Secretion of Extracellular Superoxide Dismutase From Muscle Transduced With Recombinant Adenovirus Inhibits the Growth of B16 Melanomas in Mice

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Abstract

A number of reports have described the effects of oxidative stress on tumor growth. Therefore, these experiments were designed to test the hypothesis that overexpression of extracellular superoxide dismutase (ecSOD) would inhibit the growth of tumors arising from s.c. implantation of syngenic B16-F1 melanoma cells. C57BL/6 mice were infected i.m. with adenovirus containing either β-galactosidase (Ad.IacZ) as control or the secreted extracellular isoform of SOD (Ad.ecSOD) 3 days before s.c. implantation of B16-F1 tumor cells. Serum SOD activity was elevated nearly 5-fold over control animals. Two weeks after implantation, B16-F1 tumor size was 65% smaller in mice infected with Ad.ecSOD in comparison with mice infected with Ad.IacZ. However, the presence of SOD did not affect growth rates of B16-F1 cells in vitro. Consistent with smaller tumor volume, tumors from Ad.ecSOD-infected mice also expressedless vascular endothelial growth factor (VEGF). Moreover, in vitro studies using B16-F1 cells confirm that SOD blunts oxidant-dependent VEGF expression. Importantly, CD31 expression and vessel density were markedly reduced in tumors from Ad.ecSOD-infected mice compared with controls. These data suggest that tumor oxidative stress may facilitate tumor vascularization and thus promote tumor growth.

Introduction

Nutritional factors are widely believed to be critical in tumorigenesis (1). Most of the anticancer benefits of dietary supplements such as fruits and vegetables are attributed to high concentrations of vitamin C and other potent antioxidants, such as lycopene found in tomatoes (2). It is becoming clear that supplementation with antioxidants has proven beneficial effects as lycopene found in tomatoes (2). It is becoming clear that supplementation with antioxidants has proven beneficial effects on tumorigenesis and the onset of some cancers (3–5); however, mechanisms for the anticancer effects of antioxidants are not totally clear. Moreover, while oxidative stress and free radical production in tumorigenesis is controversial, it is a likely mechanism, considering compelling epidemiological and experimental evidence in animal models (6).

The B16-F1 mouse melanoma model has been used extensively to test possible chemotherapeutic agents and is very convenient because it involves following tumor size for only 2 weeks (7, 8). It was demonstrated that B16-F1 cells cultivated in vitro and then transplanted s.c. into C57BL/6 mice produced an experimental model of tumor growth and vascularization with characteristics similar to those of the tumor of origin (9). Previous studies have shown that dietary supplementation with glycine, an anti-inflammatory amino acid, prevents increases in angiogenesis and subsequent tumor growth (10). B16-F1 melanoma tumor growth is largely dependent on expression and secretion of the angiogenic factor vascular endothelial growth factor (VEGF) by tumor cells (11). Moreover, it was recently shown that hydrogen peroxide (H2O2) up-regulated VEGF expression most likely through the oxidant-sensitive transcription factor NF-κB (12). It has also been demonstrated that treatment with antioxidant N-acetylcysteine blunted VEGF expression in a human melanoma cell line (13).

Recent evidence suggests that overexpression of gp91phox of the NADPH complex, which generates superoxide, is important for VEGF production and proliferation in NIH3T3 fibroblasts (14). Another recent report demonstrated that inducible nitric oxide synthase (iNOS)-dependent peroxynitrite formation, which also requires superoxide production, plays a critical role in B16 cell VEGF expression and tumor growth (15). These data support the hypothesis that reactive oxygen species such as superoxide, H2O2, or their reactive metabolites promote rapid tumor growth. Therefore, studies were designed to test the hypothesis that increasing extracellular superoxide dismutase (ecSOD) would inhibit growth of B16-F1 melanoma tumors in mice in vivo. To address this hypothesis, serum SOD levels were elevated by gene delivery of ecSOD using recombinant adenovirus. Using this approach, it is demonstrated that antioxidant gene transfer indeed has antitumor effects and likely blunts growth by blunting tumor vascularization.

Results

I.m. Injection of Recombinant Adenovirus Containing ecSOD Increases SOD Levels in Tumor Tissue

To address whether SOD overexpression would blunt B16 cell tumor growth, two approaches were used. The first approach was to infect mice i.m. with recombinant adenovirus containing...
either extracellular Cu/Zn SOD (Ad.ecSOD) or β-galactosidase (Ad.lacZ) before B16 cell implantation. The hypothesis was that ecSOD would be increased in serum and blunt B16 tumor growth. The second approach was to directly transfect B16 cells with CDNA of either SOD or β-galactosidase before implantation into naive mice. Here, it was hypothesized that tumor cells directly expressing SOD would have blunted growth in vivo.

