1,25-Dihydroxyvitamin D₃ Enhances the Susceptibility of Breast Cancer Cells to Doxorubicin-induced Oxidative Damage

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ABSTRACT

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the hormonal form of vitamin D, has antancer activity in vivo and in vitro. Doxorubicin exerts its cytotoxic effect on tumor cells mainly by two mechanisms: (a) generation of reactive oxygen species (ROS); and (b) inhibition of topoisomerase II. We studied the combined cytotoxic action of 1,25(OH)₂D₃ and doxorubicin on MCF-7 breast cancer cells. Pretreatment with 1,25(OH)₂D₃ resulted in enhanced cytotoxicity of doxorubicin. The average enhancing effect after a 72-h pretreatment with 1,25(OH)₂D₃ (10 nm) followed by a 24-h treatment with 1 µg/mL doxorubicin was 74 ± 9% (mean ± SE). Under these experimental conditions, 1,25(OH)₂D₃ on its own did not affect cell number or viability. 1,25(OH)₂D₃ also enhanced the cytotoxic activity of another ROS generating quinone, menadione, but did not affect cytotoxicity induced by the topoisomerase inhibitor etoposide. The anti-oxidant N-acetylcysteine slightly reduced the cytotoxic activity of doxorubicin but had a marked protective effect against the combined action of 1,25(OH)₂D₃ and doxorubicin. These results indicate that ROS are involved in the interaction between 1,25(OH)₂D₃ and doxorubicin. 1,25(OH)₂D₃ also increased doxorubicin cytotoxicity in primary cultures of rat cardiomyocytes. Treatment of MCF-7 cells with 1,25(OH)₂D₃ alone markedly reduced the activity, protein, and mRNA levels of the cytoplastic antioxidants enzyme Cu/Zn superoxide dismutase, which indicated that the hormone inhibits its biosynthesis. This reduction in the antioxidant capacity of the cells could account for the synergistic interaction between 1,25(OH)₂D₃ and doxorubicin and may also suggest increased efficacy of 1,25(OH)₂D₃ or its analogues in combination with other ROS-generating anticancer therapeutic modalities.

INTRODUCTION

The notion that 1,25(OH)₂D₃, the hormonal form of vitamin D, plays a role in the host anticancer activity rests on epidemiological evidence that shows an inverse correlation between the incidence and malignancy of breast, prostate, and colon cancer and exposure to sunlight, vitamin D intake, and circulating levels of vitamin D metabolites (1). The anticancer activity of 1,25(OH)₂D₃ was demonstrated in in vivo studies in which it retarded the growth of implanted xenografts of human breast and colon cancer, melanoma, and retinoblastoma in nude mice (1). In vitro the hormone has direct cytostatic and cytotoxic effects on cancer cell lines of various origins (1, 2). In addition, 1,25(OH)₂D₃ increases the cytotoxic action of macrophages and lymphocytes (3, 4) and potentiates the cytostatic/cytotoxic effects of immune cytokines such as TNF (5, 6) and other inflammatory mediators (7). These actions may result in enhanced anticancer activity of the immune system. Various new vitamin D analogues with low calcemic activity but significant antiproliferative and differentiation-inducing activity have now been synthesized (8–10) and may be used as anticancer drugs in the foreseeable future.

Doxorubicin, one of the most effective agents in the treatment of breast cancer, belongs to the anthracycline family of antitumor antibiotics. The quinone that is functionally common to the members of this family may undergo a one-electron reduction to the corresponding semiquinone free radical by flavin-centered reductases including microsomal cytochrome P450 reductase, NADH dehydrogenase, cytochrome b5 reductase, and xanthine oxidase (11–13). In the presence of oxygen this free radical will form superoxide anions. Superoxide dismutation, catalyzed by SOD, yields hydrogen peroxide, which undergoes the Fenton reaction to form the highly toxic OH radical. Cells are able to detoxify some of these reactive molecules before they react with vulnerable cellular targets. The enzymes catalase, SOD, and the glutathione system play a key role in the cellular defense against free radical damage. The final damage occurring in the target cell as a result of oxygen radical formation will depend on the rate of formation of ROS and on the efficacy of the cellular defense mechanisms. Another mechanism by which members of the anthracycline family cause cell death is the inhibition of topoisomerase II action that results in double-strand DNA breaks (14–16).

