Elevated Physiologic Tumor Pressure Promotes Proliferation and Chemosensitivity in Human Osteosarcoma

Saminathan S. Nathan, Gene R. DiResta, Jorge E. Casas-Ganem, Bang H. Hoang, Rebecca Sowers, Rui Yang, Andrew G. Huvos, Richard Gorlick, and John H. Healey

1Orthopaedic Surgery Service, Department of Surgery, and Departments of 2Pediatrics and 3Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York

ABSTRACT

Purpose: This study investigates the effect of constitutively raised interstitial fluid pressure on osteosarcoma physiology and chemosensitivity.

Experimental Design: We did pressure and blood flow assessments at the time of open biopsy in patients with the diagnosis of high-grade osteosarcoma and correlated this to survival and chemotherapy-associated tumor necrosis. Osteosarcoma cell lines were then evaluated for proliferative and therapeutic indices in a replicated high-pressure environment.

Results: Sixteen osteosarcomas in vivo were assessed and exhibited elevated interstitial fluid pressures (mean 35.2 ± SD, 18.6 mmHg). This was not associated with significantly impeded blood flow as measured by a Doppler probe at a single site (P < 0.12). Nonetheless, greater chemotherapy-associated necrosis and associated longer survival were seen in tumors with higher interstitial fluid pressures (P < 0.05).

In vitro, cells undergo significant physiologic changes under pressure. Osteosarcoma cell lines grown in a novel hydrostatically pressurized system had variable cell line-specific growth proportional to the level of pressure. They were more proliferative as indicated by cell cycle analysis with more cells in S phase after 48 hours of pressurization (P < 0.01). There was a significant elevation in the cell cycle–related transcription factors E2F-1 (P < 0.03) and E2F-4 (P < 0.002). These changes were associated with increased chemosensitivity. Cells tested under pressure showed an increased sensitivity to cisplatin (P < 0.00006) and doxorubicin (P < 0.03) reminiscent of the increased chemotherapy-associated necrosis seen in tumors with higher interstitial fluid pressure in the clinical study.

Conclusions: The results of this study suggest that cells in the in vivo pressurized environment are at a higher state of regenerative activity than is demonstrable in conventional cell culture systems. Variations in tumor interstitial fluid pressure have the potential to alter chemotherapeutic effects.

INTRODUCTION

Tumors in vivo have high interstitial fluid pressures (1–5). Interstitial fluid pressure results in a rapid transmembrane stress equilibration in cells. The present study investigated the biological effect that this elevated interstitial fluid pressure has on osteosarcoma cells.

Controversy exists as to the relationship between interstitial fluid pressure and blood flow in tumors. This pressure could reduce perfusion of the tumor resulting in necrosis (1). An alternative explanation is that although interstitial fluid pressures are elevated within tumors, so are pressures in the tumor microvasculature; equilibrium is maintained and blood flow is sustained (3). It has been shown that solid stress or tension derived from appositional tumor cell growth is sufficient to occlude vessels and may affect the spatial heterogeneity seen in tumor microvasculature (6–8). This solid stress may be the mechanism of reduced tumor perfusion rather than the raised interstitial fluid pressure per se.

Previous work has investigated the effect of pressure in neoplastic systems (1–4, 6, 9–12). It has been suggested that because tumor interstitial fluid pressures and microvascular pressures are similar and the resultant pressure gradient negligible, the movement of molecules across the vascular membranes would be reduced—an explanation for reduced drug delivery and hence efficacy (3, 13–15). We investigated this proposition through a clinical analysis of tumor chemotherapy–associated necrosis in relation to tumor interstitial fluid pressures.

Studies have shown that tumors experience elevated interstitial fluid pressures of 20 to 50 mmHg (1–4). This is higher than typical pressures found in normal adjacent tissues (1, 10). Cell functions, however, are traditionally assessed at 0 mmHg added pressure or atmospheric pressure. Significant differences may occur when cells are grown or tested at more physiologic “tumor pressures.” These differences could have fundamental influence on the way that cells are evaluated.