To determine the expression levels of SOD in vivo, C57BL/6 mice were injected i.m. with recombinant adenosine (3 x 10⁷ plaque-forming units [pfu]) containing either extracellular Cu/Zn SOD or β-galactosidase. The expression and secretion of ecSOD was evaluated by measuring SOD activity in serum 3, 7, 10, and 17 days after infection. Three days after infection, SOD activity in serum was increased from 3.8 ± 0.4 units/ml in Ad.lacZ-infected control animals to 14.2 ± 0.6 units/ml (P < 0.05) and remained elevated near these levels at 7 and 10 days (13.8 ± 0.9 and 11.2 ± 1.4 units/ml). After 17 days, serum SOD activity was still elevated nearly 2-fold (5.8 ± 0.4 units/ml) over basal levels.

To assess whether SOD levels were elevated in the tumors, whole extracts from tumors removed from mice were evaluated by Western blot analysis against human SOD (~19 kDa). Tumors from Ad.lacZ-infected mice exhibited no detectable human SOD (Fig. 1, lane 1). More importantly, tumors from animals infected i.m. with Ad.ecSOD contained significant levels of human SOD (lane 3). The data are consistent with serum SOD activity data, indicating that Ad.ecSOD i.m. infection results in an increase in SOD levels in serum as well as in the tumor. Tumors from animals infected i.m. with recombinant adenosine containing the intracellular cytosolic isoform of SOD (Ad.SOD1) had no detectable SOD (lane 5).

Western blot analysis was also performed on tumor tissue that was transduced with adenosine containing β-galactosidase, ecSOD, or cytosolic SOD before implantation. Two weeks after implantation, SOD was detected in tumors transduced with cDNA for the ecSOD and the cytosolic SOD (lanes 4 and 6, respectively). On the other hand, no SOD was detected in β-galactosidase-transfected tumor cells, as expected (lane 2).

To determine whether overexpression of SOD influenced the expression of other antioxidant enzymes, tumor tissue was evaluated biochemically for catalase and glutathione peroxidase activities, as described in “Materials and Methods.” While high levels of catalase activity were observed, there were no differences in catalase or glutathione peroxidase activity between the groups (data not shown).

Overexpression of ecSOD Inhibits the Growth of s.c. B16-F1 Tumors

Food consumption did not differ between the two groups at any time point studied. Body weight gains were similar for both Ad.lacZ- and Ad.ecSOD-infected animals up to 7 days after tumor implantation (Fig. 2A), demonstrating that both groups of mice received adequate nutrition. However, Ad.lacZ-infected animals significantly outgrew the Ad.ecSOD-infected animals between days 7 and 14, consistent with a significant increase in tumor size (see below).

ecSOD blunted tumor growth with the final estimated tumor volume on day 14 in the Ad.ecSOD group reaching only 1142 ± 105 mm³ compared with 5553 ± 497 mm³ for mice infected with Ad.lacZ (P < 0.05), representing a tumor about 80% smaller (Fig. 2B). After 14 days, mice were sacrificed and tumors were removed. Representative photos of tumor-bearing mice as well as tumors from Ad.lacZ- and Ad.ecSOD-infected mice that are shown in Fig. 3, A and B, clearly demonstrate a significant difference in tumor size. The tumor’s gross appearance was soft, smooth, glossy, and dark black, as described previously (16). The large tumors in Ad.lacZ-infected animals appeared to be well attached and imbedded within connective tissue and blood vessels, whereas tumors in the Ad.ecSOD-infected mice were loosely adherent to the surrounding tissue. Tumors from control mice weighed 3.6 ± 0.5 g while tumors from Ad.ecSOD-infected mice weighed significantly less by ~75% (P < 0.05; Fig. 3C).

In the second approach, B16-F1 cells were directly infected in culture with recombinant adenosine (100 pfu/cell) containing the secreted ecSOD, the intracellular isoform of Cu/Zn SOD, or β-galactosidase as control. Cells were then s.c. implanted into C57BL/6 mice, as described above, and tumor volume was evaluated for 2 weeks (Fig. 3D). Implanted tumors cells infected with Ad.lacZ in vitro resulted in tumors similar in volume to those in Ad.lacZ-infected animals injected with normal B16-F1 cells, as described above. Overexpression of either isoform of SOD in B16-F1 cells blunted tumor growth after cells were implanted into mice.

