Constitutive Activation of Akt/Protein Kinase B in Melanoma Leads to Up-Regulation of Nuclear Factor-κB and Tumor Progression

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ABSTRACT

The serine/threonine kinase Akt/protein kinase B and the pleiotropic transcription factor nuclear factor-κB (NF-κB) play important roles in the control of cell proliferation, apoptosis, and oncogenesis. Previous studies from our laboratory have shown the constitutive activation of NF-κB in melanoma cells. However, the mechanism of this activation is not clearly understood. The purpose of this study was to explore the role of Akt in the activation of NF-κB during melanoma tumor progression. Based on our observation that two of the five melanoma cell lines examined exhibit constitutive Akt activation, we evaluated Akt activation by immunohistochemistry in a series of pigmented skin lesions using an antibody specific for phospho-Akt Ser-473. Normal and slightly dysplastic nevi exhibited no significant Akt expression, in marked contrast to the dramatic Akt immunoreactivity seen in severely dysplastic nevi and melanomas (66.3% positive). When these same lesions were stained for nuclear p65, a similar expression pattern was observed. In addition, interruption of Akt activation resulted in increased apoptosis and decreased NF-κB promoter activity. These results indicate that activation of Akt kinase is linked to enhanced NF-κB nuclear localization and transactivation. We propose that activation of Akt may be an early marker for tumor progression in melanoma.

INTRODUCTION

The incidence of cutaneous melanoma has risen logarithmically in the last few decades, and melanoma accounts for the most deaths from skin cancer (1–3). Melanoma affects both relatively young and older populations, metastasizes rapidly, and is highly resistant to chemotherapy and other treatments. To develop new diagnostic strategies, it is very important to understand the events involved in the progression from dysplasia to malignancy. The transition from benign lesions to invasive, metastatic tumors occurs through a stepwise process involving changes in expression and/or function of oncopgenes or tumor suppressor genes, as well as constitutive expression of chemokine cytokines, growth factors, and metalloproteinases (reviewed in Ref. 4).

Clinically, melanocytic lesions may be divided into benign melanocytic nevi (i.e., junctional nevi, compound nevi, and dermal nevi), lesions with possible malignant potential (i.e., dysplastic nevi, congenital nevi, and lentigo maligna), and malignant lesions (i.e., primary melanoma such as superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and acral lentiginous melanoma as well as metastatic melanoma [5, 6]). For this study, we selected the following types of clinical lesions to study melanocytic tumor progression: (a) benign melanocytic nevi; (b) dysplastic nevi; (c) lentigo maligna; and (d) metastatic melanomas.

The incidence of dysplastic nevi in the general population has been estimated at 7%, with patients having dysplastic nevi exhibiting a cumulative melanoma risk of 1.9% compared with 0.04% of patients without dysplastic nevi (7). The current lifetime incidence of melanoma in the United States is estimated at 1:74 (8). The high incidence of dysplastic nevi and melanoma presents a large public health problem.

Whereas histopathology is the current gold standard for the diagnosis of atypical melanocytic lesions, interobserver correlation in the diagnosis of dysplastic nevi is variable (9, 10). The malignant potential of dysplastic nevi and early melanomas is difficult to predict with standard histological staining. Therefore, better techniques are desired. Standard immunohistochemical markers such as HMB-45 have not been useful in differentiating a dysplastic nevus from melanoma in situ (11). Some newer immunohistochemical markers such as Ki 67, proliferating cell nuclear antigen, cyclin A, matrix metalloproteinase-2, osteonectin, p16, and gp100 may be helpful prognostically in some subgroups of melanoma patients (12). However, the molecular events that correlate with the progression of benign melanocytic nevi to dysplastic nevi to malignant melanomas currently are not well understood and are vital for clarifying ambiguities in histopathology and clinical prognosis as well as offering potential new therapeutic interventions.

