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Authors
McNulty, Susan E
Tohidian, Niloufar B
Meyskens, Frank L

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Original Research Article

RelA, p50 and Inhibitor of kappa B alpha are Elevated in Human Metastatic Melanoma Cells and Respond Aberrantly to Ultraviolet Light B

SUSAN E. McNULTY, NILOUFAR B. TOHIDIAN and FRANK L. MEYSKENS Jr.

Department of Medicine and Chao Family Comprehensive Cancer Center, University of California Irvine Medical Center, 101 City Drive South, Orange, California 92868
**Address reprint requests to Prof. Frank L. Meyskins, Chao Family Comprehensive Cancer Center, Building 23, Rte 81, University of California Irvine Medical Center, 101 City Drive South, Orange, California 92868. E-mail: flmeyske@uci.edu*

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Metastatic melanomas are typically resistant to radiation and chemotherapy. The underlying basis for this phenomenon may result in part from defects in apoptotic pathways. Nuclear factor kappa B (NFκB) has been shown to control apoptosis in many cell types and normally functions as an immediate stress response mechanism that is rigorously controlled by multiple inhibitory complexes. We have previously shown that NFκB binding is elevated in metastatic melanoma cells relative to normal melanocytes. In the current study, Western blot analysis showed that, compared with normal melanocytes, melanoma cell lines have higher nuclear levels of the NFκB subunits p50 (7-fold) and RelA (5–10-fold). In response to tumor necrosis factor-alpha (TNFα), both melanocytes and melanoma cells showed increased nuclear p50 and RelA levels, but levels in melanoma cells remained higher than in melanocytes. We also found that melanoma cells expressed higher cytoplasmic levels of RelA, p105/p50 and the inhibitory protein, inhibitor of kappa B alpha (IκBα) than melanocytes. To directly test whether RelA expression has an impact on melanoma cell survival, we used antisense RelA phosphorothioate oligonucleotides and found that melanoma cell viability was significantly decreased compared with untreated or control cultures. The constitutive activation of NFκB in metastatic melanoma cell cultures may, therefore, support an inappropriate cell survival pathway that can be therapeutically manipulated.

Key words: Melanocyte, Melanoma, NFκB, Antisense, TNF, UVB

INTRODUCTION

The incidence of melanoma is increasing at the second highest rate of all human cancers, and metastatic disease is generally fatal. Metastatic melanomas are typically resistant to chemotherapy (1), with response rates of 15–20% achieved with dacarbazine, the best single agent, and reported response rates ranging from 15 to 43% with combination dacarbazine, cisplatin, carmustine, and tamoxifen, but with no survival benefit (2–4). It has been suggested that the underlying resistance of metastatic melanomas to radiation and chemotherapy may be due at least in part to over-expression of apoptosis inhibitory proteins (1).

Nuclear factor kappa B (NFκB) activation has been shown to have both pro- and anti-apoptotic functions in various cell types (5, 6). There are five mammalian NFκB/Rel family members, p50, p52, RelA, RelB, and cRel, that share a highly conserved 300-amino acid Rel homology domain containing dimerization, nuclear localization, and DNA binding regions (5–8). These proteins can form homo- and heterodimers, which bind DNA at NFκB sequences found in the promoters of a variety of genes and specific dimer pairs elicit differential induction of these genes (5, 8, 9). Several studies have suggested that NFκB transcription

Abbreviations – NFκB, nuclear factor kappa B; IκB, inhibitor of kappa B; UVB, ultraviolet light B

factors are associated with the genesis of a variety of cancers, including colon, breast, and ovarian (10, 11). The activation of NFκB by tumor necrosis factor (TNF), ionizing radiation, or daunorubicin was found to protect against apoptosis (12). Inhibition of NFκB nuclear translocation enhanced apoptotic killing by these reagents (12–16) but not by apoptotic stimuli mediated by an NFκB-independent pathway (12). Studies in RelA-deficient mice showed that the presence of this NFκB subunit is required for protection from TNFα (13, 14), and that RelA controls inducible, but not basal, transcription in NFκB-regulated pathways (14). Apparently, TNFα transmits one signal that induces cell death and another, dependent on RelA, that protects against cell death (13).

Nuclear localization and NFκB activity are tightly controlled by inhibitory proteins, including p105 and p100, which are the precursors for p50 and p52, as well as members of the inhibitor of kappa B (IκB) family, IκBα, β and ε (5, 8, 16). p105 and p100 inhibit nuclear translocation of heterodimers containing the transcriptional transactivating subunits RelA, cRel, and RelB. p50 and p52 homodimers and p50/p52 heterodimers are able to translocate to the nucleus, but lack the NFκB transactivation domain and generally act as transcriptional repressors (8). IκBα inhibits p50 heterodimers containing cRel, RelA, and RelB (5, 8, 17), while IκBβ preferentially inhibits RelA homo- and heterodimers (5, 8, 17, 18) and IκBε is associated with cRel and RelA, but not p50 (5, 19). NFκB binds to the promoters for p100/p52, p105/p50, and IκBα and transcriptionally activates their expression (8). Induction of unprocessed p105, p100, and IκB proteins by NFκB functions as a negative feedback mechanism, which leads to down-regulation of NFκB by sequestration of transactivating heterodimers as inactive cytoplasmic complexes. Induction of IκBα also facilitates export of activated RelA complexes from the nucleus, thereby terminating NFκB function (20, 21).