Collectively, these data demonstrate that SOD overexpression in B16 tumors, either by direct infection of B16-F1 cells in vitro or by systemic overexpression in vivo, blunts tumor growth, supporting the hypothesis that oxidative stress participates in the mechanisms of tumorigenesis.

ecSOD Does Not Inhibit Growth of B16-F1 Cells In Vitro

The hypothesis that smaller tumors in Ad.ecSOD-infected animals was due to inhibition of B16-F1 cell growth was tested. To determine whether the presence of SOD inhibits B16-F1 cell proliferation, cells were transduced with adenosine containing cytotoxic Cu/Zn SOD, ecSOD, or β-galactosidase, and cell

![FIGURE 1. SOD expression in B16 tumors in vivo. Male C57BL/6 mice were infected i.m. with Ad.lacZ, Ad.ecSOD, or Ad.SOD1 (lanes 1, 3, and 5, respectively) 3 days before implantation of B16-F1 melanoma cells, as described in “Materials and Methods,” or mice were implanted with cells transduced in vitro with Ad.lacZ, Ad.ecSOD, or Ad.SOD1 (lanes 2, 4, and 6, respectively) before implantation. Two weeks after implantation, tumors were harvested, and tumor homogenates were evaluated for SOD expression by 16% SDS-PAGE and Western blot (WB) analysis. A representative gel blotted for β-actin is shown to demonstrate equal loading of samples. Data are representative of three individual experiments.](Image 101x162 to 269x232)
proliferation was determined. About 90% of the cells plated were adherent after 24 h, demonstrating that SOD did not affect cell plating efficiency (data not shown). Importantly, transduced cells grew at rates similar to vehicle-treated cells regardless of SOD expression, suggesting that SOD does not directly influence B16 cell proliferation at least in vitro. Growth rates were similar to a 24-h doubling time, as reported elsewhere (10).

**ecSOD Blunts Oxidative Stress in Tumor Tissue**

Because tumor size may directly affect parameters such as tumor oxidative stress and vessel density, the following experiments compared equally sized tumors from each group to mechanistically understand how oxidative stress affects tumor growth. Tumors were harvested at two different stages: before exponential growth when tumor volume was 600–750 mm³ (i.e., days 5–7 for both groups) or when tumor volume was estimated to be ~1000 mm³ (i.e., after 9–10 days for Ad.lacZ-infected mice and after 12–14 days for Ad.ecSOD-infected mice). This approach was taken for the subsequent studies.

**FIGURE 2.** Body weight gain in mice with implanted tumor cells. Male C57BL/6 mice were infected with Ad.lacZ or Ad.ecSOD 3 days before implantation of B16-F1 melanoma cells, as described in “Materials and Methods.” Cells were implanted on day 0, and animals were evaluated for 14 days. Body weights were monitored (A) and tumor volume was determined (B), as described in “Materials and Methods.” Points, mean; bars, SEM (n = 5 per group). *, P < 0.05, repeated-measures ANOVA.

**FIGURE 3.** Tumor size. Mice were infected with either Ad.lacZ or Ad.ecSOD before implantation of B16-F1 melanoma cells. A. Representative photographs of mice 14 days after receiving B16-F1 cell implantation. B. Photographs of representative tumors from mice infected with Ad.lacZ and Ad.ecSOD. C. At sacrifice, tumors were excised and weighed. D. Cells transduced with recombinant adenovirus containing Ad.lacZ, Ad.SOD1, or Ad.ecSOD were injected into normal mice; tumors were harvested 14 days later and weighed. Columns, mean; bars, SEM (n = 5 per group). *, P < 0.05, Student’s t test.
The hypothesis that oxidants are generated in tumors was tested. Sections of tumors from both Ad.lacZ- and Ad.ecSOD-infected mice were stained for 4-hydroxynonenol, a lipid peroxidation product, as an immunohistochemical marker of oxidative stress. In tumors harvested before exponential growth, no 4-hydroxynonenol staining was observed (Fig. 4B). Larger ~1000-mm³ tumors from Ad.lacZ mice exhibited significant 4-hydroxynonenol staining (Fig. 4D). Staining was also observed in regions of inflammatory cell influx in tumors of Ad.lacZ-infected mice but to a lesser degree in Ad.ecSOD-infected mice (Fig. 4F). Equally sized larger tumors from Ad.ecSOD-infected mice had little 4-hydroxynonenol staining and did not exhibit overlapping regions of hypoxia and lipid peroxidation, consistent with the hypothesis that generation of oxidants is blunted by overexpression of ecSOD.

