Pharmacodynamics of Doxorubicin in Human Prostate Tumors

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ABSTRACT

The pharmacodynamics of doxorubicin in human prostate tumors were studied using histocultures of radical prostatectomy specimens. Drug treatment lasted 96 h. The antiproliferative effect was measured by the inhibition of DNA precursor ([3H]thymidine) incorporation, and the cytotoxic effect was measured by monitoring cells with fragmented DNA, as indicated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay. The average [3H]thymidine labeling index in 17 tumors was 39% (range, 20–56%). The antiproliferative and cytotoxic effects were concentration dependent and reached 100% at 6 and 17 μM doxorubicin. The cytotoxic concentrations were significantly higher than the antiproliferative concentrations, indicating that prostate tumors were more sensitive to the antiproliferative effect than they were to the cytotoxic effect of doxorubicin. The antiproliferative effect was inversely correlated with patient's age (P < 0.02) and weakly correlated with LI and Gleason grade (P = 0.07 and 0.06, respectively), but it was not correlated with clinical stage, prostate-specific antigen secretion, or race of patients (P > 0.12). In contrast, the cytotoxic effect was positively correlated with Gleason grade (P < 0.05) and weakly correlated with stage (P < 0.08), but it was not correlated with the other parameters (P > 0.18). The opposite correlations between the two effects with tumor grade suggest that the two effects are not coupled. A comparison of the drug concentrations required to produce 50% antiproliferative (0.06 μM) and cytotoxic (2 μM) effects and the literature data on plasma drug concentrations derived from systemic treatment suggest that there are minimal drug effects at the clinically achievable drug concentrations and that regional delivery of doxorubicin to the prostate may be necessary to provide adequate concentrations to produce antiproliferative and cytotoxic effects.

INTRODUCTION

Prostate cancer is the most common malignancy in man. About 60% of the 317,000 new cases will present with disease confined to the organ (1). Prostate cancer is a slowly progressing disease and is usually diagnosed in older men at a median age of 70.5 years, with fewer than 1% of patients being less than 50 years old. The survival rate is significantly and inversely influenced by the tumor stage at diagnosis. An emphasis on early detection and the availability of new screening techniques, in particular, the detection of PSA,3 have increased the rate of diagnosis and lowered the stage and age at the time of diagnosis (2). The current treatment options for locally confined disease include surgical radical prostatectomy, radiotherapy, and cryo-therapy, with surgery being the most common treatment modality. Neoadjuvant hormone therapy prior to radical prostatectomy is under investigation to improve the control of localized prostate cancer. Some studies showed benefits of neoadjuvant hormone therapy (3, 4), although time to progression or overall survival did not improve in another study (5).

Androgen ablation therapy and systemic chemotherapy are usually reserved for metastatic disease and are not used for local disease. Among the agents used to treat advanced prostate cancer, doxorubicin produces one of the highest combined partial and complete response rate of 29%, although complete responses are rare (6). However, doxorubicin, as a single agent or in combination with other chemotherapeutics, has not improved the survival rate (6, 7). Because chemotherapy is not commonly used in early prostate cancer, the activity of doxorubicin against early disease is not known.

This study was designed to determine the pharmacological effects of doxorubicin in surgical specimens of early-stage human prostate tumors obtained by radical prostatectomy. The drug-induced antiproliferative and cytotoxic (cell kill) effects were quantified. The effective drug concentrations were compared with the literature data on clinically achievable drug concentrations in patients to infer potential therapeutic effectiveness of neoadjuvant or adjuvant systemic doxorubicin therapy. The study was performed using histocultures of surgical specimens of human prostate tumors obtained by radical prostatectomy. The histoculture system provides advantages over monolayer cell culture system. Histocultures maintain the three-dimensional tissue structure and organization, and thus, the coexistence of tumor and stromal cells, cell-cell interaction, and inter- and intratumor heterogeneity are preserved (8). The maintenance of tissue architecture is critical because the interaction between the tumor and normal cells may be important for...
prostatic epithelial growth and response to androgen stimulation. We previously reported that human prostate tumor histocultures maintained their characteristics for at least 8 weeks, as indicated by unchanged LI and secretion of PSA and prostatic acid phosphatase (9). The clinical relevance of the human tumor histoculture system was recently demonstrated by Hoffman and colleagues (10–12). These investigators show, in retrospective and semiprpective preclinical and clinical studies, that drug responses in human tumor histocultures, using inhibition of DNA precursor incorporation or inhibition of metabolic reduction of tetrazolium dye as end points, correlates with the sensitivity, resistance, and survival of head and neck, colorectal, and gastric cancer patients to treatment with mitomycin C, doxorubicin, 5-fluorouracil, or cisplatin.