However, persistent activation of NFκB by inducers such as LPS and IL-1 leads to a loss in cellular IκBα and IκBβ (17, 22, 23), but despite rapid resynthesis of IκBα, NFκB remains induced. During prolonged NFκB stimulation, IκBβ is synthesized as an unphosphorylated protein that forms a stable complex with NFκB in the cytosol (22), but does not mask the nuclear localization signal or DNA binding domain (23). Unphosphorylated IκBβ then competes with IκBα and facilitates NFκB translocation to the nucleus (23) and is one mechanism whereby NFκB may become constitutively activated.

While the etiology of human malignant melanoma is still incompletely understood, there is substantial epidemiological evidence that ultraviolet exposure resulting in sunburn, particularly during adolescence (24), is an important risk factor. We have previously shown that NFκB expression and binding is altered in metastatic melanoma cells compared with their normal melanocyte counterparts (25). Because multiple signals activate NFκB, including UV irradiation (8), we investigated the effects of ultraviolet light B (UVB) on the expression and function of NFκB-related proteins in normal melanocyte and metastatic melanoma cell lines. NFκB functions as an immediate stress response mechanism, which is normally rigorously controlled by multiple inhibitory complexes. However, in human metastatic melanoma cells, we have found constitutive NFκB activation that is not inhibited by the normal regulatory mechanisms.

MATERIALS AND METHODS

Cell Culture

Two to five human neonatal foreskins were placed in 0.25% trypsin at 4°C overnight as has been previously described (25, 26). The tissues were scraped to recover the melanocytes, pooled and cultured in MCDB 153 (Sigma) medium containing 2% fetal calf serum, 0.3% bovine pituitary extract (Clonetics Corp.), 10 ng/ml phorbol myristate-13-acetate, 2.0 mM calcium chloride, 5 μg/ml insulin and 0.1 mM 3-isobutyl-1-methyl-xanthine (Sigma) (MCDB+). Human metastatic melanoma cells (c83-2C, c81-46A, c81-61) were cultured in F-10 (Fisher Scientific) medium containing 5% fetal calf serum and 5% newborn calf serum (F10+). Melanocyte and melanoma cultures were used in experiments between passages 4 and 8. To compare the effects of different culture conditions, cells were cultured for 24 hr prior to harvest as noted in specific experiments.

UVB Irradiation

Cell cultures were grown to subconfluence, medium was aspirated, cells were washed twice in phosphate-buffered saline (PBS), a sufficient amount to cover cells was applied and the cell cultures were exposed to UVB in a range of doses that clinically produce suberythemal (25 mJ/cm^2) to severe sunburn effects (100 mJ/cm^2). The cells were irradiated using a Stratalinker 2400 (Stratagene), which produces UVB wavelengths in the range of 280–320 nm with a peak at 302 nm.

RNA Isolation, cDNA Probes and Northern Blot Analysis

Cytoplasmic RNA was isolated by detergent lysis in RSB (25). The nuclei were pelleted by centrifugation and the supernatant was diluted in urea buffer, extracted in phenol chloroform, and precipitated with ethanol. RNA was size fractionated by denaturing formaldehyde–agarose gel electrophoresis and transferred to nylon filters by Southern transfer (27). The cDNA probes were labeled with [α-^{32}P]dCTP (NEN) using random nonamer priming (Pharmacia Biotech). The p50 cDNA probe was a 1.5-kb EcoRI insert, p65 was a 0.95-kb EcoRI insert, IκBα was a 1.2-kb EcoRI insert (gifts of John Hiscott, McGill University) (28), and an 18S rRNA probe was used (Ambion, 18S Decatemplate). The filters were hybridized with the [α-^{32}P]dCTP-labeled cDNA probes at 42°C for 2 hr in Rapid-Hyb Buffer (Amersham) and washed following the manufacturer's recommendation.

Western Blot Analysis

Normal melanocyte and metastatic melanoma cell lines were harvested at subconfluence, and cytoplasmic and nuclear

fractions were prepared by published methods (29). The cytoplasmic and nuclear proteins were quantitated by a modified Lowry assay (BioRad DC Protein Assay); aliquots separated by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were stained by Coomassie blue to verify quantitation or were transferred to nitrocellulose membranes (27). The proteins were detected using polyclonal antibodies (gift of Nancy Rice, NCI, Bethesda, MD, or Santa Cruz Biotech) (30), horseradish peroxidase linked anti-IgG (BioRad) and enhanced chemiluminescence (Pierce Supersignal).

Electrophoretic Mobility Shift Assays (EMSA)

Consensus NFκB and TFIID oligonucleotides (Santa Cruz Biotech) were labeled with [γ-^{32}P]ATP using polynucleotide kinase (27). The labeled oligonucleotide probes were incubated with nuclear extracts per manufacturer's recommendation, and the DNA–protein complexes were resolved on a 4% polyacrylamide gel and visualized by autoradiography (27). In competition experiments, 9–40-fold excess of mutant or consensus NFκB oligonucleotide was added to the reaction mixture. In antibody supershift experiments, nuclear extracts were incubated with antibodies to RelA (Santa Cruz sc-8008) and p50 (Santa Cruz sc-1190) followed by incubation with [α-^{32}P]dCTP labeled oligonucleotide (27).

Scanning Densitometry

Autoradiographs were scanned on a BioRad Model GS-700 Imaging Densitometer and analyzed with Molecular Analyst software. The relative densities of RNA bands detected by autoradiography were corrected for sample loading by normalizing each to the corresponding intensity of its 18S ribosomal RNA.