Many human solid tumor cell lines display increased nuclear NF-κB (2) levels and/or increased NF-κB transcriptional activity (13–15). NF-κB is activated in Hodgkin’s lymphoma, head and neck squamous cell carcinoma, non-small cell lung cancer, colorectal cancer, thyroid cancer, pancreatic carcinoma, leukemia, multiple myeloma, prostate cancer, and breast cancer (16–18). Our laboratory has previously shown constitutive NF-κB expression in melanoma cells (19). In most nontransformed cell types, NF-κB complexes (a heterotrimer composed of p50 and RelA/p65 subunits bound to an inhibitor, IκB) are largely cytoplasmic. On activation, the IκB proteins become phosphorylated, ubiquitinated, and subsequently degraded. This liberates NF-κB, allowing it to accumulate in the nucleus, where it enhances the transcription of specific genes. The IκB kinase, or IκK complex (M, 700,000–900,000), consists of two catalytic units, IKK-α and IKK-β, bound to a regulatory subunit IKK-γ or NEMO (20–23).

Akt/PKB, a serine/threonine kinase, is a core component of the PI3K signaling pathway that is activated through phosphorylation of Ser-473 and Thr-308/309 (24). Several studies have shown that Akt/PI3K activates the transcription of a wide range of genes, especially those involved in immune activation, cell proliferation, apoptosis, and cell survival (25). Mechanisms associated with the ability of Akt to suppress apoptosis include the phosphorylation and inactivation of many proapoptotic proteins such as Bad (26), caspase-9 (27), and the forkhead family of transcription factors (28) and activation of NF-κB (29).

Based on its role as a key regulator of cellular survival, Akt is emerging as a central player in tumorigenesis. In the last decade, evidence from different studies suggests that Akt perturbations play an important role in human malignancy. Akt1 and Akt2 amplification...
and/or mRNA overexpression was observed in human gastric cancer and 10–20% of human ovarian, breast, and pancreatic cancers, respectively (30, 31). Activation of the Akt2 kinase is reported in 40% of ovarian cancers (32, 33), whereas Akt1 kinase activity is often increased in prostate and breast cancers (34). Amplification of AKT3 has not been described. Furthermore, loss of the negative regulator of this pathway, the tumor suppressor PTEN, correlates with increased Akt activity in many types of cancers (35, 36).

The present challenge is to make sense of the network that controls apoptosis during melanoma tumorigenesis, which requires evaluating the Akt status of typical and atypical pigmented lesions, as well as cell lines. Although high expression of Akt and/or Rel/NF-kB has been demonstrated in several different tumors, the relevance of constitutive expression of these genes to the progression and metastasis of human melanomas is poorly defined. In this report, we investigated the activation of Akt and NF-kB in melanoma cell lines and melanocytic lesion tissues.

MATERIALS AND METHODS

Plasmids and Reagents. The NF-kB luciferase reporter vector contains five tandem repeats of the NF-kB element 5’ to the transcription initiation site and is contained in pUC-MCS reporter vector (Stratagene, La Jolla, CA). Rabbit anti-phospho-Akt was obtained from Cell Signaling Technology (Beverly, MA), and rabbit anti-RelA/p65 and rabbit anti-Akt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GST-IkB purified protein was also obtained from Santa Cruz Biotechnology. WM and LY294002 were obtained from Calbiochem (La Jolla, CA).

Cell Culture and Transfection. The human melanoma cell lines Hs294T, SKMel5, SKMel28, WM115, and WM164, normal RPE cells, and NHEMs were obtained and cultured as described previously (37). One day before transfection, the cells were seeded in 2-well cell culture plates to provide a final density of 40–60% confluence (~3 × 10^4 cells/well). Cells were transfected using effectene transfection reagent (Qiagen, Valencia, CA) as described previously (37). Fold stimulation was calculated for each sample by dividing the normalized luciferase activity by the value obtained from the control transfection containing empty parental expression vectors (pCMV).

Immunoblot Analysis. Whole cell extracts were obtained according to our standard protocol using radioimmunoprecipitation assay buffer, resolved on 10% SDS-PAGE, transferred to the nitrocellulose membrane, blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20, and probed with appropriate antibodies as described previously (37). The p-Akt antibody was used at a dilution of 1:500, whereas Akt was used at a 1:1,000 dilution. The antibodies were visualized with either horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) using enhanced chemiluminescence (Pierce, Rockford, IL).

Immunoprecipitation and Kinase Assays. Normal and melanoma cells were grown in 100-mm cell culture dishes. Cells were placed in serum-free media for 18–24 h. Thereafter, cells were lysed, and extracts were prepared. Samples were then immunoprecipitated with IKK-α/β antibody, and a kinase assay was performed using full-length GST-IκBα substrate, as described previously (37). The same blot was normalized with the antibody used for immunoprecipitation.

