Determinants of Paclitaxel Penetration and Accumulation in Human Solid Tumor

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ABSTRACT

The present study examined the determinants of the penetration and accumulation of [3H]paclitaxel (12–12,000 nM) in three-dimensional histocultures of patient tumors and of a human xenograft tumor in mice. The results showed 1) significant and saturable drug accumulation in tumors, 2) extensive drug retention in tumors, and 3) a slower penetration but a more extensive accumulation in the xenograft tumor compared with patient tumors. Drug penetration was not rate-limited by drug diffusion from medium through the matrix supporting the histocultures. The difference in the expression of the mdr1 P-glycoprotein did not fully account for the difference in the drug accumulation in xenograft and patient tumors. Autoradiography and imaging were used to evaluate the spatial relationship between tumor architecture, tumor cell distribution, and drug distribution as a function of time and initial drug concentration in culture medium. The tumor cell density and the kinetics of drug-induced apoptosis were also evaluated. The results indicate that a high tumor cell density is a barrier to paclitaxel penetration and that the apoptotic effect of paclitaxel enhances its penetration in solid tumors. These factors are responsible for the time- and concentration-dependent drug penetration rate, with drug penetration confined to the periphery until apoptosis and reduction of epithelial cell density occurred at 24 h, after which time paclitaxel penetrated the inner parts of the tumor.

Paclitaxel is one of the most important anticancer drugs developed in the past two decades. It has shown significant activity against human solid tumors, i.e., ovarian, head and neck, bladder, breast, and lung cancers (Rönnings et al., 1999). Paclitaxel binds to and stabilizes microtubules (Parness and Horwitz, 1981; Manfredi et al., 1982; Jordan et al., 1993; Derry et al., 1995). The intracellular concentration of paclitaxel is critical for its cytotoxic effect; drug resistance in several resistant sublines is correlated with reduced drug uptake and increased efflux compared with the sensitive parental cell lines (Bhalla et al., 1994; Jekunen et al., 1994; Lopes et al., 1993; Riou et al., 1994; Speicher et al., 1994). We and other investigators have studied the kinetics of paclitaxel uptake and efflux in monolayer cultures of human cancer cells (Jordan et al., 1996; Kang et al., 1997). The results show 1) saturable drug uptake, 2) extensive drug accumulation in cells with intracellular concentration exceeding extracellular concentration by 100- to 2300-fold, and 3) a more rapid attainment of steady state at high extracellular concentration compared with lower concentration (i.e., half-life to reach steady state is ~15 min at 1000 nM versus ~2 h at 1 nM).

Our laboratory is interested in developing regional paclitaxel therapy for localized disease, e.g., intravesical therapy for bladder cancer and i.p. therapy for ovarian cancer. In regional therapy, the drug is applied directly to the tumor-bearing organ or cavity. In contrast to systemic therapy where drug delivery to tumor cells is via the circulation, drug delivery to tumor cells during regional therapy depends on the ability of the drug to penetrate the solid tumor. A recent study of paclitaxel distribution in multicellular spheroids indicates that drug penetration is limited to the periphery, but the barriers to paclitaxel penetration are not known (Nicholson et al., 1997).

The present study evaluated the determinants of the penetration and accumulation of [3H]paclitaxel in human solid tumors at clinically relevant concentrations of 12 to 12,000 nM. We evaluated several factors, including the rate of drug diffusion to tumors, level of the mdr1 P-glycoprotein (Pgp), tissue composition, and kinetics of apoptosis. High Pgp level has been linked to decreased intracellular paclitaxel accumulation and drug resistance in several tumor cell lines (Roy and Horwitz, 1985; Bhalla et al., 1994; Speicher et al., 1994).
The present study was performed using three-dimensional histocultures of surgical specimens of head and neck and ovarian tumors from patients, and human pharynx FaDu xenograft tumor maintained in immunodeficient mice. Autoradiographic techniques and image analysis were used to visualize the time course of drug penetration and appearance of apoptotic cells and the spatial relationship between tumor architecture, tumor cell distribution, and drug penetration. We elected to use an in vitro system, instead of in vivo or in situ systems, so that the results were not confounded by the effect of blood flow on drug transfer. In addition, the clinical relevance of the histoculture system has been demonstrated in retrospective and semiprospective preclinical and clinical studies; drug response in human tumor histocultures correlates with chemosensitivity and survival of cancer patients to several chemotherapeutic drugs (Robbins et al., 1994; Furukawa et al., 1995; Kubota et al., 1995). The histoculture system retains several features of human solid tumors, i.e., three-dimensional multicellular structure and coexisting epithelial tumor cells and normal stromal tissue. As shown in this study, these features play a role in determining the rate and extent of paclitaxel accumulation in solid tumors.

