Controlling Tumor Growth by Modulating Endogenous Production of Reactive Oxygen Species

Alexis Laurent,1,5 Carole Nicco,1 Christiane Chéreau,1 Claire Goulvestre,1 Jérôme Alexandre,1,3 Arnaud Alves,1 Eva Lévy,1 François Goldwasser,1 Yves Panis,1 Olivier Soubrane,1 Bernard Weill,1 and Frédéric Batteux1

1Laboratoire d’Immunologie and 2Laboratoire de Recherche Chirurgicale, Unité Propre de Recherche de l’Enseignement Supérieur 1833, Faculté Cochin, Université Paris V; 3Service d’Oncologie Médicale, Hôpital Cochin; 4Service de Chirurgie Digestive, Centre Hospitalier Universitaire Lariboisière, Université Paris VII, Paris, France; and 5Service de Chirurgie Digestive, Centre Hospitalier Universitaire Henri-Mondor, Université Paris XII, Créteil, France

Abstract
Paradoxically, reactive oxygen species (ROS) can promote normal cellular proliferation and carcinogenesis, and can also induce apoptosis of tumor cells. In this report, we study the contribution of ROS to various cellular signals depending on the nature and the level of ROS produced. In nontransformed NIH 3T3 cells, ROS are at low levels and originate from NADPH oxidase. Hydrogen peroxide (H2O2), controlled by the glutathione system, is pivotal for the modulation of normal cell proliferation. In CT26 (colon) and Hepa 1-6 (liver) tumor cells, high levels of ROS, close to the threshold of cytotoxicity, are produced by mitochondria and H2O2 is controlled by catalase. Human- or mouse-derived N-acetylcysteine, which decreases H2O2 levels, inhibits mitogen-activated protein kinase and normal cell proliferation but increases tumor cell proliferation as H2O2 concentration drops from the toxicity threshold. In contrast, antioxidant molecules, such as mimics of superoxide dismutase (SOD), increase H2O2 levels through superoxide anion dismutation, as well as in vitro proliferation of normal cells, but kill tumor cells. CT26 tumors were implanted in mice and treated by oxaliplatin in association with one of the three SOD mimics manganese(III)-tetrakis(4-benzoic acid) porphyrin, copper(II)(3,5-diisopropylsalicylate)2, or manganese dipyridoxyl diphosphate. After 1 month, the volumes of tumors were respectively 35%, 31%, and 63% smaller than with oxaliplatin alone (P < 0.001). Similar data were gained with Hepa 1-6 tumors. In conclusion, antioxidant molecules may have opposite effects on tumor growth. SOD mimics can act in synergy with cytotoxic drugs to treat colon and liver cancers. (Cancer Res 2005; 65(3): 948-956)

Introduction
Reactive oxygen species (ROS) are natural by-products of aerobic metabolism and their production correlates with normal cell proliferation through activation of growth-related signaling pathways (1). Indeed, exposure to low levels of ROS can increase growth of many types of mammalian cells, whereas scavengers of ROS suppress normal cell proliferation in human and rodent fibroblasts (2-4). Furthermore, growth factors trigger hydrogen peroxide (H2O2) production that leads to mitogen-activated protein kinase activation and DNA synthesis, a phenomenon inhibited by antioxidant molecules (5, 6). Several observations suggest that ROS also participate in carcinogenesis. First, ROS production is increased in cancer cells and an oxidative stress can induce DNA damages that lead to genomic instability and possibly stimulate cancer progression (7). Second, elevated ROS levels are responsible for constant activation of transcription factors, such as nuclear factor-κB and activator protein 1, during tumor progression (8). Finally, the transforming capacity of ROS is illustrated by the oncogenic transformation of NIH 3T3 cells by the NADPH oxidase homologue MOX-1, which induces the production of superoxide anions (O2−; refs. 9, 10).

