Taxane-induced Apoptosis Decompresses Blood Vessels and Lowers Interstitial Fluid Pressure in Solid Tumors: Clinical Implications

Geneviève Griffon-Etienne, Yves Boucher, Christian Brekken, Herman D. Suit, and Rakesh K. Jain

ABSTRACT

Elevated tumor interstitial fluid pressure (IFP) is partly responsible for the poor penetration and distribution of therapeutic agents in solid tumors. The etiology of tumor interstitial hypertension is poorly understood. We have postulated that the solid stress generated by tumor cells growing in a confined space compresses blood vessels and increases tumor microvascular pressure and IFP. To test this hypothesis that neoplastic cell loss would decompress blood vessels and lower IFP, we induced apoptosis in tumors with paclitaxel and docetaxel. Taxanes induced the growth of the murine mammary carcinoma (MaCa-IV) and of the human soft tissue sarcoma (HSTS-26T). Taxanes assisted apoptosis and reduced the density of intact neoplastic cells in both MaCa-IV and HSTS-26T. To determine whether neoplastic cell loss decompressed blood vessels, we measured the diameter of tumor vessels in HSTS-26T tumors implanted in transparent dorsal skinfold chambers. At 48 and 96 h after paclitaxel, the diameter of tumor vessels was significantly increased. The increase in vascular diameters was associated with reductions in microvascular pressure and IFP. The changes in neoplastic cell density and IFP were also correlated. In a human glioblastoma U87, which is resistant to paclitaxel, the IFP and cellular density were not modified by paclitaxel treatment. Collectively, these results support the hypothesis that solid stress generated by neoplastic cell proliferation increases vascular resistance and IFP. The increase in vessel diameter induced by paclitaxel and docetaxel suggests that taxanes could improve tumor response by increasing the vascular surface area for delivery of therapeutic agents.

INTRODUCTION

Interstitial hypertension is a universal characteristic of solid tumors (1, 2). Elevated IFP impedes transvascular transport of large molecules (e.g., monoclonal antibodies; Ref. 3) and is believed to reduce tumor blood flow (4–6). We have shown that, due to the high permeability of tumor vessels and lack of functional lymphatics in tumors, the MVP of tumor vessels is a major determinant of IFP (7–9). MVP is dependent on arteriovenous pressure differences and the geometric and viscous resistance to blood flow. High vascular resistance in tumors is presumably the result of a decrease in vessel diameter, which, in turn, is induced by solid stress. The growth of neoplastic cells in a confined and stiff interstitial matrix can induce solid stress. We have recently estimated that solid stresses of 40–125 mmHg can be generated by neoplastic cells growing in agarose gels of varying stiffness (10). We, thus, hypothesize that a reduction in tumor cell density will decompress blood vessels and, hence, reduce the MVP and IFP. To test this hypothesis, we have used the taxanes paclitaxel and docetaxel to induce apoptosis in neoplastic cells. Taxanes are cytotoxic agents with antitumor activity in experimental and human tumors (11–14). The antiproliferative activity of taxanes is due to their ability to inhibit tubulin depolymerization and, thus, stabilize microtubules. Consequently, the cells are arrested in mitosis, and a variable proportion of cells die by apoptosis (15, 16). Three tumor lines with different elastic properties and response to paclitaxel were selected: a human glioblastoma, U87, which is resistant to paclitaxel, and a human soft tissue sarcoma (HSTS-26T) and a murine mammary carcinoma (MaCa-IV), which are responsive to paclitaxel. U87 and HSTS-26T are relatively stiff, whereas MaCa-IV is a soft tumor (17). The results show that tumor cells can compress tumor vessels and that blood vessel decompression by taxanes reduces the MVP and IFP. The increase in vessel diameter also suggests that taxanes can improve blood perfusion and drug delivery in tumors.

MATERIALS AND METHODS

Mice and Tumors. Eight- to 9-week-old male athymic NCr/nu nude (nu/nu) and C3H/Kam mice, bred and maintained in our defined flora- and specific pathogen-free animal colony, were used. Twenty four h before tumor implantation, nude mice were further immunosuppressed by whole-body irradiation with 6 Gy (0.8 Gy/min) using a Gammacell 137Cs unit (Atomic Energy Canada Limited, Ottawa, Canada). The human tumors HSTS-26T and U87 were implanted in nude mice. The murine isograft MaCa-IV was implanted in C3H mice.