Superoxides have a mediatory role in both TNF- and doxorubicin-induced cytotoxicity (11–13, 17). This common feature in conjunction with our previous finding that 1,25(OH)₂D₃ potentiates the cytotoxic effect of TNF on breast cancer and renal cell carcinoma cells (5, 6) led us to study the effect of 1,25(OH)₂D₃ on cell damage and death resulting from exposure to doxorubicin.

MATERIALS AND METHODS

Materials. Tissue culture media were purchased from Biological Industries (Beit Haemek, Israel). Tissue culture dishes were from Corning Glass Works (Corning, NY). 1,25(OH)₂D₃ was obtained from Hoffman-LaRoche Co. (Nutley, NJ, a generous gift from Dr. M. Uskokovic). 24,25(OH)₂D₃ was a gift from Teva Pharmaceutical Industries (Petah Tikva, Israel). Doxorubicin hydrochloride (Adriamycin HCl) was a gift from Pharmacia Fine Chemicals AB (Upsalla, Sweden). Potassium cyanide was from Merck (Darmstadt, Germany). Mandelone, N-acetyl-l-cysteine, NR, xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). All of the other reagents were of analytical grade. Recombinant human Cu/Zn SOD and a mouse antihuman Cu/Zn SOD mAb were a gift from General Biotechnology (Ness Ziona, Israel). Horseradish peroxidase-conjugated rabbit antinouse antibody was from Jackson, Immunoresearch Laboratories Inc. (West Grove, PA). A cDNA probe for human Cu/Zn SOD, a 453 bp BamHI fragment StuI cloned in pSODβT11, was a gift from General Biotechnology. A probe for 18S rRNA was obtained from Oncogene Research Products (Cambridge, MA). Nylon membranes (Hybond-N+) and radiolabeled nucleotides were purchased from Amersham (Buckinghamshire, United Kingdom).

Cell Culture. MCF-7 human breast cancer cells were cultured in DMEM containing 4.5 g/liter glucose and supplemented with 10% FCS and antibiotics. Cells were subcultured twice weekly and 48 h before the initiation of an experiment. Various numbers of cells were plated as follows: (a) in cytotoxicity assays 3 X 10⁴ to 1 X 10⁶ cells/well in 96-well microtiter plates; and (b)
for SOD determinations, 3 × 10^5 to 1.3 × 10^6 cells/60-mm Petri dishes. Cultures were treated 24 h after seeding and harvested or stained with NR after different incubation periods. The vehicle ethanol was added to control cultures, and its concentration never exceeded 0.08%.

Primary cardiomyocyte cultures were prepared from the ventricles of neonatal Wistar rats as described previously (18). In brief, ventricles from 1–2-day-old rats were dissociated enzymatically using RDB (Israel Institute of Biology, Ness Ziona, Israel). The dispersed cells were resuspended in growth medium (DMEM: Ham’s F12, 1:1, containing 10% FCS and antibiotics) and incubated for 45–60 min. The myocyte-enriched fraction of unsettled cells was plated onto collagen-coated 35-mm culture dishes (1 × 10^5 cells/dish). Medium was replaced with fresh growth medium 24 h and 5 days after seeding, and 1,25(OH)_{2}D_{3} or ethanol was added to the culture with the last change of medium.

Cell Viability Assays. NR uptake assay: viable MCF-7 cells were quantified by the NR dye uptake method as described previously (5). Percentage cytotoxicity was calculated by the following formula:

\[ \text{Cytotoxicity (\%)} = 1 - \frac{\text{NR uptake in drug-treated wells}}{\text{NR uptake in control wells}} \times 100 \]

Control wells contained all of the agents present in the treated wells except the cytotoxic drug doxorubicin or etoposide. Cytotoxicity in myocyte cultures was determined by assaying LDH released into the culture medium. LDH activity was determined in the culture medium and in the sonicated extracts of the cells removed from the same dish by incubation with PBS containing 5 mM EDTA (PBS/EDTA). LDH activity was determined as described by Dujovne et al. (19). Cytotoxicity was expressed as the fraction of cellular LDH activity released from the cells.

