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Activation of Nuclear Factor-κB in Human Metastatic Melanoma Cells and the Effect of Oxidative Stress[1]

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ABSTRACT

The biological basis for the general pharmacological resistance of human melanoma is unknown. A unique biochemical feature of the melanocyte is the synthesis of melanin, which leads to the generation of hydrogen peroxide and the consumption of reduced glutathione. This activity produces a state of chronic oxidative stress in these cells. We demonstrated previously that the expression of the c-*jun* family was dysregulated in metastatic melanoma cells compared with normal human melanocytes (D. T. Yamanishi *et al.*, J. Invest. Dermatol., *97:* 349–353, l991). In the current investigation, we measured the levels of two major redox response transcription factors, nuclear factor-κB (NF-κB) and activator protein-1, in metastatic melanoma cells and normal melanocytes and their response to oxidative stress. The basal DNA-binding activity of NF-κB as measured by the electrophoretic mobility shift assay in metastatic melanoma cells was increased 4-fold compared with that of normal melanocytes. This level of binding was paralleled by a 1.5- to 4-fold increase in the expression of p50 (NF-κB1), p65 (Rel-A), and IκB-α as measured by Northern blot analysis. In contrast, the expression of p75 (c-*rel*) was markedly decreased (60%) in melanoma cells compared with normal melanocytes. Following oxidative stress produced by enzyme-generated H_2O_2, free H_2O_2, or incubation with buthionine sulfoximine, NF-κB binding activity increased 1.5- to 2.5-fold in melanoma cells (buthionine sulfoximine > H_2O_2), but only slightly in normal melanocytes. In contrast, activator protein-1 binding activity was unaffected or increased in normal melanocytes in response to oxidative stress, but was either unaffected or decreased in melanoma cells. These results suggest that the redox regulation of melanoma cells at the molecular level is fundamentally different from normal melanocytes and may offer a unique avenue for preventive or therapeutic intervention as well as new insights into the pathogenesis of melanocyte transformation.

INTRODUCTION

Human melanoma is almost uniformly resistant to cytotoxic intervention (1). Although specific mechanisms of drug resistance have been described in selected cell lines (2), no uniform or unifying concept has emerged to explain the general resistance of melanoma to a wide range of chemotherapeutic agents.

In the past several years the NF-κB/Rel[3] and AP family of transcription factors have been shown to participate in the control of a diverse range of genes involved in inflammation, immunological responsiveness, development, growth control, and oncogenesis (3–6). These two families of transcriptional factors also are redox sensitive (6–10). In general, NF-κB is activated by prooxidant conditions, whereas AP-1 is stimulated by antioxidants (7, 8). The regulation of these two transcription factors is also quite different. NF-κB is inactivated in the cytoplasm by inhibitor proteins (IκB) that respond to exogenous stimuli (such as oxidative stress) by phosphorylating and releasing active dimeric complex (*e.g.*, p50/p65 and p50/p75) that translocates to the nucleus (11, 12). *De novo* synthesis of the dimers is not required for binding to DNA and gene activation to occur. In contrast, AP-1 activation requires synthesis of its precursor proteins, c-Fos and c-Jun (6). Transcription factors for both families bind to promoter regions of a large variety of genes (5, 6). We previously have shown that the expression of c-*jun* and related molecules is differentially regulated in melanoma cells compared with normal melanocytes (13).

One of the unique biochemical features of cells of melanocyte lineage is the synthesis of melanin (14, 15). Generation of H_2O_2 occurs at several points in the biochemical pathway; additionally, the synthesis of the phaeomelanin subtype requires a constant supply of cysteine via glutathione (14, 16). Both the generation of H_2O_2 and the synthesis of cysteine lowers intracellular reduced glutathione. We previously have shown that melanoma cells are considerably less efficient at handling H_2O_2-induced oxidative stress compared with melanocytes (17). It is also known that many melanomas have low catalase levels as well as abnormalities of manganese superoxide dismutase (18–21). All of these conditions favor a prooxidant intracellular state and activation of redox-sensitive genes, a result that may produce a basis for resistance to exogenous agents (20, 22). We therefore measured the levels of NF-κB and AP-1 binding activity under basal and oxidative stress conditions in melanoma