In Vitro Inhibition Studies

FITC-labeled phosphorothioate oligonucleotides (Biognostik) were added to melanocyte and melanoma cultures to determine the time course for the maximum uptake of the oligonucleotides. Based on these uptake studies, RelA antisense and control oligonucleotides were added to the melanoma cultures (2 μM) and viability was determined at 72 hr using the CellTitre96 (Promega) assay. During the last hour of incubation, a tetrazolium compound was added, which is reduced by viable cells to formazan. This colored product was quantified by spectrophotometry in replicates of four. The cell permeable synthetic p50 peptide SN50 (31–34) was added at 10–100 μg/ml to cell cultures for 6–72 hr and cell viability and apoptotic index were determined by FACS analysis. Briefly, cells were harvested, washed twice in 1 × PBS and re-suspended in binding buffer at a concentration of 1×10^6 cells/ml, of which 100 μl was incubated with 5 μl of Annexin V (conjugated to FITC) and 15 μM propidium iodide for 15 min, according to the manufacturer's protocol (Pharmingen, San Diego, CA). Cells were analyzed by flow cytometry using a Becton–Dickinson FACScan with Cell Quest software.

RESULTS

NFκB Binding is Elevated in Metastatic Melanoma Cells Relative to Normal Melanocyte Cultures

Independent pools of normal melanocytes and metastatic melanoma cell strains were grown to subconfluence and nuclear and cytoplasmic fractions were isolated. Protein concentrations were determined by modified Lowry assay and verified by Coomassie blue staining of SDS-PAGE-separated proteins. DNA binding reactions were prepared using 40 μg of nuclear protein and equal aliquots (20 μg) were incubated with ^{32}P-labeled oligonucleotides specific for either NFκB or as a loading control the basal transcriptional factor TFIID and electrophoretic mobility shift assays were performed. An excess of 4.3 μg of poly(dI)-poly(dC) (Pharmacia) was added to each reaction to eliminate non-specific binding. As shown in Fig. 1A, unstimulated melanocytes (MC) do not generate a detectable mobility shift with the labeled NFκB oligonucleotides, but unstimulated melanoma cells (81-46A, 83-2C, 81-61) do. TFIID binding, however, was comparable in melanocytes and melanomas, showing that protein loading was equivalent and that the elevation in NFκB binding is not due to a general increase in binding by transcription factors.

Cytoplasmic Levels of RelA, p105/p50, and IκBα are Elevated in Melanoma Cultures

To examine whether the elevation in NFκB binding in melanoma cells is a result of increased expression of the transactivating NFκB subunits, increased processing of precursor NFκB subunits or decreased expression of inhibitory subunits, we performed Western blot analysis on the cytoplasmic fractions of unstimulated melanocytes and melanoma cells (Fig. 1B). Cytoplasmic RelA was found to be nine times higher in melanoma cells than in melanocytes, while cytoplasmic p105 precursor and processed p50 proteins were each found at 3-fold higher levels in melanoma cells than in melanocytes. Relative to melanocytes, cytoplasmic IκBα was also found at 2–4-fold higher levels in melanomas (c81-46A, c83-2C, respectively). Cell extracts were also examined by Western blot analysis for expression of cRel and IκBβ, but these subunits were undetectable in either melanocytes or melanoma cells in both nuclear and cytoplasmic fractions (data not shown).

Because the melanocyte medium contains several known NFκB inducers (e.g. TPA), we cultured melanocytes and melanoma cells in media lacking all supplements for 24 hr prior to collection and compared protein expression with cells grown in their standard media (MCDB +, F10 +). Although melanoma cells can grow and proliferate in serum-free conditions for extended periods, the melanocytes do not tolerate the unsupplemented media conditions well. Therefore, viability, protein, and RNA expression were substantially reduced in these melanocytes. As shown in Fig. 1B, melanocytes and c83-2C melanoma cells had minor reductions in levels of unprocessed p105, but p50 levels were maintained. Under unsupplemented media conditions, melanocytes also showed increases in cytoplasmic RelA, while levels in the melanoma cells remained constant. Under

both media conditions, melanoma cells expressed higher cytoplasmic levels of RelA as compared with melanocytes. As shown in Fig. 1C, 81-61 melanoma cells were cultured in standard or unsupplemented media with similar results. To directly compare the effects of standard melanocyte and melanoma media, on RelA protein levels, melanoma cells (c81-46A, c83-2C, c81-61) were also cultured for 24 hr prior to harvest in standard melanoma medium (F10 +) or in melanocyte medium (MCDB +). Cells were also exposed to 0 or 25 mJ/cm^2 UVB, but as shown in Fig. 1D, no significant change in cytoplasmic levels of these proteins were found.

NFκB Binding and Nuclear Localization is Further Enhanced in Metastatic Melanoma Cultures Exposed to TNFα

We exposed melanocyte and melanoma cells to TNFα in order to evaluate whether there is a divergence in response between the normal and malignant cells to TNFα-induced activation of NFκB. Following a 30-min exposure to 0 ng/ml or 40 ng/ml TNFα, nuclear extracts were isolated from the melanocyte and melanoma cells. Extracts examined by Western blot analysis showed that although nuclear actin levels were comparable, RelA and p50 proteins were undetectable in the nuclear compartment in unexposed melanocytes while melanoma cultures had significant levels of nuclear RelA and p50 (Fig. 2A). Following exposure to TNFα, both melanocytes and melanomas showed increased nuclear levels of both p50 and RelA. However, the TNFα-induced increase in total nuclear abundance of these proteins was higher in melanoma than in melanocyte cultures. EMSA (Fig. 2B) conducted on the 81-46A melanoma cell nuclear extracts showed that binding to a ^{32}P-labeled consensus NFκB oligonucleotide also increased following exposure to 40 ng/ml TNFα. Competition experiments using 40-fold excess unlabeled consensus or mutant NFκB oligonucleotides indicate the NFκB-specific bands. Supershift analysis conducted by incubating the 81-46A nuclear extracts with antibodies specific for RelA and p50 prior to incubation with the labeled NFκB oligonucleotide showed that RelA and p50 are involved in the DNA binding complexes (Fig. 2B).