**MATERIALS AND METHODS**

**Chemicals and Supplies.** Doxorubicin was a gift from Pharmacia (Albuquerque, NM). Sterile pigskin collagen (Spongostan standard) was purchased from Health Designs Industries (Rochester, NY); tissue culture supplies (*i.e.*, L-glutamine, sodium pyruvate, fetal bovine serum, gentamicin, DMEM, MEM, MEM nonessential amino acids solution, and MEM vitamin solution) were from Life Technologies, Inc. (Grand Island, NY); [3H]thymidine (specific activity, 65 Ci/mmol) was from Moravek Biochemicals Inc. (Brea, CA); NTB-2 nuclear track emulsion was from Eastman Chemicals (Rochester, NY); terminal deoxynucleotidyl transferase, digoxigenin-dUTP, and antidigoxigenin-peroxidase in ApopTag for *in situ* detection of dead and dying cells were from Oncor Inc. (Gaithersburg, MD); proteinase K and mouse normal IgG were from Sigma Chemical Co. (St. Louis, MO); the liquid 3,3′-diaminobenzidine substrate kit was from BioGenex (San Ramon, CA); the mouse monoclonal antibody ER-PR8 against human PSA and Labeled Streptavidin-Biotin universal detection kit was from DAKO Corp. (Carpinteria, CA); and the PSA detection kit was from Hybritech (San Diego, CA). All chemicals and reagents were used as received.

**Tumor Procurement.** Surgical specimens of human primary prostate tumors were obtained from the peripheral zone of the prostate gland in patients who had organ-confined adenocarcinoma and who underwent radical prostatectomy. Specimens were obtained via the Tumor Procurement Service at The Ohio State University Comprehensive Cancer Center and from the neighboring Doctor’s Hospital. Tumor specimens were placed in MEM within 10–30 min after surgical excision, stored on ice, and prepared for culturing within 1 h after excision.

The histopathology of tumor specimens was established using frozen sections of tumor fragments adjacent the histocultured specimens. Tumors were graded according to the Gleason grading system, with a score of 2 for well-differentiated tumors and a score of 10 for poorly differentiated tumors (13), by pathologists at the James Cancer Hospital (Columbus, OH).

**Histoculture.** Tumor specimens were cultured as described previously (9). In brief, tumors were cut into ≤1-mm³ pieces under sterile conditions. Five to six tumor pieces were placed on a 1-cm² presoaked collagen gel and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium consisted of a 1:1 mixture of MEM and DMEM, 10% fetal bovine serum, 2 mm L-glutamine, 1 mm sodium pyruvate, 40 μg/ml gentamicin, 0.1 mm MEM nonessential amino acids, and concentrated MEM vitamin solution (100-fold concentrated; 10 ml/liter). The histocultures were fed every other day and used for doxorubicin pharmacodynamic studies on day 4.

**Detection of PSA in Media and in Histocultured Tissues.** The secretion of PSA by human prostate tumor histocultures to the culture medium collected at the end of doxorubicin exposure was measured by a sandwich immunoassay using two antibodies against two different epitope sites on PSA, performed by technicians at the Immunology Laboratory of the James Cancer Hospital (Columbus, OH). PSA expression in histocultured tumors was measured by immunohistochemistry using a universal detection kit from DAKO Corp. After sequential dewaxing and rehydration in xylene, ethanol, and water, tissue sections were boiled in a 10 mm citrate buffer (pH 6.0) in a microwave oven for 15 min, cooled at room temperature for 15 min, and washed once in PBS. Tissue sections were then incubated with the blocking solution for 10 min and subsequently with the mouse antihuman PSA (1:20 dilution in PBS containing 5 mg/ml BSA) in a humidified chamber at room temperature for 2 h. Mouse normal IgG was used as the antibody for negative controls. After washing with PBS, the tissue sections were covered with the biotin-anti-IgG linker solution, and then with peroxidase-conjugated streptavidin solution. After washing twice with PBS, tissue sections were incubated for 3–7 min with the chromogen 3,3′-diaminobenzidine and with the substrate hydrogen peroxide and then counterstained with hematoxylin, followed by dehydration and coverslipping for microscopic examination.

**Pharmacological Effects of Doxorubicin.** The antiproliferative effect was measured by the inhibition of DNA precursor ([3H]thymidine) incorporation. The cytotoxic effect was measured by monitoring dead and dying cells using the TUNEL assay. Tumor histocultures were exposed to various concentrations of doxorubicin, ranging from 0.00017 to 17 μM for 96 h. After drug treatments, the doxorubicin-containing medium was removed, and tumor histocultures were washed three times with drug-free medium. Tumors were incubated with 0.03 μM [3H]thymidine for 96 h, washed 3 times with PBS, and then fixed in 10% neutralized formalin, dehydrated, and embedded in paraffin. The embedded tumor tissues were cut onto microscope slides at a 5-μm thickness using a microtome. Two sets of the slides were collected for autoradiography and for TUNEL assay separately. Untreated controls were processed similarly.