This study tested two hypotheses through clinical and in vitro investigation. In the clinical phase, we tested whether human high-grade osteosarcomas have raised interstitial fluid pressures. The aim was to establish the magnitude of interstitial fluid pressure elevation in vivo and compare measurements with clinical data to establish a set of reference norms and characteristics specific to this sarcoma. Secondarily, we sought to show how this state of raised interstitial fluid pressure correlated with blood flow in these tumors and investigate its relation to chemotherapy-associated necrosis.
In the *in vitro* phase, we tested whether osteosarcoma cell proliferative phenotype was affected by elevated interstitial fluid pressure and whether this affected chemotherapeutic sensitivity. Considering that interstitial hypertension is a characteristic of these tumors, this study investigates the clinical significance of the elevated pressure and has important implications on the validity of conventional chemotherapy testing experiments that are done at atmospheric pressure.

**MATERIALS AND METHODS**

**Clinical Study.** Between January 1997 and June 2000, all patients with the clinical diagnosis of high-grade osteosarcoma underwent open biopsy. The Institutional Review Board granted approval for the use of clinical data (Table 1). At the time of biopsy, interstitial fluid pressure and blood flow measurements were made routinely, the specific techniques of which have been described (1). Briefly, interstitial fluid pressure was measured using a wick-in-needle probe and a Camino 420 digital pressure monitor to a sensitivity of 0.1 mmHg (Camino Laboratories, San Diego, CA). Blood flow within tumors was measured by a Vasamedics BPM 403 Laserflo laser Doppler flow perfusion monitor attached to a 0.8 mm diameter probe (TSI, Inc., Missoula, MT). Readings were taken ~1 cm from the tumor surface and from the neighboring soft tissue.

The patients were put on a 6- to 8-week course of chemotherapy. All patients who received neoadjuvant chemotherapy received cisplatin, doxorubicin, and methotrexate. One patient (case 1) received ifosfamide additionally and one received methotrexate alone (case 15). They were then restaged and underwent a definitive resection and reconstruction for their tumors. Tumors were assessed for chemotherapy-associated necrosis and graded as per published institutional pathology protocol (16–18). Briefly, this is a four-part grading system of the extent of necrosis as determined by multiple sections of postchemotherapy resection specimens, which has shown good correlation with survival. Grade 1 corresponds to necrosis in <50% of the tumor, grade 2 to 50% to 90% necrosis, grade 3 to >90% necrosis, and grade 4 to near-total necrosis. This four-part system was reclassified into the two categories of standard and good necrosis to facilitate statistical analysis. Standard necrosis was defined as <90% necrosis (grades 1 and 2) and good necrosis as defined as 90% or greater necrosis (grades 3 and 4) of the resected specimen (Fig. 1). Readings obtained at the time of biopsy were correlated with chemotheraphy-associated necrosis grade and survival. All clinical data are presented in the text as the mean ± SD. Continuous variables were assessed with Student’s *t* test.

Survival status was gleaned from a prospectively maintained institutional database (Disease Management System v 5.2, 1996, Memorial Sloan-Kettering Cancer Center, New York, NY). Data were maintained in hard copy and electronically in a spreadsheet in Microsoft Excel version 10 for Windows NT (Redmond, WA).

All statistical analyses were done using SPSS version 11.5 for Windows NT (SPSS, Inc., Chicago, IL). Statistical significance was defined as *P* < 0.05.

Table 1  Clinical data on patients with high-grade osteosarcomas for whom interstitial fluid pressure measurements were made of the tumor and surrounding normal tissue