SOD Blunts Nuclear Localization of NF-κB in B16 Tumors

Oxidants up-regulate angiogenic factors such as VEGF through a variety of mechanisms including the redox-sensitive transcription factor NF-κB (17, 18). Thus, the hypothesis that SOD blunts NF-κB activation was tested. Electrophoretic mobility shift assays (EMSA) were also performed on nuclear extracts from tumors of Ad.lacZ- and Ad.ecSOD-infected mice to evaluate NF-κB DNA binding activity (Fig. 5A). Indeed, tumors from Ad.lacZ-infected animals exhibited NF-κB DNA binding activity. Moreover, activity was significantly blunted in tumors from Ad.ecSOD-infected mice. Gel shift assay with anti-p50 and anti-65 antibodies and competition with cold probe was performed to verify the specificity of NF-κB DNA binding.

To test the hypothesis that SOD blunts oxidative stress-induced activation of NF-κB in vitro, cultured B16-F1 cells were infected with Ad.lacZ or Ad.ecSOD (multiplicity of infection [MOI] = 100 pfu/cell), as described above. Cells were then treated with xanthine oxidase (30 units/ml) in the presence of xanthine (500 μM) for 8 h to generate superoxide. Similarly, cells were treated with 400-μM H₂O₂ for 8 h. NF-κB activation was determined by EMSA (Fig. 5B). As expected, both

FIGURE 4. 4-Hydroxynonenol staining in B16-F1 melanoma cell tumors. Lipid peroxidation was measured by immunohistochemistry for 4-hydroxynonenol in tumor tissue from Ad.lacZ-infected (A, C, and E) and Ad.ecSOD-infected (B, D, and F) animals, as described in “Materials and Methods.” A-B. Tumors were evaluated before exponential growth (i.e., with an estimated volume of ~600–750 mm³) of adducts. C-F. Larger-sized tumors with an estimated tumor volume of ~1000 mm³ from Ad.lacZ and Ad.ecSOD were also evaluated, as described above. A-D. Representative photomicrographs at 40X magnification. E-F. Representative photomicrographs at 100X magnification.
xanthine/xanthine oxidase and H₂O₂ increased NF-κB DNA binding activity by 3.1- and 8.7-fold, respectively, in B16-F1 cells in vitro. Importantly, overexpression of SOD significantly blunted NF-κB activation by superoxide- and H₂O₂-induced activation of NF-κB by >60%.

Using xanthine/xanthine oxidase-treated B16-F1 nuclear extracts, supershift and competitive assays were performed to evaluate the subunit composition of NF-κB. Treatment with excess unlabeled probe nearly completely blunted DNA binding. Moreover, incubation with both p50 and p65 subunit antibodies caused a supershift in the NF-κB:DNA complex, confirming that the transcriptionally active p50:p65 heterodimer is induced by superoxide in B16-F1 cells in vitro.

Nuclear localization of NF-κB was determined immunohistochemically by staining for the active p65 subunit of NF-κB (Fig. 6, A and B). Light background staining (e.g., cytosolic localization) of p65 was observed in equally sized tumors from both Ad.lacZ- and Ad.ecSOD-infected mice. The high magnification field is shown to demonstrate the cellular distribution of p65 immunohistochemistry. Tumors from mice infected with Ad.lacZ exhibited significantly more nuclear localization of NF-κB than tumors from Ad.ecSOD-infected mice.

**ecSOD Overexpression Promotes Apoptosis in B16-F1 Tumors**

Activation of NF-κB has been associated with an anti-apoptotic effect in many cell types in response to apoptotic stimuli such as oxidative stress, transforming growth factor-β, and tumor necrosis factor-α. Because overexpression of ecSOD blunted tumor growth and inhibited maximal activation of NF-κB in large tumors, it was reasonable to hypothesize that apoptosis of B16-F1 cells compensates for the lack of tumor growth in animals overexpressing SOD. Apoptosis was evaluated by terminal deoxynucleotidyltransferase (TdT)-mediated nick end labeling (TUNEL) in equally sized tumors from Ad.lacZ- and Ad.ecSOD-infected mice (Fig. 6, C and D). In preexponential growth tumors less than ~750 mm³, minimal TUNEL staining was observed in tumors from Ad.lacZ-infected mice. In mice infected with Ad.ecSOD, TUNEL staining was slightly more prevalent but still minimal (data not shown). This is most likely due to the fact that little oxidative stress occurs at this time (see Fig. 4B). In large tumors isolated from Ad.lacZ-infected mice, minimal apoptosis was observed (Fig. 6C). On the other hand, a significant level of TUNEL staining was observed in equally sized tumors isolated from Ad.ecSOD-infected mice (Fig. 6D).

