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## ABERRANT REDOX REGULATION IN HUMAN METASTATIC MELANOMA CELLS COMPARED TO NORMAL MELANOCYTES

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**Abstract**—Melanocytes and melanoma cells contain melanin, a complex polymer that modulates redox changes in these cells. Relative intracellular hydrogen peroxide levels measured by dichlorodihydrofluorescein are similar in the two cell types, but the levels of superoxide anion measured by dihydroethidium were markedly increased in melanoma cells. Chelator-induced oxidative stress is efficiently suppressed by melanocytes without substantial recruitment of the transcription factors NF- $\kappa$ B and AP-1 as measured by electrophoretic mobility shift assay and quantitated by densitometry or by a change in frequency of apoptosis as determined by annexin V binding. In contrast, NF- $\kappa$ B in melanoma cells is strongly recruited by changes in redox status and exhibits a correlative relationship to intracellular hydrogen peroxide (but not superoxide anion). However, the response of the NF- $\kappa$ B pathway to intracellular hydrogen peroxide is anomalous, including downregulation of p65 and I $\kappa$ B $\alpha$  RNA expression (Northern blot). Additionally, recruitment of AP-1 binding in melanoma cells was directly correlated with intracellular levels of superoxide anion (but not hydrogen peroxide). Neither the degree of NF- $\kappa$ B nor AP-1 binding in melanoma cells was related to the frequency of apoptosis. The responsiveness of NF- $\kappa$ B and AP-1 recruitment to intracellular levels of hydrogen peroxide and superoxide anion without concomitant control of apoptosis provides a general mechanism by which these cells can escape noxious injury (e.g., chemotherapy). The marked enhancement of apoptosis in melanoma cells by chelators indicates, however, that this alteration can be circumvented and offers a unique therapeutic window to explore. © 2001 Elsevier Science Inc.

**Keywords**—Hydrogen peroxide, Superoxide anion, Chelator, Free radicals

### INTRODUCTION

Metastatic melanoma is among the most therapeutically refractory of all cancers [1]. The biochemical and molecular basis of this property is largely unknown, although there has been no shortage of biochemical and immunological explanations offered [2,3]. We have taken a different approach to the issue and have recently demonstrated that nuclear factor- $\kappa$ B (NF- $\kappa$ B) is constitutively activated in human metastatic melanoma cells compared to normal melanocytes [4]. Despite the marked increase of NF- $\kappa$ B binding in melanoma cells under a variety of basal and culture-supplemented conditions,

this property was further enhanced by oxidative stress produced by a number of experimental manipulations of these cells. As NF- $\kappa$ B is known to enhance survival pathways in other benign and malignant cells [5–8], we proposed that a similar situation was operative in melanoma cells and that the constitutive activation of NF- $\kappa$ B results in an ongoing and continuous stress response that affords protection against noxious agents, including chemotherapeutic and other interventions. Other investigators have shown that melanocyte-stimulating hormone inhibits NF- $\kappa$ B activation in human melanocytes and melanoma cells [9]. We have also previously shown that components of the AP-1 pathway are abnormally regulated in melanoma cells [10].

A known feature of human melanoma cells is that their antioxidant capacity is depressed with decreased catalase, glutathione-s-transferase, and MnSOD enzymatic activity and low levels of glutathione and  $\alpha$ -to-

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copherol levels compared to melanocytes [11–13]. This finding is surprising in that most tumor cells exhibit an antioxidant phenotype [14,15]. We have also previously shown that melanocytes and melanoma cells respond differentially to exogenous peroxide with melanocytes efficiently suppressing this stress and melanoma cells unable to do so [16]; surprisingly, melanoma cells were not just unable to suppress a peroxide stress, but generated a pro-oxidant response.

We therefore postulated that the handling of the redox state in melanoma cells is aberrant and in the current work have explored the effect of chelators that alter the redox status in normal melanocytes and metastatic melanoma cells. We measured the effect of chelators on levels of intracellular hydrogen peroxide and superoxide anion, degree of NF- $\kappa$ B and AP-1 binding, and frequency of apoptosis to study the relationship between each of these parameters.

## 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 Chemical Co., St. Louis, MO, USA) medium containing cysteine (42 mg/l) and methionine (44.8 mg/l) with additions of 2% fetal calf serum, 0.3% bovine pituitary extract (Clonetics Corp., San Diego, CA, USA), 10 ng/ml phorbol myristate-13-acetate, 2.0 mM calcium chloride, 5  $\mu$ g/ml insulin, and 0.1 mM 3-isobutyl-1-methyl-xanthine (Sigma Chemical Co.). Each normal melanocyte pool contained the total yield of cells from two to five Caucasian neonatal foreskins. The passage number used in these experiments was not greater than five. Metastatic melanoma cell strains (c81-46A, c83-2C, and c81-61) were cultured in F-10 medium (Fisher Scientific, Pittsburgh, PA, USA) containing cysteine (25 mg/l) and methionine (4.5 mg/l) with additions of 5% fetal calf serum and 5% newborn calf serum. The passage number used in these experiments was not higher than eight. All cell strains grow in monolayer culture; c81-46A is slow growing and morphologically resembles melanocytes, c81-61 is an intermediately growing cell strain, and c83-2C is a rapidly growing strain with the additional capacity of vertical growth *in vitro*.