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[3] The abbreviations used are: NF-κB, nuclear factor-κB; AP, activator protein; TBST, Tris-buffered saline-Tween [20 μM Tris-HCl (pH 7.6), 1377 μM NaCl, 0.5% Tween 20]; BSO, buthionine sulfoximine; EMSA, electrophoretic mobility shift assay; TPA, phorbol myristate-13-acetate.

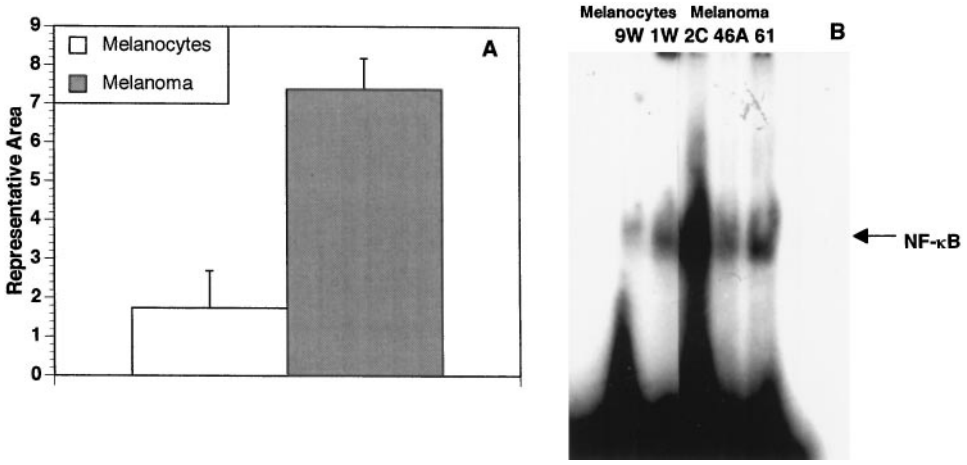

Fig. 1 EMSA of NF-κB in normal human melanocytes and metastatic melanoma cells. Nuclear proteins were extracted from cultured normal melanocytes or melanoma cells incubated with ^{32}P-labeled consensus oligonucleotides of NF-κB for 1 h and electrophoresed. *A*, laser densitometric quantitation of the protein:DNA complexes. Values for three melanoma lines (c83-2C, c81-61, c81-46A) and four separate pools of melanocytes (9W, 1W, 11W, 12W) are expressed as relative indexes; *bars*, SD. *B*, EMSA of normal melanocyte pools 9W and 1W and melanoma cell lines c83-2C, c81-46A, and c81-61.

cells and normal melanocytes as the initial basis for exploration of a drug-resistant phenotype.

MATERIALS AND METHODS

Cell Culture. Two to five human Caucasian neonatal foreskins were placed in 0.25% trypsin at 4°C overnight. The tissues were scraped to recover the melanocytes, pooled, and cultured in MCDB 153 (Sigma, St. Louis, MO) medium containing 2% FCS, 0.3% bovine pituitary extract (Clonetics Corp., San Diego, CA), 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). Each normal melanocyte pool (1W, 9W, 12W, and 11W) contained the total yield of cells from two to five Caucasian neonatal foreskins. Human metastatic melanoma cells (c83-2C, c81-61, and c81-46A) were cultured in F-10 (Fisher Scientific, Pittsburgh, PA) medium containing 5% FCS and 5% newborn calf serum (13).