**ecSOD Blunts VEGF Expression in B16-F1 Tumors**

VEGF, which is produced by B16-F1 melanoma cells, is a critical angiogenic factor involved in tumor neovascularization (11). VEGF production is also enhanced in several cell types by oxidative stress (17, 18). Thus, the hypothesis that SOD inhibits VEGF production was tested in vivo by immunohis-

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**FIGURE 5.** Oxidative stress-induced activation of NF-κB in B16-F1 cells. A. Nuclear extracts of equally sized tumors (~1000 mm³) from Ad.lacZ- and Ad.ecSOD-infected mice were evaluated for NF-κB activity using EMSA, as described in “Materials and Methods.” B. B16-F1 cells were transduced with recombinant adenovirus (MOI = 100) containing the transgenes for α-galactosidase or ecSOD. Twenty-four hours later, cells were stimulated with 30 units of xanthine to generate superoxide or with 400 mM H₂O₂ for 2 h. Nuclear extracts (20 μg) were isolated and NF-κB DNA binding activity was determined by EMSA. Nuclear extract from xanthine/xanthine oxidase (X/XO)-treated B16-F1 cells was also incubated with 100-fold excess unlabeled oligonucleotide, anti-p50 antibody (4 μg), or anti-p65 antibody (4 μg). Labeled NF-κB oligonucleotide was also added. Data are representative of three individual experiments.
tochemistry (Fig. 7) and by Western blot analysis (Fig. 8A). VEGF staining was minimal in small tumors in both Ad.lacZ- and Ad.ecSOD-infected animals. However, in larger tumors (>1000 mm³), VEGF expression was significantly elevated and was localized primarily in regions of vessel growth in tumors from Ad.lacZ-infected animals (Fig. 7A). Interestingly, VEGF staining also coincided with 4-hydroxynonenol staining, suggesting indeed that VEGF production is related to oxidative stress. While still detectable, VEGF was about 4-fold less abundant than in equally sized tumors from Ad.ecSOD-infected mice (Fig. 7B), also supporting the hypothesis that oxidative stress is important for VEGF production in tumor tissue. A similar observation was made by Western blot analysis of tumor extracts from both Ad.lacZ- and Ad.ecSOD-infected mice (Fig. 8A). Tumors from Ad.ecSOD-infected animals expressed 65% lower levels of VEGF than tumors from control mice, suggesting that VEGF expression is in part enhanced by oxidative stress.

As described above for NF-κB activation, in vitro experiments were performed to evaluate VEGF production in response to oxidative stress. Cells infected with Ad.lacZ and Ad.ecSOD were stimulated by superoxide through xanthine/xanthine oxidase or by direct addition of 400-μM H₂O₂, and VEGF production was determined by Western blot analysis (Fig. 8B). Both superoxide and H₂O₂ stimulated VEGF production by 3.4- and 4.1-fold, respectively, in control cells expressing β-galactosidase. In cells overexpressing SOD, VEGF production was elevated only 1.8-fold in xanthine/xanthine oxidase-treated cells. SOD expression has less of an effect on H₂O₂-treated cells blunting VEGF

FIGURE 6. Activation of NF-κB and apoptosis in B16-F1 melanomas in vivo. A. Nuclear localization of the p65 subunit of NF-κB in equally sized tumors (>1000 mm³) from both Ad.lacZ- and Ad.ecSOD-infected mice was evaluated by immunohistochemistry using polyclonal antibodies against the p65 subunit of NF-κB. Representative photomicrographs at 200× original magnification. Inset, photomicrograph of tumor from Ad.lacZ-infected mice at 1000× magnification. Arrow, nuclear localization; arrowhead, cytosolic localization. B. Equally sized B16-F1 tumors (>1000 mm³) were harvested from mice infected with Ad.lacZ or Ad.ecSOD, as described above. Sections were evaluated by TUNEL for apoptotic cells, as described in “Materials and Methods.”