Both media also contained penicillin (100 u/ml), streptomycin (0.1 mg/ml), bicarbonate (1.2 mg/ml), and phenol red indicator. No glutathione or mercaptoethanol was added to either media. In all experiments, cells were treated at 50–70% confluency and fresh media and drug added simultaneously for various times (24–72 h). Final

density ranged from 70–90% confluency. No change in media pH (7.6–7.8) was recorded.

### *Chemicals*

Metal chelators included: 1,10 phenanthroline (OP), pyrrolidine dithiocarbamate (PDTC), deferoxamine mesylate (DEF). OP primarily binds metals with a +2 valence, DEF primarily binds metals with a +3 valence [17], and PDTC can bind both +2 and +3 valences [18]. Also used were ferrous ammonium sulfate and ferric sulfate. All chemicals were purchased from Sigma Chemical Co.; unbuffered stock solutions (OP, 39.6 mg/ml; PDTC, 16.4 mg/ml; all others, 10 mg/ml) were made fresh for each experiment.

### *Northern blot analysis*

This measurement was done following our prior protocol [4,19]. Total RNA was isolated by detergent lysis (0.65% NP40, 10 mM Tris-Cl, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>) followed by phenol-chloroform extraction and ethanol precipitation. Ten micrograms of RNA was size fractionated on denaturing formaldehyde agarose gels and transferred to nylon filters by capillary blotting. Blots were exposed to <sup>32</sup>P-labeled cDNA probes and hybridized at 42°C for 2 h using Rapid-Hyb Buffer (Amersham, Braunschweig, Germany). Autoradiographs were quantified by densitometry using Molecular Analyst software (Bio-Rad, Richmond, CA, USA). The NF- $\kappa$ B1 (p50), RelA (p65), and I $\kappa$ B $\alpha$  cDNA probes [20] were a generous gift from John Hiscott of McGill University.

### *Electrophoretic mobility shift assay*

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 PMSF, Sigma Chemical Co.) for 2 h at 10°C. Five microgram samples of nuclear protein were incubated with 600,000 cpm of <sup>32</sup>P-labeled consensus oligonucleotides of AP-1 (sense strand; 5'-CGC TTG ATG AGT CAG CCG GAA-3') or NF- $\kappa$ B (sense strand; 5'-AGT TGA GGG GAC TTT CC AGG C-3') (Promega, Madison, WI, USA). Following a 1 h incubation, samples were electrophoresed in a low ionic strength polyacrylamide gel. Quantitation of protein:DNA complexes were accomplished by densitometry as described for Northern analysis. Consistent loading was determined by Coomassie Blue (Sigma Chemical Co.) staining of protein on a SDS polyacrylamide gel and normalized to binding activity.

### Reactive oxygen probes

Relative intracellular hydrogen peroxide and superoxide anion levels were determined based on methods adapted from those previously described [21,22] using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (5  $\mu$ M) and dihydroethidium (10  $\mu$ M), respectively (Molecular Probes, Eugene, OR, USA). These concentrations were based on pilot studies and were optimal in our system, as has been found by others [23,24]. Hydrogen peroxide: (as indicated in the manufacturer's protocol) 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate diffuses through cell membranes and is hydrolyzed by intracellular esterase to DCF, which remains trapped within cells, reacts with hydrogen peroxide and generates the fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence intensity is proportional to the amount of peroxide produced by the cells.

Superoxide anion: dihydroethidium (HE) is the sodium borohydride-reduced form of ethidium bromide that is permeable to viable cells. HE can be directly oxidized to ethidium bromide by superoxide anion, which then fluoresces. The red fluorescence is proportional to the intracellular superoxide anion levels. Cells were incubated with the appropriate probe for 15 min, harvested, and analyzed by flow cytometry using a Becton Dickinson FACScan with Cell Quest software (Becton Dickinson, Mountain View, CA, USA). Hydrogen peroxide (DCF) was analyzed by using an excitation of 488 nm and filter of 530 nm, while superoxide anion (HE) was analyzed using an excitation of 488 nm and filter of 585 nm.

### Statistical analysis

Measurements from various assay systems (p50, p65, and I $\kappa$ B $\alpha$  RNA expression, AP-1 and NF- $\kappa$ B DNA-binding activity, intracellular hydrogen peroxide and superoxide anion levels, and apoptosis) were compared under identical test conditions (Fe<sup>++</sup>, Fe<sup>+++</sup>, OP, OP + Fe<sup>++</sup>, OP + Fe<sup>+++</sup>, PDTC, PDTC + Fe<sup>++</sup>, DEF, DEF + Fe<sup>++</sup>, and DEF + Fe<sup>+++</sup>) utilizing linear regression. Total reactive oxygen species (ROS) was calculated by addition of intracellular hydrogen peroxide and superoxide anion values and divided by two. Differences were considered statistically significant at the  $p < .05$  level. All correlations compared a minimum of three separate experiments per point with standard error of the mean indicated.