Determination of SOD Activity. Cell extracts were prepared by sonication of cell suspensions in lysis buffer containing Tris-HCl [20 mM (pH 8.5)], NaCl (140 mM), Mg acetate (1 mM), CaCl_{2} (1 mM), Triton X-100 (0.1% v/v), and protease inhibitors (phenylmethyl-sulfonyl-fluoride, 0.2 mg/ml and Protosol 0.07 units/ml). Sonicated cell extracts were centrifuged at 20,000 × g for 30 min, and supernatants were used for the determination of SOD activity. SOD activity was determined by the method of Elstner and Heupel (20) adapted to 96-well microtiter plates. The method is based on the inhibition of nitrite formation from hydroxylammoniumchloride by superoxides formed in the xanthine/xanthine-oxidase superoxide generation system. SOD activity was calculated using semi-logarithmic calibration curves with recombinant Cu/Zn SOD. The fraction of SOD activity that was inhibited by KCN (5 mM) was attributed to Cu/Zn SOD and the residual activity to Mn SOD. Protein content was determined by the method of Lowry to 96-well microtiter plates.

Electrophoresis and Immunoblot Analysis. Immunoblot analysis was performed as described previously (6). SDS sample buffer was added to cell extracts prepared as for the determination of SOD activity. Samples were boiled and centrifuged before SDS PAGE under reducing conditions using 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with anti-Cu/Zn SOD mAb. Detection was carried out by horseradish peroxidase-conjugated rabbit antimouse antibody and enhanced chemiluminescence. Protein content of cell extracts in SDS sample buffer was measured by the method of Minamide and Bamburg (21), and equal loading of transfer was verified by staining of the nitrocellulose membranes with Poncave S.

Northern Blot Analysis. Total RNA was isolated by acid guanidium thiocyanate phenol/chloroform extraction (22). RNA of individual cultures was size-fractionated on 1% agarose-formaldehyde gels (20 μg/lane), transferred to nylon membranes, UV-irradiated, and subjected to hybridization with a cDNA probe for human Cu/Zn SOD that was random-prime labeled with α-^{32}PdCTP using commercial kits. Hybridizations were performed overnight at 42°C in 5% SSC, 50 mM phosphate buffer (pH 6.5), 5% Denhard’s solution, 0.1% SDS, 50% formamide, and 0.2 mg/ml salmon sperm DNA. The hybridized membranes were rinsed with 2 × SSC/0.1% SDS twice for 30 min at room temperature and in 0.1 × SSC/0.1% SDS twice for 30 min at 60°C and were exposed at −70°C to Kodak X-OMAT-AR film using intensifying screens.

The absorbance of the bands was quantified by a soft laser scanning densitometer (SLR-2D/1D, Biomed Instruments Inc., Fullerton, CA). A comparison between lanes was corrected by normalization to 18S rRNA measured by hybridization with an oligoprobe specific for 18S rRNA that was^{32}P-labeled with T4 polynucleotide kinase and γ-^{32}PATP.

Statistical Analysis. Statistical analysis was performed by Student’s t test and two-way ANOVA. The cutoff point for significance is defined as P = 0.05.

RESULTS

Pretreatment of the human breast cancer cell line, MCF-7 with 1,25(OH)_{2}D_{3} (10 nm) for 72 h resulted in a marked enhancement of the cytotoxic effect of doxorubicin (Fig. 1A). The effect of the

Fig. 1. The effect of 1,25(OH)_{2}D_{3} on doxorubicin cytotoxicity. MCF-7 cells were plated onto 96-well microtiter plates (4 × 10^3 cells/well). Doxorubicin was added 96 h later. 1,25(OH)_{2}D_{3} was added at various times and various concentrations before the exposure to doxorubicin. Cytotoxicity was assessed 24 h after doxorubicin addition by NR uptake. Treatment with the hormone alone resulted in 5.7 ± 0.1% or 15.7 ± 0.4% reduction in NR uptake for cultures treated with 10 nm or 100 nm of 1,25(OH)_{2}D_{3}, respectively. Results are expressed as mean ± SD of four parallel cultures. Statistical significance was judged by Student’s t test: * p < 0.01; ** p < 0.001. A, 1,25(OH)_{2}D_{3} (10 nm) was added 72 h before doxorubicin. B, cultures treated with vehicle; ■ cultures treated with 1,25(OH)_{2}D_{3}, B, 1,25(OH)_{2}D_{3} was added 72 h before doxorubicin (1 μg/ml). The results are expressed as percent enhancement of cytotoxicity in cultures treated with 1,25(OH)_{2}D_{3} and doxorubicin as compared with the cytotoxicity (9.9 ± 0.5%) in cultures treated with doxorubicin alone. C, 1,25(OH)_{2}D_{3} (10 nm) was added at various times before doxorubicin (2 μg/ml). Results are expressed as in B. The cytotoxicity of doxorubicin alone was 30.0 ± 1.4%.