Immunohistochemistry and Immunofluorescence. Paraffin-fixed tissue samples were obtained from melanoma and normal tissues of patients using protocols approved by the Vanderbilt University Institutional Review Board. All melanocytic lesion diagnoses on tissues used in these studies were made independently by dermatopathologists who were not involved in the study. Criteria for the diagnosis of dysplastic nevi were those adopted by the 1992 NIH Consensus Conference (38). This diagnosis requires both (a) architectural disorder and (b) melanocytic atypia, which is classified as mild, moderate, or severe. Sections were placed on glass slides and deparaffinized with xylene. The antigen was unmasked by heating samples in 10 mM sodium citrate buffer (pH 6.0) for 5 min, the reaction was quenched using hydrogen peroxide, and samples were then immunostained for p-Akt using rabbit anti-p-Akt antibody (1:50; Cell Signaling Technology) or for activated RelA/p65 with mouse anti-RelA/p65 (1:50, Chemicon, Temecula, CA). The ABC biotin/avidin reagent kit was used to visualize the immunolocalization of antigen using Novared substrate, and cell contents were counterstained with hematoxylin. For immunofluorescence, primary antibodies were used at the following dilutions: rabbit anti-p-Akt (1:50; Cell Signaling Technology); and mouse anti RelA/p65 (1:100; Chemicon). All primary antibody incubations were performed in a moisture chamber overnight at 4°C. Secondary antibodies were donkey anti-rabbit Texas red and goat antimmunoe Alex 488. The slides were fixed with Vectashield and sealed with clear nail polish. Stained sections were viewed and photographed using the fluorescence microscope. The excitation wavelength was 549 nm for Texas red and was 488 nm for Alex488.

Measurement of Apoptosis. Subconfluent melanoma Hs294T, SKMel28, and WM115 cells and normal RPE cells were transfected with either vector control or Akt dominant negative constructs. Alternatively, cells were incubated with the PI3K inhibitor, WM (100 nM). The cells were then collected and stained with fluorescein-conjugated annexin V and propidium iodide, using the TACS-Annexin V-FITC kit in accordance with the manufacturer’s instructions (Boehringer Mannheim, Basel, Switzerland). After staining, the cells were sorted by flow cytometry with a FACScan (Becton Dickinson, Franklin Lakes, NJ). Annexin V-positive stained cells were counted as apoptotic cells. Cells that were propidium iodide positive but annexin V negative were not counted as apoptotic cells.

Statistical Analysis. Student’s t test for paired samples was used to determine statistical significance of the transfection data. Differences were considered statistically significant at P ≤ 0.05.

RESULTS

Akt/PKB Phosphorylation and Activity Are Constitutive in Some Melanoma Cells. To determine whether Akt/PKB is activated in melanoma cells, five different metastatic melanoma cell lines obtained from American Type Culture Collection were studied. Cells were deprived of serum overnight, and then expression of activated Akt was assessed by immunoblotting cell lysates using phospho-specific antibodies against phosphorylated Ser-473 (Fig. 1A). Two of the five melanoma cell lines, Hs294T and WM115, were highly phosphorylated at Ser-473 as compared with normal controls. In vitro kinase assays using glycogen synthase kinase-3α/β as a substrate were performed to verify that phosphorylated Akt/PKB is enzymatically active. Fig. 1B shows that the highest levels of Akt/PKB enzymatic activity are found in the

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**Fig. 1.** Akt is constitutively activated in Hs294T and WM115 melanoma cell lines. RPE, NHEMs and melanoma cells were cultured in the absence of serum overnight, and the next morning, whole cell extracts were prepared for immunoblotting. A, the expression of both activated Akt and total Akt was determined using anti-phospho-Akt (Ser-473) and anti-Akt antibodies. B, in vitro kinase assays of Akt immunoprecipitates from normal and melanoma cells. Akt activity was determined using Akt kinase assay with glycogen synthase kinase-3α/β as a substrate. This figure is representative of three separate experiments.

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Hs294T and WM115 cells, which have the highest levels of phosphorylated Akt/PKB. When combined, the data from the phosphorylation and kinase assay experiments show that there is constitutive activation of Akt/PKB in two of the five melanoma cell lines.