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor location</th>
<th>Histology</th>
<th>Size* (cm)</th>
<th>Normal tissue IFP (mmHg)</th>
<th>Tumor IFP (mmHg)</th>
<th>Status at last review</th>
<th>Survival (mo)</th>
<th>Chemotherapy response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>8</td>
<td>Distal femur</td>
<td>Chondroblastic</td>
<td>95</td>
<td>–1.9</td>
<td>61.2</td>
<td>Alive</td>
<td>60</td>
<td>Good</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>9</td>
<td>Proximal tibia</td>
<td>Not otherwise specified</td>
<td>86</td>
<td>2.8</td>
<td>18.2</td>
<td>Alive</td>
<td>82</td>
<td>Standard</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>10</td>
<td>Proximal humerus</td>
<td>Not otherwise specified</td>
<td>183</td>
<td>3.1</td>
<td>31</td>
<td>Expired</td>
<td>18</td>
<td>Standard</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>11</td>
<td>Distal femur</td>
<td>Giant cell rich</td>
<td>75</td>
<td>9.5</td>
<td>18.3</td>
<td>Alive</td>
<td>73</td>
<td>Good</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>11</td>
<td>Distal femur</td>
<td>Not otherwise specified</td>
<td>96</td>
<td>3.8</td>
<td>35.1</td>
<td>Alive</td>
<td>51</td>
<td>Standard</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>13</td>
<td>Distal tibia</td>
<td>Telangiectatic</td>
<td>97</td>
<td>2</td>
<td>10</td>
<td>Expired</td>
<td>33</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>15</td>
<td>Sacrum</td>
<td>Chondroblastic</td>
<td>30</td>
<td>4.4</td>
<td>17</td>
<td>Expired</td>
<td>13</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>15</td>
<td>Distal femur</td>
<td>Not otherwise specified</td>
<td>90</td>
<td>10.5</td>
<td>34.1</td>
<td>Expired</td>
<td>32</td>
<td>Standard</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>17</td>
<td>Proximal tibia</td>
<td>Not otherwise specified</td>
<td>25</td>
<td>3</td>
<td>31</td>
<td>Alive</td>
<td>91</td>
<td>Good</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>17</td>
<td>Proximal tibia</td>
<td>Not otherwise specified</td>
<td>70</td>
<td>1.4</td>
<td>32.5</td>
<td>Alive</td>
<td>61</td>
<td>Standard</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>18</td>
<td>Proximal tibia</td>
<td>Giant cell rich</td>
<td>66</td>
<td>5.1</td>
<td>68.9</td>
<td>Alive</td>
<td>68</td>
<td>Good</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>19</td>
<td>Distal femur</td>
<td>Malignant fibrous histiocytoma</td>
<td>55</td>
<td>8.8</td>
<td>31.9</td>
<td>Alive</td>
<td>61</td>
<td>Standard</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>20</td>
<td>Proximal tibia</td>
<td>Chondroblastic</td>
<td>95</td>
<td>–0.4</td>
<td>59.9</td>
<td>Expired</td>
<td>66</td>
<td>Good</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>22</td>
<td>Proximal humerus</td>
<td>Malignant fibrous histiocytoma</td>
<td>25</td>
<td>0.5</td>
<td>38.9</td>
<td>Alive</td>
<td>66</td>
<td>Standard</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>36</td>
<td>Distal femur</td>
<td>Dedifferentiated</td>
<td>130</td>
<td>–13.7</td>
<td>14</td>
<td>Alive</td>
<td>86</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>61</td>
<td>Pubic bone</td>
<td>Radiation sarcoma</td>
<td>85</td>
<td>3.9</td>
<td>61.9</td>
<td>Expired</td>
<td>8</td>
<td>NA</td>
</tr>
</tbody>
</table>

**NOTE.** Tumor pressures were significantly elevated over normal tissue pressures (*P* < 0.00001).

Abbreviations: IFP, interstitial fluid pressure; NA, not applicable.

*Size was expressed as the greatest dimension seen on imaging or pathologic specimens.

†All patients in this series underwent chemotherapy. Chemotherapy response assessment was only available for patients who underwent neoadjuvant therapy.
Cell Culture System. All experiments were done using a novel cell culture system, the Opticell (donated by Biocrystal, Westerville, OH), which was modified to permit cell culture in a pressurized environment (19). Cells are grown as monolayers within the system and pressurized using a fluid column that was adjusted to provide 0, 20, or 50 mmHg of static gauge pressure. Growth media for propagation of cells was identical in all experiments and consisted of 10 mL MEM-α with 20% FCS and penicillin and streptomycin.

The human osteosarcoma cell lines, HOS, SaOS, and U2OS (American Type Culture Collection, Manassas, VA), were grown at three pressures: 0 mmHg (comparable with conventional culture systems), 20 mmHg (an intermediate osteosarcoma pressure observed in clinical studies), and 50 mmHg (the higher pressures observed in these studies; refs. 1, 3, 10). Cells were seeded at $1.5 \times 10^5$ cells into each chamber and separate chambers were provided for the three time points of 24, 48, and 72 hours of pressurization. This cell seeding density ensured that the cells would not reach confluence by 72 hours and influence the results of cell cycle analysis due to contact inhibition. Cells were systematically assessed for growth by taking the average cell counts over five prescribed 100 power fields.