NFκB Binding in Melanocyte and Melanoma Cell Lines is not Increased by 25 mJ/cm^2 UVB

UVB is another classical inducer of NFκB activation. In order to evaluate the UVB response in melanocytes and melanoma cells, cultures were exposed to UVB irradiation

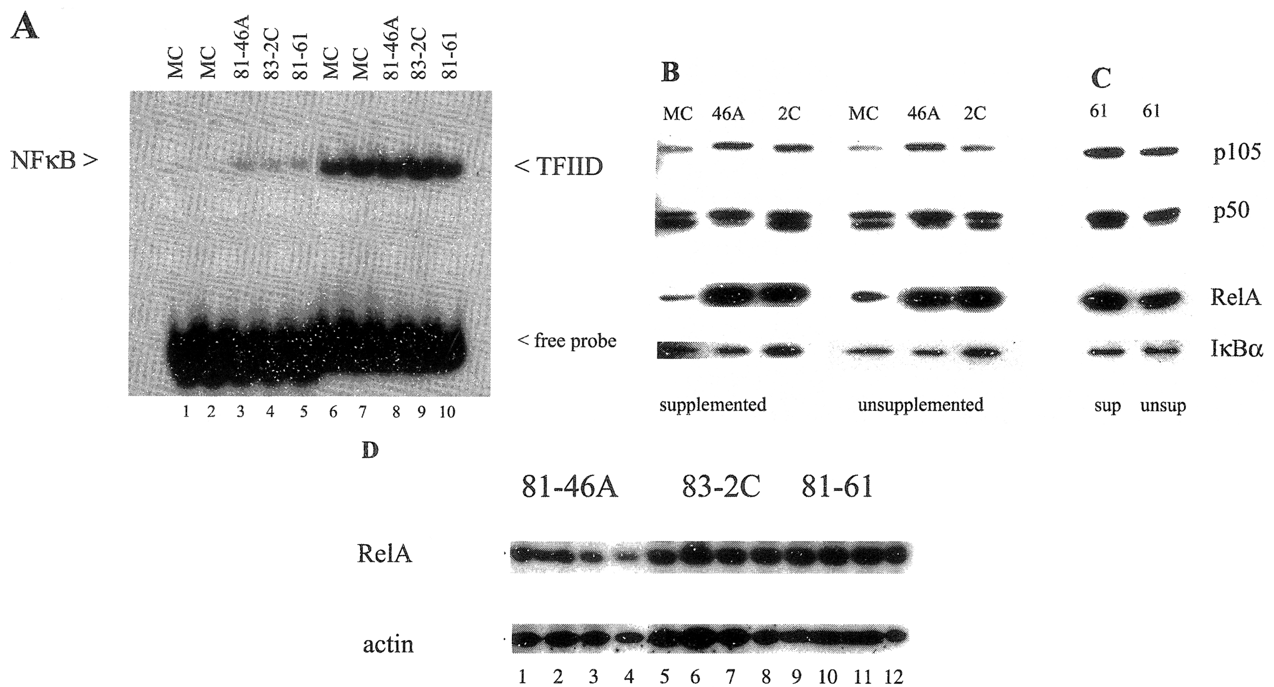

Fig. 1. NFκB binding and expression is elevated in metastatic melanoma cells relative to normal human melanocytes. (A) Nuclear extracts from melanocyte (MC) and metastatic melanoma cells (c81-46A, c83-2C, c81-61) were incubated with ^{32}P-labeled consensus NFκB (lanes 1–5) or consensus TFIID (lanes 6–10). Oligodeoxynucleotides and electrophoretic mobility shift assays were performed as a measure of DNA binding activity. In this experiment, an excess of 4.3 μg of poly(dI)-poly(dC) was added per reaction to eliminate non-specific binding. (B) MC and metastatic melanoma cells c81-46A, c83-2C (46A, 2C) and (C) c81-61 (61) were incubated in media containing standard supplements as described in Materials and Methods, or in medium containing no supplements for 24 hr prior to collection. Cytoplasmic extracts were separated by SDS-PAGE and Western blot analysis was performed to measure expression of NFκB subunits and inhibitors. RelA, p50 and its precursors, p105 and IκBα, are shown. (D) Melanoma cells c81-46A (lanes 1–4), c83-2C (lanes 5–8) and c81-61 (lanes 9–12) were cultured in standard F10 + melanoma medium (lanes 1, 2, 5, 6, 9, 10) or in standard MCDB + melanocyte medium (lanes 3, 4, 7, 8, 11, 12) for 24 hr prior to collection and exposed to 0 mJ/cm^2 (lanes 1, 3, 5, 7, 9, 11) or 25 mJ/cm^2 (lanes 2, 4, 6, 8, 10, 12) UVB 30 min prior to collection. Cytoplasmic extracts were separated by SDS-PAGE and Western blot analysis was performed using antibodies specific for RelA. The blot was reprobed with antibodies specific for actin as a measure of protein loading.

