Novel Action of Paclitaxel against Cancer Cells: Bystander Effect Mediated by Reactive Oxygen Species

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Abstract

Generation of reactive oxygen species (ROS) has been observed in cancer cells treated with paclitaxel, but the underlying mechanisms and therapeutic implications remain unclear. In the present study, we showed that paclitaxel promoted ROS generation through enhancing the activity of NADPH oxidase (NOX) associated with plasma membranes. Treatment of breast cancer cells caused an increased translocation of Rac1, a positive regulatory protein of NOX, to the membrane fraction. The paclitaxel-induced ROS generation occurred rapidly within several hours of drug exposure, with O2•− and H2O2 accumulation mainly outside the cells while the intracellular ROS remained unchanged. Importantly, the increase in extracellular ROS caused lethal damage to the bystander cancer cells not exposed to paclitaxel, as shown by two different methods using coculture systems where the bystander cells were differentiated from the paclitaxel-treated cells by fluorescent or radioactive labeling. This cytotoxic bystander effect was also observed with other microtubule-targeted agents vincristine and taxotere but not with 5-fluorouracil or doxorubicin. This toxic bystander effect was enhanced by CuZnSOD that converts O2•− to H2O2 and was abolished by a catalase that eliminates H2O2. Furthermore, paclitaxel was able to induce an almost complete inhibition of proliferation of the bystander cells in the coculture system. Our study revealed a novel mechanism by which paclitaxel induces toxic bystander effect through generation of extracellular H2O2 from the membrane-associated NOX. This may contribute to the potent anticancer activity of paclitaxel and provide a novel basis to improve the clinical use of this important drug. [Cancer Res 2007;67(8):3512–17]

Introduction

Paclitaxel is a microtubule-targeted agent widely used in cancer therapy. Its primary cellular effect is to cause abnormal stabilization of the dynamic microtubule polymerization, leading to the failure of mitosis. In addition, paclitaxel also alters other cellular functions that involve microtubules, such as intracellular signaling and organelle transport and locomotion (1). Recent studies showed that paclitaxel is able to induce early reactive oxygen species (ROS) production in cancer cells, and hydrogen peroxide (H2O2) was found to be involved in paclitaxel-induced cancer cell death in vitro and in vivo (2–4). However, the mechanisms of paclitaxel-induced oxidative stress remain unknown. H2O2 mainly comes from the dismutation of superoxide (O2•−), a reaction catalyzed by superoxide dismutases (SOD). Most cellular O2•− is produced by mitochondrial respiratory chain as a product of aerobic metabolism (5). Another significant source of O2•− is NADPH oxidases (NOX), which are mainly associated with plasma membrane and produce O2•− outside the cells (6). NOX activity is regulated by the assembling of active enzyme complex from catalytic subunits gp91phox, p22phox, and Rac1 and other components. The translocation of Rac1 from cytosol to the plasma membrane seems to be a crucial step in NOX activation and O2•− production (7). The objectives of the present study were to determine the source or subcellular location of the ROS production induced by paclitaxel and to evaluate the possible contribution of ROS in mediating its cytotoxic effect.

Materials and Methods

Materials and cell culture. Dihydroethidine, N-acetyl-3,7-dihydroxyphenoxazine, and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Molecular Probe (Eugene, OR), and 1H-thymidine was purchased from Amersham (Piscataway, NJ). Mouse anti-Rac1, mouse anti-p-actin, and rabbit anti-p22phox antibodies were from BD Biosciences (San Diego, CA), Sigma (St. Louis, MO), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Human breast cancer cell line MCF7 was cultured in a 1:1 mix of DMEM and F12 medium. Human lung cancer cell line H1299, human leukemia cell line HL-60, and the mitochondrial respiratory-defective HL60/C6F clone were cultured in RPMI 1640. All media were supplemented with 10% fetal bovine serum (FBS). The HL60/C6F cell culture medium was also supplemented with 1 mmol/L sodium pyruvate, 50 mmol/L uridine, and an additional 2.7% glucose.

