Tumor Necrosis Factor-α Is a Potent Endogenous Mutagen that Promotes Cellular Transformation

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Abstract

Tumor necrosis factor-α (TNF-α) is an important inflammation cytokine without known direct effect on DNA. In this study, we found that TNF-α can cause DNA damages through reactive oxygen species. The mutagenic effect of TNF-α is comparable with that of ionizing radiation. TNF-α treatment in cultured cells resulted in increased gene mutations, gene amplification, micronuclei formation, and chromosomal instability. Antioxidants significantly reduced TNF-α–induced genetic damage. TNF-α also induced oxidative stress and nucleotide damages in mouse tissues *in vivo*. Moreover, TNF-α treatment alone led to increased malignant transformation of mouse embryo fibroblasts, which could be partially suppressed by antioxidants. As TNF-α is involved in chronic inflammatory diseases, such as chronic hepatitis, ulcerative colitis, and chronic skin ulcers, and these diseases predispose the patients to cancer development, our results suggest a novel pathway through which TNF-α promotes cancer development through induction of gene mutations, in addition to the previously reported mechanisms, in which nuclear factor-κB activation was implicated. (Cancer Res 2006; 66(24): 11565-70)

Introduction

The cytokine tumor necrosis factor-α (TNF-α) is a major mediator of inflammation. TNF-α was discovered by virtue of its capacity to inhibit tumor growth. Recently, there is increasing evidence that TNF-α may act as an endogenous tumor promoter. Direct evidence for the involvement of TNF-α in malignancy comes from observations that disruption of the TNF-α signaling pathway could significantly attenuate chemically induced skin tumor formation (1, 2).

Previous studies have indicated that exposure to environmental carcinogen and chronic inflammation are two important underlying conditions for sporadic human tumor development, with the latter accounting for ~20% of human cancers (3). Despite numerous observations of inflammation-associated tumorigenesis in lung, liver, stomach, bowel, colon, bladder, and skin, the molecular and cellular mechanisms linking chronic inflammation to tumorigenesis has not been completely elucidated. Many reports have indicated a role for the inflammatory cytokine TNF-α, which is suggested to promote inflammation-associated cancer development by activating the transcription factor nuclear factor-κB (NF-κB) signaling (4, 5). NF-κB-activation has been shown to inhibit the death of precancerous or transformed cells during the development of inflammation-associated cancers (6–8). Other studies suggested that TNF-α mediates tumor promotion via a protein kinase Cα– and activator protein-1–dependent pathway (9).

In this study, we explored an additional pathway through which that TNF-α can induce carcinogenesis (e.g., the ability of TNF-α to induce genetic instability). Although TNF-α is not known to damage DNA directly and there is no published report of other cytokine-induced instability, our study revealed that TNF-α is a potent mutagen that causes DNA damage through the induction of reactive oxygen species (ROS). Consistent with the mutagenicity of TNF-α exposure is its ability to transform normal mouse embryo fibroblasts, a process that was suppressed by antioxidants. This finding suggested a novel mechanism through which inflammation can cause cancer. Apparently, the combined effects of NF-κB activation and mutagenesis are both important for driving malignant transformation of cells at the sites of inflammation.

Materials and Methods

Cell culture, chemicals, and animals. 379.2 cells are $p53^{-/-}$ colon carcinoma HCT116 cells, which were kindly provided by Dr. Bert Vogelstein of Johns Hopkins University (Baltimore, MD). 379.2 cells were cultured in McCoy 5A medium supplemented with 10% fetal bovine serum (FBS). L929 cells were maintained in DMEM supplemented with 10% equine serum. 10T1/2 and BALB/3T3 are mouse embryonic fibroblasts obtained from Cell Culture Facility of Duke University Comprehensive Cancer Center (Durham, NC). They were maintained in DMEM supplemented with 10% FBS. Recombinant human and mouse TNF-α were purchased from R&D Systems, Inc (Minneapolis, MN). *N*-acetyl-L-cysteine (NAC), vitamin C, vitamin E, actinomycin D, rotenone, and dichlorofluorescein diacetate (DCFDA) were purchased from Sigma, Inc. (St. Louis, MO). C57BL/6 mice were purchase from Charles River Laboratories, Inc. (Wilmington, MA). NF-κB1 (tm1Bal/J) knockout mice were purchase from The Jackson Laboratory (Bar Harbor, ME).