**Autoradiography.** Tumor sections on microscope slides were deparaffinized, rehydrated, and stained with hematoxylin. Slides were then coated with a thin layer of NTB-2 nuclear track emulsion and exposed for 7–10 days in the dark in a cold room. Slides were developed, dehydrated, and then coverslipped. The [3H]thymidine-labeled cells were scored, and the LI was calculated as (labeled cells)/(total cells).

**TUNEL Assay.** Procedures were carried out as described in the manufacturer’s instructions for the ApopTag detection kit. Tissue sections on slides were deparaffinized, rehydrated, and incubated with proteinase K (20 μg/ml) in PBS for 15 min at room temperature, followed by 4 washes with distilled water for 2 min each. This protease treatment is necessary to make DNA fragments accessible for reaction with enzyme and substrate. Endogenous peroxidase was quenched by incubating tissue
sections with 2% hydrogen peroxide in PBS for 5 min at room temperature, followed by two washes with PBS for 5 min each. Equilibration buffer from the kit was applied directly onto tissue sections and incubated for 10 min at room temperature. Terminal deoxynucleotidyl transferase-containing solution from the kit was then applied and incubated in a humid chamber at 37°C for 1 h. After the enzyme reaction, slides with tissues were incubated with 37°C-prewarmed stop solution for 10 mm at room temperature, followed by three washes with PBS for 5 mm each. Antidigoxigenin-peroxidase from the kit was applied and incubated for 30 mm at room temperature, followed by 3 washes with PBS for 5 mm each. Between processes, excess liquid was tapped off gently, and the volume of applied liquid was enough to cover the whole section. The tissue was blotted around carefully. The slides were kept in a humid chamber so that no concentration changes occurred during the incubation periods. The tissue sections were counterstained with hematoxylin, dehydrated, and coverslipped. The TUNEL-positive cells were scored.

**Pharmacodynamic Data Analysis.** The drug concentration-effect relationships were analyzed by the Hill equation, shown in Eqs. A (antiproliferative effect) and B (cytotoxic effect; Ref. 14):

\[
E_{\text{antiproliferative}} = 100\% \left(1 - \frac{C^n}{K_1^n + C^n}\right) \quad (A)
\]

\[
E_{\text{cytotoxic}} = \frac{(100\% - E_{\text{control}} \times C^n)}{K_1^n + C^n} + E_{\text{control}} \quad (B)
\]

where \(E_{\text{antiproliferative}}\) is the LI of doxorubicin-treated specimens expressed as a percentage of the LI from the untreated control, C is the drug concentration, \(K_1\) and \(K_2\) are the drug concentrations at 50% effect, \(n\) is a curve shape parameter, and \(E_{\text{control}}\) is the fraction of dead cells in untreated control. Values for IC\(_{50}\) or IC\(_{90}\) (drug concentrations needed to produce 50 or 90% inhibition of DNA synthesis, respectively) and LC\(_{50}\) or LC\(_{90}\) (drug concentrations needed to induce 50 or 90% cell death, respectively) were determined.

**Statistical Analysis.** Differences in mean values between groups were analyzed using Student’s t test. The software used for statistical analysis (TTEST procedures) was from SAS (Cary, NC).

**RESULTS**

**Histocultures.** Twenty tumors, obtained from 20 patients, were studied. Of these, 17 tumors (or 85%) were successfully cultured. A successful culture was defined as having ≥40 tumor cells per microscopic field and having a LI of ≥5%. Table 1 shows the patient and histopathological characteristics of the cultured tumors. All tumors were from chemotherapy-naive patients who had not received adrogen ablation. The average control LI was about 39%. This value is similar to the average value of 41% in 34 human prostate tumors, shown in our previous study (15). Fig. 1A shows the presence of [\(^3\)H]thymidine-labeled tumor cells in an untreated control tumor specimen. The cultured tumors secreted PSA, as shown by the positive immunohistochemical staining (Fig. 1B) and the presence of PSA in culture medium. The PSA concentration in

Table 1  Patients and tumor characteristics and tumor sensitivity of tumors to 96-h doxorubicin treatments

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<tr>
<th>Tumor no.</th>
<th>Age (yr)</th>
<th>Race</th>
<th>Stage</th>
<th>Gleason grade</th>
<th>Control PSA in medium (ng/ml)</th>
<th>Control LI (%)</th>
<th>IC(_{50}) (nM)</th>
<th>IC(_{90}) (nM)</th>
<th>Control TUNEL index (%)</th>
<th>LC(_{50}) (nM)</th>
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\(^a\) NA, not applicable.