**Immunostaining of p-Akt in Normal and Melanoma Tissues.** To determine whether activated Akt is present in the biopsies of pigmented lesions at various stages of tumor progression, immunohistochemical staining of 29 paraffin-embedded tumor specimens in various stages of tumor progression was performed using a phospho-specific anti-AKT antibody. As shown in Fig. 2, melanoma and severely dysplastic nevi showed enhanced Akt immunoreactivity (Fig. 2, A–E), in contrast to a mildly dysplastic specimen (Fig. 2, F and G). Fig. 2H represents a negative control. Some melanoma lesions did not exhibit immunostaining for Akt (Fig. 2I), suggesting that there may be subgroups of melanoma that do not exhibit activation of this pathway. Interestingly, phosphorylated Akt was located in the tumor cell membrane, the cytoplasm, and the nucleus, as supported by the previously reported observation that Akt transiently associates with plasma membrane of stimulated cells, which is followed by translocation to the nucleus (39). The normal nevus lesions were largely negative for phospho-Akt.

**Constitutive NF-κB (RelA/p65) Activation Is Observed in Melanoma Cells.** Immunostaining for RelA/p65 was done using an antibody that is directed against the nuclear localization signal, and thus immunofluorescence is a valid test to detect nuclear NF-κB as well as RelA/p65 not bound to IκB in the cytoplasm. The active RelA/p65 was displayed by melanoma tumor tissues and severely dysplastic nevi (Fig. 3, A–E) and not by normal nevi or in nevi with mild to moderate dysplasia (Fig. 3, F and G). A melanoma lesion negative for RelA/p65 immunostaining is shown in Fig. 3H.

The Akt and RelA/p65 activation status of the 29 patients tested is summarized in Table 1. The normal patients were mainly negative for both RelA/p65 and phosphorylated Akt staining. Of 11 melanoma cases examined, 7 (63.6%) were positive for both p-Akt and nuclear p65. Interestingly, the p-Akt and RelA/p65 staining was almost negative for normal nevi, increased with progression of melanocytic lesion atypia, and was most apparent in metastatic melanoma lesions.

**Relationship between p-Akt and RelA/p65 Expression in Melanoma Lesions.** To determine whether p-Akt and activated RelA/p65 colocalized, double immunofluorescence staining was used. This was accomplished using Texas red-conjugated donkey antirabbit antibody and Alexa 488-conjugated goat antimouse to stain p-Akt and RelA/p65 antibody, respectively. Colocalization of p-Akt and activated RelA/p65 was observed in tumor tissues (Fig. 4) and melanoma cell lines (Fig. 5), and this colocalization was largely nuclear because p-Akt and activated p65 translocate to nucleus (40). In addition, we found that the normal nevi exhibited no staining, but in cultured NHEMs, a low level of staining was observed in a few cells, probably due to the in vitro proliferation of these cells. These observations suggest that Akt activation is associated with NF-κB activation in melanoma. Moreover, this event parallels the degree of atypia observed in pigmented lesions (Figs. 4 and 5).

**PI3K Is Required for Activation of Akt and Thus NF-κB in Melanoma Cells.** To address the roles of the PI3K pathway in NF-κB activation by Akt, the effects of pharmacological PI3K inhibitors LY294002 and WM on Akt phosphorylation and NF-κB activity were examined. Fig. 6A shows that both LY294002 (5, 10, and 20 μM) and WM (100 and 200 nM) almost completely inhibited the phosphorylation of Ser-473 on Akt in a dose-dependent manner in the Hs294T cell line, whereas there was no effect in RPE cells (data not shown). Native Akt/PKB levels were not affected by these treatments. Thus, Akt/PKB phosphorylation in the Hs294T melanoma cell line is PI3K dependent. Furthermore, to test whether NF-κB activation in melanoma cell lines is PI3K dependent, the effect of these inhibitors was examined on NF-κB promoter luciferase activity in Hs294T and WM115 melanoma cell lines (Fig. 6B). A dose-dependent decrease in reporter luciferase activity in both melanoma cell lines was observed. Thus, for these two melanoma cell lines, NF-κB activity is constitutively activated, and this activation is dependent on the PI3K-Akt pathway.