Paclitaxel and Docetaxel. Paclitaxel (Taxol; Sigma Chemical Co., St. Louis, MO) was initially dissolved in absolute ethanol with an equal volume of Cremophor EL (Sigma), sonicated for 1 h, and stored at 4°C for up to 1 week. The final paclitaxel solution was prepared by diluting the stock solution 4 times with sterile physiological saline and injected within 10 min of preparation.

Docetaxel was provided by Dr. M. C. Bissery (Taxotere; Rhône Poulenc Rorer, Vitry sur Seine Cedex, France). The stock solution (50 mg/ml) in absolute ethanol was stored at −20°C. The final solution was obtained by mixing 1 volume of the stock solution with 1 volume of polysorbate 80 (Sigma) and 18 volumes of 5% glucose solution, and it was injected within 10 min of formulation. Both drugs were injected at a dose of 40 mg/kg body weight into the tail vein. In the control groups, only the drug carrier (Cremophor or polysorbate 80) was injected.

IFP and MVP Measurements. IFP was measured with the “wick-in-needle” technique (18). The pressure in each tumor was the average of two IFP measurements. IFP was measured at 9, 24, 48, 72, and 96 h (up to 120 h with HSTS-26T) after paclitaxel or docetaxel injections. IFP was measured at two different times in the same tumor (a) 9 and 48 h, (b) 24 and 72 h, and (c) 96 and 120 h after paclitaxel and docetaxel injections and in untreated tumors. The initial IFP was measured at a tumor size of ~250 mm³. There were 7–10 mice per group. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed on a heating pad to maintain the body temperature at 37°C.

The MVP was measured in vessels at the surface of HSTS-26T tumors (8 × 8 mm) with the micropuncture technique and a servo-null system (7). The MVP was also measured using the same technique at 1–2 mm from the tumor surface. The skin overlying the tumors was stretched due to tumor growth, which permitted the visualization of vessels located at the skin-tumor interface. Micropipettes were easily introduced in superficial vessels through the skin. MVP was measured in two to four vessels per tumor. Following the measurement, a small volume of Evans blue dye (0.05% by weight) was injected to...
verify the location of the micropipette in the lumen of the vessel. MVP was measured before and 24 h after paclitaxel injection.

Measurement of MABP. MABP was measured by inserting a PE10 polyethylene catheter in the left carotid artery in treated and untreated controls (19).

Estimation of Hydraulic Conductivity. Slices of untreated or treated (24 and 96 h after paclitaxel treatment) tumors were perfused in an in vitro flow chamber (20). Tumors were excised and immediately placed in isotonic saline at room temperature. Tissue slices (~1.4 mm thick) were cut from the central part of the tumor using two surgical blades mounted with a fixed separation of

Table 1 Mean vessel diameter, vascular density, and RBC velocity in untreated and paclitaxel-treated HSTS-26T tumors

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Density (cm/cm²)</th>
<th>Diameter (μm)</th>
<th>RBC velocity (mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h (9)</td>
<td>124.6 ± 18.7 (89.3–145.7)</td>
<td>5.7 ± 1.5 (3.7–8.4)</td>
<td>0.09 ± 0.00</td>
</tr>
<tr>
<td>48 h (6)</td>
<td>127.7 ± 18.8 (112.7–143.5)</td>
<td>8.3 ± 2.7 (5.5–11.8)</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>96 h (6)</td>
<td>118.3 ± 44.9 (74.4–174.27)</td>
<td>10.5 ± 4.4 (6.1–15.8)</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

* Time after paclitaxel treatment.

** n, number of animals.

" Mean ± SD.

" Range.

\* P < 0.05 vs. 0 h.
Mouse serum filled the flow cell and a 10-cm portion of the inflow and outflow lines. A constant hydrostatic pressure head (~7 mmHg) was applied across the tissue slice, the flow was measured by recording with a charge coupled device camera (AVC-D7; Sony, Tokyo, Japan) linked to a stereomicroscope (SMZ-U; Nikon, Tokyo, Japan), the movement of an air bubble that was introduced in the capillary flow meters. Capillary flow was analyzed off-line. Hydraulic conductivity measurements were obtained by a least squares nonlinear regression of the integral of a monoexponential function to the experimental data.