Whereas, under certain circumstances, ROS promote cell proliferation, they can also induce apoptosis. Indeed, most anticancer drugs kill their target cells at least in part through the generation of elevated amounts of intracellular ROS. ROS can stimulate proapoptotic signal molecules, such as apoptosis signal regulating kinase 1, c-Jun-NH2-kinase, and p38 (11, 12); activate the p53 protein pathway; or engage the mitochondrial apoptotic cascade (13). The various ROS can exert different effects according to their nature and to their intracellular level, which is determined by both their production rate and the activity of antioxidant enzymes. Using pharmacologic modulators of the ROS pathways, we first investigated the source and nature of ROS produced in nontransformed cells and in established tumor cell lines and then determined their respective contribution to various cellular signals. Finally, we investigated the consequences of ROS modulation in association with cytolytic drugs in vitro and in vivo in mice with implanted tumors.

Materials and Methods

Animals. BALB/c female mice (for CT-26 tumors) or C57/BL6 female mice (for Hepa 1-6 tumors) between 6 and 8 weeks of age were used in all experiments (Ifla Credo, L’Aubresles, France). Animals received humane care in compliance with institutional guidelines.

Chemicals and Cell Lines. All chemicals were from Sigma (Saint Quentin Fallavier, France) except for mangafodipir (Teslascan, Amersham Health, Amersham, United Kingdom), oxaliplatin (Eloxatine, Sanofi-Pharma, Paris, France), and paclitaxel (Taxol, Bristol-Myers Squibb, Rueil Malmaison, France). CT26 (mouse colon carcinoma), Hepa 1-6 (mouse liver hepatoma), and NIH 3T3 (mouse fibroblast) were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM/Glutamax-I supplemented with 10% heat-inactivated FCS and antibiotics (Life Technologies, Cergy Pontoise, France). All cell lines were routinely tested to rule out Mycoplasma infection of cells.

Cellular Production of O2− and H2O2. Cells (2 × 104 per well) were seeded in 96-well plates (Costar, Corning, Inc., Corning, NY) and incubated

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

Requests for reprints: Frédéric Batteux, Laboratoire d’Immunologie, Pavillon Hardy, Hôpital Cochin, 75679 Paris cedex 14, France. Phone: 33-1-58-41-20-07; Fax: 33-1-58-41-20-08, E-mail: frederic.batteux@cch.ap-hop-paris.fr.

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for 48 hours with various concentrations of copper(II)(3,5-diisopropylsalicylate)2 (CuDIPS), manganese(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP), N-acetyl-L-cysteine (NAC), reduced glutathione (GSH), amino-triazol (ATZ), buthionine sulfoximine (BSO), catalase, or culture medium alone. Cellular production of O$_2^-$ was evaluated with the use of nitroblue tetrazolium reduction technique (14). The level of intracellular H$_2$O$_2$ was assessed spectrofluorimetrically (Victor2, Perkin-Elmer, Paris, France) by oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Leiden, the Netherlands). O$_2^-$ and H$_2$O$_2$ productions were reported to the amount of proteins in each sample (bovine serum albumin microbiuret assay, Pierce, Bezons, France).

**Determination of Enzymatic Activities.** The SOD activities of tumor or normal cells and tissues were evaluated with the use of nitroblue tetrazolium reduction technique as previously described by Beauchamp and Fridovich (14). The catalase activities of tumor or normal cells and tissues were determined at 25°C by UV spectroscopy at 240 nm according to Aebi (15). Levels of GSH were measured by the method of Baker et al. (16). Antioxidant enzyme activities and GSH content of cells and tissues were reported to the amount of proteins in each sample.

**In vitro Cell Proliferation and Viability Assays.** Cells (2 × 10$^4$ per well) were seeded in 96-well plates (Costar) and incubated for 48 hours with various amounts of pharmacologic modulators of antioxidant enzymes as indicated in the figure captions. Cell proliferation was determined by pulsing the cells with [3H]thymidine (1 Ci/well) during the last 16 hours of culture. Cell viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan. The absorbance at 550 nm was recorded in each well with the use of an ELISA microplate reader. Results are expressed as % viable cells ± SE versus cells in culture medium alone (100% viability). Cell viability was controlled in all cases by the crystal violet assay.

**In vitro Cytostatic and Cytotoxic Effects of Exogenous H$_2$O$_2$.** Cells (5 × 10$^4$ cells/well) were seeded as above in culture medium supplemented or not with 400 mol/L NAC, or 400 mol/L ATZ (a catalase inhibitor) or 400 mol/L BSO, which depletes GSH. H$_2$O$_2$ oxidative stress was elicited by the addition of serial dilutions of H$_2$O$_2$ (Sigma) to the cells for 48 hours. The cytostatic and the cytotoxic effects of H$_2$O$_2$ were evaluated by thymidine incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction.