**IKK Activity Is Not Affected by PI3K Inhibitors.** To explore the possibility of IKK being involved in NF-κB activation, the effect of treatment with the PI3K inhibitors LY294002 and WM on IKK activity was evaluated in Hs294T melanoma and RPE control cells. IKK-α and IKK-β were immunoprecipitated from the treated samples,
and then an IKK kinase assay was performed using GST-IκBα as a substrate. As shown in Fig. 7, there was no difference between control and treated samples in the phosphorylation of GST-IκBα for either Hs294T or RPE cells. Thus, it appears that the PI3K-Akt activation pathway does not affect the IKK activity leading to IκBα phosphorylation, ubiquitination, and degradation.

**PI3K-Akt Pathway Promotes Survival of Melanoma Cells.** To test whether active Akt/PKB promotes cellular survival of melanoma cell lines, transient transfection and pharmacological approaches were used to alter Akt/PKB activity and assess apoptosis. Melanoma cells expressing constitutively active Akt/PKB were either transiently transfected with dominant negative Akt or treated with WM (100 nM) for 48 h. Typical morphological changes of apoptosis were observed, including membrane blebbing, increased refractoriness, and chromatin condensation (data not shown). Other evidence for WM-induced apoptosis included increased Annexin V binding to cells as assessed by flow cytometry. Apoptosis was potentiated when Akt activation was inhibited in the Hs294T and WM115 cells by about 30–35%, but neither dominant negative Akt nor WM (100 nM) had substantial effects on the SKMel28 cell line, which exhibited the lowest level of Akt/PKB activity (Table 2). Thus, WM not only inhibits Akt phosphorylation and activity but also increases melanoma cell apoptosis in relative proportion to the level of endogenous Akt/PKB activity. Therefore, the PI3K-Akt pathway is involved in promoting cell survival by activating NF-κB and preventing apoptosis.

| Table 1 | Immunostaining for phosphorylation of Akt and activated p65/RelA in normal and melanoma patients |
|---|---|---|
| Patient no. | Histological diagnosis* | p-Akt staining | p65 staining |
| 1 | Intradermal nevus | − | + |
| 2 | Intradermal nevus | + | − |
| 3 | Intradermal nevus | − | + |
| 4 | Intradermal nevus | + | − |
| 5 | Intradermal nevus | − | ND |
| 6 | Intradermal nevus | − | ND |
| 7 | Intradermal nevus | − | + |
| 8 | Intradermal nevus | − | − |
| 9 | Compound nevus | − | + |
| 10 | Compound nevus | − | − |
| 11 | Lentigo maligna | + | +/− |
| 12 | Dysplastic nevus mild | + | + |
| 13 | Dysplastic nevus moderate | + | − |
| 14 | Dysplastic nevus severe | + | + |
| 15 | Dysplastic nevus moderate/severe | + | ND |
| 16 | Dysplastic nevus moderate/severe | + | + |
| 17 | Severe dysplastic nevi | + | ND |
| 18 | Metastatic melanoma | + | + |
| 19 | Metastatic melanoma | + | + |
| 20 | Metastatic melanoma | + | + |
| 21 | Metastatic melanoma | + | + |
| 22 | Metastatic melanoma | − | ND |
| 23 | Metastatic melanoma | − | − |
| 24 | Metastatic melanoma | − | − |
| 25 | Metastatic melanoma | + | + |
| 26 | Metastatic melanoma | + | + |
| 27 | Metastatic melanoma | + | + |
| 28 | Metastatic melanoma | + | + |
| 29 | Metastatic melanoma | + | + |

* Represents patients in different stages of melanoma tumor progression.

**DISCUSSION**

In recent years, there has been an explosion of information describing the roles of Akt (PKB) in signaling pathways essential in the regulation of cell growth, survival, and apoptosis. It is overexpressed as well as activated in numerous human malignancies (41). However, whether Akt overexpression and/or constitutive activation has any role in the development of melanomas is not known. In this study we have shown not only that Akt is constitutively activated in human melanomas but also that it enhances cell survival through NF-κB activation, hence playing a key role in melanoma development. In addition, we have provided suggestive evidence that Akt can be used as a diagnostic marker of melanoma progression. Our immunohistochemical studies of various stages of human melanomas suggest a possible correlation between enhanced Akt activation, tumor progression, and malignancy.