### Apoptosis

Concentrations of chelators chosen for these studies were based on the results of pilot dose-response experi-

Table 1. Ratio of Intracellular Superoxide Anion and Hydrogen Peroxide in Metastatic Melanoma Cells Compared to Normal Melanocytes

Reactive oxygen species	Melanoma/melanocyte ratio		
	24 h	48 h	72 h
Superoxide anion	3.3 (0.04)	4.3 (0.027)	4 (0.04)
Hydrogen peroxide	1.1 (0.68)	0.57 (0.16)	0.96 (0.95)

*P* values (shown in parentheses, unpaired *t*-test) from three separate experiments compared basal fluorescent units, measured by dichlorofluorescein (DCF) and dihydroethidium (HE) under standard culture conditions.

ments utilizing the minimum concentration that produced the maximum apoptosis. Cells were treated with 1,10 phenanthroline (OP, 10  $\mu$ M), pyrrolidinedithiocarbamate (PDTC, 1  $\mu$ M), deferoxamine mesylate (DEF, 76  $\mu$ M), and ferrous sulfate or ferric sulfate (51  $\mu$ M) for 72 h, and analyzed for apoptosis by Annexin V binding according to the manufacturer's protocol (Pharmingen, San Diego, CA, USA). Briefly, cells were harvested, washed twice in 1  $\times$  PBS, and resuspended in binding buffer at a concentration of 1  $\times$  10<sup>6</sup> cells/ml, of which 100  $\mu$ l was incubated with 5  $\mu$ l of Annexin V (conjugated to FITC) and 15  $\mu$ M propidium iodide for 15 min at room temperature. Cells were analyzed by flow cytometry using a Becton Dickinson FACScan with Cell Quest software. The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for Annexin V while remaining impermeable to propidium iodide. A similar result was seen by FACS analysis when apoptosis was determined by the percentage of cells in the sub G1 population (data not shown).

## RESULTS

### Intracellular levels of reactive oxygen species

Using the molecular probes DCF and HE we measured, respectively, the basal intracellular levels of hydrogen peroxide and superoxide anion in normal melanocytes and metastatic melanoma cells cultured in their standard media (Table 1). The ratio of superoxide anion in melanoma compared to melanocytes was increased 3- to 4-fold while hydrogen peroxide was decreased as much as 43%. As the composition of the two media are quite different in terms of reducing equivalents, we also tried the experiment in which the media were switched. However, melanoma cells cultured in melanocyte media resulted in nearly a 50% decrease in viability within 24 h. A similar decrease in viability of melanocytes was also noted when melanocytes were grown in melanoma medium.

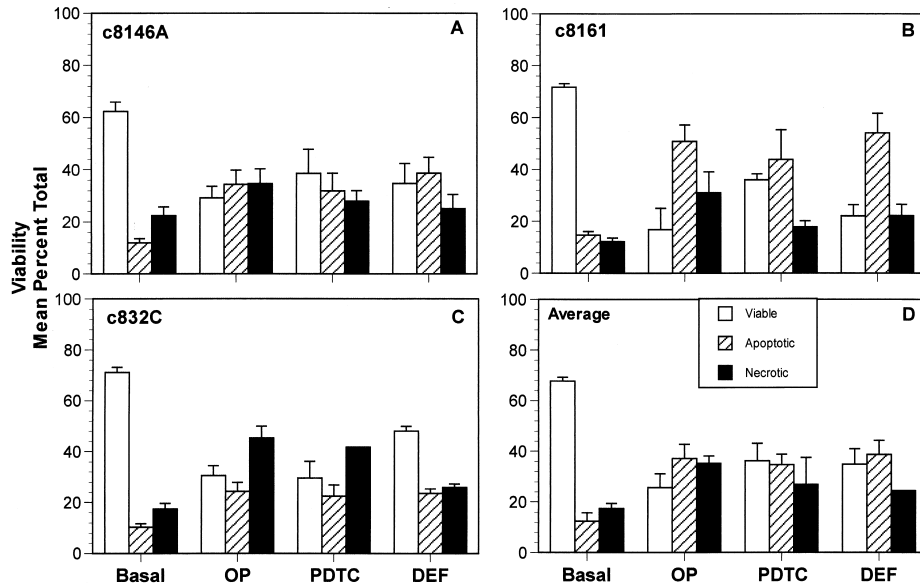


Fig. 1. Effect of heavy metal ion chelators on viability in metastatic melanoma cells. Mean values of viable, apoptotic, and necrotic as compared to basal following 72 h of OP, PDTC, or DEF. Metastatic melanoma cell lines: (A) c81-46A; (B) c81-61; (C) c83-2C. (D) Mean values of the three melanoma cell lines.

#### *Effect of heavy metal ion chelators and ferrous and ferric ions on apoptosis*

All three chelators (OP, PDTC, DEF) tested produced a substantial increase in apoptosis ranging from 2- to 4-fold in the different melanoma cell lines compared to untreated control (Figs. 1A–1C). On average, apoptosis in the melanoma cell lines was increased 3.5-fold by the chelators (Fig. 1D). The addition of ferric or ferrous ions alone to normal melanocytes had little or no effect on basal or chelator-modulated apoptosis, while the addition of ferric ion increased the basal rate of apoptosis in melanoma cells about 50% (Fig. 2). The addition of ferrous ion decreased (15–40%) chelator-induced apoptosis in melanoma cells. The addition of ferric ion also resulted in a decrease (25–60%) in chelator-induced apoptosis, but only with DEF or PDTC while having no effect in the presence of OP. In contrast, in normal melanocytes the addition of ferric ion (but not ferrous ion) enhanced (~25%) apoptosis.