Materials and Methods

Chemicals and Reagents. Paclitaxel was a gift from the Bristol Myers Squibb Co. (Wallfording, CT). 3H-paclitaxel (specific activity 19.3 Ci/mmol) was supplied by the National Cancer Institute (Bethesda, MD). Cefotaxime sodium was purchased from Hoechst-Roussel (Somerville, NJ); gentamicin was purchased from Solo Park Laboratories (Franklin Park, IL); fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco’s modified Eagle’s medium, nonessential amino acids, t-glutamine, and trypsin were purchased from Gibco Laboratories (Grand Island, NY); Solvable tissue gel solubilizer and Atombild scintillation fluid were purchased from Amersham; Ultorfilm was purchased from Leica (Deerfield, IL); autoradiographic supplies were purchased from Kodak (Rochester, NY). Dynamic tissue culture supplies were purchased from DuPont Biotechnology systems (Boston, MA); LKB 2208 Ultrofilm was purchased from Leica (Deerfield, IL); autoradiographic supplies were purchased from Kodak (Rochester, NY); and monoclonal antibody (JSB-1) and polyclonal antibody (ab-1) against paclitaxel were purchased from Biogenex (San Ramon, CA) and Oncogene (Cambridge, MA), respectively.

Cell Culture and Tumor Procurement. FaDu cells were obtained from American Type Culture Collection (Manassas, VA). Culture medium was MEM supplemented with 9% heat-inactivated FBS, 2 mM glutamine, 0.1 mM nonessential amino acids, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime sodium. Cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating. Cells with greater than 90% viability, as determined by trypan blue exclusion, were used for tumor implantation. Cells were centrifuged and resuspended in Matrigel (1:1, v/v), a solubilized tissue basement membrane preparation extracted from the Engelbreth-Holmswarm mouse tumor that has been shown to support the growth of human tumors in immunodeficient mice (Kleinman, 1990). The tumor establishment was achieved by s.c. injecting 10⁶ cells (0.1–0.2 ml) with an 18-gauge needle at left and right sides of the upper back. The tumor was removed when it reached a size of 0.5 to 1 g and used for experiments.

Human head and neck and ovarian tumors were obtained via the Tumor Procurement Service at the Ohio State University Comprehensive Cancer Center. All tumor specimens were obtained during surgery and placed in Hank’s balanced salt solution within 10 min after surgery and maintained at 4°C until use.

Histocultures. Tumor specimens were dissected into 1-mm³ pieces under sterile conditions within 6 h after procurement. Five to six pieces were placed on a 1-cm² presoaked collagen gel and incubated in 6-well plates. Tumors were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium consisted of MEM (for FaDu xenograft tumor) or MEM/Dulbecco’s modified Eagle medium (1:1; for patient tumors) supplemented with 9% heat-inactivated FBS, 2 mM glutamine, 0.1 mM nonessential amino acids, 90 μg/ml gentamicin, and 90 μg/ml cefotaxime sodium. After 2 to 4 days, tumor histocultures were used to study the kinetics of drug penetration.

Drug Uptake and Efflux in Histocultures. Tumor histocultures were incubated for 4 ml of medium containing 12 to 12,000 nM mixture of radiolabeled and unlabeled paclitaxel. The final concentration of [3H]paclitaxel was 2.6 nM at 0.05 μCi/ml or 5.2 nM at 0.1 μCi/ml. The paclitaxel concentrations used are within the range of clinically achievable concentrations in plasma (i.e., up to 13,000 nM, Kearns et al., 1995). For the efflux study, tumor histocultures were incubated with paclitaxel for 24 h, the longest time before substantial apoptosis occurs (Au et al., 1998), and then transferred to new plates and maintained in drug-free medium. At predetermined times, 100 μl of medium was taken from each well and the histocultures were removed from the plates, blot-dried on a filter paper, and weighed. One hundred microliters of medium or tumor samples were mixed with 0.5 ml of Solvable tissue/gel solubilizer, incubated at 50°C in an oven overnight, and analyzed for total radioactivity using liquid scintillation counting. A preliminary study determined that 95% of the radioactivity in culture medium, analyzed by high-pressure liquid chromatographic fractionation using a previous described method (Royer et al., 1995), was represented by paclitaxel and its epimerization product, 7-epitaxol. The ratio of 7-epitaxol to paclitaxel in culture medium containing FaDu cells was affected by the incubation time and the drug concentration in the medium, increasing from 2% at 3 h to 72% in 24 h and from 7% at 100 nM to 25% at 5000 nM after 24 h. Because 7-epitaxol has microtubule binding affinity and cytotoxicity similar to those of paclitaxel (Ringel and Horwitz, 1987), the total radioactivity was expressed in paclitaxel equivalents. Drug concentration in tissue was calculated as (drug amount) divided by (tissue weight) and was expressed in molar terms.

For each tumor category, i.e., patient head and neck tumors, patient ovarian tumors, and FaDu xenograft tumors, three tumors were used per experiment, and 30 to 35 histocultures were used for each concentration and each time point. The design of experiments using patient tumors was dictated by the size of the specimens. On some occasions, specimens from an individual patient were sufficient only to study drug uptake and efflux at one or more, but not all, drug concentrations. A total of seven head and neck tumors and three ovarian tumors were used. For the FaDu xenograft tumor, specimens from individual animals were sufficiently large that each tumor was used for studying uptake and efflux at all four drug concentrations.