In situ cell counts were first made at 24 hours after seeding—this corresponds to the zero-hour time point in all studies. Cells were then pressurized as described above after the cells had gained attachment and begun to grow. They were then assessed at the completion of the time points. Net growth was expressed as percentage increment in cell number over the baseline zero-hour time point. Cell growth was normalized in this manner to account for the different growth rates seen in different cell lines. This data is expressed in Fig. 2. Each data point represents the mean of three experiments and the error bars represent the SE.

Cells were then released from culture using 3 mL 0.25% trypsin-EDTA and counted with a hemocytometer (Bright-Line, Horsham, PA) using trypan blue stain (Fisher Scientific,

![Fig. 1](https://example.com/figure1.png)

**Fig. 1** Good chemotherapy-associated tumor necrosis as defined in the text was associated with higher interstitial fluid pressure within tumors (A). This greater necrosis was not due to a reduction in blood flow secondary to raised interstitial fluid pressures—blood flow was not consistently related to differences in interstitial fluid pressure (B). Similarly, when comparing size and blood flow to pressure (IFP, interstitial fluid pressure), there was no apparent relationship (C).
Chicago, IL) non-uptake as a marker of cell viability. This data was used as corroborative data for the high-power-field data. The experiment was done in triplicate. Hence, data for each experiment were derived from 27 cassettes—three pressures done at three time points in triplicate.

**Cell Cycle Analysis.** The HOS and U2OS cell lines were used as they showed different growth characteristics under pressure. These were pressurized for 24, 48, and 72 hours. Cells were then prepared for cell cycle analysis. They were fixed in 66% methanol and 33% PBS, then treated with 100 units/mL RNase and DNA fluorescent labeled with 0.05 mg/mL propidium iodide. Morphologic cell cycle parameters were measured by flow cytometry using the FACScalibur System (BD Biosciences, Franklin Lakes, NJ). Data were analyzed using FlowJo 4.3.1 (Tree Star, Ashland, OR). Cell cycle analysis was done by investigating the FL2-A profile of the cells identified to be in cycle (Fig. 3). Each histogram in Fig. 3 represents the concatenated data of three experiments. The Y axis represents the percentage of the total event frequency (% of max). FL2-A on the X axis is the fluorescence of propidium iodide as read by the flow cytometer. Figure 3B represents the percentage of cells in S phase at the three time points at 0 and 20 mmHg, derived from the Dean-Jett-Fox cell cycle algorithm (20). These data were tested for significance using Student’s t test. All experiments were done in triplicate. Each data point represents the mean of three experiments with error bars reflecting the SE.

**Quantitative Real-time PCR.** The E2F family of genes is involved in the transition of cells in G1-S phase (21). Their expression was used as a corroborative test for the proliferative effects of pressure in the system. HOS cells were pressurized for 72 hours, released from culture, and immediately treated with RNA lysis buffer. RNA was extracted with the Stratagene RNA Isolation kit (La Jolla, CA). The Promega Reverse Transcription System (Madison, WI) was used to synthesize cDNA. Standardization, validation, and analysis of the assays have been previously described (22). Briefly, 5 µL cDNA were added to 20 µL of separate aliquots of master mix containing primer and probe sequences for E2F-1 and E2F-4 and the reference housekeeping gene β-actin. Real-time PCR with the iCycler (Bio-Rad, Hercules, CA) was done. Cycling parameters were as follows: one 10-minute hot start at 94°C; 45 cycles of 30-second denaturation at 94°C; 1 minute of annealing at 55°C; 30-second extension at 72°C; and a final 5-minute extension at 72°C. Each sample was run in triplicate for both the target gene and the housekeeping gene. These data were normalized to the gene expression of a control known to express E2F-1 and E2F-4. The control used was a laboratory grown osteosarcoma cell line designated M187, which was treated in the same way and in the same PCR reaction in a 96-well plate. Significance testing was done with the Wilcoxon two-sample test (Fig. 3C). Each data point represents the mean of six real-time PCR results with error bars reflecting the SE.