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VITAMIN D ENHANCES DOXORUBICIN-INDUCED OXIDATIVE DAMAGE

Table 1 The protective effect of N-acetyl-L-cysteine on doxorubicin cytotoxicity

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Doxorubicin</th>
<th>Doxorubicin + 1,25(OH)2D3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without NAC</td>
<td>With NAC</td>
</tr>
<tr>
<td>1</td>
<td>11.2 ± 2.2</td>
<td>11.2 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>12.3 ± 0.3</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>17.1 ± 0.9</td>
<td>16.6 ± 1.4</td>
</tr>
<tr>
<td>4</td>
<td>26.3 ± 1.8</td>
<td>23.3 ± 1.6</td>
</tr>
<tr>
<td>5</td>
<td>19.1 ± 2.3</td>
<td>15.7 ± 0.6</td>
</tr>
</tbody>
</table>

* NAC, N-acetyl-L-cysteine.

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**Table 2** The effect of 1,25(OH)2D3 on etoposide-induced cytotoxicity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etoposide (μM)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>16.7 ± 1.9</td>
</tr>
<tr>
<td>50</td>
<td>20.7 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>27.6 ± 2.2</td>
</tr>
<tr>
<td>Doxorubicin (μg/ml)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.4 ± 1.8</td>
</tr>
</tbody>
</table>

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The hormone was reproduced in 16 independent experiments: the cytotoxic effect of doxorubicin alone (1 μg/ml) was 18.6 ± 3.4% (mean ± SE), and was enhanced by 74 ± 9% (mean ± SE; P = 2.4 × 10-9, paired t test). Fig. 1B illustrates the dose dependence of the enhancing effect of 1,25(OH)2D3. Maximal enhancement was attained at a concentration of 10 nM at which the hormone on its own had only a marginal effect on NR uptake. The results in Fig. 1C show that the enhancement of doxorubicin-induced cytotoxicity increased with the duration of pretreatment with 1,25(OH)2D3 and was already evident after 48-h exposure to the hormone. The specificity of the enhancing effect of 1,25(OH)2D3 was ascertained by showing that treatment of the cells with another dihydroxylated vitamin D metabolite—24,25(OH)2D3—had no effect on doxorubicin-induced cytotoxicity (data not shown).

Thiols, and particularly glutathione, constitute a major cellular protective mechanism against ROS. N-acetyl-L-cysteine is a thiol-containing free radical quencher that also serves as a glutathione precursor. N-acetyl-L-cysteine had a protective effect against doxorubicin-induced cytotoxicity when added concomitantly with the drug (Table 1). It is noteworthy that the extent of protection increased markedly in the presence of 1,25(OH)2D3 which suggests a mediatory role for ROS in the enhancing effect of the hormone.

To further substantiate the involvement of ROS, we examined the effect of the hormone on the cytotoxic activity of another superoxide generating quinone, menadione (23). As seen in Fig. 2, 24-h treatment of MCF-7 cells with 1,25(OH)2D3 (100 nM) increased their susceptibility to menadione-induced cytotoxicity (P < 1 × 10-9, two-way ANOVA). The effect of the hormone was reproducible in five independent experiments; the cytotoxic effect of menadione (15 μM) was enhanced by 73.0 ± 21.4% (mean ± SE; P = 3 × 10-3, paired t test). We have also observed a significant shift to the left of the dose-response curve for menadione after treatment with 10 nM 1,25(OH)2D3 (P = 3.3 × 10-5, two-way ANOVA; data not shown). Treatment with 24,25(OH)2D3 at 10 nM or 100 nM did not result in any potentiation of cell-killing by menadione.

The cytotoxic effect of doxorubicin may also be caused, at least in part, by the inhibition of topoisomerase II. To examine the possibility that cytotoxicity caused by such an inhibitory effect is also potentiated by 1,25(OH)2D3, we studied the effect of the hormone on the activity of the well-known topoisomerase II inhibitor etoposide (24). It is apparent from the results in Table 2 that in cultures parallel to those in which 1,25(OH)2D3 enhanced the cytotoxicity of doxorubicin, it had no effect on etoposide-induced cell death.