Gene amplification assays. Frequency of gene amplification was evaluated as described previously (10). Please refer to Supplementary Data.

Analysis of chromosomal aberrations. Chromosome aberrations were scored as described previously (11). Please refer to Supplementary Data.

Micronucleus assay. Cells were plated in the six-well plates 24 hours before treatment. After TNF-α treatment, cytochalasin B (5 μg/mL; Sigma) was added to the medium and cultured for 48 hours. Then, the medium was removed, and the cells were rinsed with PBS and fixed by Carnoy fixative (methanol/acetic acid, 3:1) for 5 minutes. The air-dried cells were then immersed in the 2× sodium chloride-sodium citrate buffer with 0.1% NP40 for 1 minute and then stained in 5 μg/mL acridine orange (Sigma-Aldrich) in PBS for 2 to 5 minutes with gentle shaking. Then, cells were washed with PBS, and the micronuclei were scored under a fluorescent microscope.

ROS assay. After treatment, freshly made DCFDA (final concentration of 2–5 μmol/L) was added to medium for 40 to 60 minutes. Cells were washed by PBS thrice and detached. The same number of cells in each well was transferred to one well in a 96-well black fluoroimmunoassay plate (Bellco Biotechnology, Vineland, NJ), and the fluorescence was quantified by FLUOstar Galaxy fluorescence plate reader (BMG Labtechnologies, Durham, NC).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Hydrodynamic delivery of DNA. Mice to be injected were weighed and the volumes of solution to be injected were calculated for each mouse individually based on 0.09 mL/g body weight. Four micrograms of N1-TNF-α mammalian expression plasmid were added to the calculated volume of solution and the DNA solution was injected to each mouse through tail vein over a period of 5 seconds by means of a sterile 3-mL syringe and 27 G1/2 needle.

Immunohistochemistry. Using a Leica CM 1850 cryotome (Meyer Instruments, Inc. Houston, TX), serial sections were cut into 12-μm frozen sections and adhered to poly-L-lysine–coated slides (Polysciences, Inc, Warrington, PA). The sections were fixed in 4% paraformaldehyde in PBS (pH 7.2) for 1 hour. The slides were washed with PBS for 2 × 5 minutes. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 15 minutes, tumor sections were blocked with 10% donkey serum for 25 minutes. Sections were incubated in primary mouse monoclonal antibody 8-hydroxydeoxyguanine (8-OHdG; dilution 1:2,000; marker for DNA oxidation and oxidative stress, mouse monoclonal, Japan Institute for the Control of Aging, Shizuoka, Japan) overnight at 4°C. After rinsing for 3 × 5 minutes with PBS, biotinylated donkey anti-mouse antibody (Jackson Immunoresearch, West Grove, PA) was applied for 30 minutes at room temperature. The slides were washed with PBS for 3 × 5 minutes followed by application of an avidin-biotin complex (Vectastain avidin-biotin complex method kit, Vector Laboratories, Inc., Burlingame, CA). Location of the reaction was visualized with NovaRED kit (Vector Laboratories). Omission of the primary antibody served as negative control.

Transformation assay. Mouse embryonic fibroblast cells were seeded in 10-cm dishes (35 dishes per group) in different numbers (as determined empirically). Twelve hours after seeding, cells were treated with TNF-α in the presence or absence of antioxidants for 2 weeks, during which TNF-α and medium were changed every three days. At the end of TNF-α treatment, cells were grown in fresh medium without TNF-α for additional 3 weeks. Then, cells were fixed and stained with 0.5% crystal violet in 80% methanol. Foci larger than 3 mm were identified as transformed foci.

Results

The mutagenic ability of TNF-α is comparable with that of ionizing radiation. We studied the ability of TNF-α as a mutagen in comparison with that of ionizing radiation (IR). IR is a potent mutagen and carcinogen. It is known to induce gene amplification, chromosome aberrations, and micronuclei formation. After a series of experiments, we found that TNF-α significantly induced gene amplification, chromosome aberrations, and micronuclei in various cells (Fig. 1). The mutagenic potency of TNF-α is comparable with that of 3 Gy γ-IR (Fig. 1).