#### *Effect of heavy metal ion chelators on intracellular reactive oxygen species in melanocytes and melanoma cells*

The addition of chelators to different melanoma cell lines produced different changes in intracellular hydrogen peroxide and superoxide anion (Fig. 3). All three chelators decreased both hydrogen peroxide and superoxide anion in c81-61 melanoma cells (Fig. 3B). In contrast, the chelators increased intracellular hydrogen

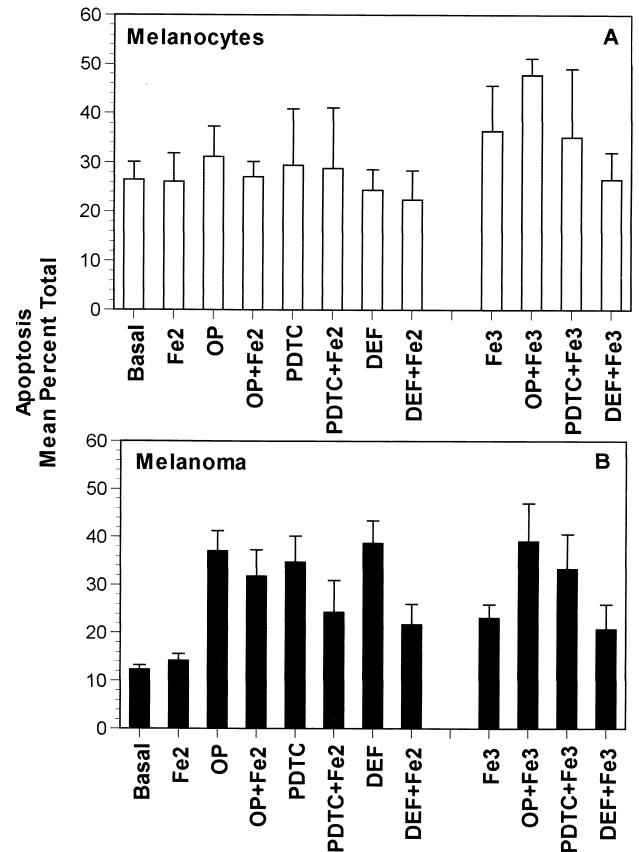


Fig. 2. Effect of ferrous and ferric ions on chelator-induced apoptosis in normal human melanocytes and metastatic melanoma cells. Mean values of apoptosis in three melanoma cell lines (B) as compared to normal human melanocytes (A) following 72 h of OP, PDTC, or DEF with ferrous ammonium sulfate ( $\text{Fe}^{+2}$ ) or ferric sulfate ( $\text{Fe}^{+3}$ ).

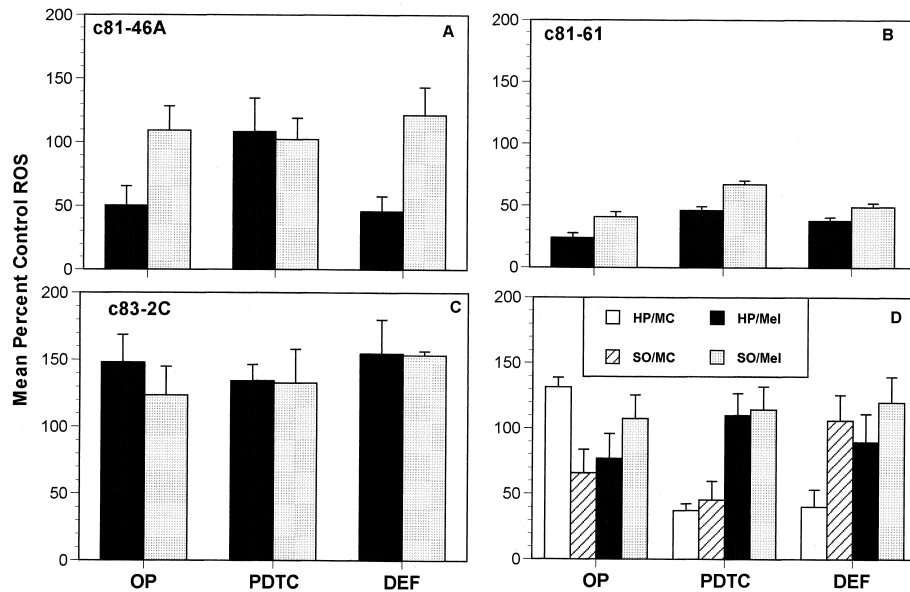


Fig. 3. Effect of chelators on intracellular hydrogen peroxide and superoxide anion levels in normal human melanocytes and metastatic melanoma cells. Mean values of intracellular hydrogen peroxide (HP) and superoxide anion (SO) as compared to basal following 72 h incubation with OP, PDTC, or DEF utilizing DCF and HE probes, respectively. Metastatic melanoma cell lines (A) c81-46A; (B) c81-61; (C) c83-2C. (D) Mean intracellular hydrogen peroxide and superoxide anion values of the three melanoma cell lines: HP/Mel and SO/Mel, respectively. Mean intracellular hydrogen peroxide and superoxide anion values of normal human melanocytes: HP/MC and SO/MC, respectively.