The clinical usefulness of doxorubicin is often limited by its accumulating damage to the myocardium. ROS seem to be the major mediators of the cytotoxic action of doxorubicin on the nondividing cardiomyocytes (25). Cardiomyocytes contain active receptors to 1,25(OH)2D3 (26) and, therefore, are a potential target for the hormone. We monitored cytotoxicity in primary cultures of rat cardiomyocytes by the release of LDH to the extracellular medium. Cardiomyocytes were treated with 1,25(OH)2D3 (10 nM) or vehicle for 72 h and for an additional 24 h with doxorubicin (1 μg/ml). Doxorubicin-induced cytotoxicity was 12.2 ± 2.3% (mean ± SD; n = 6 independent cultures) in control as compared to 23.8 ± 4.7% in hormone-treated cultures. Thus, treatment with 1,25(OH)2D3 significantly (P = 8 × 10-4, unpaired t test) exacerbated the damage caused by doxorubicin in these cells as well.

Enzymatically generated ROS play a mediatory role in the action of cytotoxic quinones (11–13, 23). The enhancing effect of 1,25(OH)2D3 on ROS-dependent cytotoxicity may be due to an increase in ROS generation or to a decrease in cellular antioxidant or repair mechanisms. Two enzymes with SOD activity—the cytoplasmic Cu/Zn SOD and the mitochondrial Mn SOD—have a major role in the antioxidant cellular defense against superoxide formation resulting from exposure to cytotoxic quinones. Indeed, an inverse correlation

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Fig. 2. The effect of 1,25(OH)2D3 on menadione cytotoxicity. MCF-7 cells were plated onto microtiter plates (1 × 10³ cells/well) and incubated in the presence (○) or absence (□) of 1,25(OH)2D3 (100 nM) for 24 h. Menadione was then added to the cultures for an additional 24 h. Cytotoxicity was assessed by NR uptake. Data are expressed as the mean ± SD of three parallel cultures. The difference between the two dose-response curves was significant, P = 1 × 10⁻⁶ as judged by two-way ANOVA. 1,25(OH)2D3 on its own had no significant effect (5.7%) on MCF-7 cell number and viability. Results represent one of three comparable experiments.

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was reported between (a) SOD levels and target cell susceptibility to the cytotoxic action of doxorubicin in various cellular systems including MCF-7 cells (27, 28) and (b) increasing of SOD activity inhibited doxorubicin-induced cytotoxicity (29, 30). We, therefore, assessed the effect of 1,25(OH)2 D3 on SOD activity in MCF-7 cells. The results in Table 3 show that 96-h treatment with the hormone alone (100 nM) markedly reduced SOD activity. By the use of cyanide, a specific inhibitor of Cu/Zn SOD, we were able to attribute the change in SOD activity to this enzyme. Only a minor fraction (6–23%) of SOD activity was not inhibited by cyanide and may, thus, be due to Mn SOD activity. Under our experimental conditions, we did not find a consistent effect of 1,25(OH)2 D3 on this residual fraction. Immuno- blot analysis of the same cell extracts used for SOD activity assay revealed (Fig. 3) that the reduction in SOD activity is associated with decreased protein levels of Cu/Zn SOD and, therefore, is probably not due to deactivation of the enzyme. As previously shown for a variety of human cells (31), two mRNA transcripts of Cu/Zn SOD were detected in MCF-7 cells (Fig. 4). These two transcripts (0.7 kb and 0.9 kb) are transcribed from the same gene and differ in the length of their 3'-untranslated region (31). The level of both transcripts declined following treatment with 1,25(OH)2 D3 (Fig. 4). This reduction was very pronounced after 96-h treatment with 1,25(OH)2 D3 and already apparent after 48-h treatment with the hormone. It is noteworthy that under identical conditions—namely, 48-h treatment of MCF-7 cells with 100 nM 1,25(OH)2 D3—there was no change in cell-cycle distribution as determined by flow cytometric analysis (data not shown).