Vessel Diameter and Blood Velocity. HSTS-26T tumors were transplanted on the striated skin muscle in the dorsal skin fold chamber of SCID mice instead of nude mice. SCID mice were used because the skin of nude mice cannot hold a dorsal skin fold chamber for several weeks. To test whether the effect of paclitaxel on tumor IFP was similar in SCID and nude mice, we grew HSTS-26T tumors s.c. in SCID mice. At 48 h following paclitaxel, IFP decreased by 54%, which is comparable to the IFP decrease in HSTS-26T transplanted in nude mice (see “Results”).

The chamber preparation, tumor implantation, and measurements of functional vessel density (defined as total length of vessels per unit area), vessel diameter, and RBC velocity were performed as described previously (21). The development of the tumor neovasculature was characterized 2–3 weeks after tumor implantation with an intravital microscope (Axioplan, Zeiss, Oberkochen, Germany). Each parameter was measured in randomly selected vessels before paclitaxel injection, as well as 48 and 96 h after paclitaxel injection. In the untreated group, vascular parameters were also measured at 0, 48, and 96 h.

Data points, means of 7–10 mice; bars, SD. * P < 0.05 (ANOVA multiple comparison test).
Immunohistochemistry. The tumors were collected, formalin-fixed, and paraffin-embedded. Tissue blocks were cut into 4-μm sections that were stained for TUNEL or anti-CD31. At different times (9–120 h) after treatment with taxanes, apoptotic cells were detected by using the nick end labeling technique (Apoptag-Peroxidase kit; Oncor Inc., Gaithersburg, MD). Briefly, dewaxed sections were rehydrated and treated with 0.2% proteinase K (Sigma) for 30 min at 37°C before endogenous peroxidase was blocked with 2% hydrogen peroxide for 5 min. Slides were incubated with terminal deoxynucleotidyl transferase enzyme and digoxigenin-labeled nucleotides for 1 h at 37°C followed by an incubation with antidigoxigenin-peroxidase for 30 min at room temperature. Slides were then developed with diaminobenzidine and counterstained with hematoxylin. Five fields of nonnecrotic areas (0.067 mm² per field) were randomly selected in each histological specimen. The number of apoptotic and intact tumor cells per mm² was determined.

For vessel density, tumor sections were stained with the anti-CD31 monoclonal antibody (clone MEC13.3; PharMingen, San Diego, CA) using the avidin-biotin peroxidase procedure (Vectastain; Vector Laboratories, Inc., Burlingame, CA). Dewaxed sections were rehydrated and treated with 0.1% trypsinase (Sigma) for 30 min at 37°C before endogenous peroxidase was blocked with 2% hydrogen peroxide for 5 min. The slides were placed in normal rabbit serum for 20 min and then incubated overnight at 4°C with anti-CD31 diluted 1:50 in normal rabbit serum. The slides were then incubated with rabbit antirat biotinylated antibody, followed by peroxidase-conjugated avidin. After the diaminobenzidine reaction, the section were counterstained with hematoxylin. In each section histologically recognizable blood vessels were counted at a magnification of ×200. Cluster of endothelial cells, with or without a lumen, were considered as individual vessels. Microvessel counts were expressed as the number of vessels per field.

Statistical Analysis. All measured values are presented as the mean ± SD. Significant differences between groups were evaluated by ANOVA or the Mann-Whitney U test. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Tumor Growth Is Inhibited by Taxanes. Treatment with paclitaxel and docetaxel significantly inhibited the growth of MCA-IV and HSTS-26T (Fig. 1). U87 treated with paclitaxel grew at the same rate as control tumors, whereas docetaxel retarded the growth of U87 (Fig. 1). In accordance with in vitro studies, docetaxel was found to be a more potent cytotoxic agent than paclitaxel (23).