Analysis of Drug Uptake and Efflux Kinetics. Results for drug uptake and accumulation showed that the drug concentration in histocultures increased with time and reached a pseudo-steady state with respect to the concentration in culture medium. The rate of paclitaxel uptake in tumors involves multiple kinetic processes, i.e., movement from media to collagen gel matrix and to surrounding tissue gel solubilizer, incubated at 50°C in an oven overnight, and analyzed for total radioactivity using liquid scintillation counting. A preliminary study determined that 95% of the radioactivity in culture medium, analyzed by high-pressure liquid chromatographic fractionation using a previous described method (Royer et al., 1995), was represented by paclitaxel and its epimerization product, 7-epitaxol. The ratio of 7-epitaxol to paclitaxel in culture medium containing FaDu cells was affected by the incubation time and the drug concentration in the medium, increasing from 2% at 3 h to 72% in 24 h and from 7% at 100 nM to 25% at 5000 nM after 24 h. Because 7-epitaxol has microtubule binding affinity and cytotoxicity similar to those of paclitaxel (Ringel and Horwitz, 1987), the total radioactivity was expressed in paclitaxel equivalents. Drug concentration in tissue was calculated as (drug amount) divided by (tissue weight) and was expressed in molar terms.

Results for drug efflux indicate that the tumor concentration declined to a pseudo-steady-state level at 48 h. During efflux, the free drug, including the drug dissociated from binding sites, travels sequentially from intracellular space to interstitial space by diffusion and/or transport by Pgp to collagen gel matrix and to surrounding culture medium. Drug retention in tumors is determined by its binding to macromolecules and the rate of efflux is determined by the...
dissociation of drug from binding sites. The pseudo-steady state attained during efflux reflects a slow dissociation of paclitaxel from binding sites.

Several of the processes involved in drug uptake and efflux are dependent on time and drug concentration. For example, the paclitaxel binding sites (Derry et al., 1995) and the Pgp-mediated efflux (Jang et al., 1998) are saturable. In addition, drug transfer in interstitial space is dependent on the cellularity and, therefore, on the time- and concentration-dependent drug-induced apoptosis (see Results). Because of the complexity of the system, we were not able to identify the rate constants for the individual kinetic steps in the uptake and efflux processes. In an attempt to obtain an estimate of the differences for the different tumor types, we analyzed the tumor concentration-time profiles as monoexponential kinetic processes to obtain the time for the tumor concentration to reach 50% of the pseudo-steady-state level during uptake ($T_{1/2\text{,\;uptake}}$) and efflux ($T_{1/2\text{,\;efflux}}$), respectively (eqs. 1 and 2). It is noted that the apparent $T_{1/2}$ for uptake and efflux obtained by this method were not true $T_{1/2}$, as would be expected for monoexponential kinetic processes, but represented hybridized rate constants of the multiple kinetic processes outlined above. In eqs. 1 and 2, $C_{\text{Tumor, ss, uptake}}$ and $C_{\text{Tumor, ss, efflux}}$ are the drug concentrations in tumor histocultures at steady state during uptake and efflux processes, respectively. $C_{\text{Tumor, initial}}$, in eq. 2 is the drug concentration at the beginning of the efflux experiment, when the drug-loaded tumors were placed in drug-free medium.

$$C_{\text{Tumor}} = C_{\text{Tumor,ss, uptake}} \cdot \left(1 - e^{-\frac{ln2}{T_{1/2,\text{uptake}}}}\right)$$ (1)

$$C_{\text{Tumor}} = C_{\text{Tumor,ss, efflux}} + (C_{\text{Tumor, initial}} - C_{\text{Tumor, ss, efflux}}) \cdot e^{-\frac{ln2}{T_{1/2,\text{efflux}}}}$$ (2)

**Autoradiographic and Image Analysis of Paclitaxel Penetration in Histocultures.** We evaluated the rate of $[^{3}H]$paclitaxel penetration in tumors and the spatial relationship between drug penetration, tumor architecture, and tumor cell distribution using autoradiographic techniques and image analysis. The autoradiographic method was as described previously (Lesser et al., 1995). After incubation with $[^{3}H]$paclitaxel (0.231 and 2.31 µCi/ml, corresponding to 12 and 120 nM, respectively) for 1 h to 3 days, tumor histocultures were collected and washed twice by dipping in ice-cold drug-free medium. Tissue samples were mounted on cryostat chucks with embedding matrix (O.C.T. Compound, Miles Inc., Elkhart, IN) and cut into 10-µm-thick sections in a cryostat at −20°C. Sections were thaw-mounted on a glass slide and heat-fixed on a slide warmer for 15 min. The slides containing the tissue sections were placed against tritium-sensitive film (Ultronfilm) in an X-ray cassette and exposed for 1 to 2 weeks at room temperature. The films were developed for 3 to 5 min at room temperature (D-19 Developer), placed in a stop bath for 30 s, immersed in fixer for 3 min, and exposed to running room-temperature water for 15 min. The films were then rinsed in Photo-Flo 200 and allowed to air dry. Separately, the tissue section slides were stained with H&E.

Image analysis was then used to capture the autoradiographic image, where the grains indicated the location of the radiolabeled drug, and the histologic image of the tissue section slide stained with H&E, which showed the tissue structure and distribution of tumor cells. The threshold for the autoradiographic image was adjusted to minimize the background signal. The autoradiographic image was overlaid on the histologic image to visualize the distribution of $[^{3}H]$paclitaxel in tumor histocultures.