**Measurement of O$_2^-$ Production by Flow Cytometry.** Cells were treated with either 10 μmol/L rotenone (an inhibitor of mitochondrial complex I), 10 μmol/L antimycin (an inhibitor of mitochondrial complex III), 10 μmol/L diphenyleneiodonium (an inhibitor of NADPH oxidase), or

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**Table 1.**

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<th>O$_2^-$ (pmol/min/μg)</th>
<th>CT 26</th>
<th>Hepa 1-6</th>
<th>NIH 3T3</th>
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<tr>
<td>Culture medium</td>
<td>3.71 ± 0.085†††††</td>
<td>1.21 ± 0.027†††††</td>
<td>0.36 ± 0.002</td>
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<td>NAC (400μM)</td>
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<td>GSH (400μM)</td>
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<td>1.17 ± 0.058</td>
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<td>BSO (400μM)</td>
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<td>1.12 ± 0.002</td>
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<td>CAT (100μL)</td>
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<td>ATZ (400μM)</td>
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<td>0.33 ± 0.002</td>
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<td>MnTBAP (100μM)</td>
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<td>0.81 ± 0.008***</td>
<td>0.21 ± 0.009***</td>
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<tr>
<td>CuDIPS (100μM)</td>
<td>2.39 ± 0.139****</td>
<td>0.91 ± 0.020****</td>
<td>0.22 ± 0.012****</td>
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**Figure 1.** Production of O$_2^-$ and H$_2$O$_2$ in nontransformed and tumor cells. **Dotted line,** basal level of H$_2$O$_2$. Note the change of scale for NIH 3T3. O$_2^-$ and H$_2$O$_2$ productions were reported to the amount of proteins in each sample and expressed as means ± SE pmol/min/μg for O$_2^-$ and arbitrary units/μg for H$_2$O$_2$ (A and B). Antioxidant enzyme activity and glutathione content of tumor and nontransformed cells and tissues were measured and reported to the amount of proteins in each sample (C). Data from at least three independent experiments have been pooled. Statistical significance: *, versus culture medium alone (basal level); †, versus NIH 3T3 cells.
10 μmol/L allopurinol (an inhibitor of xanthine oxidase) for 30 minutes in culture medium. Cells were washed in HBSS and resuspended in HBSS containing 10 μmol/L DHE (Molecular Probes) at 37°C for 30 minutes. After two washes, cells were resuspended in 500 μL HBSS. Cells (20,000 events per sample) were analyzed by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA).

**Immunoblotting of Cell Lysates.** Cells were treated or not with 400 μmol/L NAC for 24 hours then lysed in ice-cold 10 mmol/L Tris buffer, pH 7.5, with protease inhibitors, 25 mmol/L NaF, 0.5 mmol/L sodium orthovanadate, and 1% Triton X-100. Forty micrograms of cell lysate were analyzed by immunoblotting after 10% SDS-PAGE with the use of anti–extracellular signal-regulated kinase 2 monoclonal antibody from Santa Cruz (Le Perray en Yvelines, France), and anti p-ERK monoclonal antibody from New England Biolabs (Saint Quentin en Yvelines, France).

**DNA Strand Break Analysis.** The pcDNA3.1 plasmid DNA (Invitrogen) was incubated with oxaliplatin at a molar ratio of 0.50 in a final volume of 50 μL. MnTBAP (5 μmol/L), CuDPBi (5 μmol/L), MnDPDP (5 μmol/L), or NAC (5 mmol/L) was added into the mixture. O$_2^-$ was generated with the use of 200 μmol/L xanthine (Sigma) and 1 unit of xanthine oxidase (Sigma) in the dark at 37°C for 24 hours. Then, 10 μL aliquots were electrophoresed on a 0.8% agarose gel and revealed by ethidium bromide staining. The gels were analyzed with the use of a scanner densitometer (Vilber Lourmat, Marnes-la-Vallée, France).