Taxane-induced Apoptosis Reduces Neoplastic Cell Density. To determine whether tumor growth inhibition was related to taxane-induced apoptosis and a resulting decrease in neoplastic cell density, we counted apoptotic and intact neoplastic cells on TUNEL-stained tissue sections. In MCA-IV, paclitaxel and docetaxel induced a rapid increase in the number of apoptotic cells that was close to control values at 48 h (Fig. 2). Neoplastic cell density was significantly reduced by taxane treatment (Fig. 2). The extent and kinetics of apoptosis induction in MCA-IV (from 9 to 24 h) were comparable to previous findings for MCA-IV (15, 16). In HSTS-26T, the number of apoptotic cells increased at early time points, and the neoplastic cell number decreased with time (Fig. 2). The number of apoptotic cells increased slightly following taxane in U87, but no significant difference in apoptosis or cell density was found between treated and control tumors.

Paclitaxel Increases the Diameter of Tumor Vessels. To assess whether neoplastic cell loss would decompress blood vessels, we measured vessel diameter in HSTS-26T transplanted in dorsal skin fold chambers. Vascular density and RBC velocity were also measured in the same tumor preparation. Paclitaxel increased blood velocity; however, there was no change in vessel density (Table 1). The vascular diameter of treated tumors was increased significantly at 48 and 96 h (Table 1), whereas that of untreated control group did not change significantly (data not shown). The doubling of vascular diameter at 96 h indicates that the vascular surface area increased by 2-fold. The increase in RBC velocity and vessel diameter suggests that blood flow increased by severalfold (~3–5-fold). Increases in vascular surface area and perfusion are likely to increase drug delivery in tumors responding to taxanes.

Relationship between Tumor Blood Vessels and Neoplastic Cells. Modifications in vascular architecture and the relationship between tumor cells and blood vessels were characterized histologically. An antibody against CD31 was used to reveal blood vessels. Microvessel counts were made in regions of high vessel density (hot spots). Similar to the intravitaly measured vascular density in HSTS-26T, taxane treatment did not modify the vascular counts on histo-
vascular endothelium (Fig. 3). In untreated tumors, neoplastic cells often appeared in contact with the lumen that were observed in regions occupied by intact neoplastic cells as well as by cellular debris. Thus, solid stress induced by the neoplastic cell density between docetaxel-treated and untreated U87 tumors (data not shown). Paclitaxel did not decrease the IFP of U87 tumors, whereas the IFP of MCa-IV decreased over the period of 9–96 h (P < 0.05). In contrast, the IFP of MCa-IV decreased over the period of 9–96 h (P < 0.05; Fig. 4). In MCa-IV, IFP decreased significantly following paclitaxel and docetaxel treatments. The IFP of HSTS-26T was significantly reduced from 24 to 96 h after paclitaxel and docetaxel injections (Fig. 4). In contrast to MCa-IV and HSTS-26T, there was a significant delay that preceded the IFP reduction in U87 treated with paclitaxel; IFP decreased by 33 and 42% at 72 and 96 h, respectively (data not shown). Paclitaxel did not decrease the IFP of U87 tumors, which do not respond to paclitaxel. In tumors with a growth delay induced by paclitaxel or docetaxel, MVP reduction is the likely cause of the IFP decrease.

To evaluate for possible correlations between IFP and changes in cellular density and tumor growth, IFP was measured with the wick-in-needle technique in MCa-IV, U87, and HSTS-26T at different times after a single dose of 40 mg/kg paclitaxel or docetaxel. Because of probable changes in pressure with tumor growth, the IFPs of untreated and treated tumors were measured at the same time points. The IFP of HSTS-26T and U87 was independent of tumor growth. In contrast, the IFP of MCa-IV decreased over the period of 9–96 h (P < 0.05; Fig. 4). In MCa-IV, IFP decreased significantly following paclitaxel and docetaxel treatments. The IFP of HSTS-26T was significantly reduced from 24 to 96 h after paclitaxel and docetaxel injections (Fig. 4). In contrast to MCa-IV and HSTS-26T, there was a significant delay that preceded the IFP reduction in U87 treated with docetaxel; IFP decreased by 33 and 42% at 72 and 96 h, respectively (data not shown). Paclitaxel did not decrease the IFP of U87 tumors, which do not respond to paclitaxel. In tumors with a growth delay induced by paclitaxel or docetaxel, MVP reduction is the likely cause of the IFP decrease.