**Image Analysis of Tumor Composition.** The fractions of stromal tissue and tumor cells in each histoculture were measured using image analysis. Briefly, stromal and tumor cells of a 100× magnification field were outlined with the computer mouse. The size of each of these regions was determined via image analysis by counting the number of pixels in the region. For each tumor histoculture, 50 to 100 images were processed per tumor, and the fractions of the tumor represented by tumor cells, stromal tissue, and interstitial space were calculated.

**Paclitaxel Diffusion from Culture Medium to Tumor Histocultures.** This study determined the rate of drug diffusion from culture medium into the collagen gel matrix supporting the histocultures, to evaluate whether slow drug diffusion contributed to the slow drug penetration into solid tumors. Collagen gel pieces 1 cm² were pre-soaked and placed in a well of a 6-well plate containing 4 ml of complete culture medium. No tumors were added. After incubation for 3 to 4 days, the medium was replaced with 4 ml of 120 nM $[^{3}H]$paclitaxel-containing medium and incubated at 37°C for 24 h. At predetermined times, 100 µl of medium was removed from each well. For the sampling of medium trapped in the porous collagen gels, one piece of collagen gel was transferred to a new plate and the medium was obtained by squeezing the gel with a pair of forceps. These procedures required less than 20 s. The radioactivity in medium was determined.

**Detection of Pgp.** The expression of Pgp was measured by immunohistochemical methods, using procedures described previously (Toth et al., 1994). Briefly, tissue sections were dewaxed and rehydrated sequentially in xylene, ethanol, and water. Tissue sections were boiled in a 0.1 M citrate buffer, pH 6.0, in a microwave oven, then cooled and washed in PBS. The tissue sections were incubated with Dako blocking solution for 10 min and subsequently with the following antibody solutions for 2 h: a mouse anti-human Pgp antibody (JSB-1, 1:200 dilution) and a rabbit anti-human Pgp polyclonal antibody (ab-1, 1:100 dilution). JSB-1 does not cross-react with MDR3 (Schinkel et al., 1991). The incubation was carried out in a humidified chamber at room temperature. The antibodies were diluted in PBS containing 5 mg/ml BSA. For negative controls, we used mouse IgG as the primary antibody. For positive controls, we used human adrenal gland, which shows high Pgp expression (Pavelic et al., 1993). After washing with PBS, the tissue sections were covered with the linker solution, and then with peroxidase-conjugated.
TABLE 1
Uptake of paclitaxel into histocultures of FaDu xenograft and patient tumors

The concentration-time profiles of paclitaxel in tumor histocultures obtained after incubation with different initial drug concentrations in culture medium (C_{medium}), as depicted in Fig. 1, were analyzed for the time for the tumor concentration to reach one-half of the pseudo-steady-state level (T_{1/2, uptake}) and for the tumor-to-medium concentration ratio at steady state. The statistical significance of the differences among patient (head and neck and ovarian, n = 6) and FaDu (n = 3) tumors, at equal initial medium concentrations, were analyzed by the two-tailed unpaired Student’s t test. Note the different units used for the medium and tumor concentrations. Mean ± S.D. (n = 3). Note that for head and neck tumors, some experiments used different tumors for the different initial C_{medium} (see Materials and Methods).

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Initial C_{medium}</th>
<th>Steady-State C_{medium}</th>
<th>Steady-State Ctumor</th>
<th>Steady-State C_{medium}-to-C_{tumor} Ratio</th>
<th>T_{1/2, uptake}</th>
<th>Mass Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and neck patient tumor</td>
<td>12 nM</td>
<td>10.9 ± 1.0</td>
<td>1.06 ± 0.21</td>
<td>97.8 ± 15.2</td>
<td>24.2 ± 3.4</td>
<td>91.2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>120 nM</td>
<td>83.0 ± 0.7</td>
<td>5.77 ± 1.52</td>
<td>64.5 ± 18.4</td>
<td>19.8 ± 4.5</td>
<td>83.8 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>1,200 nM</td>
<td>925 ± 25</td>
<td>22.0 ± 6.2</td>
<td>23.1 ± 7.1</td>
<td>10.4 ± 3.8</td>
<td>87.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>12,000 nM</td>
<td>9,870 ± 69</td>
<td>341 ± 125</td>
<td>34.8 ± 14.5</td>
<td>5.81 ± 2.31</td>
<td>91.5 ± 3.5</td>
</tr>
<tr>
<td>Ovarian patient tumor</td>
<td>12 nM</td>
<td>10.6 ± 0.7</td>
<td>1.32 ± 0.31</td>
<td>109 ± 22</td>
<td>18.4 ± 9.6</td>
<td>88.8 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>120 nM</td>
<td>86.4 ± 2.3</td>
<td>2.86 ± 1.28</td>
<td>32.8 ± 14.2</td>
<td>11.1 ± 6.6</td>
<td>84.3 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>1,200 nM</td>
<td>870 ± 2.6</td>
<td>30.1 ± 11.7</td>
<td>32.3 ± 13.7</td>
<td>14.2 ± 7.3</td>
<td>85.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>12,000 nM</td>
<td>10,500 ± 920</td>
<td>211 ± 80</td>
<td>19.8 ± 8.4</td>
<td>7.17 ± 3.81</td>
<td>93.3 ± 4.5</td>
</tr>
<tr>
<td>FaDu xenograft</td>
<td>12 nM</td>
<td>9.09 ± 0.27</td>
<td>1.01 ± 0.21</td>
<td>124 ± 25</td>
<td>35.3 ± 2.0</td>
<td>92.5 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>120 nM</td>
<td>89.7 ± 13.3</td>
<td>9.51 ± 2.88a</td>
<td>115 ± 38a</td>
<td>27.8 ± 3.7</td>
<td>86.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>1,200 nM</td>
<td>887 ± 131</td>
<td>48.8 ± 6.6a</td>
<td>53.7 ± 14.8a</td>
<td>18.9 ± 3.2a</td>
<td>89.4 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>12,000 nM</td>
<td>8,910 ± 267</td>
<td>433 ± 15a</td>
<td>48.5 ± 0.5a</td>
<td>21.6 ± 6.9a</td>
<td>90.2 ± 5.4</td>
</tr>
</tbody>
</table>