peroxide (40–55%) and superoxide anion (20–75%) in c83-2C melanoma cells (Fig. 3C). The effect on c81-46A was more complex, with intracellular hydrogen peroxide unaffected or decreased by up to 50% by chelator addition but superoxide anion unaffected (Fig. 3A). The effect of chelators on intracellular hydrogen peroxide and superoxide anion in normal melanocytes seemed dependent on the type of chelator used. Both DEF and PDTC bind +3 valence metals, and hydrogen peroxide in melanocytes decreased 75% under these conditions. In contrast, OP (binds +2 valence) resulted in a 25% increase in hydrogen peroxide. Both OP and PDTC bind metals with +2 valence, and superoxide was decreased (35–55%) in melanocytes. DEF, which binds +3 valence metals, had no effect on superoxide anion levels in melanocytes.

#### *Effect of heavy metal ion chelators on NF- $\kappa$ B and AP-1 binding in melanocytes and melanoma cells*

The trivalent chelators markedly increased (DEF > PDTC) while OP had no effect on NF- $\kappa$ B binding in melanoma cells (Fig. 4). In contrast, the chelators DEF and PDTC had no effect on NF- $\kappa$ B binding in normal melanocytes, while OP enhanced NF- $\kappa$ B binding 2- to 3-fold. In contrast, the chelators had no effect on AP-1 binding in melanocytes and uniformly decreased AP-1 binding in melanoma cells.

#### *Correlative relationship of intracellular hydrogen peroxide and superoxide anion levels to NF- $\kappa$ B and AP-1 binding and apoptosis*

The levels of ROS, transcription factor binding and NF- $\kappa$ B family members were simultaneously measured under different levels of oxidative stress (see legend to Fig. 5). The comparative relationships of intracellular levels of hydrogen peroxide and superoxide anion to p50, p65, and I $\kappa$ B $\alpha$  RNA expression, NF- $\kappa$ B and AP-1 binding activity, and apoptosis were analyzed and the significant correlations shown in Figs. 5A, 5B, 5D, 5F, and 5G, as well as the nonsignificant correlations between superoxide anion/NF- $\kappa$ B and hydrogen peroxide/AP-1 (Figs. 5C, 5E, and 5H). The data is not shown for other correlations that were nonsignificant (detailed below). In all cases the data is expressed as mean percent of the appropriate control as described in Materials and Methods.

NF- $\kappa$ B binding was linearly related to hydrogen peroxide levels (Fig. 5A;  $p = .017$ ) as was total ROS (Fig. 5D;  $p = .052$ ), but not to superoxide anion (Fig. 5E;  $p = .33$ ). Hydrogen peroxide levels were inversely related to p65 (Fig. 5B;  $p = .002$ ) and possibly to I $\kappa$ B $\alpha$  (Fig. 5C;  $p = .08$ ) RNA expression. There was also no significant correlation between hydrogen peroxide and p50 RNA levels. There was no apparent relationship of intracellu-