Assays of ROS production. Intracellular O2•− was measured by flow cytometry using dihydroethidine as described (5). SOD-inhibitable reduction of cytochrome c was used to detect extracellular O2•− release as previously described (8). Briefly, MCF7 cells in 96-well plates at 80% confluence were incubated with 100 nmol/L paclitaxel for 1 h in Hank's buffer saline (HBS). Cytochrome c (110 mmol/L) and catalase (70 units/mL) were then added, with or without 100 units/mL CuZnSOD. Absorbance was measured at 550 nm after a 20-min incubation. The A550 values were converted to nanomoles of cytochrome c reduced, using a net extinction coefficient of 2.1 × 105 (mol/L)−1 cm−1. O2•− concentration was calculated from the difference in the amounts of cytochrome c reduced in the absence and presence of CuZnSOD. Extracellular H2O2 was determined by fluorometry in 96-well plates using N-acetyl-3,7-dihydroxyphenoxazine as a cell-impermeable probe as previously described (9). The cells were incubated with 100 nmol/L paclitaxel, 50 mmol/L Amplex red, and 0.1 units/mL horseradish peroxidase in serum-free medium for 60 min at 37°C. Fluorescence was measured using excitation at 530 nm and detection at 590 nm. Because this assay has a high fluorescent background, samples containing the same medium and Amplex red reagents without cells were run in parallel as blank control for subtraction of the nonspecific fluorescent background from the test samples. The net values were then used to calculate extracellular H2O2 concentrations using a H2O2 standard curve determined under the same conditions.
Assay of NOX activity associated with cell membranes. Cells were scrapped and suspended in ice-cold buffer containing 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 100 mmol/L sucrose, and a cocktail of protease inhibitors. The cell suspension was homogenized with 15 strokes in a glass tissue homogenizer. The samples were centrifuged at 8000 × g at 4°C for 5 min to remove unbroken cells and nuclei. The supernatants were centrifuged again at 100,000 × g for 30 min to separate the membrane fraction (pellet) and the cytosolic fraction (supernatant) as described (10). Protein concentrations were determined using a BCA Protein Assay kit (Pierce, Rockford, IL) with bovine serum albumin as standard. Generation of O₂⁻ in the membrane fraction was measured by lucigenin chemiluminescence in 100 μL of HBS containing 100 μmol/L NADPH, 50 μmol/L lucigenin, and 1.75 μg of cell membrane proteins (11). After a 5-min incubation at 37°C, chemiluminescence was measured using a luminomter (Turner Designs, Sunnyvale, CA) for 1 min. The signal was normalized and expressed as arbitrary light units per microgram protein per minute.

Immunoblotting of membrane-associated proteins. Cell membrane fractions were isolated as described above, and the associated proteins (20 μg) were resolved by electrophoresis on 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blotted for molecules of interest with anti-Rac1 (1:2,000), anti-p22phox (1:500), and anti-actin (1:10,000) antibodies overnight. The bound primary antibodies were detected using proper horseradish peroxidase–conjugated secondary antibodies followed by detection with a SuperSignal enhanced chemiluminescence kit (Pierce). For sequential blotting, the membranes were stripped with a stripping buffer (Pierce) followed by reblotting with proper antibodies.