TNF-α–elevated ROS. We hypothesized that the observed TNF-α–induced genetic instability may be caused by elevated ROS, which was shown to be induced by TNF-α exposure (12, 13). Production of ROS can occur at a slow rate wherever there is oxygen in high concentration. Small quantities of ROS are inevitable by-products of the electron transport chain in mitochondria. Normally, ROS are kept at very low level by enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase. However, the defenses against ROS accumulation may be overwhelmed by excessive ROS production that can lead to cellular damages. To examine whether TNF-α induces excessive intracellular ROS, we analyzed ROS level in various cell lines by DCFDA-based ROS assay. A significant increase in ROS (about 2- to 3-fold increase) was detected ~6 hours after treatment with TNF-α in BALB/3T3 and L929 cells (Fig. 2). Moreover, antioxidant treatment significantly decreased ROS in both cell lines. Vitamin E and NAC seemed to be more potent in decreasing ROS. Therefore, we chose them as antioxidants in subsequent experiments.

Where do the TNF-α–induced ROS species come from? As mitochondria are the sites of cellular respiration and normally

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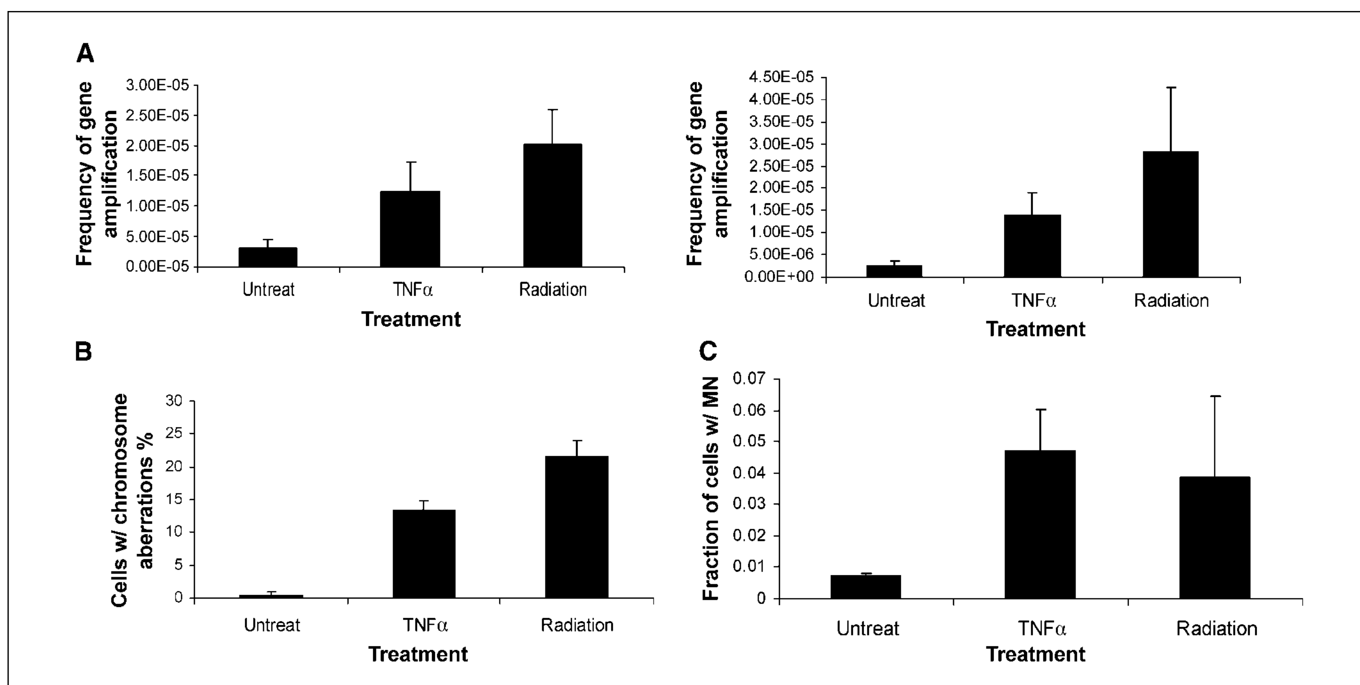