![Fig. 3. The effect of 1,25(OH)2 D3 on Cu/Zn SOD protein content in MCF-7 cells. MCF-7 cells were treated for 96 h with 1,25(OH)2 D3 (100 nM). Cell extracts were prepared, subjected to SDS-PAGE, immunoblotted with a mAb for human Cu/Zn SOD, and visualized by enhanced chemiluminescence.](image)

**Table 3** The effect of 1,25(OH)2 D3 on SOD activity

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Total SOD (units/µg protein)</th>
<th>Cu/Zn SOD (units/µg protein)</th>
<th>Mn SOD (units/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>1,25D3</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>104 ± 4</td>
<td>83 ± 4</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>2</td>
<td>91 ± 10</td>
<td>27 ± 3</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>3</td>
<td>149 ± 20</td>
<td>104 ± 6</td>
<td>117 ± 17</td>
</tr>
<tr>
<td>4</td>
<td>127 ± 19</td>
<td>37 ± 10</td>
<td>118 ± 17</td>
</tr>
</tbody>
</table>

* 1,25D3: 1,25(OH)2 D3.
* a P < 0.01 compared to cultures in the absence of the hormone as judged by unpaired Student’s t test.
* b P < 0.05 compared to cultures in the absence of the hormone as judged by unpaired Student’s t test.
* c P < 0.01 compared to cultures in the absence of the hormone as judged by unpaired Student’s t test.

Fig. 4. The effect of 1,25(OH)2 D3 on Cu/Zn SOD mRNA levels in MCF-7 cells. Cells were treated for 48 and 96 h with 1,25(OH)2 D3 (100 nM). Total RNA was subjected to Northern blot analysis with specific probes to Cu/Zn SOD mRNA and 18S rRNA as described in “Materials and Methods.” A, autoradiogram of the Northern blots. The results from two parallel independent cultures are presented for each treatment group. B, quantification of mRNA levels by normalization with rRNA levels as assessed by laser densitometry.
DISCUSSION

The main finding of this work is that treatment with 1,25(OH)\(_2\)D\(_3\) increases the susceptibility of breast cancer cells to the cytotoxic action of the commonly used anticancer drug doxorubicin. The two main mechanisms responsible for the cytotoxic action of doxorubicin are thought to be superoxide production and the inhibition of topoisomerase activity (11–16). Our results are consistent with the notion that 1,25(OH)\(_2\)D\(_3\) selectively enhances the ROS-mediated cytotoxic pathway. This conclusion is based on the use of antioxidants and model compounds. We have found that the thiol antioxidant and glutathione precursor, N-acetyl-L-cysteine, had only a small protecting effect against doxorubicin on its own, which indicated that the ROS-dependent mechanism has only a minor role under these conditions, although ROS generation as a result of doxorubicin treatment was directly demonstrated in MCF-7 cells (32). However, the protective effect of N-acetyl-L-cysteine was markedly increased in the presence of 1,25(OH)\(_2\)D\(_3\), which implies that the interaction between the 1,25(OH)\(_2\)D\(_3\) and doxorubicin is ROS-dependent. Further evidence to this point was obtained by the use of model compounds that exert their cytotoxic effect predominantly by one of the mechanisms of doxorubicin. We found that 1,25(OH)\(_2\)D\(_3\) enhanced the action of menadione, a compound that shares with doxorubicin the quinone moiety responsible for ROS generation (23), but had no effect on the cytotoxicity of etoposide, the inhibitor of topoisomerase II. This selective effect also rules out the possibility that enhancement of doxorubicin-induced cytotoxicity is caused by the hormonal modulation of the MDR1 gene product, P-glycoprotein, which is known to reduce the intracellular levels of both doxorubicin and etoposide (33). Another strategy to distinguish between the different mechanisms of action of doxorubicin is by examining a target cell in which the ROS-mediated cytotoxic mechanism of the drug predominates. A case in point is the damage caused by doxorubicin to cardiomyocytes (25), which contain active receptors to 1,25(OH)\(_2\)D\(_3\) (26). The finding that 1,25(OH)\(_2\)D\(_3\) also exacerbates doxorubicin-induced damage in these cells supports the notion of a mediatory role for ROS in the interaction between the two agents.