Assessment of cytotoxic bystander effect using fluorescent probes. For cell labeling, we used a fluorescent probe CMFDA, which passes cell membranes freely. Once inside the cells, it is converted to cell-impermeable green fluorescent product. After loading, cells remain fluorescent for several days and proliferate for at least four cell divisions (12). MCF7 cells were first incubated with 5 mmol/L CMFDA at 37°C for 30 min in serum-free medium, which was then replaced by fresh medium (with 10% FBS without CMFDA) for another 30 min before the cells were trypsinized for coculture with unlabeled MCF7 cells pretreated with 100 mmol/L paclitaxel for 3 h in six-well plates (50% confluence). Paclitaxel was removed by extensive washing with fresh medium, and the CMFDA-labeled cells in fresh medium were added to each well in the presence or absence of catalase or SOD as indicated. As a negative control, CMFDA-labeled cells were cocultured with untreated MCF7 cells. The ratio of CMFDA-labeled cells to unlabeled drug-treated cells was ~ 1:3. After 48 h of coculture, cell death was assessed by direct propidium iodide (PI) staining (1 μg/mL PI in 1 mL PBS per well). The dead cells with compromised membrane integrity became permeable to PI and their nuclei appeared either red (paclitaxel treated) or yellow (CMFDA-labeled cells, indicative of bystander effect). Cell images were captured using a Nikon fluorescence microscope (Eclipse TE300). To evaluate the ROS effect on the growth of bystander cells, the number of CMFDA-labeled cells was directly counted in 24-well plate under fluorescent microscope 4 h after plating (baseline cell number) and 48 h after coculture. In each well, cells were counted in three separate areas of 0.4 mm² per field. Each condition was done in triplicate. Thus, the cell density was calculated as the mean of nine separate measurements.

Measurement of toxic bystander effect by 3H-thymidine labeling. MCF7 cells (5 × 10⁴ per mL) were incubated with 2 μCi/mL 3H-thymidine for 48 h, washed twice with PBS, and trypsinized for coculture with unlabeled MCF7 cells (50% confluence) pretreated for 3 h with paclitaxel (50–500 mmol/L), taxotere (100–500 mmol/L), vincristine (300 mmol/L), 5-fluorouracil (5-FLU; 1 mmol/L), and doxorubicin (3 μmol/L) in a similar fashion as described above. Nonadherent cells were removed after overnight incubation. After a 96-h coculture, radioactivity in the culture medium released from the bystander cells was measured using a scintillation counter. The level of radioactivity in the medium was indicative of cell death from the 3H-thymidine–labeled population (13).

Statistical analysis. Student’s t test was used to evaluate the statistical differences of the experimental values between two samples to be compared.

Results and Discussion

Paclitaxel induces NOX activation and extracellular release of O₂⁻ and H₂O₂. We first examined the effect of paclitaxel on intracellular ROS levels in MCF7 cells. As shown in Fig. 1A, exposure of cells to a toxic concentration of paclitaxel (100 mmol/L) for 8 h did not cause any increase in cellular O₂⁻. In contrast, paclitaxel caused a significant increase in extracellular O₂⁻ and H₂O₂ at 1 h (Fig. 1B). This H₂O₂ increase was detectable as early as 30 min (but not at 15 min) and continued to increase linearly up to 90 min. Addition of exogenous CuZnSOD further increased the extracellular H₂O₂ by 45% in the paclitaxel-treated cells, suggesting an increased conversion of O₂⁻ to H₂O₂ by the enzyme. SOD showed no effect on extracellular H₂O₂ when cells were not exposed to paclitaxel (data not shown). Because neither CuZnSOD nor O₂⁻ is able to pass cellular membranes, these data suggest that O₂⁻ production in the paclitaxel-treated cells was directly released outside the cells where it is subsequently dismutated into H₂O₂.

The ability of paclitaxel to induce elevation of extracellular H₂O₂ was also observed in a different human cell line HL-60 (Fig. 1C). Interestingly, using a subclone of HL-60 (C6F cells) completely defective in mitochondrial respiration (5), we showed that although the overall ROS generation was low due to respiration defect, paclitaxel caused a similar fold of increase in extracellular H₂O₂ in this cell culture (Fig. 1C). These data suggest that mitochondrial respiratory chain was not a significant source of paclitaxel-induced ROS generation, and that membrane-associated NOX would be the likely site of ROS production. These observations prompted us to further measure the activity of NOX in membrane fractions of MCF7 cells incubated with or without paclitaxel. As shown in Fig. 1D, exposure of MCF7 cells to 100 nmol/L paclitaxel induced a 2-fold increase in NOX activity, as evidenced by an in vitro luminescence assay. Similar results were obtained with human lung cancer H1299 cells (data not shown). Immunoblotning showed that paclitaxel induced an increase in Rac1 protein (a positive regulator of NOX enzyme complex) in the membrane fraction of MCF7 cells (Fig. 1D), consistent with the activation of membrane-associated NOX.