* P < .05.

Fig. 2. Kinetics of paclitaxel efflux in patient and xenograft tumor histocultures. Tumor histocultures were treated with paclitaxel for 24 h at 120 and 1200 nM and then transferred to drug-free medium. The drug concentration remaining in tumors was determined. Patient head and neck tumors (♦), patient ovarian tumors (□), FaDu xenograft tumors (○). Data represent the mean ± S.D. of three individual tumors. For each tumor, we used 30 to 35 histocultures for each concentration and each time point. Not that for head and neck tumors, some experiments used different tumors for the different initial C_{medium} (see Materials and Methods).

Results

Apparent T_{1/2} for Paclitaxel Uptake and Efflux. As discussed above, the apparent T_{1/2} for uptake and efflux were hybrid rate constants for multiple kinetic processes. The T_{1/2} values were used to obtain a relative ranking of the rates in different tumor types. This information was then used to design additional studies to evaluate the spatial relationship between drug penetration, tumor architecture, and tumor cell distribution as a function of time and drug concentration.

Accumulation of Paclitaxel in Tumor Histocultures. Figure 1 shows the increase of paclitaxel concentration in histocultures of patient tumors (head and neck, ovarian) and xenograft tumor. Table 1 summarizes the data. For all three tumor types, the drug concentration in tumor histocultures increased with time, reaching a pseudo-steady state between 48 to 72 h, with <5% increase in the next 24 to 48 h. During this time period, the drug concentration in the medium decreased by about 25%. Analysis of the mass balance indicates that about 90% of the dose was accounted for. The tumor-to-medium concentration ratios at steady state ranged from 10 to 120, indicating significant drug accumulation in tumors.

The steady state paclitaxel concentration in tumors increased whereas the steady state tumor-to-medium concentration ratio decreased with the initial drug concentrations in culture medium, although the relationships were not linear. In general, T_{1/2, uptake}, which is the time to reach 50% of the pseudo-steady-state level, decreased with increasing initial medium concentration (P < .01, regression analysis). These data indicate that drug accumulation is partly saturable and show a more rapid attainment of steady state at higher initial extracellular drug concentration.

Efflux of Paclitaxel from Histocultures. Figure 2 and Table 2 compare the kinetics of drug efflux from patient and xenograft tumors. In all three tumor types, the drug concentration declined to a pseudo-steady-state level at 48 h. The extent of efflux was also dependent on the initial concentration, ranging from 29 to 60% at 120 nM and from 41 to 81% at 1200 nM in the first 24 h. The decreases in drug concentration in the next 48 h was severalfold lower, ranging from 1 to 12% at 120 nM and from 3 to 13% at 1,200 nM. T_{1/2, efflux} is the time to reach 50% of the pseudo-steady-state level, ranged from 3 to 7.5 h. The decreases in tumor concentrations were accompanied by increases in medium concentrations. The tumor-to-medium concentration ratios ranged from 400 to 4000 at 24 h and from 250 to 2700 at 72 h. These ratios exceed the steady-state tumor-to-medium concentration ratios achieved during the uptake study (i.e., 20–120) by 8- to 38-fold, indicating that a sink condition was maintained during the efflux study. Hence, the high steady-state tumor-to-medium concentration ratios indicate a significant retention of the drug in tumors, i.e., 19 to 71% of initial drug concentration was retained after 24 h, and 16 to 72% retained after 72 h. In general, the fractions retained and the tumor-to-medium concentration ratios attained at the lower initial medium concentration of 120 nM were significantly higher than those attained at the higher initial concentration of 1200 nM (P < .05, unpaired two-tailed Student’s t test). But the differences between the apparent T_{1/2, efflux} at these two
TABLE 2
Paclitaxel efflux from histocultures of FaDu xenograft and patient tumors

Tumors were incubated with paclitaxel for 24 h. After replacing the drug-containing medium with drug-free medium, the drug concentration remaining in histocultures at 24 and 72 h post-treatment were analyzed to determine the time to reach 50% of the pseudo-steady-state level ($T_{1/2, \text{efflux}}$). The statistical significance of the differences among patient (head and neck and ovarian, $n = 6$) and FaDu ($n = 3$) tumors, at equal initial medium concentrations, were analyzed by unpaired $t$ test. Note the different units used for the medium and tumor concentrations. Mean ± S.D. ($n = 3$). Note that some of the efflux experiments needed different tumors as in the uptake experiments reported in Table 1.