**In vivo Antitumor Activity of Antioxidant Treatments.** CT-26 or Hepa 1-6 tumor cells (2 x 10$^6$) were injected s.c. into the back of the neck of BALB/c or C57/B16 mice, respectively. When the tumors reached a mean size of 200 to 500 mm$^3$, the animals received a single injection of either 20 mg/kg oxaliplatin (Eloxatine) or of vehicle alone. Mice were then injected or not with 10 mg/kg MnDTPB, 10 mg/kg MnTBAP, or 10 mg/kg CuDIPS or with 150 mg/kg NAC i.p. (three injections weekly at the same dosages for 1 month). Tumor size was measured with a vernier caliper every 3 days. Tumor volume was calculated as follows: $TV (mm^3) = (L \times W^2)/2$, where $L$ is the longest and $W$ the shortest radius of the tumor in millimeters. Results are expressed as means of tumor volumes ± SE (n = 15 in each group). In five mice implanted with CT26 cells, biopsies have been done every 15 days for 45 days. Tumor cells were then compared in vitro with cells from the

![Figure 2. Measurement of intracellular generation of O$_2^-$ by flow cytometry.](image-url)
original CT26 line in terms of ROS production, proliferation rate, intracellular GSH content, and sensitivity to oxaliplatin.

Statistical Analysis. The statistical significance of differences between experimental treated groups and untreated controls was analyzed by $\chi^2$ test for incidence data and by Student’s $t$ test for comparison of means. A level of $P < 0.05$ was accepted as significant. * or $\dagger\dagger: P < 0.05$; ** or $\dagger\dagger\dagger: P < 0.01$; *** or $\dagger\dagger\dagger\dagger: P < 0.001$ versus controls.

Results

Levels of ROS and of Antioxidant Enzymes in Normal and Tumor Cells. Both CT-26 and Hepa 1-6 cell lines produced higher amounts of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ than NIH 3T3 fibroblasts ($P < 0.001$; Fig. 1A and B). SOD activity was 3-fold lower in CT26 and Hepa 1-6, and catalase activity 2-fold (CT26) and 3-fold (Hepa 1-6) lower than in NIH 3T3. The level of GSH was 2- to 3-fold higher in NIH 3T3 cells than in Hepa 1-6 and CT26 cells, respectively (Fig. 1C). Differences in terms of antioxidant enzyme concentrations between tumor and nontransformed cells were still greater when Hepa 1-6 and CT-26 cell lines were compared with normal liver (SOD, 44.0 ± 4.4 units/mg; catalase, 43.3 ± 2.0 units/mg; GSH, 2.4 ± 0.1 nmol/mg).

Contribution of the Various Antioxidant Pathways to ROS Production. MnTBAP and CuDIPS, mimics of MnSOD (17) and of Cu/ZnSOD (18), respectively, decreased $\text{O}_2^-$ production in all the cell types tested ($P < 0.001$); however, they increased intracellular $\text{H}_2\text{O}_2$ ($P < 0.001$). This effect resulted from the dismutation of $\text{O}_2^-$ into $\text{H}_2\text{O}_2$ that accumulated because of the weak catalase-like activity of the SOD mimics (Fig. 1A and B). $\text{H}_2\text{O}_2$ production decreased in a dose-dependent manner in all cell types incubated with the thiol-related compound NAC that displays a catalase-like and a glutathione reductase–like activity (Fig. 1B; ref. 19). The concentration of $\text{H}_2\text{O}_2$ decreased in NIH 3T3 cells treated by GSH and increased in NIH 3T3 cells treated with BSO, an agent that depletes the intracellular pool of GSH (Fig. 1B). By contrast, inhibition of catalase by ATZ increased $\text{H}_2\text{O}_2$ production in both tumor cell lines. ATZ had a milder effect (50% increase) in NIH 3T3 cells. Adding exogenous catalase decreased $\text{H}_2\text{O}_2$ level by 37% in CT26 and 33% in Hepa 1-6 tumor cells but had no effect in nontransformed cells (Fig. 1B).