Of the five human melanoma cell lines tested, two had a high level of constitutive Akt phosphorylation and activity (Hs294T and WM115). To determine whether the differences in phospho-Akt observed among cell lines are a cell-specific phenomenon or whether they reflects possible differences among human melanoma lesions, 29
melanocytic lesions providing a spectrum of tumor progression were examined by immunostaining using phospho-specific anti-Akt: junctional, compound, and dermal nevi (benign); dysplastic nevi (with cytologic atypia graded as mild, moderate, or severe; precancerous); lentigo maligna; and metastatic melanoma. When evaluating precancerous lesions (dysplastic nevi), it is often assumed that the greater the degree of melanocytic cytologic atypia, the greater the risk for malignant transformation of a given lesion, although the significance of the degree of atypia has not been established for prognosis of the risk of a single lesion progressing to a melanoma or of the patient developing a melanoma (42). Interestingly, the phosphorylation and activation of Akt increased in a stage-specific manner, with maximum activation in metastatic melanoma lesions and melanoma cell lines. Thus, we found good correlation between the progression of atypia of the melanocytic lesions based on immunostaining with the phospho-specific anti-Akt antibody. Our data suggest a possible role for Akt in the transformation of nevi into melanoma at the critical juncture where the nevus becomes atypical. In addition, as observed with melanoma cell lines, the melanoma lesions fell into two groups: (a) those with high phospho-Akt; and (b) those negative for phospho-Akt. Taken together, the observations suggest the possibility for high and low phospho-Akt subtypes of melanoma. The low phospho-Akt group of melanoma lesions would presumably use Akt-independent signals in association with NF-κB activation and transformation. In this regard, we have previously demonstrated NF-κB-inducing kinase (NIK)-dependent NF-κB constitutive activation in certain human melanoma cell lines (37). In support of our report, others have shown that in primary glial cells, Akt activation converts anaplastic astrocytoma...
ROLE OF AKT IN MELANOMA TUMOR PROGRESSION

Fig. 6. A. Akt/PKB is constitutively activated in a PI3K-dependent manner in melanoma cells. Hs294T cells were either left untreated (control) or cultured for 3 h in the presence of increasing concentrations of the PI3K inhibitor LY294002 or WM. Cells were lysed, and the activity of Akt/PKB was determined by immunoblotting with the anti-phospho-Akt (Ser-473) antibody. The same blot was reprobed with anti-Akt antibody as a control for protein loading. This figure is a representative of three separate experiments.

B. Akt activates NF-κB in a PI3K-dependent manner. The Hs294T and WM115 cells were cotransfected with NF-κB luciferase reporter construct and the RSV-β-galactosidase expression construct and either left untreated or treated with increasing concentrations of LY294002 (10 and 20 μM) or WM (50 and 100 nM) for 10–12 h. Cells were then harvested, and luciferase and β-galactosidase activity were measured. The relative luciferase activity represents the luciferase activity of the sample that was normalized by β-galactosidase activity from three different experiments. The results are reported as the mean ± SD of inhibition, considering 1.0 as the relative luciferase activity of the cells transfected with corresponding empty vector. * P ≤ 0.05.

Fig. 7. IKK activity is not affected by the PI3K inhibitors LY294002 or WM. Whole cell lysates of cells treated with LY294002 and WM were immunoprecipitated with IKK-α, and then IKK kinase assays were performed on these immunoprecipitates using GST-IκBα substrate (1 μg/reaction). This figure is a representative of three separate experiments.

Table 2 Exposure of dominant negative Akt (AKT DN) or WM leads to increased apoptosis in melanoma cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Control</th>
<th>AKT DN</th>
<th>WM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKMe128</td>
<td>1</td>
<td>1.04 ± 0.26</td>
<td>1.16 ± 0.33</td>
</tr>
<tr>
<td>WM 115</td>
<td>1</td>
<td>5.91 ± 0.43</td>
<td>4.31 ± 0.35</td>
</tr>
<tr>
<td>Hs294T</td>
<td>1</td>
<td>2.95 ± 0.61</td>
<td>3.32 ± 0.50</td>
</tr>
</tbody>
</table>

Fig. 8. A model for Akt-induced activation of NF-κB. Akt can activate NF-κB through three potential and overlapping mechanisms (growth factor and/or cytokine activation of the PI3K/Akt pathway, constitutive activation of the PI3K/Akt pathway can lead to NF-κB activation, and PTEN can be mutated and/or absent). NF-κB can be activated by direct phosphorylation by Akt or by phosphorylation of IKK-α, followed by IKK-α phosphorylation of p65, and Akt can activate NF-κB through the IKK/IκB/NF-κB pathway. Upon activation, NF-κB can activate a wide variety of genes, including those involved in apoptosis and cell survival.

to glioblastoma multiforme (43). Also, the Akt activation in mammary glands imparts survival signals in association with tumor progression (44). Taken together, these observations imply that increased Akt activity might be a common requirement for oncogenic transformation of primary cells to metastatic tumors.