Figure 1. Mutagenicity of TNF-α in comparison with IR. *A,* gene amplification: L929 (*left*) or 379.2 (*right*) cells were treated with TNF-α (*TNFa*) or 3 Gy γ-irradiation. One or 2 weeks after TNF-α treatment or 4 days after radiation, the frequency of *N*-(phosphonacetyl)-L-aspartic acid–resistant cells was determined. *B,* chromosome aberrations: 379.2 cells were treated with TNF-α for 4 to 6 hours or 3 Gy γ-rays. Four hours after irradiation or immediately after TNF-α treatment, cells were incubated with colcemid for 2 hours to prepare metaphase spreads. Chromosome aberrations examined included chromatid break, double minutes, ring chromosome, and dicentric chromosome. *C,* Micronuclei (*MN*): 10T1/2 MEFs were treated with TNF-α for 6 hours or 3 Gy γ-ray. Then, cells were grown in the presence of cytochalasin B for 2 days before they were fixed and scored for micronuclei.

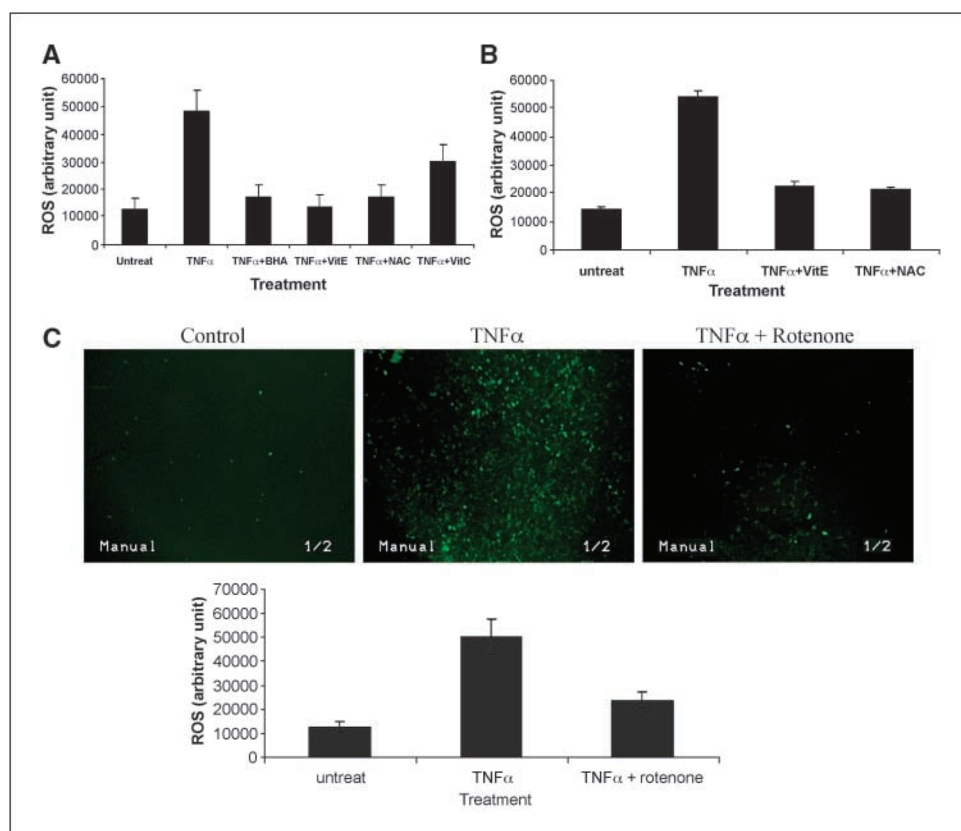

Figure 2. ROS assay in BALB/3T3 (*A*) and L929 (*B*) cells. Cells were treated or left untreated with TNF-α for 6 hours. Various antioxidants were added to the medium for 30 to 60 minutes before TNF-α treatment. At the end of TNF-α treatment, DCFDA was added and incubated with cells in the dark for 1 hour before measuring the fluorescence by the FLUOstar Galaxy fluorescence plate reader. *C,* inhibitor of mitochondrial respiration prevents generation of ROS. L929 cells were treated with TNF-α (50 ng/mL), in the absence or presence of rotenone (100 nmol/L) for 5 hours. *Top,* fluorescence micrographs of DCFDA-stained cells; *bottom,* quantitative determination (through the use of a fluorescence plate reader) of DCFDA fluorescence after 1 hour of DCFDA treatment.

produce small amount of ROS, it is possible that exposure to TNF-α leads to elevated ROS production/leakage from the mitochondria. To examine this possibility, we used an inhibitor of complex I of the mitochondrial electron transport chain, rotenone. We found that TNF-α–induced increase of ROS level was significantly suppressed by rotenone treatment ($P < 0.01$), indicating that mitochondria are an important source for TNF-α–induced ROS (Fig. 2*C*).