One mechanism that could underlie the enhancing effect of 1,25(OH)\(_2\)D\(_3\) on the cytotoxic quinones is a reduction in the cellular capacity to withstand the oxidative damage exerted by these agents. SOD is the first line of defense of the cellular antioxidant system against the oxidative damage mediated by superoxide radicals. It removes superoxides by catalyzing the dismutation of two superoxide radicals to yield hydrogen peroxide and oxygen. SOD diminishes the damage caused by superoxide-producing agents as long as the removal of the generated hydrogen peroxide by glutathione peroxidase and catalase does not become rate limiting (34). The relatively mild oxidative capabilities of superoxide radical and hydrogen peroxide belie the severity of their direct oxidative effects on biomolecules. Therefore, it has been assumed that their cytotoxic activity may be caused by their ability to generate the highly toxic hydroxyl radical by reductive decomposition of hydrogen peroxide catalyzed by reduced metals (Fenton reaction). To maintain an ongoing Fenton reaction, an electron source must be available to regenerate the reduced metals. Superoxides play a crucial role in this regeneration process (35). SOD activity, thus, has a dual effect on Fenton reaction, stimulatory by increasing the supply of hydrogen peroxide and inhibitory by decreasing the rate of reduced metal generation. In MCF-7 cells, the addition of SOD or metal chelators protected cells against doxorubicin-induced cytotoxicity, and resistance to doxorubicin cytotoxicity was associated with increased Mn SOD activity, which indicated the dominance of the metal-reducing effect (28–30).

The reduction in the cellular activity of the constitutive enzyme Cu/Zn SOD by 1,25(OH)\(_2\)D\(_3\) on its own could thus underlie the potentiation of doxorubicin cytotoxicity by the hormone. Because Cu/Zn SOD protein and mRNA levels were also decreased in 1,25(OH)\(_2\)D\(_3\)-treated cells, the reduction in SOD activity is probably due to decreased biosynthesis of Cu/Zn SOD rather than to its inactivation or enhanced degradation rate. The effect of 1,25(OH)\(_2\)D\(_3\) on Cu/Zn SOD mRNA was detected after 48-h exposure to the hormone when no change in cell cycle distribution was observed, which ruled out the possibility that this hormonal modulation of SOD is secondary to cell cycle arrest.

1,25(OH)\(_2\)D\(_3\) and doxorubicin that act synergistically in vitro to reduce the viability of breast cancer cells may act in concert on tumor cells in the course of chemotherapy inasmuch as the hormone may be produced by activated tumor-associated macrophages (36, 37). The potentiating effect of 1,25(OH)\(_2\)D\(_3\) on doxorubicin-induced cytotoxicity was observed both in MCF-7 breast cancer cells and in rat cardiomyocytes. Because damage to the myocardiun is usually the clinical feature limiting the use of doxorubicin, our results suggest that concomitant treatment with vitamin D analogues may not increase the therapeutic selectivity of the drug. However, elevated 1,25(OH)\(_2\)D\(_3\) levels in the tumor milieu may be achieved by the administration of the inactive precursor 25-hydroxyvitamin D which can be locally converted to the hormonal form by tumor-associated macrophages. It is plausible that 1,25(OH)\(_2\)D\(_3\) will also increase the cytotoxicity of other superoxide generating quinones like mitomycin C, an effect that may be of clinical interest since, in contrast to doxorubicin therapy, cardiotoxicity is a rare side effect in mitomycin C therapy (38).

The synergistic interaction between 1,25(OH)\(_2\)D\(_3\) and cytotoxic quinones may result from the reduced levels of SOD activity in the hormone-treated cells. It thus seems likely that the exposure of cells to 1,25(OH)\(_2\)D\(_3\) alone reduces the cellular antioxidant capacity and consequently the cells’ ability to withstand assault mediated by various ROS-generating cytotoxic agents including the immune cytokine TNF (17). Moreover, because ROS are also produced during the course of normal cell metabolism and this production is probably increased in tumors (39), the reduced cellular capacity to neutralize these agents may account to some extent for the anticancer activity of active vitamin D metabolites even in the absence of cytotoxic agents. It remains to be seen to what extent the potentiation of the cytotoxic action of ROS-induced cell damage by 1,25(OH)\(_2\)D\(_3\) is a general phenomenon that may be exploited in anticancer chemotherapy and radiotherapy.

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1,25-Dihydroxyvitamin D₃ Enhances the Susceptibility of Breast Cancer Cells to Doxorubicin-induced Oxidative Damage

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