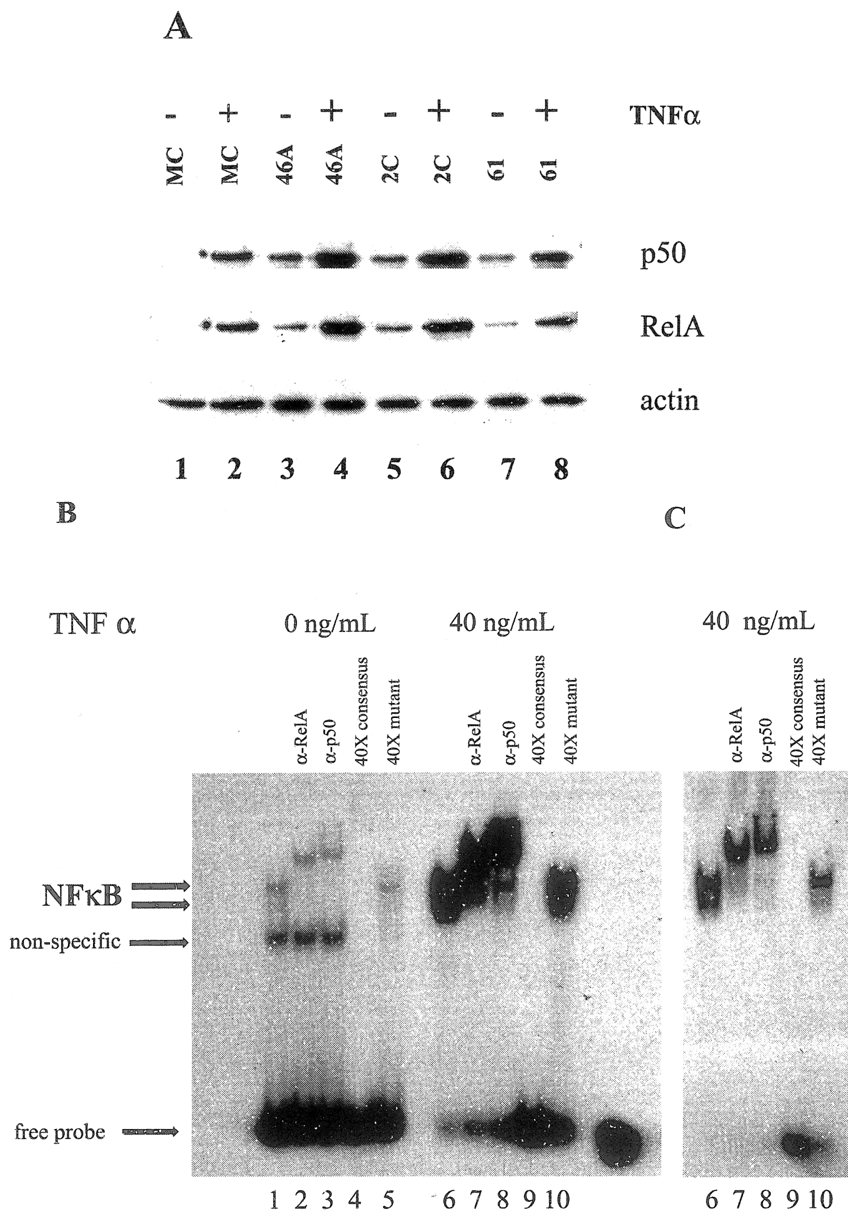

Fig. 2. TNFα induces increased nuclear localization of p50 and RelA and increased DNA binding by NFκB. (A) Melanocyte (lanes 1, 2) and melanoma cells c81-46A (lanes 3, 4), c83-2C (lanes 5, 6), and c81-61 (lanes 7, 8) were incubated with 0 ng/ml (−) or 40 ng/ml (+) TNFα for 30 min prior to collection. Nuclear extracts from the melanocyte (MC) and metastatic melanoma cell cultures were isolated, separated by SDS-PAGE and Western blot analysis performed to measure expression of the NFκB subunits RelA and p50. Western blot analysis was also performed using antibodies directed against actin to verify equivalent protein loading in each lane. (B) 81-46A melanoma cells were exposed to TNFα at a dose of 0 or 40 ng/ml for 30 min prior to collection. Electrophoretic mobility shift assays were performed using melanoma c81-46A cell nuclear extracts incubated with ^{32}P-labeled consensus NFκB oligonucleotides in the presence of a 20-fold excess of unlabeled non-specific oligodeoxynucleotide competitor. Competition assays were also performed using 40-fold excess unlabeled consensus (lanes 4 and 9) or mutant (lanes 5 and 10) NFκB oligodeoxynucleotides to confirm NFκB binding specificity. Supershift analysis was performed by addition of anti-RelA (lanes 2 and 7) or anti-p50 (lanes 3 and 8) antibodies to the nuclear extracts prior to incubation with the oligodeoxynucleotides as a measure of DNA binding by RelA and p50 containing dimers. The gel was exposed to film for 5 hr for autoradiography. (C) The film was re-exposed for 2 hr to allow visualization of the individual bands in the TNFα exposed cells. Only the TNFα lanes are shown.