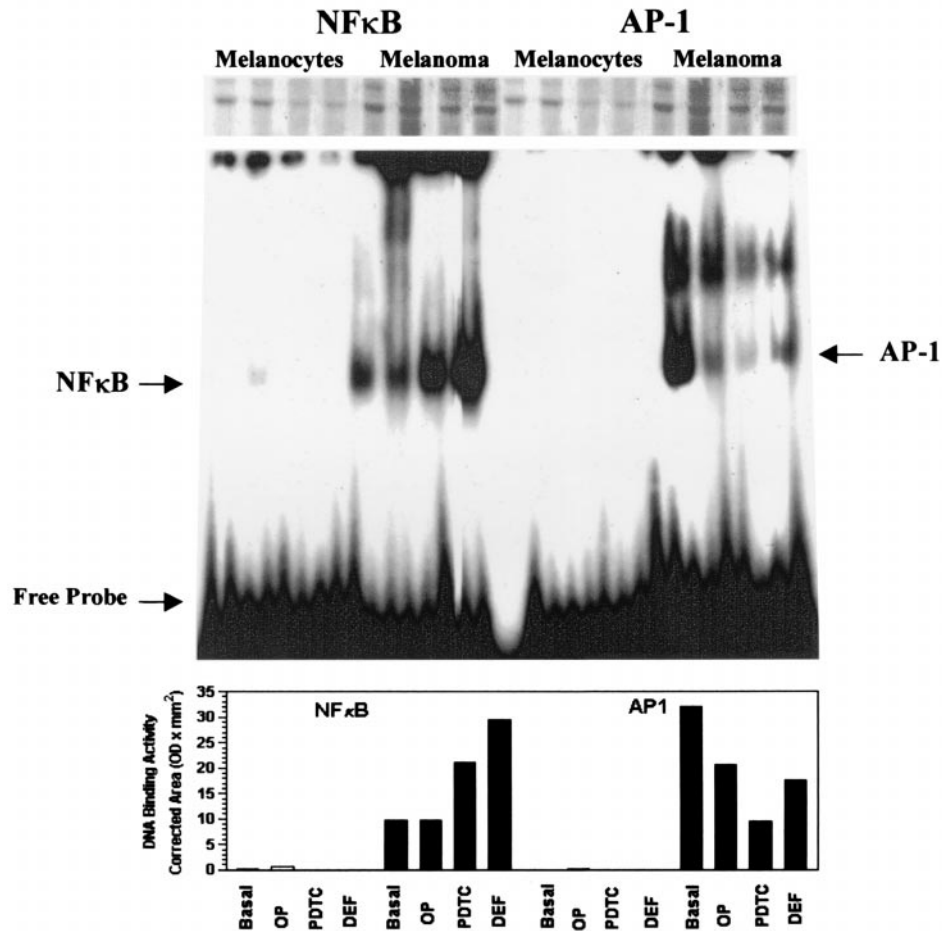


Fig. 4. Representative electrophoretic mobility shift assay (EMSA) showing NF- $\kappa$ B and AP-1 DNA-binding activity in normal human melanocytes and metastatic melanoma cells. Cells were treated for 72 h with OP, PDTC, or DEF. Coumassie staining of protein was done for normalization.

lar hydrogen peroxide levels or of overall NF- $\kappa$ B binding to apoptosis.

In the correlative studies of AP-1 and ROS the following findings were established. There was no significant relationship between intracellular hydrogen peroxide and AP-1 binding, but there was a strong direct relationship between superoxide anion and AP-1 binding (Fig. 5G;  $p = .02$ ) as there was for total ROS and AP-1 binding (Fig. 5F;  $p = .028$ ). However, AP-1 binding was not related to the frequency of apoptosis.

These results suggest that NF- $\kappa$ B is regulated by hydrogen peroxide and AP-1 by superoxide levels in melanoma cells, but that the relationship between these ROS and transcription factors and apoptosis has been abrogated.

#### DISCUSSION

Our overall conclusions from these studies are that melanocytes rapidly sequester reactive oxygen species,

thereby preventing both a significant recruitment of redox-sensitive transcription factors and initiation of apoptosis. Since reactive oxygen species are important cellular messengers [25,26], such a control mechanism is probably central to the effective suppression of unscheduled proliferation of melanocytes. In contradistinction, our data suggest that intracellular levels of superoxide anion are high in melanoma cells resulting in an overall intracellular oxidative stress that leads to facilitation of a constitutive stress response in melanoma cells mediated by the NF- $\kappa$ B pathway. However, the response is aberrant and neither apoptosis nor cessation of growth occurs. Other studies suggest that this process is mediated by a constitutively elevated RelA expression (S. McNulty and F. Meyskens, unpublished data). We speculate that the consequence of this situation is that a mutated phenotype is generated or selected for in the presence of continuously high superoxide anion levels and abnormal NF- $\kappa$ B regulation.