FIGURE 7. VEGF expression in B16-F1 melanoma. Equally sized tumor tissue (>1000 mm³) from Ad.lacZ- and Ad.ecSOD-infected animals was evaluated for VEGF expression by immunohistochemical analysis. Representative photomicrographs at 40× original magnification.
A VEGF expression in vivo

Ad.lacZ  Ad.ecSOD  p21

WB: VEGF

B VEGF expression in vitro

Ad.lacZ  Ad.EC-SOD  control  XNO  H2O2  control  XNO  H2O2

WB: VEGF  β-actin

FIGURE 8. Oxidative stress-induced VEGF production in vitro. A, Western blot analysis of VEGF expression was performed, as described in "Materials and Methods," using tumor tissue homogenates. B, B16-F1 cells were infected with recombinant adenovirus (MOI = 100) containing the transgenes for β-galactosidase or ecSOD. Twenty-four hours later, cells were stimulated with 30 units of xanthine in the presence of 500-μM xanthine to generate superoxide or with 400-mM H2O2 for 8 h. Total extracts including supematant and cell lysates were separated by 18% SDS-PAGE and analyzed by Western blot for VEGF expression. Western blot analysis for β-actin expression was also performed to determine equal loading. Data are representative of three individual experiments.

expression to a 2.8-fold increase over control levels. These data support the hypothesis that oxidative stress increases VEGF production in B16-F1 cells, which is consistent with other recent reports.

Overexpression of ecSOD Blunts Vessel Density

Tumor growth is often dependent on formation of new blood vessels for supply of oxygen and nutrients (19). Thus, the effect of ecSOD on vessel density of the equally sized tumors (i.e., nearly 1000 mm³) was assessed histologically (Fig. 9). The H&E staining clearly demonstrates a higher number of neovessels in tumors from Ad.lacZ-infected mice than those from Ad.ecSOD-infected mice.

Sections were stained for CD31 expression to directly assess vessel density within the tumor (Fig. 9, C and D). Indeed, tumors from mice infected with Ad.ecSOD exhibited less staining for CD31, consistent with the hypothesis that SOD blunted growth of new blood vessels. Importantly, tumor sections were scored for vessel density by densitometric analysis of CD31 expression, which was assessed by imaging 100 random microscope fields across the entire section as reported previously (20, 21). The histogram shows the proportion of fields with the given percentage of vessel area/tumor area. A leftward shift in the histogram is observed for the tumors from Ad.ecSOD-infected mice, suggesting a decrease in vessel density compared with tumors from Ad.lacZ-infected mice.

Discussion

SOD Blunts B16-F1 Tumor Growth in Vivo

Data presented here clearly demonstrate, for the first time, that overexpression of ecSOD inhibits the growth of B16-F1 tumor cells implanted s.c. in mice (Figs. 2 and 3). These data are consistent with the hypothesis that B16-F1 melanoma tumor growth is dependent on tumor oxidative stress. In addition, these data are consistent with two recent findings that iNOS and gp91phox, two oxidant-generating enzymes, participate in VEGF production and tumorigenesis (14, 15). These findings also suggest that inflammation may be an important aspect of oxidative stress in tumor tissue. Lipid peroxidation was indeed observed in regions of inflammatory cell influx in tumors of Ad.lacZ-infected mice but to a lesser degree in Ad.ecSOD-infected mice (Fig. 4F). More necrosis is present in Ad.lacZ tumors than in the Ad.ecSOD tumors. It is also apparent in some Ad.lacZ tumors that there is an increase in inflammation that often corresponds with an increase in necrosis (Figs. 4 and 7). Thus, oxidant production may be in part due to infiltrating neutrophils and macrophages, which produce radical species and growth factors that may stimulate tumor growth and vascularization.

It is commonly known that rapidly growing tumors become hypoxic (22, 23). Moreover, it is also noted in some tumors that oxidants are generated at areas, which border hypoxic regions in a hypoxia-reoxygenation-dependent mechanism (24). Indeed, it is confirmed here that tumors of both Ad.lacZ- and Ad.ecSOD-infected mice are hypoxic but that only tumors from Ad.lacZ-infected mice exhibited lipid peroxidation as a marker of oxidative stress (Fig. 4D). However, as mentioned above, it is not unlikely that other sources of oxidant production such as inflammatory cells containing gp91phox (NADPH) and iNOS contribute to oxidative stress in tumors.

SOD Minimizes VEGF Expression by B16-F1 Cells

B16-F1 melanoma tumor growth in vivo has been shown to be dependent on vascularization of the implanted tumor (25). VEGF, which is expressed by B16-F1 cells, is critical for neovascularization of growing melanomas (13, 26). It was recently shown that inhibition of VEGF signaling using neutralizing antibodies against VEGF suppressed B16-F1 melanoma growth as well as microvascular density within the tumor (11). In the studies reported here, tumors from mice overexpressing ecSOD had significantly less VEGF production and tumorigenesis (13, 26). It was also suggested that inflammation may be an important aspect of VEGF production in the B16-F1 cell (Figs. 7 and 8), which is a potential mechanism for vascularization and tumor growth. It should be noted, however, that other factors such as basic fibroblast growth factor are also important for tumor vascularization and growth.
SOD Blunts Oxidant-Dependent Activation of NF-κB in B16-F1 Cells

Data presented here clearly demonstrate that overexpression of ecSOD blunts the production of VEGF by melanoma cells in vivo (Fig. 7). These data support the hypothesis that oxidant production is important for the production of VEGF (17, 18). Interestingly, it was recently shown that treatment of VEGF-producing cells with H₂O₂ significantly enhanced VEGF production in vitro (12).