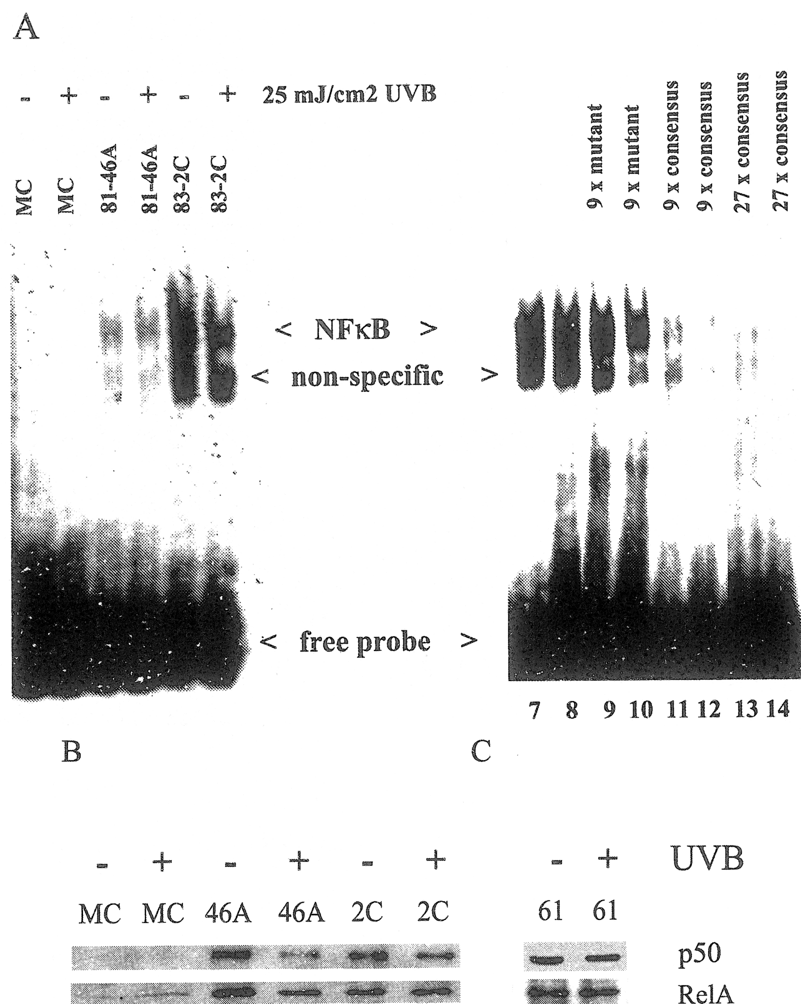

Fig. 3. Effect of UVB exposure on NFκB binding in melanocyte and melanoma cells. Melanocyte (MC) and metastatic melanoma (c81-46A and c83-2C) cell cultures were exposed to UVB at doses of 0 (−) or 25 mJ/cm^2 (+) and collected 30 min later. (A) Nuclear extracts were isolated and electrophoretic mobility shift assays were performed using consensus NFκB oligodeoxynucleotides. Competition assays were also performed using c83-2C nuclear extracts in the presence of 9-fold excess mutant (lanes 9, 10) or 9-fold (lanes 11, 12) and 27-fold (lanes 13, 14) excess consensus NFκB unlabeled oligodeoxynucleotides to confirm NFκB binding specificity. (B) Nuclear extracts from control (−) and UVB-exposed (+) MC and 81-46A and 83-2C melanoma cells (46A, 2C) were separated by SDS-PAGE and Western blot analysis was performed using antibodies directed against p50 and RelA. (C) Nuclear extracts from control (−) and UVB-exposed 81-61 melanoma cells were separated by SDS-PAGE and Western blot analysis was performed using antibodies directed against p50 and RelA.

at doses of 0 or 25 mJ/cm^2 and EMSAs were performed on nuclear extracts. As shown in Fig. 3A, in the absence of UVB irradiation, melanoma cells (81-46A and 83-2C) have elevated NFκB binding relative to melanocytes. When collected 30 min after exposure to 25 mJ/cm^2 UVB, no significant change in NFκB binding was found. Competition experiments using 9–27-fold excess unlabeled consensus or 9-fold excess mutant NFκB oligonucleotides indicate the NFκB-specific bands.

Analysis of the Nuclear Localization of the NFκB Subunits, p50 and RelA, and the Effect of UVB

Because UVB exposure has been shown to activate NFκB binding in other cell types, we examined nuclear extracts from melanocytes and melanoma cells by Western blot analysis for the presence of p50 and RelA proteins, as shown in Fig. 3B. As determined by scanning densitometry of replicate blots, melanoma cells were found to have substantially higher levels of p50 (more than 7-fold) and RelA (5–10-fold) localized to the nuclear compartment than melanocytes. Following a 25 mJ/cm^2 dose of UVB, the 81-46A melanoma cells exhibited a loss (60%) in nuclear RelA (Fig. 3B) and both 81-46A and 83-2C cells had decreases (60% and 50%, respectively) in p50. In contrast, melanocytes showed modest increases in p50 and RelA. However, relative to melanocytes, nuclear p50 and RelA protein levels remained elevated in melanoma cells, despite UVB irradiation. Melanoma cells 81-61 were also exposed to 0 mJ/cm^2 or 25 mJ/cm^2 UVB. As shown in Fig. 3B, no significant change in nuclear RelA or p50 was noted.

Northern Blot Analysis of the Effects of UVB on RNA Expression of RelA in Normal Melanocytes and Melanoma Cells

We found that the c81-46A and c83-2C melanoma cells expressed significantly higher RelA RNA than melanocytes. To examine whether the aberrant response of melanoma cells to UVB is found at the level of RNA expression, we used Northern blot analysis to examine changes in expression of RelA. The blot was stripped and reprobed for 18S rRNA and the relative densities of the RelA bands were normalized to their 18S bands (Fig. 4A). Exposure to UVB resulted in a 1.5-fold increase in RelA expression in the normal melanocytes, but resulted in 20% and 35% decreases, respectively, in 81-46A and 83-2C melanoma cells at 30 min. We also specifically investigated the effects of TPA (10 ng/ml phorbol myristate-13-acetate) and IBMX (0.1 mM 3-isobutyl-1-methyl-xanthine) on RelA RNA expression in melanocytes and melanoma cells. Melanoma cells c81-46A and c83-2C showed, respectively, 3-fold ($P < 0.007$) and 9-fold ($P < 0.0001$) higher levels of RelA RNA than melanocytes, regardless of exposure to UVB or culture conditions.