Northern Blot Analysis. Total RNA was isolated by detergent lysis followed by phenol-chloroform extraction and ethanol precipitation. Ten μg of RNA were size fractionated on denaturing formaldehyde agarose gels and transferred to nylon filters by capillary blotting. Blots were exposed to ^{32}P-labeled cDNA probes and hybridized at 42°C for 2 h, using Rapid-Hyb Buffer (Amersham, Arlington Heights, IL). Autoradiographs were quantified by densitometry, using Molecular Analyst software (Bio-Rad, Emeryville, CA). NF-κB1 (p50) RNA expression was detected using a 1.5-kb *Eco*RI insert, c-Rel (p75) was detected using a 2.34-kb *Eco*RI insert, Rel A (p65) was detected using a 0.95-kb *Eco*RI insert, and IκB was detected using a 1.190-kb *Eco*RI insert (23).

EMSA. Nuclear proteins were extracted from detergent-lysed cells by dialysis with hypertonic buffer (20 mM HEPES, 3.5 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride; Sigma) for 2 h at 10°C. Consistent loading was determined by Coomassie Blue (Sigma) staining of protein after SDS-PAGE. Samples of nuclear protein (5 μg) were incubated with 600,000 cpm of ^{32}P-labeled consensus oligonucleotides of AP-1 or NF-κB (Promega, Madison, WI). Following a 1-h incubation, samples were electrophoresed in a low ionic strength polyacrylamide gel. Quantitation of protein:DNA complexes was accomplished by densitometry as described for Northern analysis.

Western Analysis. Nuclear proteins were isolated as described above. Five μg of nuclear protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% milk in TBST [20 μM Tris-HCl (pH 7.6), 1377 μM NaCl, 0.5% Tween 20], incubated with rabbit polyclonal antibodies to IκB-α (1:1000 dilution in 3% milk; Ref. 24), and then incubated with horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed in TBST between steps. Immune-complexed IκB-α proteins were detected using SuperSignal enhanced chemiluminescence (Pierce, Rockford, IL).

Oxidative Stress Treatment. H_2O_2 was quantitated by the oxidation of ferrous iron (Fe^{+2}) to ferric iron (Fe^{+3}) and reacted with xylene orange, producing a colorimetric change that was detected by absorbance at 560 nm (25). Glucose (present in all media at 1.0 mg/ml) and glucose oxidase (Boehringer Mannheim, Indianapolis, IN) were used to enzymatically generate H_2O_2 in cells. Exposure for 15 min to 10.0 U/ml glucose oxidase enzyme-generated H_2O_2 was equivalent to 0.012 mM free H_2O_2. Exposure for 1 min to 0.001% free H_2O_2 was equivalent to 0.3 mM H_2O_2. Buthionine sulfoximine (BSO) was preincubated with the cells at a concentration of 10 mM for 24 h. All chemicals were from Sigma.

RESULTS

EMSA of NF-κB in Normal Human Melanocytes and Melanoma Cells. The DNA-binding activity of NF-κB from three different pools of normal melanocytes and three different metastatic melanoma cell lines under basal culture conditions, as measured by EMSA and quantitated by laser densitometry, is shown in Fig. 1*A*. The DNA-binding activity of NF-κB from melanoma cells, as measured by EMSA, was on average 4-fold higher than the DNA-binding activity of NF-κB from normal melanocytes. A representative blot of the DNA-binding activity of NF-κB from normal melanocytes and metastatic melanoma cells is shown in Fig. 1*B*. The DNA-binding activity of NF-κB

