inhibition of PI3K and MEK1/2 results in decreased proliferation, increased apoptosis, and reduced tumor angiogenesis. **A,** Ki67 staining of control (*top*) or Ly294002/U0126-treated tumors (*bottom*). **B,** cleaved caspase 3 staining of control and Ly294002/U0126-treated tumors. **C.** CD31 staining of control and Ly294002/U0126-treated tumors. Number of positive cells/field or vessels/field is reported. Statistically significant difference between ethanol-treated and Ly294002/U0126-treated tumors was evaluated by Student's *t* test (*, $P < 0.002$; ¶, $P < 0.05$; #, $P < 0.001$). Values are the average ± SD of five randomly chosen fields of five different slides per treatment group. *Bars,* 50 μm.

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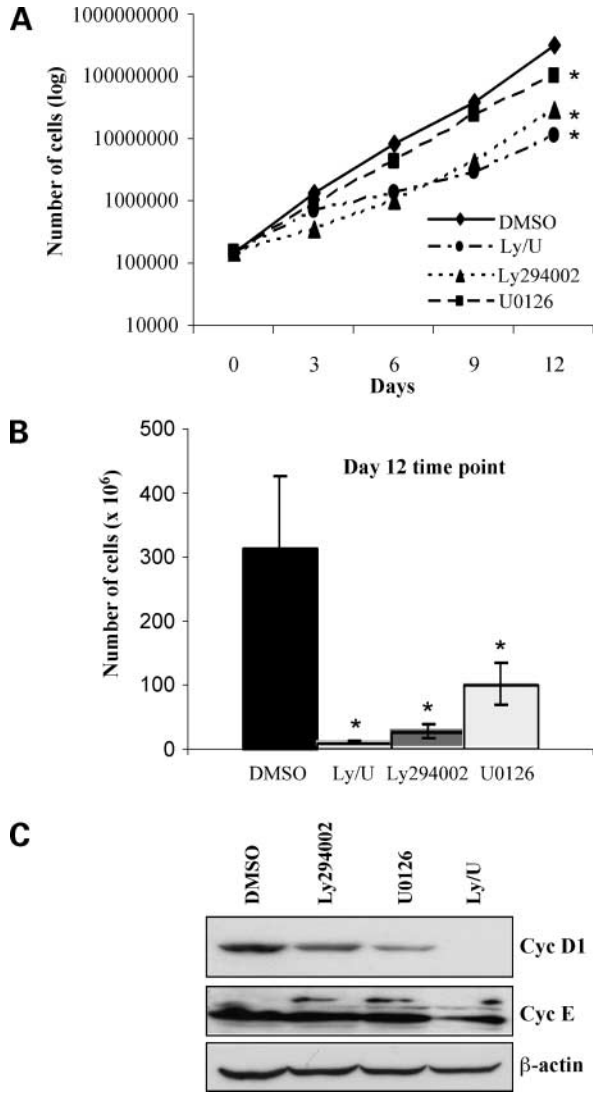

Figure 4. Ly294002 and U0126 treatments inhibit melanoma cell growth *in vitro*. **A,** 10^5 1984-1 cells/plate (60 mm dishes) were treated with the inhibitors for 72 h (Ly294002, 50 μmol/L; U0126, 10 μmol/L). Cells were counted every 3 d using an electronic particle counter. The number of cells is represented on a log scale. **B,** number of cells at the final time point represented on a linear scale. All treatments inhibit cell growth significantly with respect to controls (Student's *t* test, $P < 0.05$). *Columns,* average of three independent experiments; *bars,* SD. **C,** cells treated with Ly294002 and U0126 show decreased expression of cyclin D1 and a slight decrease of cyclin E. β-Actin was used as a loading control.

Most melanoma cells express VEGF and bFGF, which have been shown to contribute to melanoma development and progression by both supporting tumor growth and angiogenesis (22). Reduced vascularization in the TPRas tumors correlates with reduced VEGF and bFGF expression *in vitro* in 1984-1 cells treated with the chemical inhibitors or overexpressing dominant negative PI3K and dominant negative MEK1/2 (Fig. 6A and B). This inhibition in angiogenic factor productions is likely to depend on the effect of the inhibitors, both chemical and genetic, on the transcription factor HIF1α, whose expression has been shown, in other tumor models, to be, at least in part, controlled by the PI3K/Akt and Raf/MEK/Erk cascades (23–26).

Discussion

In this study, we show that topical treatment with the PI3K and MEK1/2 inhibitors, Ly294002 and U0126, appreciably reduces the incidence of melanoma in the TPRas mouse model and also contributes to the regression of the tumor.