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Initial $C_{\text{medium}}$</th>
<th>At 24 h Post-Treatment</th>
<th>At 72 h Post-Treatment</th>
<th>$T_{1/2, \text{efflux}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$nM$</td>
<td>$nM$</td>
<td>$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>Head and neck patient</td>
<td>120</td>
<td>1.13 ± 0.17</td>
<td>1.70 ± 0.44</td>
<td>1560 ± 514</td>
</tr>
<tr>
<td>Ovarian patient</td>
<td>1,200</td>
<td>7.69 ± 2.55</td>
<td>4.38 ± 1.99</td>
<td>550 ± 88</td>
</tr>
<tr>
<td>FaDu xenograft</td>
<td>120</td>
<td>0.989 ± 0.228</td>
<td>3.88 ± 1.27</td>
<td>4359 ± 1230a</td>
</tr>
</tbody>
</table>

$^a P < .05$. 

Determinants of Drug Penetration into Three-Dimensional Cultures. Of the patients, between patient and xenograft tumors, the profile of drug uptake and accumulation during the uptake period is depicted in Figure 3. The concentration-dependent differences in the apparent $T_{1/2, \text{efflux}}$ between patient and xenograft tumors are shown in Table 2. The apparent $T_{1/2, \text{efflux}}$ is inversely related to drug concentration. As shown below, the concentration-dependent differences in apparent $T_{1/2, \text{efflux}}$ are apparent. On the other hand, there are no differences in the apparent $T_{1/2, \text{efflux}}$ between patient and xenograft tumors. As shown below, the concentration-dependent differences in apparent $T_{1/2, \text{efflux}}$ are apparent. On the other hand, there are no differences in the apparent $T_{1/2, \text{efflux}}$ between patient and xenograft tumors.
diffusion from the culture medium surrounding the collagen gel matrix to the medium surrounding the histocultures. The results are shown in Fig. 5. Quickly (i.e., <12 min) after adding drug solution to the medium, the drug concentration in the culture medium trapped in the collagen gel matrix ($C_{gel}$) was ~50% of the initial medium concentration. $C_{gel}$ then increased gradually at a slower rate to reach a steady state at 12 h. The increase in $C_{gel}$ was accompanied by a gradual decrease in the medium concentration. Because $C_{gel}$ rose much faster with time than the drug concentrations in the histocultures, we conclude that the slight delay in drug diffusion from the medium through the collagen gel matrix was not the rate-limiting factor for drug penetration into tumor histocultures during the initial time points. The rea-

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**Fig. 3.** Autoradiographic analysis of $[^3]H$ paclitaxel penetration in patient tumor histocultures treated with 120 nM paclitaxel. Histocultures of a head and neck patient tumor were treated with 120 nM $[^3]H$ paclitaxel. Top: Histologic images (stained with H&E), Magnification, 25x. Bottom: images of autoradiographic film overlaid on histologic images. Magnification, 25x.

**Fig. 4.** Autoradiographic analysis of $[^3]H$ paclitaxel penetration in FaDu xenograft tumor histocultures treated with 120 nM paclitaxel. Histocultures of FaDu tumor were treated with 120 nM $[^3]H$ paclitaxel. Top: Histologic images (stained with H&E), Magnification, 25x. Middle: images of autoradiographic film overlaid on histologic images, Magnification, 25x. Bottom: enlargement of the indicated boxed region of the slide in the middle panel, to demonstrate the presence of apoptotic cells (indicated by white dots), Magnification, 400x. The fractions of apoptotic cells were ~30% and ~50% at 24 and 72 h, respectively.
The treated tumors was sively increasing with time. The fraction of apoptotic cells in there was a significant reduction in tumor cell density and graft tumor histocultures treated with 120 nM paclitaxel, time, for the xenograft tumor. Figure 4 shows that in xenograft tumor histocultures treated with 120 nM paclitaxel, there was a significant reduction in tumor cell density and increase in apoptotic cells, first detected at 24 h and progressively increasing with time. The fraction of apoptotic cells in the treated tumors was ~30% at 24 h and increased to ~50% at 72 h, whereas the untreated controls showed <5% apoptotic cells (data not shown). The onset of apoptosis and expansion of interstitial space at 24 h coincided with the abrupt increase in drug penetration, i.e., the drug penetrated >80 cell layers during the second 24 h as opposed to 15 cell layers during the first 24 h. To confirm this, we evaluated the drug penetration in the xenograft tumor treated with 12 nM paclitaxel. We have shown in a separate study that 12 nM paclitaxel causes minimal apoptosis and 120 nM causes significant apoptosis in patient tumors (Gan et al., 1996). The second reason for selecting these two concentrations was that they are the concentrations at which the biggest difference between drug accumulation in xenograft and patient tumors was observed, i.e., the 12-nM concentration resulted in only 20% higher drug accumulation in the xenograft tumor than in patient tumors, whereas the 120-nM concentration resulted in 136% higher accumulation in the xenograft tumor (Table 1). Figure 6 shows that 12 nM paclitaxel did not significantly reduce cellularity or increase apoptotic cells; the fraction of apoptotic cells remained relatively constant at ≤7%. Under these conditions, drug penetration was restricted to the periphery. Collectively, our data indicate that drug-induced apoptosis, by disrupting the tissue architecture and reducing the cellularity, enhances the penetration of paclitaxel into solid tumors.