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**Figure 3.** Effects of exogenous $\text{H}_2\text{O}_2$ on the proliferation and the viability of nontransformed and tumor cells. Cell proliferation was expressed as %cpm ± SE. Viability was expressed as percent ± SE versus cells in culture medium alone (100% viability). Data from at least three independent experiments have been pooled. Differences between untreated and antioxidant-treated cells were always significant with at least three concentrations of $\text{H}_2\text{O}_2$ except for BSO-treated CT26, BSO-treated Hepa 1-8 tumor cells, and ATZ-treated nontransformed NIH 3T3 cells (not significant) (A). Effects of antioxidant molecules on the in vitro proliferative rate of nontransformed and tumor cells. Cell proliferation expressed as %cpm ± SE versus (dotted line) cells in culture with medium alone. Data from at least three independent experiments have been pooled. Statistical significance is indicated for the highest concentrations of antioxidant molecules (B). Inhibition of mitogen-activated protein kinase expression by NAC, Cells were treated (+) or not (−) with 400 $\mu$mol/L NAC for 24 hours. In the presence of NAC, pERK, the phosphorylated form of ERK, is decreased (C). Model proposed to explain the opposite effects of various concentrations of intracellular $\text{H}_2\text{O}_2$ on cellular proliferation of nontransformed and tumor cells. In normal cells, the basal level of $\text{H}_2\text{O}_2$ is low and its increase is first associated with cell growth. In tumor cells, the high level of $\text{H}_2\text{O}_2$ is associated with rapid cell growth. However, any further increase in intracellular $\text{H}_2\text{O}_2$ inhibits tumor cell proliferation, whereas any decrease in intracellular ROS increases tumor cell growth (D).
Origin of ROS in Normal and Tumor Cells. Mitochondria are the main source of ROS in nonphagocytic cells (20) but other cytosolic enzymatic systems, such as NADPH oxidase (9) or xanthine oxidase (21), can generate $O_2^{.-}$. Rotenone and antimycin increased the production of $O_2^{.-}$ in CT26 or Hepa 1-6 tumor cells but not in NIH 3T3 cells (Fig. 2). By contrast, neither the NADPH-dependent oxidase inhibitor diphenyleneiodonium nor the xanthine oxidase inhibitor allopurinol could inhibit the production of $O_2^{.-}$ by tumor cells, whereas diphenyleneiodonium inhibited ROS production in NIH 3T3 fibroblasts (Fig. 2).

ROS Level Controls the Proliferative Rate of Normal and Tumor Cells. Treating nontransformed NIH 3T3 fibroblasts with low amounts of exogenous $H_2O_2$ increased their proliferative rate ($P < 0.01$ versus untreated cells; Fig. 3A). In contrast, incubation with increasing amounts of exogenous $H_2O_2$ or with molecules that increase intracellular $H_2O_2$ production, such as BSO or SOD mimics, resulted in growth arrest and cell death (Fig. 3A). Inhibition of the basal production of intracellular $H_2O_2$ by NAC, GSH, and, to a lesser extent, catalase, also decreased the proliferative rate (Fig. 3B). This phenomenon was associated with the inhibition of the p42/p44 mitogen-activated protein kinase pathway by NAC in NIH 3T3 cells (Fig. 3C).

Adding increasing amounts of exogenous $H_2O_2$ to CT26 or Hepa 1-6 tumor cells in culture led to a dose-dependent decrease in proliferation and to cell death (Fig. 3A). The cytostatic and cytotoxic effects of $H_2O_2$ were amplified by the catalase inhibitor ATZ but not by BSO. In addition, the incubation of tumor cells with SOD mimics consistently increased intracellular $H_2O_2$ concentration through the dismutation of $O_2^{.-}$ and abrogated cell proliferation (Fig. 3B). By contrast, NAC and catalase, the two most potent inhibitors of $H_2O_2$ production, significantly increased the proliferation rates of Hepa 1-6 and CT26 tumor cells. Among the other antioxidant