The Ras downstream signaling cascades, PI3K/Akt and Raf/MEK/Erk, regulate survival and growth pathways that are normally shared by both normal and tumor cells (27, 28). The regulation of these functions in physiologic conditions is strictly controlled by the availability of extracellular signals that converge on Ras and its effectors. In pathologic situations, such as neoplasms, these molecules can be constitutively activated, rendering cells able to grow indefinitely and to survive proapoptotic stimuli (29). A tumor cell that acquires such a phenotype is often resistant to common cytotoxic drugs and radiation. Down-regulation of PI3K/Akt and/or Raf/MEK/Erk pathways may therefore contribute to the inhibition of tumor development and progression, and improve responses to common therapies (30, 31).

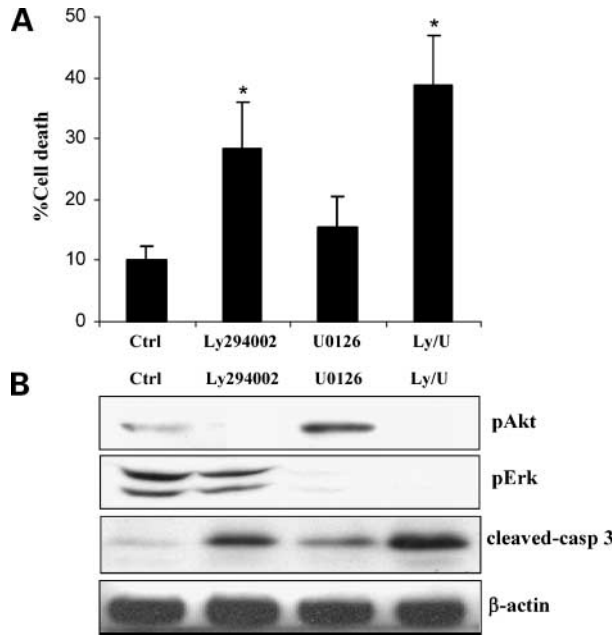

Figure 5. Inhibition of PI3K induces apoptosis in 1984-1 melanoma cells *in vitro*. **A,** 10^6 1984-1 cells/plate (100 mm dishes) were treated with Ly294002 (50 μmol/L) and U0126 (10 μmol/L) for 24 h. Cells were then fixed and permeabilized in 100% ethanol and prepared for flow cytometry. The cell cycle distribution was analyzed and apoptotic cells were quantified as the fraction of cells with a hypodiploid amount of DNA (sub-G_1). *Columns,* average percentage of apoptotic cells from three independent experiments; *bars,* SD. Significance was calculated by Student's *t* test ($P < 0.05$). **B,** protein lysates were screened for cleaved caspase 3 as a measure of activation of the apoptotic pathway. Inhibition of phosphorylation of Akt and Erk was used as an indication of the efficacy of the treatments. β-Actin was used as a loading control.

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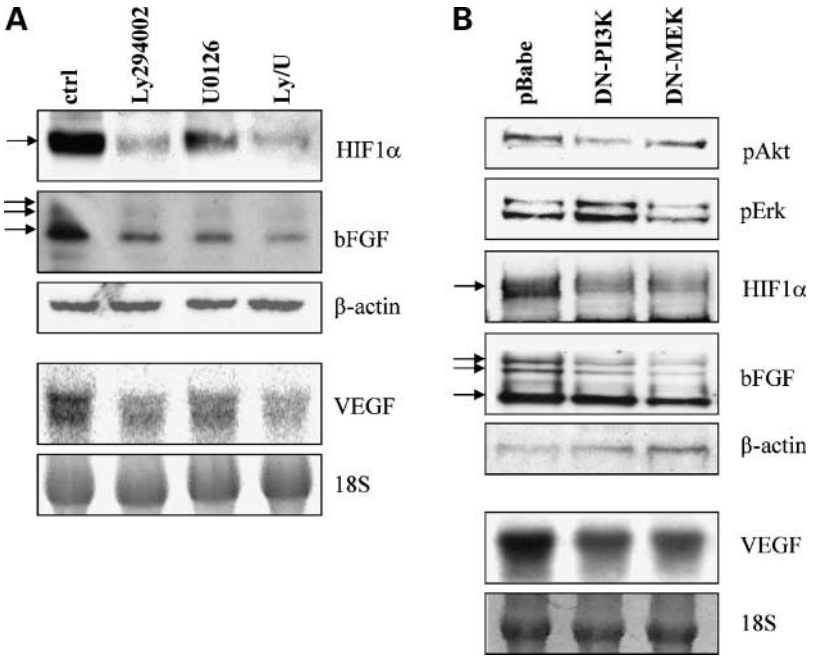

Figure 6. Inhibition of PI3K and MEK1/2 decreases angiogenic factor expression. **A,** protein lysates from 1984-1 cells treated with DMSO, 50 μmol/L of Ly294002, or 10 μmol/L of U0126. **B,** lysates from 1984-1 cells overexpressing the dominant negative constructs. Cell lysates were screened for HIF1α protein expression and bFGF (*arrows*). Anti-β-actin was used as a loading control. Total RNA from 1984-1 cells treated as described above or expressing the dominant negative constructs for PI3K and MEK1/2 were analyzed for the expression of VEGF. Ribosomal 18S was used as a loading control.