**Determinants of Drug Accumulation in Patient and Xenograft Tumors.** We evaluated whether a difference in Pgp expression in tumors might have caused the differential drug accumulation. Only tumors that were stained by two Pgp antibodies and showed Pgp proteins in at least two-thirds of the histocultures were considered Pgp-positive. By these criteria, the xenograft tumor, three head and neck tumors, and two ovarian tumors were Pgp-positive, whereas four head and neck tumors and one ovarian tumor were Pgp-negative. We compared the accumulation of paclitaxel in these tumors, at two initial medium concentrations, 120 and 12,000 nM. The results are shown in Table 3. The xenograft tumor showed a higher accumulation than the Pgp-positive patient tumors. Within the patient tumors, Pgp expression did not always result in a lower drug accumulation. For example, although the Pgp-positive patient tumors showed a trend of lower drug accumulation compared with the Pgp-negative patient tumors at the higher drug concentration of 12,000 nM, the difference was small (i.e., average of <25%) and not statistically significant. Furthermore, no difference between the two groups was observed at the lower drug concentration of 120 nM. These data indicate that Pgp expression, although it might have contributed to the lower drug accumulation in some tumors, is not the major determinant of drug accumulation and did not fully account for the 50 to 100% difference in drug accumulation between patient and xenograft tumors.

Another possible cause of the differential drug accumulation between patient and xenograft tumors is the tissue composition. The extensive accumulation of paclitaxel in cells (Jordan et al., 1996; Kang et al., 1997) and our finding of a preferential drug localization in regions of histocultures that had a high cell density (Figs. 3, 4, and 6) suggest that cell density is critical for determining the extent of drug accumulation. Figure 7 compares the cell density in xenograft and patient tumors. The results showed that xenograft tumors contained more tightly packed tumor cells, fewer stromal cells, and less interstitial space compared with patient tumors. The image analysis results of five randomly selected tumors (50–100 images/tumor) for each tumor type indicate a significantly higher volume fraction of tumor cells in the xenograft tumor compared with human head and neck tumors (i.e., 79 ± 7% versus 51 ± 18%, P < .05, unpaired two-tailed Student’s t test), and conversely a significantly lower fraction of interstitial space in the xenograft tumor. The relationship between drug penetration, interstitial space, and cellularity is represented in eq. 3. This equation describes the diffusion coefficient (D) of a drug in a gel structure as a function of interstitial space (c) and tortuosity (τ), where $D_w$ is the diffusion coefficient in the extracellular fluid (Schultz and Armstrong, 1978; Fox and Wayland, 1979). A larger fraction of interstitial space and/or a decrease in tortuosity would result in a more rapid drug diffusion. Accordingly, we conclude that the time- and drug concentration-dependent penetration of paclitaxel in solid tumors is due to changes in tissue architecture as a result of its pharmacologic effect, i.e., apoptosis.

$$D = D_w \cdot \frac{c}{τ}$$

(3)
The magnitude of the difference in cell density between the xenograft and patient tumors (i.e., 60%) is within the range of the difference of drug accumulation between these tumors (i.e., 70–100%). The higher density of epithelial cancer cells in the xenograft tumor correlates with the slower drug penetration rate and the higher drug accumulation. These data suggest cellularity as a major determinant of the rate and extent of paclitaxel penetration and accumulation in solid tumors.

**Discussion**

The two key findings of the present study are 1) a high tumor cell density is a barrier to paclitaxel penetration, and 2) the apoptotic effect of paclitaxel enhances its penetration in solid tumor. These factors are responsible for the time- and concentration-dependent drug penetration rate, with drug penetration confined to the periphery until apoptosis and reduction of tumor cell density occurred after 24 h, at which time paclitaxel penetrated the inner parts of the tumor. This also explains the characteristics of drug efflux. Drug efflux was measured after 24 h treatment with paclitaxel at concentrations sufficient to induce apoptosis. Hence, during efflux, the drug travels in the already-created interstitial space and is not subjected to delay as is the case during drug uptake. The role of apoptosis in drug efflux rate is also supported by the concentration-dependent drug loss at 24 h post-treatment. In all three tumor types, the fraction of drug remaining in tumors was reduced by increased drug concentration, which resulted in a higher apoptotic fraction and a lower cellularity. For example, the fraction remaining in

**Fig. 6.** Autoradiographic analysis of [³H]paclitaxel penetration in FaDu xenograft tumor histocultures treated with 12 nM paclitaxel. Histocultures of FaDu tumor were treated with 12 nM [³H]paclitaxel. Top: Histologic images (stained with H&E), Magnification, 25×. Middle: images of autoradiographic film overlaid on histologic images, Magnification, 25×. Bottom: enlargement of the indicated boxed region of the slide in the middle panel, to demonstrate the presence of apoptotic cells (indicated by white dots), Magnification, 400×. Very few apoptotic cells (7% of total cells) were detected throughout 72 h.