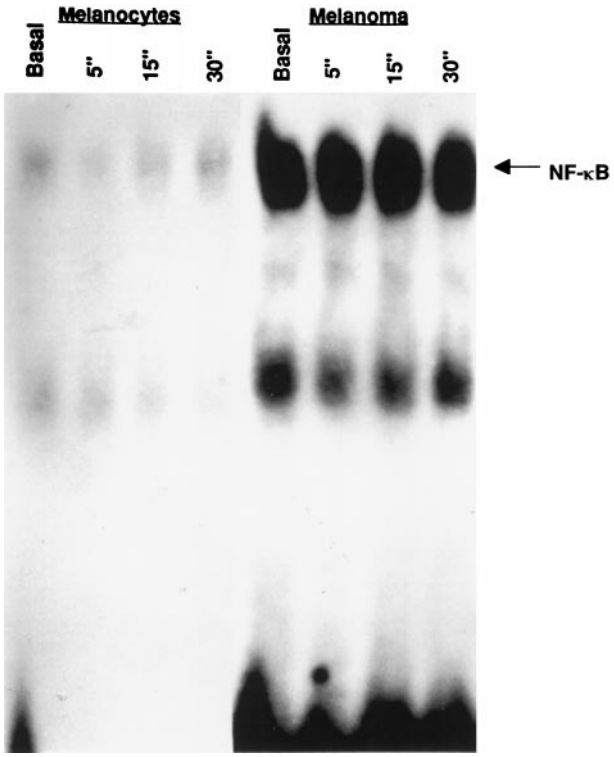

Fig. 2 EMSA of normal melanocyte pool 1W and metastatic melanoma cell line c83-2C following incubation with cycloheximide (10 μg/ml) for 1 h before nuclear extraction at times indicated.

from normal melanocytes and melanoma cells was unaffected by preincubation with cycloheximide (Fig. 2).

Northern Blot Analysis of p50, p65, p75, and IκB-α in Normal Human Melanocytes and Melanoma Cells. The expression of p50, p65, p75, and IκB-α by three pools of normal melanocytes and three metastatic melanoma cell lines, as measured by Northern blot analysis and quantitated by laser densitometry, is shown in Fig. 3*A*. A representative blot of p50, p65, p75, and IκB-α RNA expression by normal melanocytes and melanoma cells is shown in Fig. 3*B*. The expression of p50, p65, and IκB-α was on average 2-, 4-, and 1.5-fold greater, respectively, in melanoma cells compared with melanocytes, whereas expression of p75 was markedly decreased (60%).

EMSA of NF-κB in Normal Human Melanocytes and Melanoma Cells following Oxidative Stress. The basal DNA-binding activity of NF-κB was much higher in melanoma cells compared with normal melanocytes. However, binding did increase in melanoma cells following exposure to free or enzyme-generated H_2O_2 or incubation with BSO (Fig. 4, *A* and *B*). Free H_2O_2 or incubation with BSO caused a modest increase in binding in normal melanocytes, but it was considerably less than that seen in melanoma cells. A representative blot of the DNA-binding activity of NF-κB from normal melanocytes and metastatic melanoma cells following oxidative stress is shown in Fig. 4*C*.

EMSA of AP-1 in Normal Human Melanocytes and Melanoma Cells following Oxidative Stress. The basal DNA-binding activity of AP-1 was higher in melanoma cells