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Potential Role of Interstitial Matrix. Modifications in IFP did not always track neoplastic cell density. At 9 h, in MCa-IV there was a significant decrease in cell density without a change in IFP, whereas in HSTS-26T treated with docetaxel, the reduction in IFP was not associated with a decrease in cellular density (Figs. 2 and 4). In HSTS-26T, at 120 h following paclitaxel, the IFP increase was not associated with an increase in neoplastic cell density. Because of the high hydraulic conductivity of tumor vessels, IFP decreases rapidly (within seconds) following a decrease in MVP (3). Thus, the rapid equilibration of MVP and IFP cannot explain the difference in IFP response. Modifications in vessel diameter, MVP, and IFP following a change in neoplastic cell density are dependent on the solid stress and elasticity of the interstitial matrix and vascular wall. The rate of solid stress build-up or release will be a function of the elasticity and the magnitude of matrix deformation. Thus, even a slight deformation of a stiff matrix could induce high levels of solid stress, whereas a soft matrix will respond poorly to solid stress changes resulting from tumor cell growth or cell loss. Stress-strain tests have shown that MCa-IV responds poorly to an applied stress. In comparison, the matrix of HSTS-26T is relatively elastic (17). The soft matrix of MCa-IV might explain the time delay in IFP response following neoplastic cell loss. The stiffer matrix of HSTS-26T could respond relatively quickly, even to small changes in cellular density or in the volume of individual cells. At 9 h following docetaxel, the IFP decrease was associated with a small reduction in cellular density (which was not significant) and a 2.5-fold increase in the number of apoptotic cells. Apoptosis can reduce the volume of individual cells by 40–50% (25). Small changes in cell volume could be significant because neoplastic cells represent >80% of the volume of HSTS-26T tumors. Thus, in HSTS-26T, small changes in cellular volume combined with the relatively small volume of the interstitial matrix could lead to a significant expansion of the matrix and diameter of blood vessels. The differences in IFP response between MCa-IV and HSTS-26T tumors suggest that the transmission of solid stress produced by neoplastic cell proliferation in a confined space is highly dependent on the physical properties of the interstitial matrix.

Clinical Implications. The findings of this study have both prognostic and therapeutic implications. Our work and the work of others have shown that tumor IFP measurements can predict tumor response in patients (26–28). In patients with cervical carcinomas, increases in IFP during radiation were associated with a poor response, whereas a decrease in IFP was indicative of a good response (26, 27). The mechanisms responsible for the changes in tumor IFP during treatment are poorly understood. This study provides insight into the effect of solid stress and the interrelationships among neoplastic cells, the tumor vasculature, and IFP. The increase in vascular diameter without a change in tumor vascular density indicates that taxane increases blood flow and the vascular surface area for exchange in tumors. Thus, taxanes could improve the delivery of therapeutic agents to tumors. Milas et al. (29) have shown that paclitaxel significantly increases the partial pressure of oxygen in tumors and enhances tumor response to radiation under air-breathing but not under hypoxic conditions. The increase in tumor pO2 could be due to an increase in oxygen delivery and/or a decrease in oxygen-consuming cells. The combination of paclitaxel and herceptin (antibody against the HER2/neu receptor that blocks tumor cell proliferation) significantly improves the tumor response of mammary carcinomas overexpressing HER2/neu as compared to paclitaxel or herceptin alone (30). Thus, the improvement of tumor vascular parameters by taxanes could be a significant mechanism contributing to improved tumor response to antitumor agents. Increases in tumor vascular diameters and blood flow have also been induced by other chemotherapeutic agents and radiation (31, 32). Of course, the more efficient vascular network could also improve the delivery of nutrients making the tumor microenvironment more favorable for growth (31). In conclusion, our data demonstrate that the reduction in tumor MVP and IFP with taxanes is associated with significant increases in the diameter of tumor blood vessels. Modifications in neoplastic cell density are most likely responsible for the changes in vascular architecture and pressure. The transmission of solid stress generated by proliferating neoplastic cells is also dependent on the relative stiffness of the interstitial matrix.

ACKNOWLEDGMENTS

We thank Sylvie Roberge and Julia Kahn for excellent technical assistance and Tim Padera, Leo Gerwick, and Mark Heijn for helpful comments.

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