**TABLE 3**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Pgp Status</th>
<th>120 nM</th>
<th>12,000 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>FaDu</td>
<td>+</td>
<td>3</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>Patient</td>
<td>+</td>
<td>3</td>
<td>30 ± 11</td>
</tr>
<tr>
<td>Patient</td>
<td>–</td>
<td>3</td>
<td>38 ± 3</td>
</tr>
</tbody>
</table>

* Comparison between FaDu xenograft and Pgp-positive patient tumors, *P* < .05.  
* Comparison between FaDu xenograft and Pgp-negative patient tumors, *P* < .05.
human head and neck tumors decreased from 55% at 120 nM drug concentration to 19% at 1200 nM.

Several previous studies have proposed that drug penetration into three-dimensional spheroids is dependent on drug binding characteristics. Drugs that do not bind to macromolecules and can cross cell membranes readily penetrate spheroids (Nederman and Carlsson, 1984; Nederman et al., 1988; Erlanson et al., 1992). For example, 5-fluorouracil is evenly distributed in thyroid cancer cell spheroids within 15 min (Nederman and Carlsson, 1984). Drugs such as doxorubicin and paclitaxel, which bind to cellular macromolecules, remain localized in the periphery of spheroids (Nederman et al., 1981; Durand, 1989, 1990; Erlanson et al., 1992; Nicholson et al., 1997). The initial confinement of paclitaxel to the periphery of human solid tumor histocultures is in agreement with the data in spheroids. The major difference between our results and the spheroid results is the demonstration of the penetration of paclitaxel into the inner cell layers in tumor histocultures after a delay and after apoptosis and reduction in epithelial cell density have occurred. It is not known whether the same occurs in the spheroids.

We also observed extensive and saturable drug accumulation, extensive drug retention, and preferential drug localization in epithelial tumor cells compared with stromal tissues and interstitial space. The preferential drug localization in epithelial tumor cells is in agreement with the extensive binding of paclitaxel to intracellular macromolecules such as microtubules (Parness and Horwitz, 1981; Manfredi et al., 1982). The saturable and substantial drug accumulation and the extensive drug retention in tumor histocultures are qualitatively similar to the findings in monolayer cultures (Jordan et al., 1996; Kang et al., 1997). The differences between the two culture systems are mainly quantitative, i.e., the monolayer cultures show a more rapid uptake rate ($T_{1/2,\text{uptake}}$ of <2 h versus >20 h) and a higher accumulation (steady-state tumor-to-medium ratio of 100–2300 versus 20–120). Our present results show that the longer $T_{1/2,\text{uptake}}$ in histocultures is due to the delay in drug penetration to the inner cell layers. The lower drug accumulation in histocultures is partly due to the limited drug localization in stromal tissue and interstitial space, which are present in histocultures but not in monolayers. However, this difference in tissue composition does not entirely account for the up to 20-fold difference in drug accumulation in the two culture systems because the stromal tissue and interstitial space constitute only 20 to 50% of the volume of histocultures. Theoretically, the different cell types used in the previous (i.e., HeLa cells) and present study (FaDu cells and patient tumors) may introduce biological variations, such as binding affinity and the number of binding sites, resulting in the different drug accumulation. More studies are needed to identify the cause of the differential drug accumulation in two-dimensional monolayer cultures and three-dimensional histocultures.

Our results showed a more rapid attainment of a pseudosteady-state level at higher extracellular concentrations, consistent with the findings in monolayer cultures (Jordan et al., 1996; Kang et al., 1997). This is likely the result of the saturation of intracellular binding sites, which is achieved more readily at higher drug concentrations. Other possible causes are the changes in tissue composition and tumor cellularity which occurred as a function of drug concentration and time. Our data also indicate no consistent differences in drug retention between Pgp-positive and Pgp-negative tumors and that the expression of Pgp does not necessarily result in lower drug retention. This may be due in part to saturation of the Pgp-mediated efflux at higher drug concentration; a separate study in our laboratory has shown that the Pgp-mediated efflux in the mdr1-transfected human breast tumor cells was diminished at paclitaxel concentrations above 500 nM (Jang et al., 1998). Another possible cause is the higher paclitaxel-induced apoptosis in Pgp-positive tumors as compared with Pgp-negative tumors, as we have observed in earlier studies using patient tumors (Gan et al., 1996, 1998). A higher apoptotic cell fraction would lead to a more rapid drug uptake due to decreased cellularity and a lower retention due to the reduced number of binding sites.
In summary, results of the present study indicate that 1) the penetration of paclitaxel in tumors is more rapid but the accumulation is lower as the density of epithelial tumor cells decreases, 2) drug-induced apoptosis enhances drug penetration into the inner cell layers of solid tumors, 3) the concentration-dependent drug penetration rate is related to the concentration-dependent apoptotic effect, and 4) the time-dependent drug penetration is related to the kinetics of apoptosis. Our study was designed to examine the barriers and determinants of paclitaxel penetration and accumulation and hence used an in vitro system, which excludes blood perfusion. Our findings are important to an understanding of the penetration of paclitaxel in solid tumors under in vitro conditions and when the drug is delivered directly to tumor-bearing organs. Extrapolation of these results to in vivo conditions during regional therapy or systemic i.v. therapy, where paclitaxel is removed and/or delivered to the tumor via the systemic circulation, would require consideration of other factors, such as tumor vasculature.

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