Figure 4. Production of $O_2^{.-}$ and $H_2O_2$, and intracellular levels of GSH in CT26 tumor cells exposed to increasing amounts of oxaliplatin. Differences between oxaliplatin-treated and untreated cells were always significant ($P < 0.01$). MnTBAP and CuDIPS significantly decreased $O_2^{.-}$ production ($P < 0.001$). Antioxidant treatment always affected significantly the production of $H_2O_2$ versus untreated cells ($P < 0.01$). Differences in GSH levels between antioxidant-treated and untreated cells were always significant ($P < 0.01$) except for ATZ and chloramphenicol acetyltransferase. Similar data were observed with Hepa 1-6 cells (not shown) (A). Effects of antioxidant treatment on the cytostatic properties of oxaliplatin. CT26 cells were cultured as above with oxaliplatin alone or in association with CuDIPS or MnTBAP at dosages of 0 μmol/L (D0), 100 μmol/L (D1), 50 μmol/L (D2), and 25 μmol/L (D3); or of NAC, GSH, ATZ, and BSO at the dosages of 0 μmol/L (D0), 400 μmol/L (D1), 200 μmol/L (D2), and 100 μmol/L (D3); or of catalase at dosages of 0 units (D0), 100 units (D1), 50 units (D2), and 25 units (D3). Cell proliferation was determined by thymidine incorporation. Data from at least three independent experiments have been pooled. At 5 μmol/L oxaliplatin, differences between antioxidant-treated (D1) and untreated (D0) cells were always significant ($P < 0.001$) except for ATZ and chloramphenicol acetyltransferase. Similar data were observed with Hepa 1-6 cells (not shown) (B).
molecules tested, mannitol and, to a lesser extent, seleno-L-methionine also stimulated the growth of tumor cell lines (Fig. 3B). Pro-proliferative effects of H$_2$O$_2$ were also observed on nontransformed human umbilical vein endothelial cells, and antiproliferative effects on A549 human lung carcinoma cell line (Supplementary Figs. 1 and 2) and on tumor cell lines with various mutations of the p53 gene (Supplementary Fig. 3).

The Effects of Anticancer Drugs Are Modulated by H$_2$O$_2$. In tumor cells cultured with various concentrations of the platinum antitumoral compound oxaliplatin (22), a dose-dependent increase in ROS production associated with a decrease in proliferation was observed upon addition of SOD mimics and BSO. Opposite effects were induced by NAC and GSH (Fig. 4). The catalase pathway was not involved in the antitumoral activity of oxaliplatin. The inhibition of the cytostatic and cytotoxic activities of oxaliplatin by NAC and their increase by SOD mimics was further investigated with the use of other anticancer molecules known to augment intracellular levels of H$_2$O$_2$ in tumor cells, such as paclitaxel (23) and 5-fluorouracil (5-FU; ref. 24). Incubating tumor cells with both drugs in association with increasing concentrations of NAC resulted in a dose-dependent decrease in the cytostatic and cytotoxic effects of paclitaxel or 5-FU on tumor cells versus incubation with paclitaxel or 5-FU alone (Fig. 5A). Reciprocal effects of SOD mimics MnDPDP, MnTBAP, and CuDIPS were observed.

H$_2$O$_2$ Is Responsible for DNA Damages. Oxaliplatin alters DNA structure as shown by the loss of the supercoiled form (form I DNA) and by the increase in the nicked circular form (form II DNA). H$_2$O$_2$ generated by oxaliplatin in association with that produced by metalloporphyrin SOD mimics through O$_2$$^-$-dismutation can increase the alteration of DNA structure as previously described (25). Indeed, the damages caused to DNA by oxaliplatin in vitro were increased by the addition of SOD mimics, whereas the association of NAC and oxaliplatin was antagonistic (Fig. 5B).

ROS Modulate Antitumoral Activity of Cytotoxic Drugs In vivo. When MnTBAP, CuDIPS, or MnDPDP (another SOD mimic; ref. 26) was repeatedly injected into BALB/c mice with CT26 tumors, the volumes of tumors were respectively 59% ($P < 0.01$), 28% (not significant), and 54% ($P < 0.01$) smaller than in untreated controls after 1 month (Fig. 6A). In animals treated by the association of oxaliplatin and either MnTBAP, CuDIPS, or MnDPDP, the volumes of tumors were respectively 35%, 31%, and 63% smaller after 1 month than with oxaliplatin alone ($P < 0.001$). Injecting NAC alone resulted in 44% increase in tumor volumes after 1 month compared with untreated mice ($P < 0.01$). NAC infusion into
oxaliplatin-treated mice completely abrogated the beneficial effect of oxaliplatin because, at 1 month, the volumes of tumors were similar to those in the absence of oxaliplatin. The same effects were observed in immunocompetent C57/BL6 mice injected with Hepa 1-6 cells (Fig. 6B). In implanted tumors iteratively biopsied for 45 days, time-dependent increases in GSH content ($P < 0.01$), $O_2^-$ and $H_2O_2$ production ($P < 0.001$ in both cases), and a time-dependent decrease in proliferation rate ($P < 0.001$) were observed at day 45 versus original cell line.