**Chemosensitivity Testing in a Conventional System.** HOS cells were pressurized at 0 and 20 mmHg for 72 hours, depressurized to atmospheric pressure, and then trypsinized and seeded at 2,000 cells per well in a Costar 96-well plate
They were allowed 24 hours to gain attachment. Serial dilutions of doxorubicin were immediately applied to the wells at a dose of 100 to 0.00128 μg/mL. These were allowed an exposure time of 24 hours and then washed with PBS and 200 μL growth media replaced for a further 24 hours. Twenty-five microliters of Alamar blue dye (BioSource International, Camarillo, CA) were added for a further 8 hours and metabolic activity read with a Cytofluor plate reader (Applied Biosystems, Foster City, CA). Metabolic activity was expressed as per manufacturer recommendations (BioSource International) as described previously (23). A similar set of experiments was done to assess for cisplatin sensitivity under pressure. Student’s t test was used to assess for significance between the results of chemosensitivity at 0 and 20 mmHg (Fig. 4). Each data point represents the mean of six readings with error bars reflecting the SD.

Chemosensitivity Testing in a Pressurized System. To investigate chemosensitivity in a pressurized system, 1.5 × 10^4 HOS cells were seeded in each of 16 Opticell chambers. These were incubated for 24 hours and baseline counts were made at the end of 24 hours. Serial dilutions of doxorubicin were added at a dose of 0, 0.16, 1, and 8 μg/mL. Similarly, serial dilutions of cisplatin were added at doses of 0, 0.16, 1, and 2 μg/mL. Each chamber received a single concentration of the drug. Two sets were provided for each drug at 0 and 20 mmHg. Pressurization proceeded for 72 hours as described above. At the end of this
time point, in situ cell growth over the identical five high-power fields recorded after the first 24 hours were made. The observer was blinded to the identity of the sample being read. Cell viability was expressed as a ratio between the 72-hour and baseline 24-hour counts. This was then normalized to the readings for cells that were not exposed to chemotherapy (i.e., 0 readings for cells that were not exposed to chemotherapy baseline 24-hour counts. This was then normalized to the viability was expressed as a ratio between the 72-hour and paired data, mean blood flow in the tumors was 1.7 ± 1.3 mL/min/100 g as opposed to 12.5 ± 22.2 mL/min/100 g in normal tissue. This difference was not statistically significant (P < 0.12). In two patients, blood flow in the soft tissues was remarkably high (33.6 and 76.8 mL/min/100 g), and in one patient (case 6) tumor blood flow in a telangiectatic osteosarcoma was atypically high (15.3 mL/min/100 g). Removal of these skewed data points further narrowed the differences between blood flow in tumors and that in normal tissue. There was no apparent association between size and interstitial fluid pressure (Fig. 1C) on linear regression analysis (R^2 = 0.002) or between size and blood flow (R^2 = 0.002).

RESULTS

Between January 1997 and June 2000, 20 patients with the clinical diagnosis of osteosarcoma were entered into the study. Survival data were last acquired in May 2004. Four patients were excluded from the study—two had low-grade juxtacortical osteosarcomas, one had a round cell Ewing’s family tumor-like lesion and was lost to follow-up, and one had a telangiectatic osteosarcoma that was ruptured at the time of biopsy and yielded unreliable data. Of the remaining 16 cases (Table 1), the data set was complete except that tumor blood flow could not be obtained in one patient and blood flow in normal tissue could not be obtained in four patients.

There were seven male and nine female patients. Mean age was 19 ± 13 years. Six tumors were in the distal femur, five in the proximal tibia, two in the proximal humerus, one in the distal tibia, one in the pubic bone, and one in the sacrum. Mean size as per the longest dimension was 81 ± 40 cm. There were six osteosarcomas of mixed subtype and three chondroblastic, two giant cell rich, two malignant fibrous histiocytoma—like, one postradiation osteosarcoma, and one dedifferentiated juxtacortical osteosarcoma. Necrosis data were available in the 12 patients who underwent neoadjuvant chemotherapy—five had a good response and seven had a standard response. At last follow-up, there were six deaths in the cohort. Median survival was 61 (range, 8 to 91 months) months.