Antioxidant suppressed TNF-α–induced genetic aberrations. ROS can generate DNA damage directly or through activation of topoisomerases (14, 15). Elevated ROS level in TNF-α–treated cells correlated with increased instability. The frequency of gene amplification in TNF-α–treated L929 cells is about four times that of nontreated cells. Antioxidant vitamin E reduced gene amplification for ∼3-fold (Fig. 3*A*). Similar finding were observed in 379.2 cells (Fig. 3*A*). A more direct line of evidence for the involvement of ROS in TNF-α–induced DNA damage is TNF-induced chromosomal aberrations, mostly chromatid breaks. In the metaphase spreads prepared right after TNF-α treatment, 25% to 30% cells had chromosomal aberrations, whereas very few (<1%) had aberrations in nontreated cells. Vitamin E protected the chromosomes from being damaged by TNF-α–induced ROS (Fig. 3*B*). Similarly, TNF-α–induced micronuclei formation was also decreased by antioxidant (Fig. 3*C*).

TNF-α induces oxidative stress and DNA damage *in vivo*. It has been discovered that TNF-α plays critical roles in inflammation-initiated carcinogenesis. Experiments were conducted to examine whether TNF-α can induce oxidative DNA damage *in vivo*. We overexpressed TNF-α in mice by hydrodynamic DNA transfer. TNF-α can be expressed mainly in liver as well as other tissues, such as spleen, etc. These tissues secrete TNF-α and the concentration of TNF-α in peripheral blood was about 2 to 3 ng/mL as shown in Fig. 4*A*. 8-OHdG is a marker for oxidative stress and a type of nucleotide damage as well. Forced expression of TNF-α induced significantly increased levels of 8-OHdG in the liver tissue. On the other hand, antioxidants, such as vitamin E and NAC, significantly decreased the nucleotide damage caused by oxidative stress (Fig. 4*B*). This indicates that TNF-α can cause DNA damages through induction of ROS *in vivo* as well as *in vitro*, consistent with the hypothesis that TNF-α oxidative DNA damage might be involved in TNF-α–induced carcinogenesis.

NF-κB is an important mediator of TNF-α–induced signaling and activates the expression of antioxidant molecules, such as manganese superoxide dismutase (MnSOD; ref. 16). Therefore, it is possible that the status of NF-κB could influence the observed TNF-α–induced DNA damage. To determine the effect of NF-κB on TNF-α–induced DNA damage, We overexpressed TNF-α in wild-type (WT) and NF-κB1 (tm1Bal/J) knockout mice by hydrodynamic gene transfer. Immunohistochemical staining of the livers showed no difference in the level of 8-OHdG between the WT and NF-κB1 knockout mice (Supplementary Fig. S1). These results indicate that NF-κB does not play a significant role in TNF-α–induced DNA damages at relatively early time points when the tissues were examined. Apparently, the ROS-mediated DNA damage we observed occurred before NF-κB–mediated MnSOD expression can influence it.