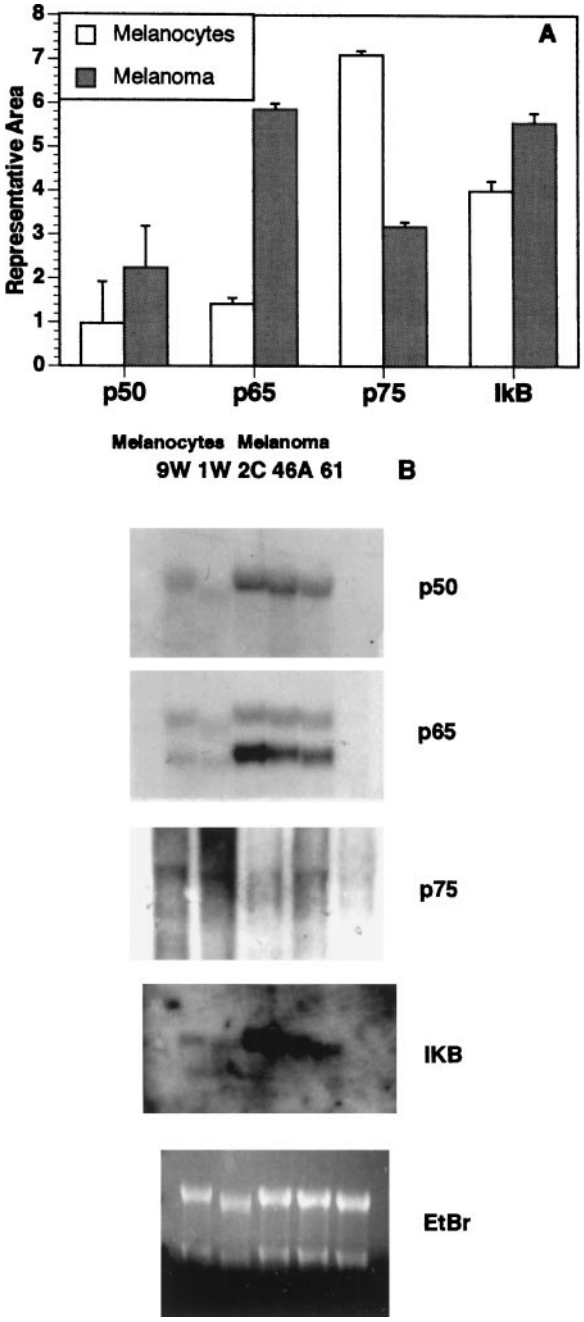

Fig. 3 Northern blot analysis of p50, p65, p75, and IκB-α expression by normal human melanocytes and metastatic melanoma cells. *A*, total RNA was isolated, size fractionated, and transferred to nylon filters. Blots were hybridized to ^{32}P-cDNA probes and quantitated by laser densitometry. Values are expressed as relative indexes; *bars*, SD. *B*, Northern blot of normal melanocyte pools 9W and 1W and melanoma cell lines c83-2C, c81-46A, and c81-61.

compared with normal melanocytes (Fig. 5*A*). Free H_2O_2, enzyme-generated H_2O_2, or incubation with BSO produced no effect or an enhancement of the DNA-binding activity of AP-1 in normal melanocytes, whereas the DNA-binding activity in melanoma cells was unaffected or decreased (Fig. 5, *A* and *B*).