Paclitaxel causes cytotoxic bystander effect mediated by H₂O₂. We speculated that the extracellular accumulation of ROS induced by paclitaxel might be toxic to bystander cells. To test this possibility, we first treated MCF7 cells with 100 nmol/L paclitaxel for 3 h and removed the drug by washing with fresh medium. A separate dish of MCF7 cells was prelabeled with a nontoxic green dye CMFDA (without paclitaxel) and then trypsinized for coculture with the washed MCF7 cells pretreated with paclitaxel (Fig. 2A). Adherent dead cells with compromised membrane integrity were revealed by directly staining with PI (red) in the culture dish. As shown in Fig. 2B, dead cells from the paclitaxel-treated population exhibited red fluorescent nuclear staining, whereas dead cells from the CMFDA-labeled bystander cells showed yellow nuclear staining due to colabeling with green (CMFDA) and red (PI) dyes. Quantitation of multiple samples showed that among the CMFDA-labeled bystander cells, the mean percentage of PI-labeled cells increased over 10-fold from 0.3% in the sample cocultured with control (untreated) cells to 3.6% in the sample cocultured with paclitaxel-treated cells (Fig. 2C; P < 0.01). It should be noted that this percentage probably underestimated the true percentage of dead cells because only the adherent cells were counted, and direct PI staining mainly revealed dead cells in the late stage. Nevertheless, among all
PI-labeled cells (red + yellow), the percentage of yellow cells was 21 ± 7%, suggesting that a significant portion of cell death was due to the bystander effect.

To further investigate the role of ROS in mediating cytotoxic bystander effect, the paclitaxel-treated cells and the CMFDA-labeled cells were cocultured in the presence or absence of catalase or CuZnSOD in the culture medium. Catalase (2,000 units/mL), a specific scavenger of H$_2$O$_2$, effectively prevented the cytotoxic bystander effect, as evidenced by a significant decrease in percentage of cells with yellow nuclear staining among the CMFDA-labeled cells (Fig. 2C; $P = 0.003$). In contrast, addition of CuZnSOD (100 units/mL) to the coculture did not prevent the cytotoxic bystander effect. Instead, SOD caused a slight (but statistically insignificant) increase in cell death among the CMFDA-labeled bystander cells (Fig. 2C; compare column 4 with column 5; $P = 0.3$). This slight increase likely reflected an increase of H$_2$O$_2$ in the medium due to conversion of superoxide to H$_2$O$_2$ catalyzed by SOD. Neither catalase nor SOD caused any significant change in the number of dead cells with red nuclear staining, indicating that neither enzyme was able to alter the direct cytotoxic effect of paclitaxel in MCF7 cells pretreated with the drug.

The ability of exogenous catalase in the culture medium to abolish the cytotoxic bystander effect strongly suggests that this effect was mediated by H$_2$O$_2$ and was not due to the possible presence of residual paclitaxel after washing.