To determine whether constitutive Akt activation in our system is PI3K dependent and also to test its role as a potential cell survival pathway, we used PI3K-specific pharmacological inhibitors LY294002 and WM as well as overexpression of a dominant negative mutant construct of Akt. We show here that Akt activation is PI3K dependent; moreover, Akt activation increases the survival of melanoma cells. In support of our results, a previous study in one melanoma cell line (G361) demonstrated that overexpression of the dominant negative p85 subunit of PI3K induced apoptosis (45). In addition, N-Cadherin dependent Akt activation has also been shown to enhance cell growth and survival in melanoma cells, though the mechanism involves signaling through beta-catenin (46).

Based on our observation that Akt and NF-κB activation in melanoma tissues showed a similar pattern, our data suggest that NF-κB is a significant downstream target of the signaling pathway initiated by constitutive up-regulation of the PI3K/Akt pathway in melanoma cells.

The IKK/IκBα/NF-κB pathway is the major mechanism for NF-κB activation (Fig. 8). To determine whether PI3K/Akt-dependent NF-κB activation also modulates NF-κB activation in melanoma lesions, we investigated the role of IKK phosphorylation of IκBα. No change in IKK phosphorylation of IκBα was observed after treatment with PI3K inhibitors, suggesting that IKK/IκBα degradation is not required for NF-κB activation (Fig. 8). Similar observations showing that activated Akt does not stimulate endogenous IKK activity directed toward IκBα degradation have been reported (47). Thus, it is possible that IKK activity (possibly independent of IκBα phosphorylation) or some change in a structural aspect of IKK might be required for Akt stimulation of RelA/p65 phosphorylation and transactivation.

Direct phosphorylation of p65 by Akt has been the subject of controversy in literature. The transactivation domain (TAD1) region of p65 does not contain a strong consensus sequence for Akt phos-
phorylation (RAXX/S-T-bulky hydrophobic; Ref. 48). However, IKK-α does have a perfect Akt phosphorylation consensus sequence; therefore, Akt may potentially phosphorylate IKK-α, which subsequently phosphorylates RelA/p65 at Ser-529. Currently, it is not clear whether Akt can directly phosphorylate p65 or whether Akt only phosphorylates IKK-α to phosphorylate RelA/p65 (Fig. 8). Additional studies are required to answer these questions.

The constitutive Akt activation in severely dysplastic nevi and melanoma lesions could be due to either autoocrine cytokine stimulation, loss of function PTEN mutation, or gain of functional mutation in Akt and PI3K. However, previous studies have shown that in human melanoma, pleckstrin homology domain mutations or mutations of the activation-associated phosphorylation sites at codons 308 and 473 of PKB/Akt do not play a major role in melanoma carcinogenesis (49, 50). We have observed loss of PTEN in melanoma cell lines with higher Akt expression, and this could very well be the case in human melanoma samples with high Akt activation. These studies are currently under way. We hypothesize that activation of Akt, whether mediated by PTEN inactivation or growth factor signaling activation, might represent an important common pathway in the pathogenesis or progression of melanoma.

In summary, the studies in this report suggest that constitutive Akt activation plays an important role in human melanoma. Although it may not be essential for initiation of melanoma, Akt activation facilitates melanoma progression, possibly by enhancing cell survival through up-regulation of NF-κB and escape from apoptosis. These studies also provide compelling evidence for use of activation of Akt as an important prognostic marker of melanoma progression. In addition, Akt activation in severely dysplastic nevi and metastatic melanoma and not in normal or mild to moderate dysplastic nevi suggests that a therapeutic window may exist in patients whereby antagonism of Akt activity might block progression of premalignant lesions. Akt activates a number of diverse downstream proliferative and anti-apoptotic pathways and thus is a promising target for future molecular based therapy.

REFERENCES


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