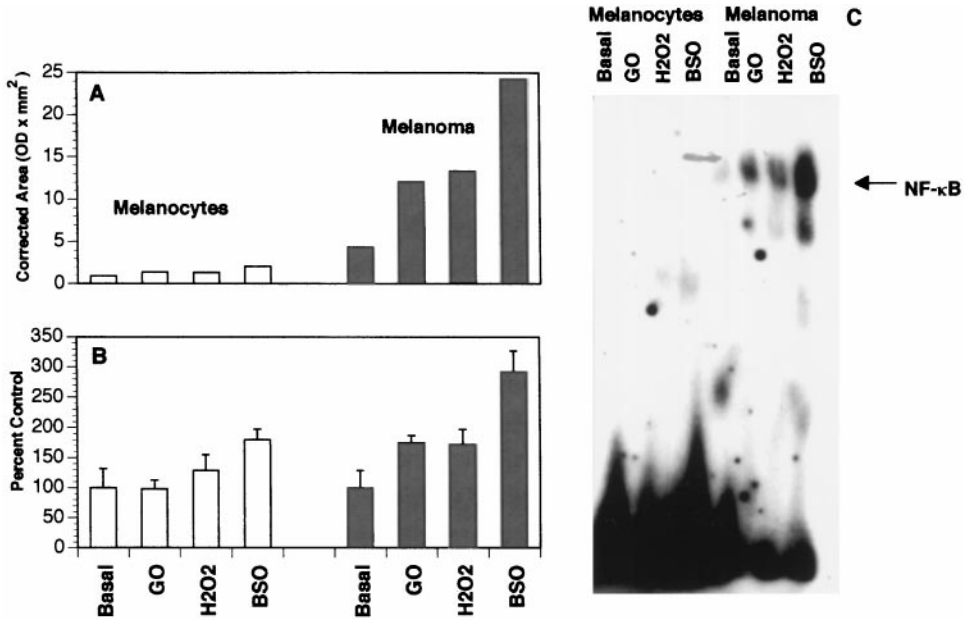

Fig. 4 EMSA of NF-κB in normal human melanocytes and metastatic melanoma cells following oxidative stress. *A*, cells were incubated with BSO, free H_2O_2, or enzyme-generated H_2O_2 as described in "Materials and Methods." Laser densitometer was used to quantitate the protein:DNA complexes shown in *C*. *B*, summary of densitometry data expressed as percentages of control from three melanocyte pools (1W, 9W, 12W) and three melanoma cell lines c83-2C, c81-46A, and c81-61. *C*, EMSA of normal melanocyte pool 1W and melanoma cell line c81-46A performed as described in legend to Fig. 1.

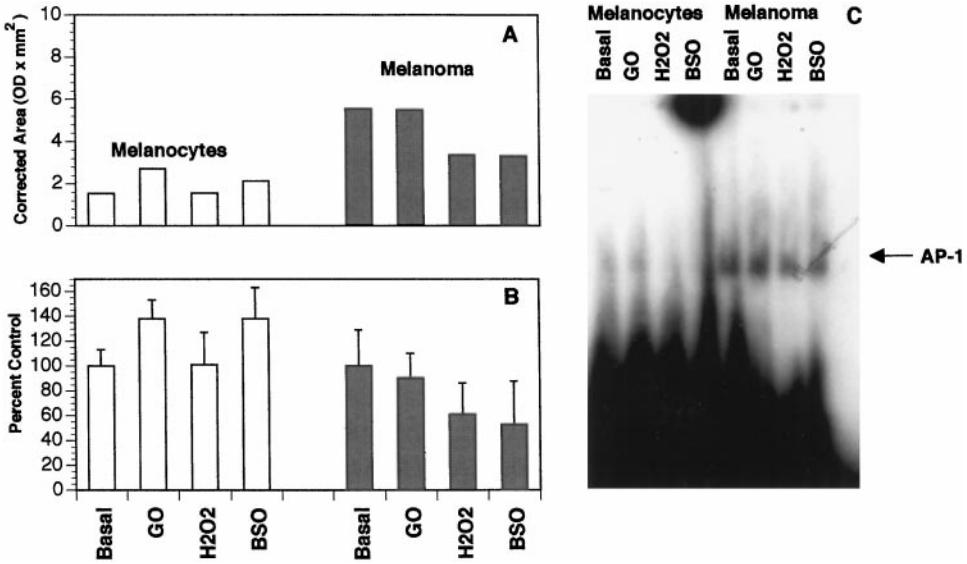

Fig. 5 EMSA of AP-1 in normal human melanocytes and metastatic melanoma cells following oxidative stress. *A*, cells were incubated with BSO, free H_2O_2, or enzyme-generated (glucose oxidase) H_2O_2 as described in "Materials and Methods." Laser densitometer was used to quantitate the protein: DNA complexes shown in *C*. *B*, summary of densitometry data expressed as percentages of control from three normal melanocyte pools (1W, 9W, 12W) and three melanoma cell lines (c83-2C, c81-46A, c81-61). *C*, EMSA of normal melanocyte pool 9W and melanoma cell line c83-2C; conditions were as for Fig. 1 except a ^{32}P-labeled AP-1 oligonucleotide consensus sequence was used.

A representative blot of the DNA-binding activity of AP-1 from normal melanocytes and metastatic melanoma cells following oxidative stress is shown in Fig. 5*C*.

Western Analysis of Nuclear IκB-α. When cultured under standard conditions, the nuclear abundance of IκB-α was increased 2- and 11-fold, respectively, in c81-61 and c83-2C melanoma cell lines relative to the normal melanocyte cell line 95-1W. When exogenous signals for IκB-α degradation were withheld from the medium, the nuclear levels of IκB-α increased in these cell lines relative to standard culture conditions. Normal melanocytes were deprived of TPA for 24 h, resulting in a 9-fold increase in nuclear IκB-α relative to cells cultured in the presence of TPA. Melanoma cell lines were deprived of serum for 24 h, resulting in a 6- and 1.6-fold increase in nuclear IκB-α in c81-61 and c83-2C when compared with serum-starved cells.