The mechanism by which oxidants increase VEGF production is not clear; however, it is known that VEGF production can be regulated through NF-κB, which is activated by oxidants, as well as hypoxia-inducible factor under certain conditions (12, 27–29). Thus, it is hypothesized that oxidants activate transcription factors that up-regulate the expression of VEGF in B16 cells. It is demonstrated here using in vitro experiments that SOD overexpression blunts superoxide and to a lesser degree H₂O₂-induced NF-κB activation (Fig. 5) and VEGF production (Fig. 8) by B16-F1 cells. These data, however, do not suggest that activation of NF-κB or the production of VEGF is necessary for B16 tumor growth, nor do they suggest that their inhibition by SOD overexpression is central to the antitumor properties of the antioxidant. However, these data do support a plausible hypothesis that redox-sensitive transcription factors may regulate the expression of angiogenic factors including VEGF.

FIGURE 9. Evaluation of B16-F1 melanoma vessel formation. A. Vessel density in tumors from Ad.lacZ- and Ad.ecSOD-infected mice was demonstrated in H&E-stained sections of formalin-fixed tumors. B. Immunohistochemical analysis of CD31 expression. Representative photomicrographs at 40× original magnification. E. Vessel density is defined as the percentage of vessel area (CD31)/tumor area in 100× fields. Columns, number of fields with a given vessel density; bars, fields with higher microvessel density. One hundred total fields were scored per tumor.
In conclusion, these data are consistent with the hypothesis that oxidant production contributes to tumor growth, consistent with recent findings that vitamin E inhibited hepatic tumor growth in c-myc/transforming growth factor-α transgenic mice (30). Moreover, these findings demonstrate the efficacy of an antioxidant gene delivery and suggest that it may be a clinically effective anticancer treatment. This is an intriguing finding, because the i.m. injection of adenovirus is an effective yet simple approach that overcomes the challenges of targeting specific tumor cells.

**Materials and Methods**

**Recombinant Adenoviral Vector Preparation**

All recombinant adenoviral vectors were produced by the Viral Vector Core of the Center for Alcohol Studies in conjunction with the Gene Therapy Center at the University of North Carolina at Chapel Hill. The adenoviral seed stock of Ad.EcSOD was the kind gift from Dr. Beverly Davidson of the University of Iowa. Each vector was constructed from plasmids containing human cDNA for the extracellular (secreted) isoform of SOD under the control of the cytomegalovirus promoter to regulate transcription. The cytomegalovirus promoter was also used to regulate the expression of the bacterial β-galactosidase reporter. The recombinant E1-deleted adenoviral vectors were originally generated, as described by Zwacka et al. (31). Virus was harvested from human embryonic kidney 293 cell lysates by CsCl gradient centrifugation after freeze/thawing. Viral titers were determined by plaque assay (32) and demonstrated a particle:pfu ratio of ~20:1. Neither of these recombinant vectors have been shown to have adverse effects in rodents at the titers used in these studies.

**B16-F1 Cell Culture**

The B16-F1 murine melanoma cell line was purchased from the University of North Carolina Tissue Culture Facility (original source: ATCC CRL 6322) and was maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) + 10% fetal bovine serum (Sigma, St. Louis, MO) + 20-mM HEPES (Life Technologies) + penicillin and streptomycin (100 IU/ml and 100 µg/ml, respectively). The B16-F1 variant was established by Fidler through in vitro selection (33). S.c. implantation of both B16 and B16-F1 cells yield tumors that are similar in gross and histological features (16).

B16-F1 cells were plated at a density of 5000 cells/ml in RPMI 1640 (Life Technologies) + 10% fetal bovine serum + 20-mM HEPES + penicillin and streptomycin. For growth studies, cells were transduced in vitro with recombinant adenovirus (MOI = 100 pfu/cell) containing β-galactosidase, human cytosolic Cu/Zn SOD, or extracellular isoform of SOD. Cells were washed with PBS, collected after brief trypsinization, and counted at daily intervals.