Effect of UVB on Expression of p105/p50 and IκBα RNA

We also examined melanocyte and melanoma cells by Northern blot analysis for changes in RNA expression of p105/p50 and IκBα following UVB exposure. This blot was also reprobed for 18S rRNA, but since it had been stripped and reprobed four times, the end lanes were compromised. The data is therefore presented qualitatively. In Fig. 4B we show that, overall, the c83-2C melanoma cells expressed substantially higher levels of p50 RNA than melanocytes. However, melanocytes exposed to UVB exhibited small increases in p50 RNA while in the c83-2C melanoma cells,

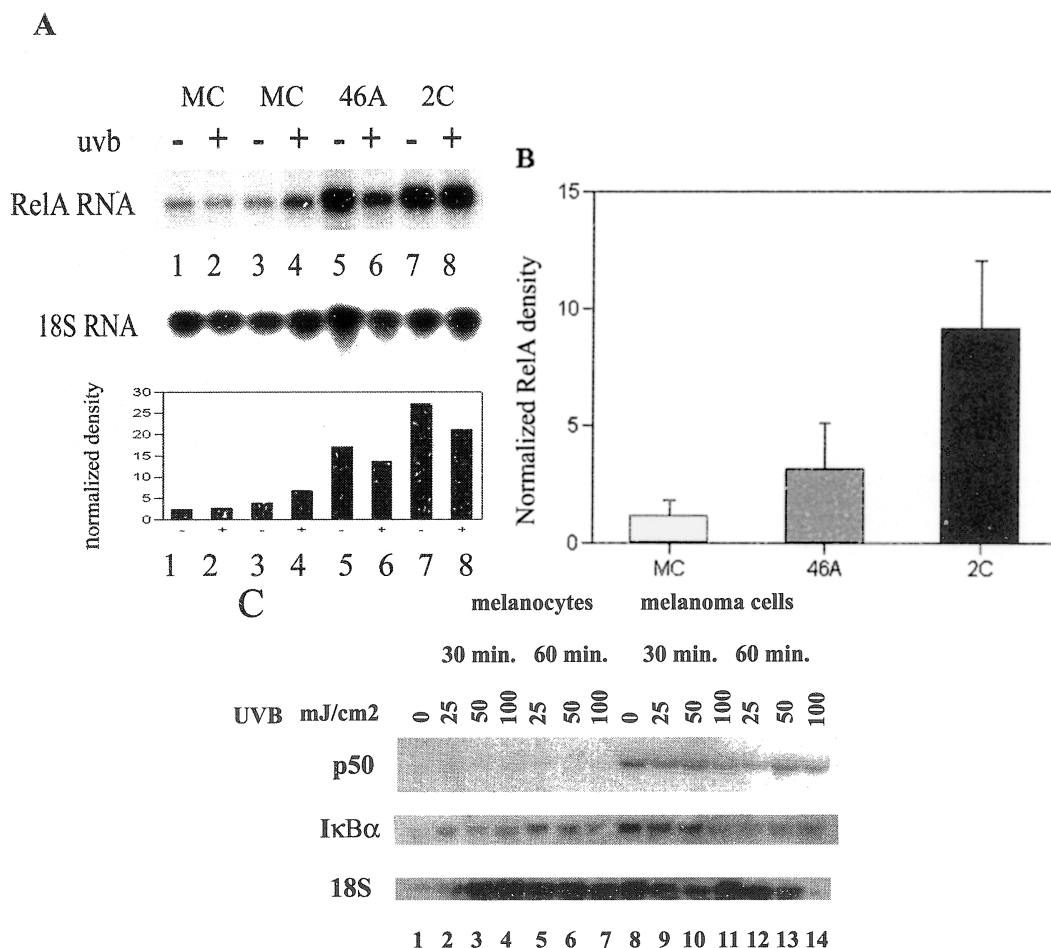

Fig. 4. Northern blot analysis of the effect of UVB on RNA levels for RelA, p105/p50, and IκBα. Melanocytes (MC) and melanoma cells were exposed to UVB and cytoplasmic RNA was isolated as described in Materials and Methods. RNA was fractionated by denaturing formaldehyde-agarose gel electrophoresis and examined with the relevant cDNA as described in Materials and methods. (A) MC and c81-46A and c83-2C metastatic melanoma cells (46A, 2C) were exposed to 0 (−) or 25 mJ/cm^2 (+) UVB and 30 min later RNA was collected. Northern blot analysis was performed using a ^{32}P-labeled RelA cDNA probe. The blot was stripped and reprobed using a ^{32}P-labeled 18S rRNA probe. Relative densities were normalized to the 18S band. (B) Melanoma cells (81-46A and 83-2C) express higher levels of RelA RNA than normal melanocytes regardless of culture conditions or UVB exposure. Data is compiled from specific experiments examining the effects of TPA, IBMX, and UVB. (C) Melanocytes (lanes 1–7) and c83-2C cells (lanes 8–14) were exposed to increasing intensities of UVB irradiation (0, 25, 50, and 100 mJ/cm^2) and collected 30 or 60 min later. Northern blot analysis was performed using ^{32}P-labeled cDNA probes for p105/p50, IκBα, and 18S rRNA.