We show that Ly294002 and U0126 significantly delay melanoma development and reduce the overall incidence of melanoma in TPRas mice, with the greatest effect seen when drugs were applied in combination. Inhibition of tumor development may be due in part to the cytostatic function exerted by the two drugs. In fact, we observed a reduced number of proliferating cells both *in vivo* and *in vitro*.

Previous data obtained in a xenograft model showed no effect of U0126 on melanoma proliferation *in vivo* (16). This is in apparent disagreement with the results obtained here in which both drugs seem to exert a similar inhibitory effect on melanoma development. In the previous study, Ly294002 and U0126 were applied on an already established melanoma cell line injected in SCID mice. Here, the drugs are delivered to melanocytes that were initiated by the presence of Ras and DMBA treatment but not yet transformed. Proliferation is essential for the establishment and perpetration of mutations. Both PI3K and MAPK contribute to Ras-dependent cell proliferation, therefore, it is very likely that both pathways are necessary in the initial steps of melanomagenesis.

Interestingly, established TPRas melanomas also respond to Ly294002 and U0126. The response ranges between complete regression and partial regression, with treatment using both drugs having the greatest effect. The response may depend on the tumor size when treatments are initiated. In fact, not surprisingly, tumors with an average size of 150 mm^3 tended to respond better than tumors that were double the size by showing a higher rate of regression.

We have previously observed that although inhibition of MEK1/2 did not inhibit tumor growth in a xenograft model for melanoma, treatment with U0126 contributed to the inhibition of tumor progression in part by reducing invasion and angiogenesis (16). In the studies on the TPRas model, we observed that both treatment with U0126 and Ly294002 contributed similarly to tumor regression (both total and partial) confirming the role of MAPK and PI3K pathways in the tumor maintenance. Nevertheless, we also observed that the percentage of melanomas that progressed in the U0126 group was higher compared with the Ly294002 group. Ly294002, therefore, more efficiently inhibits tumor growth and progression compared with U0126 in this system. A possible explanation for this phenomenon is that whereas the MEK1/2 inhibitor mainly exerts a cytostatic effect on these tumors, blocking PI3K results in both cell growth arrest and increased apoptosis.

Treatments with these drugs resulted in the inhibition of tumor angiogenesis. Treated tumors showed a dramatic decrease in the number of vessels with respect to vehicle-treated melanomas, and this correlates with a strong decrease in VEGF and bFGF expression in 1984-1 cells treated with the inhibitors or expressing dominant negative constructs for PI3K and MEK1/2.

Both angiogenic factors are important mediators of the tumor ''angiogenic switch'' and have been found to be highly expressed in human melanomas as well as being associated with melanoma progression (32). Oncogenes such as Ras can increase the expression of both VEGF and bFGF. This effect is likely due to increased stabilization/activity of HIF1α, a transcription factor that plays an essential role in tumor angiogenesis and tumor progression (33). Both PI3K and MAPK have been shown, in other tumor models, to be implicated in the regulation of HIF1α function downstream of Ras (23–26). Indeed, we have observed that treatments with the chemical inhibitors or the dominant negative molecules reduce HIF1α protein, and this correlates with the reduction in VEGF and bFGF

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expression in 1984-1 Ras-dependent melanoma cells. Altogether, these effects on proliferation, survival, and angiogenesis contribute to the overall response of Ras-dependent melanomas to the treatments.

Great efforts have been put into the development of new drugs aimed at targeting specific oncogenes that are necessary for tumor development, maintenance, and progression. In melanomas that present BRaf mutations, inhibition of the molecule by small interfering RNA or by chemical inhibitors results in decreased proliferation and increased apoptosis (34, 35). On the other hand, we have shown that Akt-dependent melanomas respond well to rapamycin, a specific inhibitor of mTOR, a downstream target of Akt (36).

In this study, we present evidence that Ras-dependent melanomas require the activity of both PI3K/Akt and Raf/MEK/Erk pathways for their development and maintenance. We show that to achieve a maximal response, such tumors should receive combined treatment with the inhibitors of these molecules. We also show that a topical approach virtually eliminates the systemic toxicity that would likely occur when using inhibitors of pathways crucial for both normal and neoplastic cells.

Taken together, these findings underline the importance of choosing a therapeutic strategy that better counteracts the tumorigenic effect of specific oncogenes. We propose that combined treatment with Ly294002 and U0126 may represent a very promising new therapeutic approach in the prevention and treatment of human melanoma.

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