Numerous authors have suggested that tumor cells are

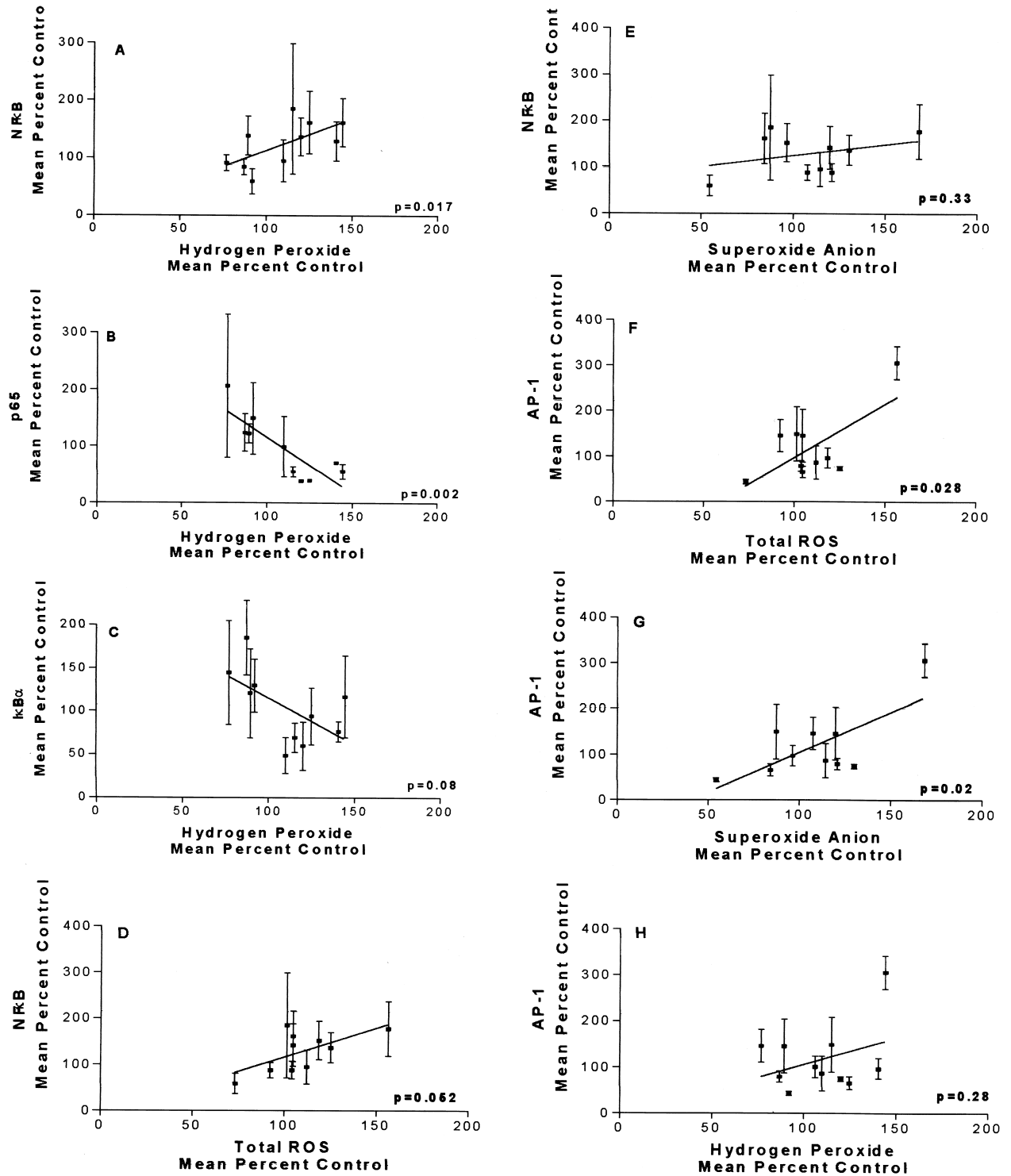


Fig. 5. Relationship of intracellular hydrogen peroxide, superoxide anion alone, or total reactive oxygen species (ROS) in metastatic melanoma cells to AP-1 and NF- $\kappa$ B binding, p65 and  $\text{I}\kappa\text{B}\alpha$  RNA expression. Melanoma cells were exposed to different chelators and cations, and the intracellular levels of hydrogen peroxide and superoxide anion were assessed by fluorescent probes using flow cytometry. Transcription factor binding was simultaneously assessed, under the same conditions, by EMSA; NF- $\kappa$ B family members (p50, p65,  $\text{I}\kappa\text{B}\alpha$ ) were assessed by Northern blotting. Both the EMSA binding and Northern blotting were quantitated by laser densitometry. All experimental measurements were converted to mean percentage of appropriate control as described in Materials and Methods. The mean percent of control was taken for each stress condition and the relationship between the paired means was analyzed by linear regression with  $p$  values indicated.



under chronic oxidative stress and that intracellular hydrogen peroxide and superoxide anion have an important role in apoptosis. [27,28]. However, comparisons of reactive oxygen species in the normal and malignant phenotype and their effect on NF- $\kappa$ B and AP-1 and apoptosis have not been performed systematically [29]. Since cells of different lineage have a diversity of mechanisms to control growth and to mediate oxidative stress, comparisons of redox changes between the normal and malignant phenotype of cells and the effect on apoptosis is important. Others have observed that different cell types produce a range of levels of extracellular hydrogen peroxide products [30–32]. In contrast, the relative amount of hydrogen peroxide in melanoma cells decreased with time while intracellular superoxide anion in melanoma cells seemed to accumulate and was 3- to 4-fold that of normal melanocytes (Table 1). We have demonstrated elsewhere that melanoma cells quench an exogenous peroxidative stress poorly and, in fact, paradoxically generate a pro-oxidant response to oxidant stress, most likely mediated by superoxide anion [16]. This oxidant stress was resisted by melanocytes and no apoptosis occurred while apoptosis was markedly increased in melanoma cells.

These results are of particular significance since it has been shown that melanoma cells produce large amounts of extracellular superoxide anion [33], suggesting that these cells are under constant oxidative stress from a high level of intracellular generation of this ROS. Others have suggested that hydroquinone precursors to melanin formation can serve as a redox cyler [34–36], particularly in the presence of iron [37]. We also have direct evidence from study of the electro-chemical response of melanin that the species involved is superoxide anion (P. Farmer and F. Meyskens, unpublished data); an observation made by others using a different biochemical approach [35].

Apoptosis in normal melanocytes was remarkably impervious to the effects of chelator stress including the addition of ferrous and ferric ion, which should enhance Fenton and Haber-Weiss (particularly in the presence of increased superoxide anion) reactions, respectively. The response to chelator addition by melanocytes was accompanied by a substantial decrease in the intracellular hydrogen peroxide and superoxide anion levels, but there was no significant change of either NF- $\kappa$ B or AP-1 binding activity (Fig. 4). In contrast, melanoma cells underwent increased apoptosis in response to chelators and were variable in their ability to appropriately manage intracellular reactive oxygen species (Fig. 3). Consequently, in melanoma cells NF- $\kappa$ B was recruited for binding and AP-1 binding was decreased by chelators and correlated with oxidative stress (Fig. 4). Chelators have also been shown to inhibit the growth and induce

apoptosis in Kaposi sarcoma cells, but ROS or transcription factors were not measured [38].