**Discussion**

Tumor cells are under a higher oxidative stress than normal cells (2, 27). In this report, we first show that tumor cells differ from nontransformed cells not only in the level but also in the origin of ROS produced and in the pathways involved in their control.

In nontransformed cells, we observed that ROS essentially originate from the cytosolic NADPH and are controlled by the GSH system. Our data are in agreement with the previous observation that Mox-1, a homologue of the gp91 phox, the catalytic moiety of the NADPH oxidase, increases $O_2^-$ and $H_2O_2$ generation in NIH 3T3 cells, the latter species being responsible for an increased mitotic rate, cell transformation, and tumorigenicity (9, 10). Similarly, as previously described (28), treating nontransformed fibroblasts or human umbilical vein endothelial cells with low amounts of exogenous $H_2O_2$ increased the proliferative rate of NIH 3T3 cells. The intracellular targets of $H_2O_2$ involved in cell growth are multiple. They include p42/p44 and p38 mitogen-activated protein kinase, p70S6K, signal transducers and activators of transcription, Atk/protein kinase B and phospholipase D signaling pathways, as well as direct inhibition of protein tyrosine phosphatase-1B (I, 8). The effects of ROS on the growth of nontransformed NIH 3T3 cells are finely regulated because detoxification of intracellular $H_2O_2$ by NAC decreased the proliferative rate. This already observed phenomenon is related to the suppression of cell cycle progression into $G_1$ phase by inhibition of the mitogen-activated protein kinase pathway (29).

The situation is quite different in established tumor cell lines, such as CT26 and Hepa 1-6, in which the increased generation of ROS resulted from both an elevated mitochondrial production and a profound decrease in the activity of antioxidant enzymes. No increase in the proliferative rates was observed whatever the amounts of exogenous $H_2O_2$ added to the cultures. On the contrary, adding increasing amounts of exogenous $H_2O_2$ or increasing its intracellular levels with the use of SOD mimics led to a dose-dependent decrease in proliferation and to cell death. Similar data have been obtained in A549 human lung carcinoma cell line and in cell lines with various $p53$ gene mutations, suggesting that ROS modulation induced by SOD mimics or NAC are not linked with a particular $p53$ status. Conversely, detoxification of $H_2O_2$, especially through the catalase pathway, stimulated tumor cell proliferation. Those data are in line with previous observations that MnSOD or Cu/ZnSOD gene transfection inhibits growth of glioma (30) and pancreatic tumor cell lines (31), a phenomenon that can be reverted by cotransfection of the catalase gene (32, 33). Our data are also in agreement with the report by Hussain et al. (34), showing that $p53$ is associated with up-regulation of MnSOD resulting in ROS overproduction and apoptosis, a phenomenon inhibited by overexpression of catalase.

Although $H_2O_2$ directly controls tumor cell proliferation, $OH^-$, which results from the conversion of $H_2O_2$ via the Fenton reaction, is also involved in that control. Indeed, addition of mannitol, a compound known to detoxify $OH^-$ (35), stimulated the growth of tumor cell lines. Along with catalase, the glutathione pathway could also be involved in the regulation of the intracellular

Figure 6. In vivo antitumor activity of antioxidant molecules. Mice bearing CT26 or Hepa 1-8 tumors received a single injection of either oxaliplatin or vehicle alone. Mice were then injected or not with MnDPDP, MnTBAP, or CuDIPS, or with NAC i.p. for 1 month (three injections weekly). Tumor size was measured every 3 days at the time of injections. Mean of tumor volumes ± SE (n = 15 in each group).
concentration of H$_2$O$_2$ and tumor cell growth. Indeed, adding seleno-$\text{-}$methionine, a molecule that stimulates glutathione peroxidase activity, augmented the proliferative rate of the colon carcinoma cell lines as previously described (36). However, adding reduced GSH or depleting intracellular GSH with BSO had weaker effects on the proliferation of tumor cells than exogenous catalase, probably because of the indirect effects of those molecules on the intracellular concentration of H$_2$O$_2$.