TNF-α induces cellular transformation. Genetic instability is a hallmark of cancers. Increased instability can result in accumulation of mutations in critical genes, such as oncogenes and tumor suppressor genes (17). The consequence is the outgrowing of malignant cells and cancer development. To test whether TNF-α–induced genetic instability leads to oncogenic transformation, we carried out transformation assays. Treatment

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with TNF-α over 3 weeks induced transformation in mouse embryo fibroblasts. Antioxidants, NAC, and vitamin C decreased cellular transformation by at least 2-fold (Fig. 4C). Cells of 10 transformed clones were expanded and inoculated s.c. into 10 nude mice, respectively. Tumor growth was observed in all these 10 mice.

Discussion

In this study, we discovered that the inflammatory cytokine, TNF-α, directly causes DNA damages and leads to cellular transformation through the induction of ROS. This study brings up two new concepts, a mechanism through which a cytokine can induce genetic instability and the involvement of the TNF-α–mediated DNA damage pathway (separate from the NF-κB signaling) in inflammation-associated carcinogenesis.

DNA damages can be induced by conventional mutagens, such as radiation and chemicals, or endogenously from errors in DNA replication or ROS produced from cell metabolism. In this study, we found that endogenous cytokine TNF-α is a potent mutagen by virtue of its ability to induce ROS. It sheds new light into the roles of cytokine-induced genetic instability in cancer development. It is likely that other cytokines (e.g., Fas ligand) that can induce ROS may also be involved in carcinogenesis through the induction of ROS (18, 19). It is also likely such cytokine-induced ROS mechanisms my be involved in carcinogenesis induced by chemical mutagens and IR as they can clearly induce an inflammatory response.

There have been several reports indicating that TNF-α–activated NF-κB signaling was an important link between inflammation and carcinogenesis. Here, we provide further evidence for the essential role of TNF-α in inflammation-associated cancers. In parallel to the NF-κB signaling, TNF-α causes DNA damages and promote transformation. Therefore, TNF-α drives tumor development by promoting the accumulation of mutations and survival of precancerous or transformed cells.

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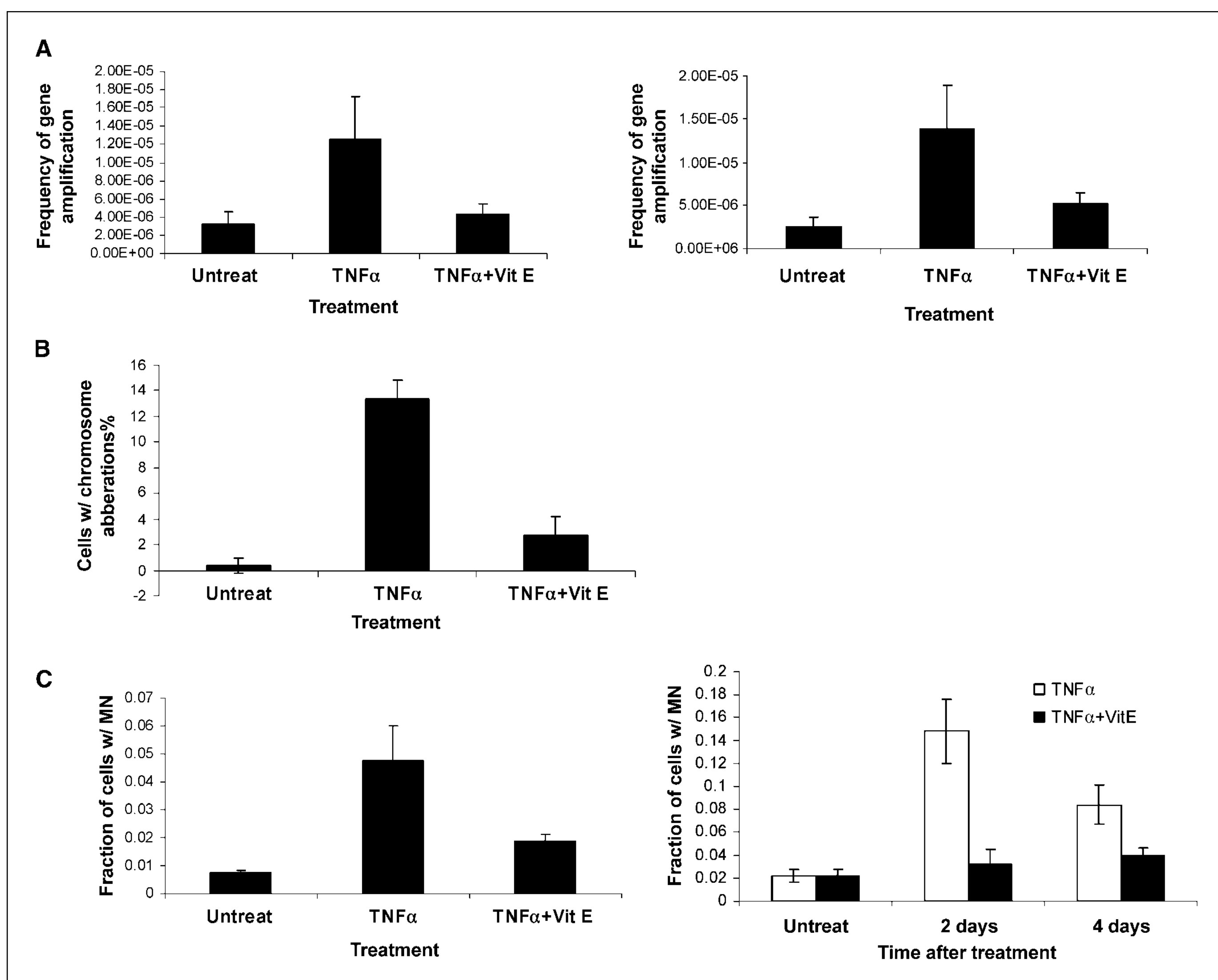