These current results, together with our previous observations [4], suggest that metastatic melanoma cells have lost the ability to perform a key function of the melanocytic lineage: the effective sequestration and inactivation of reactive oxygen stress without the concomitant transcription factor recruitment and activation. We have shown elsewhere that NF- $\kappa$ B regulation in melanoma cells responds to ultraviolet B aberrantly (S. McNulty and F. Meyskens, unpublished data), suggesting that our results may have etiologic importance as well.

The analysis of correlative relationships in melanoma cells between intracellular ROS (hydrogen peroxide and superoxide anion) and NF- $\kappa$ B binding, the RNA expression of p50, p65, and I $\kappa$ B $\alpha$ , and the relationship of modulation of these molecules to apoptosis was informative (Fig. 5). A major feature of these studies is that we have determined the quantitative and correlative relationship between intracellular ROS and the NF- $\kappa$ B and AP-1 system, while prior studies including our own investigations [16,4] have largely been qualitative. A limitation of these comparisons is that they have been made at only one time point; however, such comparisons require the generation of a very large amount of data. Nevertheless, we have performed preliminary studies and the data suggest that the chelator effect on ROS occurs rapidly and on mitochondrial depolarization slowly (and similar to its effect on melanocytes). The effect of NF- $\kappa$ B binding takes 48–72 h to be fully expressed while the effect on AP-1 binding is immediate and sustained (unpublished data). Neither of these observations would alter the conclusions regarding the various positive correlations described between various ROS and NF- $\kappa$ B or AP-1, and the absence of correlation between NF- $\kappa$ B or AP-1 and apoptosis. However, strictly evaluated these conditions are only valid for the 72 h time point after chelator addition.

An increase in intracellular hydrogen peroxide was related in a dose-response fashion to NF- $\kappa$ B binding (Fig. 5A). These results confirm and extend our observations that melanoma cells exposed to extracellular oxidative stress resulted in an increase in NF- $\kappa$ B binding even though NF- $\kappa$ B was constitutively activated in these cells [4]. If the NF- $\kappa$ B system was responding normally in melanoma cells, p50 and I $\kappa$ B $\alpha$  RNA expression should have increased while p65 expression is not regulated by NF- $\kappa$ B. However, no relationship between hydrogen peroxide and p50 RNA expression was evident and surprisingly both p65 and I $\kappa$ B $\alpha$  expression were inversely related to intracellular hydrogen peroxide levels (Figs. 5B and 5C). These results can not be easily explained but suggest a fundamental abnormality in NF- $\kappa$ B regulation in melanoma cells.

Many possibilities come to mind including, most prominently, the presence of a NF- $\kappa$ B repressor and/or an increase in RelA (p65) expression or processing. Shattuck-Brandt et al. [39] have shown that enhanced degradation of I $\kappa$ B $\alpha$  may contribute to endogenous activation of NF- $\kappa$ B in Hs294T melanoma, while Soldatenkov et al. [40] have shown that inhibition of Slimb sensitizes melanoma cells for apoptosis. Whether these observations or the current findings are related to the high levels of antiapoptotic proteins present in melanoma cells is unknown but would be worth exploring [41]. The observations that NF- $\kappa$ B binding is constitutively activated in melanoma cells [39,4,42] and that NF- $\kappa$ B binding and apoptosis are unrelated indicate a basic disruption of this important pathway in controlling growth in melanoma cells.

Analysis of the interaction between the NF- $\kappa$ B system and superoxide anion indicate that this ROS does not have a significant role in regulating NF- $\kappa$ B in melanoma cells. In contrast, intracellular superoxide anion and AP-1 binding were strongly correlated. However, an interaction between AP-1 binding and the frequency of apoptosis was not evident. In the few systems in which the relationship of superoxide anion and AP-1 have been studied, there has been either no effect or AP-1 binding has increased apoptosis. Our prior work demonstrated that the regulation of AP-1 and its subunits was very abnormal in melanoma cells [10]. Since an intact Fas R/Fas L system (as are other proteins) is important for control of cell death, [43] the failure of AP-1 to be related to apoptosis could be mediated via this pathway as well.

It is of interest that hydrogen peroxide and superoxide anion have been shown to have differential effects on apoptosis in other cell systems [44]. The responsiveness of NF- $\kappa$ B and AP-1 recruitment to intracellular levels of hydrogen peroxide and superoxide anion without concomitant control of apoptosis provides a general mechanism by which these cells can escape noxious injury (e.g., chemotherapy). However, binding assays of transcription factors represent only a first approximation of biological activity. We plan in the future to perform reporter assays, as well as measurements of effector molecules, to better assess the mechanistic relationship of redox changes to apoptosis and biological responsiveness. On the other hand, the marked enhancement of apoptosis in melanoma cells by chelators indicates that this abnormality can be circumvented and offers a unique therapeutic window to explore.

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