Bystander cytotoxicity induced by microtubule-targeted agents. A different coculture method was then used to confirm the cytotoxic bystander effect of paclitaxel and to further evaluate other anticancer agents with different mechanisms of action. In this coculture system, the bystander cells were first labeled with $^3$H-thymidine for two cell cycles (48 h) and washed free of unincorporated $^3$H-thymidine before trypsinization for coculture with MCF7 cells pretreated with paclitaxel, vincristine, 5-FU, or doxorubicin. After 96 h of coculture, the radioactive DNA in the medium released from dead cells as a consequence of toxic bystander effect was quantified by liquid scintillation counting. As shown in Fig. 3A, the bystander toxic effect of paclitaxel was again observed, indicated by a significant increase of radioactivity released from the bystander cells. Interestingly, the microtubule destabilization agent vincristine also caused a substantial increase (32%) of radioactivity released from the bystander cells. Using the fluorescent double labeling coculture system described above, 300 nmol/L vincristine induced a significant increase of bystander...
cells death with yellow nuclei (2.3 ± 0.6% in treated sample compared with 0.7 ± 0.1% in control, \( P = 0.02 \)). We also tested various concentrations of paclitaxel (50–500 nmol/L) and taxotere (100–500 nmol/L) for the bystander cell killing activity and showed significant bystander effect in most concentrations tested (Fig. 3B). In contrast, a highly toxic concentration of 5-FU (1 mmol/L) or doxorubicin (3 μmol/L) did not cause cell death in the bystander cells detected by the radioactive assay (Fig. 3A), or by using CMFDA and PI double staining method (data not shown).

**Paclitaxel induces potent inhibition of bystander cell proliferation.** Based on the observation that H\(_2\)O\(_2\) released from the paclitaxel-treated cells was able to cause lethal damage to the bystander cells, we reasoned that it is possible that in addition to their severe toxic effect, the released ROS might also affect the proliferative capacity of the bystander cells. To test this possibility, the bystander cells were first labeled with CMFDA (green) and then cocultured with unlabeled MCF7 cells pretreated with or without paclitaxel in a similar fashion as described above. The density of the green cells (number of cells per mm\(^2\)) was then monitored over a 48-h period to evaluate their growing rate. As shown in Fig. 4A, the density of the green cells increased significantly 48 h after coculture with the control MCF7 cells, indicating that the CMFDA-labeled cells were viable and capable of proliferation, consistent with previous observation (12). Quantitation of multiple samples from separate culture wells

![Diagram](image_url)

**Figure 2.** Induction of cell death by paclitaxel in neighboring untreated cells. A, experimental design used to assess the bystander effect of paclitaxel. MCF7 cells labeled with CMFDA were cocultured with MCF7 cells pretreated with or without 100 nmol/L paclitaxel for 3 h. Drug was removed by repeat washing before coculture. B, after 48 h of coculture, cell killing was evaluated by PI staining and examined under fluorescence microscopy (×40 objective) as described in Materials and Methods. Dead cells from the paclitaxel-treated population exhibited red fluorescent nuclear staining, whereas dead cells from the CMFDA-labeled bystander cells showed yellow nuclear staining (arrow). C, quantitation of cell killing in adherent CMFDA-labeled cells cocultured with control or paclitaxel-treated cells in the presence or absence of 100 units/mL CuZnSOD or 2,000 units/mL catalase as indicated. Cell death was expressed as the percentage of PI-labeled cells counted from multiple fields (n = 9). Columns, mean of three independent experiments; bars, SD. *, \( P < 0.01 \); #, \( P = 0.003 \).

**Figure 3.** Bystander cytotoxicity induced by anticancer agents. A, MCF7 cells labeled with \(^{3}H\)-thymidine were cocultured with MCF7 cells pretreated with or without 100 nmol/L paclitaxel, 300 nmol/L vincristine, 3 μmol/L doxorubicin, or 1 mmol/L 5-FU for 3 h as described in Materials and Methods. After 96 h, the level of radioactivity released in the culture medium was measured. Radioactivity levels are expressed as percentage of radioactivity compared with the control (\(^{3}H\)-thymidine–labeled cells cocultured with untreated cells). Columns, mean of three independent experiments; bars, SD. **, \( P = 0.01 \). B, bystander effects of various concentrations of paclitaxel and taxotere were measured by the radioactivity release assay in a similar fashion as in (A). Columns, mean of four experiments; bars, SD. *, \( P = 0.05 \).
showed that the green cell density increased from 317 ± 26 to 598 ± 42 cells per mm² at 48 h after seeding to the coculture dish containing control (untreated) MCF7 cells (Fig. 4B). Importantly, when the green cells were coculture with MCF7 cells pretreated with 100 nmol/L paclitaxel for 3 h (drug was removed by washing with fresh medium before coculture), the proliferation of these bystander cells was almost completely inhibited (Fig. 4A and B). Similar results were obtained using the human lung cancer H1299 cells (data not shown). Thus, paclitaxel was able to induce a potent inhibition on proliferation of the bystander cells. These results were consistent with previous observations that exposure of cancer cells to H₂O₂ suppresses cell proliferation and induces cell death, even at micromolar concentration (14).