Cytotoxic drugs induce cellular stress responses and the generation of ROS. However, whether this phenomenon participates in the antitumoral activity of those drugs has remained unclear thus far. To address this issue, we have examined the combined effects of cytotoxic molecules with several modulators of ROS pathways. As already observed with cisplatin (37), oxaliplatin increased ROS production and decreased CT26 and Hepa 1-6 cell proliferation in a dose-dependent manner. Whereas the GSH system was not involved in the control of the basal proliferative rate of tumor cells, it modulated the ROS production and the antitumoral activity of oxaliplatin as shown by the decrease in ROS levels and the increase in tumor cell proliferation following addition of exogenous GSH. Several lines of evidence suggest that the antagonistic effects of GSH and oxaliplatin involve intracellular mechanisms and do not result from extracellular interaction of GSH with oxaliplatin. Indeed, in our study, tumor cells depleted of GSH by BSO had an increased sensitivity to oxaliplatin. Moreover, when exogenous GSH was added with oxaliplatin to tumor cells in culture, the initial increase in ROS levels was similar to that observed when cells were incubated with oxaliplatin alone. Those data confirm that platinum-induced oxidative stress involves oxidation of cellular components and depletion of intracellular GSH (37, 38). The inhibition of the cytostatic and cytotoxic activities of oxaliplatin by NAC and their increase by SOD mimics was further investigated with the use of other anticancer molecules known to augment intracellular levels of H$_2$O$_2$ in tumor cells. Incubating CT26 and Hepa 1-6 cells with the taxane-related compound paclitaxel (23) or with 5-FU (24), with increasing concentrations of NAC, resulted in a dose--dependent decrease in the cytostatic and cytotoxic effects of paclitaxel and 5-FU on tumor cells versus incubation with individual drugs alone. Reciprocal effects were observed with SOD mimics MnTBAP and CuDIPS that generate H$_2$O$_2$ through $O_2^\cdot^{-}$ dismutation.

DNA is the main target of platinum-related anticancer drugs. H$_2$O$_2$ generated by those drugs in association with that produced by metalloporphyrin SOD mimics can alter DNA structure (25). Indeed, the damages caused to DNA by oxaliplatin in vitro were increased by the addition of SOD mimics, whereas the association of NAC and oxaliplatin was antagonistic. The same was observed in vivo in murine models of colon cancers. During tumor growth, H$_2$O$_2$ production increased whereas ex vivo proliferation rates decreased, and the GSH content augmented whereas an ex vivo resistance to oxaliplatin developed. Consequently, the three SOD mimics studied decreased, whereas NAC increased tumor growth when given alone in mice with implanted tumors. In addition, when oxaliplatin was associated, the three SOD mimics potentiated, whereas NAC abrogated the antitumoral effect of oxaliplatin.

This observation should stimulate further clinical investigations because NAC is widely used after surgery as a mucolytic agent in patients operated for cancers. Our observation is in line with a previous large human study that showed an increased incidence in lung cancer in heavy smokers treated with antioxidant molecules (39). In conclusion, the growth of normal cells is triggered when those cells are submitted to oxidant signals directed to growth-related genes up to a critical threshold beyond which ROS become cytotoxic. Because in tumor cells, the level of endogenous ROS is close to that threshold, the simultaneous exposure to ROS-generating agents and to cytotoxic drugs dramatically increases the rate of cell death. Those data have clinical implications in humans. Indeed, antioxidant molecules probably play a protective role against cancer in healthy individuals by preventing DNA damages linked to the oxidative stress. However, once cancer cells have emerged, a cancer-promoting effect can result from the administration of agents that decrease intracellular H$_2$O$_2$ levels, whereas an anticancer activity is exerted by agents that tend to increase that level.

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**References**


8. Gupta A, Rosenberger SF, Bowden GT. Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignantly progressed mouse keratinocyte cell lines. Carcinogenesis 1999;20:2063–73.


Controlling Tumor Growth by Modulating Endogenous Production of Reactive Oxygen Species

Alexis Laurent, Carole Nicco, Christiane Chéreau, et al.


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