\(^b\) P < 0.00001 between IC\(_{50}\) and LC\(_{50}\).

\(^c\) P < 0.0001 between IC\(_{90}\) and LC\(_{90}\).
The cultured medium varied by ~130-fold, reflecting the intertumor variation (Table 1).

**Antiproliferative Effect.** The 96-h doxorubicin treatment produced a sigmoidal, concentration-dependent inhibition of LI, reaching complete inhibition in all 17 tumors (Fig. 1C). Fig. 1C also shows the micrographs of untreated control and doxorubicin-treated tumors. The drug-treated tumor showed a nearly complete inhibition of DNA synthesis but no morphological difference, as compared to the control. Among the 17 tumors, the doxorubicin concentrations that caused 50 and 90% inhibition of DNA synthesis (IC$_{50}$ and IC$_{90}$, respectively) showed 68- and 18-fold ranges, respectively, indicating substantial intertumor variation.

The antiproliferative effect of doxorubicin was inversely correlated with the age of patient ($P < 0.02$) and with the LI of untreated controls and Gleason grade ($P = 0.07$ and 0.06, respectively), but it had no correlation with tumor stage, PSA secretion, or race of patients ($P > 0.12$). Although age has been related to the incidence and progression of prostate cancer, the reason for its inverse correlation with the antiproliferative effect of doxorubicin is not apparent.

**Cytotoxic Effect.** The 96-h doxorubicin treatment produced a sigmoidal, concentration-dependent cytotoxic effect (Fig. 2). Doxorubicin induced 100% cell death in all 17 tumors. In comparison, untreated controls showed insignificant cell death at 3.1 ± 2.5%. Fig. 1D shows the cells labeled by the TUNEL assay, which identifies cells with fragmented DNA. Interestingly, the dead and/or dying cells did not show the typical apoptotic features, such as chromosome condensation and apoptotic bodies (16).
Inhibition of 96-h cumulative [H]thymidine LI (\(\text{H}^\text{thymidine LI}\)) was expressed (0.18 for all cases). Treatment option is via regional drug delivery, such as direct sigmoidal lines are computer-fitted lines, according to Eqs. A and B. Speculate that the partial responses observed clinically with \(0\) was assessed by TUNEL as a percentage of total tumor cells. The more chemoresistant than are the early-stage tumors. We as a percentage of untreated controls. Induction of apoptosis or necrosis \(\text{prostate tumors obtained via radical prostatectomy. Despite the deprivation therapy before radical prostatectomy for prostate cancer.}

The results demonstrate that doxorubicin was able to completely Pathol... to determine the potential value of neoadjuvant therapy. The results demonstrate that doxorubicin was able to completely inhibit DNA synthesis and induce cell death in histocultures of prostate tumors obtained via radical prostatectomy. Despite the 13-fold higher intertumor variations for the antiproliferative effect than for cytotoxic effect, there was a correlation between the IC and LC values. This indicates a parallel tumor sensitivity to the two effects. The lower IC values, as compared to the LC values, indicate a higher tumor sensitivity to the antiproliferative effect than the cytotoxic effect of doxorubicin. The drug concentrations needed to produce 50 and 90% antiproliferative and cytotoxic effects are between 60 and 4200 nM. These concentrations are 17-fold higher than the effective concentrations in monolayer cultures of human prostate cancer cells (17), probably due to biological differences between continuous cell lines and human tumors and/or differences in culture conditions (monolayer versus three-dimensional).

The clinically achievable plasma concentrations of doxorubicin during a 96-h infusion vary from 22 to 178 nM (18–20), which is about the same as the average IC\(_{90}\) but is much lower than the average values of IC\(_{90}\), LC\(_{50}\), and LC\(_{90}\) (660, 2100 and 4,200 nM, respectively). This study was performed using radical prostatectomy specimens, which typically represent early-stage disease. Although the pharmacodynamics of doxorubicin in advanced prostate tumors have not been studied, the general chemoresistance of advanced tumors suggests that the advanced tumors are likely to be more chemoresistant than are the early-stage tumors. We speculate that the partial responses observed clinically with systemic doxorubicin therapy correlate with inhibition of proliferation. Complete drug responses would likely require a cytotoxic effect. Extrapolation of the results of this study suggests that the reasons for the ineffectiveness of doxorubicin therapy in the treatment of advanced prostate cancer are inadequate drug delivery and tumor chemoresistance.

In conclusion, results of this present study suggest that neoadjuvant or adjuvant systemic doxorubicin therapy has limited value in treating early-stage prostate cancer. An alternative treatment option is via regional drug delivery, such as direct intraprostatic injection, to provide high drug concentration in the prostate tissue to achieve appreciable antitumor effect.

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