**Animals and Treatments**

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6–8 weeks of age (18–20 g). Three days before implantation of tumor cells, mice were infected with adenovirus (3 × 10⁹ pfu/25 g body weight i.m.) containing the gene for either ecSOD, an isof orm of SOD that is glycosylated and secreted, or β-galactosidase as control. Cells in log-phase growth (24–48 h after plating) were harvested by brief trypsinization and 5 × 10⁵ cells were injected s.c. in mice in 0.15-mI PBS. Animals were maintained on tap water and diets ad libitum, and food consumption and body weights were measured throughout the study. All animal treatments followed guidelines established by the Institutional Animal Care and Use Committee.

Palpable tumors were measured in two perpendicular diameters using digital calipers, and the radius was estimated by dividing the mean diameter by 2. Tumor volume, which correlates well with tumor weight (34), was calculated using the formula $v = 4/3\pi r^3$, assuming that the tumors were spheres (35). Fourteen days after implantation of cells, mice were sacrificed under pentobarbital anesthesia (60 mg/kg) and tumors were excised and weighed. In separate studies, equally sized tumors from both groups were harvested before exponential growth (i.e., <750 mm³) and during the growth phase (~1000 mm³). Sections of tumors were formalin fixed or frozen in liquid nitrogen for further studies.

**Histology and Immunohistochemistry**

Formalin-fixed tumor sections were stained with H&E to estimate vascular density. For immunohistochemical staining for VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), CD31 (Santa Cruz Biotechnology), the p65 subunit of NF-κB (Santa Cruz Biotechnology), and 4-hydroxynonenol (Alpha Diagnostic International, San Antonio, TX), formalin-fixed tumor sections were deparaffinized and rehydrated by standard protocols and were incubated with specific primary antibodies. Staining was visualized using the horseradish peroxidase-conjugated DAKO staining system (DAKO Corp., Carpinteria, CA).

**TdT-Mediated Nick End Labeling**

*In situ* cell death detection by the TUNEL was performed, as described previously (36). Tumor sections were incubated in the TdT buffer (25-mM Tris-HCl, 200-mM sodium cacodylate, 5-mM cobalt chloride, and 250-µg/ml BSA [pH 6.6]) for 15 min and then in 50 µl of TdT buffer containing 10 units of TdT and biotinylated dUTP (1 nmol) in a humidified chamber at 37°C for 60 min. The biotinylated dUTP molecules incorporated into nuclear DNA were visualized with FITC-conjugated streptavidin (1:100; room temperature for 30 min) using fluorescence microscopy.

**SOD, Catalase, and Glutathione Peroxidase Activity**

SOD activity was measured in serum and in tumor tissue from the reduction of ferricytochrome c, as described previously (37, 38). Catalase activity was measured in liver homogenate, as described by Aebi (39). Glutathione peroxidase activity was determined spectrophotometrically, as previously described by Flohe and Günzler (40).

**Western Blot Analysis**

Tumor tissue or cultured B16-F1 cells were homogenized in lysis buffer containing 25-mm HEPES, 150-mM NaCl,
2 mM EDTA, 1.5 mM MgCl₂, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM Na₃VO₄, 10 μg/ml leupeptin, and 10-mg/ml aprotinin. Homogenate was separated by 16% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-VEGF antibody (1:1000 dilution, Santa Cruz Biotechnology) or β-actin (1:2000 dilution, DAKO) followed by horseradish peroxidase-conjugated secondary antibody. Protein was visualized by radiography using ECL Western Detection Reagent (Amersham Life Sciences, England).

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were isolated as described by Dignam et al. with minor modifications (41). Binding conditions for NF-κB were characterized and EMSA was performed, as described elsewhere (42). Briefly, nuclear extracts from tumors (10 μg) were preincubated for 10 min on ice with 1-μg poly(deoxyinosinic-deoxyctydilic acid) and 20-μg BSA (Pharmacia/Biotech, Piscataway, NJ) and 2 μl of a 32P-labeled DNA probe (10,000 counts/min/μl, Cerenkov) containing 1 ng of double-stranded oligonucleotide in a total volume of 20 μl. Mixtures were incubated for 20 min on ice and resolved on 5% polyacrylamide (29:1 cross-linking) and 0.4× Tris-borate EDTA gels. After electrophoresis, gels were dried and exposed to X-OMAT LS Kodak film. Data were quantified by scanning autoradiograms with GelScan XL (Pharmacia, Uppsala, Sweden).

**Statistics**

ANOVA and repeated-measures ANOVA followed by the appropriate post hoc tests or Student’s t tests were used for data analysis. Two-sided tests were used in all cases and a P value of <0.05 was defined before the study as a significant difference between groups.

**References**


31. Zwicka, R. M., Dudas, L., Epperly, M. W., Greenberger, J. S., and...