Several anticancer agents, such as arsenic trioxide, doxorubicin, bleomycin, cisplatin, 5-FU, and paclitaxel, have been shown to induce ROS generation in cancer cells (15, 16). Various mechanisms have been described, including respiratory chain disruption, redox cycling, or p53-mediated mitochondrial oxidase activation (5, 15, 16). To date, the mechanism responsible for ROS induction by paclitaxel remains elusive. Our study showed that at a clinically relevant concentration, paclitaxel is able to activate the membrane-associated NOX, leading to extracellular release of O₂, which is subsequently converted to H₂O₂ (spontaneous and/or SOD catalyzed). Plasma membrane-associated NOX is the main source of extracellular O₂ and also seems to be the main source of ROS production induced by paclitaxel. The ability of paclitaxel to cause ROS generation in the mitochondrial respiration-defective cells (HL-60/C6F clone) suggests that mitochondria respiratory chain is not the site where paclitaxel induces ROS production. The observation that paclitaxel treatment was followed by an increased translocation of Rac1 to the membrane fraction, a crucial step in active NOX assembly (7), further suggests that activation of NOX may be a key mechanism of action. This result is in line with previous report showing that microtubules play a key role in Rac1 transport, and that the polymerization state of microtubules affects the Rac1 location to the membranes (17). It is possible that the disturbance of microtubule polymerization by paclitaxel, taxotere, or vincristine render Rac1 stabilized in the active NOX complex, leading to increased ROS generation. Interestingly, we observed that higher concentrations of paclitaxel did not proportionally cause further increase in ROS generation (data not shown), suggesting that the drug-induced disturbance of microtubule polymerization state enhanced ROS production due to stabilization of active NOX, which could not be further activated by higher drug concentrations. This may also explain why the elevated bystander cell killing effect was not linearly concentration dependent (Fig. 3B).

Although the bystander effect of ionizing radiation has been well described (18), there are few reports of such effect with chemotherapy agent. By using two different methods, we showed that paclitaxel displayed cytotoxic bystander effect in neighboring cancer cells. By comparing the number of dead cells with yellow or red nuclear staining, we estimated that about 20% of cell death occurred in the bystander cells. Because the ratio of paclitaxel-treated cells and bystander cells in the coculture was 3:1 (25% bystander cells), it seemed that paclitaxel killed nearly the same proportion of cells in the population directly exposed to the drug and in those submitted to bystander effect under our experimental conditions. In addition, paclitaxel was able to almost completely inhibit the proliferation of the bystander cells. Taken together, these data suggested that the bystander effects, both lethal damage and inhibition of proliferation, may play a significant role in the antitumor activity of paclitaxel. It is worth noting that paclitaxel, like most anticancer drugs, has limited ability to reach tumor cells that are distant from blood vessels (19). Because H₂O₂ can diffuse relatively far from its production origin, it may act on cells that are not reached by paclitaxel. It was recently reported that SOD mimics that increase H₂O₂ production are able to increase the antitumor activity of paclitaxel (4, 14). Our findings that NOX activation by paclitaxel leads to bystander effects against cancer cells open new perspectives to improve the therapeutic activity of paclitaxel and potentially other microtubule-targeted agents.

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