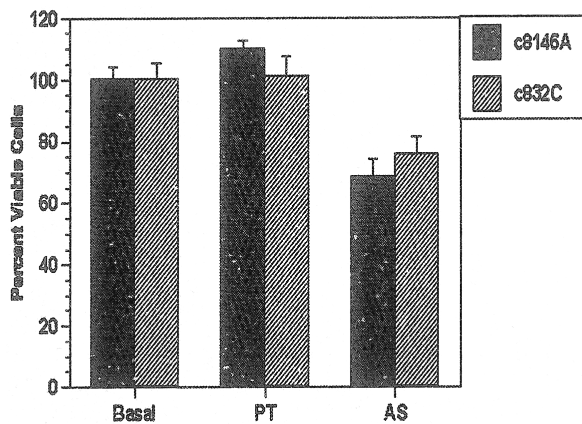

Fig. 5. Antisense RelA oligonucleotides, but not control oligonucleotides decrease viability in melanoma cultures. Metastatic melanoma cells (c81-46A, c83-2C) were incubated with 2 μM control (PT) or RelA antisense (AS) phosphorothioate oligonucleotides for 72 hr. During the last hour of incubation a tetrazolium compound was added which is reduced by viable cells to formazan. This colored product was quantified by spectrophotometry in replicates of four.

exposure to UVB slightly depressed p50 RNA expression. Nonetheless, at all doses the p50 expression in c83-2C cells was higher than in melanocytes. IκBα RNA levels were also slightly higher in c83-2C melanoma cells than melanocytes, but following UVB exposure, melanocytes exhibited small increases in IκBα RNA, while in c83-2C melanoma cells, IκBα RNA was slightly decreased following UVB.

The Effect of RelA and p50 Inhibitors on Percent Viability in Melanoma Cells

Metastatic melanoma cells were incubated with FITC-labeled phosphorothioate oligonucleotides and maximum cellular uptake was determined by immunofluorescent microscopy to be 72 hr. RelA antisense and matched control phosphorothioate oligonucleotides (2 μM) were added to melanoma cell cultures. The 81-46A and 83-2C melanoma cultures were unaffected by the control phosphorothioate oligonucleotides, but viability decreased 25–30% when exposed to the RelA antisense oligonucleotides (Fig. 5). SN50, a cell permeable recombinant peptide (31–34) that selectively inhibits translocation of p50-containing dimers, was added to cell cultures at 10–100 μg/ml for 6–72 hr and cell viability and apoptosis were measured by FACS analysis. There was no significant difference in cell viability between SN50 and control peptide cultures (data not shown).

DISCUSSION

Our data indicate that NFκB regulation in melanoma cells is fundamentally altered in terms of RNA expression, protein compartmentalization and DNA binding of p50 and RelA. Experiments with TNFα, a known inducer of NFκB signaling, showed that both melanocyte and melanoma cells respond as anticipated with increased nuclear localization of p50 and RelA, indicating that NFκB proteolytic processing is functional in both cell types. Shattuck-Brandt and Richmond reported altered IκBα processing in the HS294T melanoma cell line that resulted in decreased IκBα levels and thus higher NFκB binding (35). We had previously found that IκBα RNA and nuclear levels of IκBα protein were higher in several of our melanoma cell lines relative to normal melanocytes (25). In this study, we confirmed that IκBα RNA is elevated in melanoma cells relative to normal human melanocytes and that cytoplasmic levels of the IκBα protein are 2–4-fold higher in c81-46A and c83-2C melanoma cells. However, despite the elevation in IκBα RNA and cytoplasmic and nuclear protein that we observed in the melanoma cells, NFκB binding was not fully inhibited. We also examined nuclear and cytoplasmic extracts for the IκBβ protein, which responds to persistent NFκB activation. However, we did not detect IκBβ in either cytoplasmic or nuclear fractions in either melanocytes or melanoma cells. The elevated expression of IκBα that we observed in melanoma cells could reflect a futile cycling of the inhibitory pathway in the presence of constitutive NFκB activation.

We found that expression of p50 and IκBα RNAs in response to UVB is inversely related in normal melanocytes and malignant melanoma cells. In melanocytes, UVB exposure resulted in increased expression of p50 and IκBα RNAs as expected, but surprisingly in the melanoma cells, UVB irradiation resulted in decreased expression. UV radiation is thought to activate cellular signal transduction pathways through a variety of mechanisms, including generation of reactive oxygen species (16, 36–38), activation of tyrosine kinases (39–41), direct induction of receptor clustering (40–44), and DNA damage-dependent processes (43, 45). However, in contrast to the melanoma cells, UV exposure normally activates NFκB binding in other cell types (8, 44–46), including normal melanocytes. Photoxidative stress generated by visible light in photoreceptor cells down-modulates the NFκB proteins p50, RelA, and IκBα by caspase-1-dependent proteolysis (47) and such a mechanism could explain the reduction in nuclear p50 protein in melanoma cells after UVB exposure. However, the fact that cytoplasmic RNA levels of p50 and IκBα are also reduced within 1 hr of UVB exposure in melanoma cells suggests that the alteration in melanoma cells is upstream of conventional post-translational regulatory mechanisms. p53 is also activated by UVB and has been shown to compete with NFκB for a limiting pool of transcriptional coactivators, CBP/p300, resulting in inhibition of NFκB transactivation. Competitive inhibition of NFκB by p53 following UVB exposure could also account for the decreased expression of p50 and IκBα RNA observed in melanoma cells (48–50).

Huang et al. have also reported high constitutive NFκB binding in A375SM and A375-C28 metastatic melanoma cells (51), confirming our original observations (25). Dominant negative IκBα constructs were used to alter NFκB activation and to define some of the consequences of this manipulation (51). As we are interested in potentially useful therapeutic agents to alter RelA/NFκB effects, we used RelA antisense oligonucleotides and found that relative to controls, viability decreased significantly in melanoma cultures, suggesting that this and other pharmacological agents, which impinge on the NFκB pathway, can be exploited for chemotherapeutic purposes. Alterations in RelA expression and function may enhance the ability of melanoma cells to

circumvent normal apoptotic controls; thus allowing survival of genetically damaged cells. Future studies to identify the underlying mechanism(s) for the defect may provide deeper insights into diagnostic, therapeutic, and chemopreventive strategies.

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