Figure 3. TNF-α–induced mutations were suppressed by antioxidants. *A,* L929 (*left*) and 379.2 (*right*) cells were treated with TNF-α in the presence or absence of the antioxidant, vitamin E (*VitE*). About 7 days later when the cells recovered, frequencies of amplification of *cad* gene were quantified by *N*-(phosphonacetyl)-L-aspartic acid selection. *B,* 379.2 cells were treated with TNF-α in the presence or absence of the vitamin E. Metaphase spreads were prepared immediately after 6 hours of treatment with TNF-α. *C,* 10T1/2 (*left*) and BALB/3T3 (*right*) cells were left untreated and treated by TNF-α alone or by TNFa and vitamin E for 6 hours. Immediately or 2 days after treatment, cells were grown in the presence of cytochalasin B for 2 days before being fixed and scored for micronuclei.

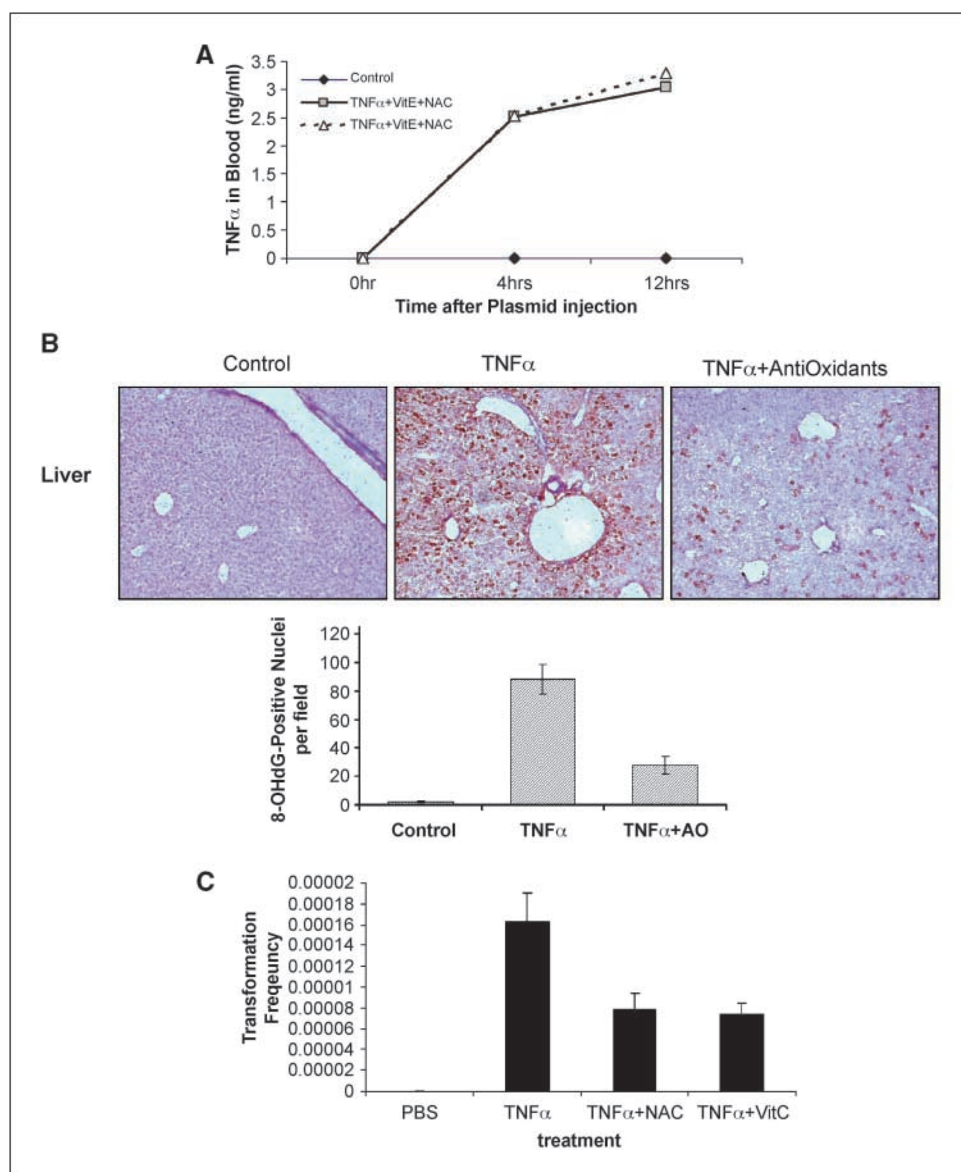

Figure 4. TNF-α–induced oxidative stress and DNA damages *in vivo. A,* expression of TNF-α in mice by hydrodynamic delivery of N1-TNF-α expression plasmid. Four micrograms of DNA in a calculated volume were injected into each mouse over a period of 5 seconds. Blood was drawn from each mouse at 4 and 12 hours after injection, and the concentration of TNF-α in blood was measured by the Quantikine TNF-α immunoassay ELISA kit (R&D Systems). *B,* induction of 8-OHdG by TNF-α in mouse liver tissue. Mice were sacrificed 7 hours after injection, and tissues were removed and frozen in liquid nitrogen and subsequently stained with an antibody specific to 8-OHdG. *Top,* the staining of liver tissue; *bottom,* quantification of the 8-OHdG–positive cells. *C,* frequency of TNF-α–induced oncogenic transformation in mouse embryonic fibroblasts.

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Given the important physiologic roles that TNF-α plays *in vivo*, it is indeed surprising to find that TNF-α can also be a potent endogenous mutagen. How does the organism use the normal antitumor, anti-infectious agent roles of the TNF-α while keeping its mutagenic properties at bay? The answer may lie in two levels of TNF-α regulation/response. One is that the TNF-α receptor is not present in all cells. Second is that cells that suffered extensive oxidative damage may be eliminated by a functional *p53* gene. Indeed, TNF-α–induced chromosome aberrations were found most prominently in $p53^{-/-}$ cells. In *p53* normal cells, it was rare to find cells with chromosomal aberrations after TNF-α treatment probably because the cells suffering DNA damages by TNF-α treatment were arrested in cell cycle or undergo P53-induced apoptosis by the effect of P53.

In summary, our data suggest that ROS may play a significant role in mediating TNF-α–induced carcinogenesis through the induction of oxidative DNA damage. This additional mechanism may have important implications for both cancer etiology and cancer prevention.

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