DISCUSSION

Our investigations demonstrate that the transcription factor NF-κB is constitutively activated in melanoma cells compared with normal melanocytes and is further increased under conditions of oxidative stress. In contrast, the binding activity of the transcription factor AP-1 was unaffected or decreased in melanoma cells in response to oxidative stress. These results suggest that the expression and redox control mechanisms involving

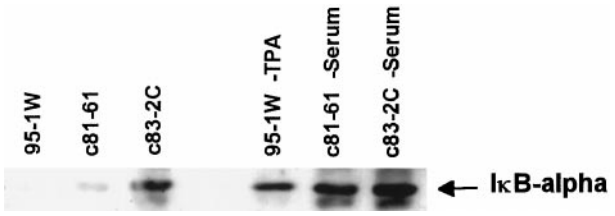

Fig. 6 Western analysis of nuclear IκB-α. Cells were incubated in the presence or absence of TPA (*95-1W*) or serum (*c81-61, c83-2C*). Nuclear proteins were isolated and analyzed as described in "Materials and Methods."

these transcription factors are altered during transformation of the melanocyte to its malignant counterpart. The quantitative levels of AP-1 and NF-κB were affected by culture conditions, but the qualitative relationship between levels was preserved, (data not shown); this is important in as much as the growth requirements of normal melanocytes and melanoma cells require different culture additives.

High expression of NF-κB and *rel*-related proteins has been shown in other tumor cell lines, although interpretation has been limited because the normal phenotype has not been studied (26). The increased expression of p50, p65, and IκB-α in melanoma cells could be the result of autoregulatory induction by the higher NF-κB activity rather than a cause of the increased NF-κB binding activity. The basis for increased NF-κB binding activity is generally unknown; however, Shattuck-Brandt and Richmond (27) showed recently that in Hs294T melanoma cells, enhanced degradation of IκB-α contributed to the endogenous activation of NF-κB. Our data in two other melanoma lines and in melanocytes indicate that IκB-α is present in the nuclear protein and is increased by the withdrawal of serum or TPA (Fig. 6). This suggests that in general, a more complex regulatory mechanism than enhanced degradation of IκB-α underlies the enhanced NF-κB binding activity in melanoma cells.

The differential response of NF-κB and AP-1 to oxidative stress is not surprising in that NF-κB usually is activated by prooxidant conditions, whereas AP-1 binding activity is inhibited (7, 8). However, the differential responses of NF-κB and AP-1 in normal melanocytes and melanoma cells to oxidative stress are striking and suggest a fundamental difference in redox regulation at the transcriptional level between the normal and malignant phenotypes. Preliminary studies suggest that the levels of endogenous reactive oxygen species are higher in melanoma cells compared with melanocytes and many other transformed and normal cells.[4]

Because many toxic agents, including chemotherapeutic drugs and radiation, generate reactive oxygen species, manipulation of these transcription factors may be useful in effecting a therapeutic outcome (28, 29).

The parallel constitutive elevation of NF-κB binding activity and p50, p65, and IκB-α expression as well as the decrease in p75 expression in melanoma cells compared with normal melanocytes may also offer specific targets for small molecule modulation or antisense inhibition, in particular because experiments in other systems suggest specific roles for the NF-κB/Rel subunits (28, 29). For example, antisense inhibition of Rel A/NF-κB activity can block cellular adhesion to the extracellular matrix and inhibit *in vivo* tumorigenicity in nude mouse models (28). Whether the imbalance in NF-κB/Rel factors in melanoma cells is cause or effect has not been determined by the current study, but exploration of the role of these subunits in oncogenesis of the melanocyte should be informative. Results from this current investigation plus our recent observation that melanoma cells quench reactive oxygen species considerably less effectively than normal melanocytes should also offer unique targets for intervention (16).

[4] Unpublished data.

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