Mean interstitial fluid pressure in this cohort of osteosarcomas was 35.2 ± 18.6 mmHg. This was significantly different from the interstitial fluid pressure in the surrounding normal soft tissue, which was 2.7 ± 5.6 mmHg (P < 0.00001). Of the paired data, mean blood flow in the tumors was 1.7 ± 1.3 mL/min/100 g as opposed to 12.5 ± 22.2 mL/min/100 g in normal tissue. This difference was not statistically significant (P < 0.12). In two patients, blood flow in the soft tissues was remarkably high (33.6 and 76.8 mL/min/100 g), and in one patient (case 6) tumor blood flow in a telangiectatic osteosarcoma was atypically high (15.3 mL/min/100 g). Removal of these skewed data points further narrowed the differences between blood flow in tumors and that in normal tissue. There was no apparent association between size and interstitial fluid pressure (Fig. 1C) on linear regression analysis (R^2 = 0.002) or between size and blood flow (R^2 = 0.002).

Interstitial fluid pressures correlated with the extent of chemotherapy-associated necrosis rates. In patients with good necrosis, interstitial fluid pressure was 47.9 ± 21.9 mmHg in contrast to those with standard response who had a reading of 31.7 ± 6.5 mmHg (Fig. 1A). This was statistically significant (P < 0.05). Tumor necrosis was not apparently associated with reduced blood flow in this group of 12 patients. Blood flow in tumors with good necrosis was 1.3 ± 0.9 mL/min/100 g, whereas that in tumors with standard necrosis was 1.9 ± 1.6 mL/min/100 g (P < 0.3). There was no apparent association (R^2 = 0.0002) between tumor interstitial fluid pressure and blood flow on linear regression analysis (Fig. 1B).

Good necrosis was associated with longer survival (median 68 months), whereas standard necrosis (median 61 months) was associated with poorer survival (P < 0.06).

In deciding what pressure to replicate for the in vitro portion of the study, we used the “inherent” tumor interstitial fluid pressure defined as the difference between the tumor interstitial fluid pressure and the soft tissue interstitial fluid pressure. This had the theoretical effect of reducing the possible error resulting from the tumors being from different locations in the body (in reference to central venous pressure). Accordingly, the inherent pressures recorded were 32.6 ± 19.1 mmHg. The actual range was 8.0 to 63.8 mmHg. Consequently, the pressures chosen for in vitro assessment were 0, 20, and 50 mmHg, respectively, consistent with other work in the field (1–4).

**Fig. 4** Conventional chemosensitivity testing (done at atmospheric pressure) of HOS cells with cisplatin (A) and doxorubicin (B) showed that there was a marginal, although statistically significant, difference in cytotoxicity (P < 0.05) in the clinically relevant range of 0.04 to 4 μg/mL with pressurized cells showing increased chemosensitivity.
Figure 2 shows the percentage incremental growth of the three osteosarcoma cell lines under pressure. Cells grown at 0 mmHg exhibited growth that proceeded in an exponential fashion as found in traditional culture systems. Cell lines grown under pressure exhibited two distinct patterns of proliferation. The HOS and SaOS cell lines grown under gauge pressures of 20 and 50 mmHg proliferated at relatively suppressed rates of growth with respect to pressure. The U2OS cell line proliferated at rates that exceeded growth at 0 mmHg.

The cells grown under 20 mmHg pressure were phenotypically distinct from cells grown at atmospheric pressure (0 mmHg). Figure 3A shows the cell cycle analysis as determined by propidium iodide FL2-A fluorescence of HOS cells grown at two pressures. This showed that whereas the cells began at 24 hours with similar rates of proliferation as indicated by a similar proportion of cells in S phase, at 48 and 72 hours, the cells under pressure were at a higher proliferative state with a greater number of cells in S phase ($P < 0.01$). Figure 3B shows the percentage of cells in S phase at the three time points at two pressures. This shows the significant increase in cells in S phase at 48 and 72 hours as described above. Similar results were found with the U2OS cell line grown under pressure (data not shown). Figure 3C shows the relative expression of E2F-1 and E2F-4 at 0 and 20 mmHg compared with the M187 cell control. It shows a statistically significant up-regulation of E2F-1 ($P < 0.03$) and E2F-4 ($P < 0.002$) under pressure. This accounts for the greater fraction of cells in S phase under pressure as described above. We analyzed the pre-G1 fraction of the cells that seemed to have conflicting responses to pressure as shown in Fig. 2. Despite both cell lines exhibiting a more proliferative phenotype, U2OS exhibited less death while HOS exhibited more death under pressure (Fig. 3D). This relative fragility of the cells accounted for the comparatively increased growth under pressure with U2OS and the comparatively suppressed growth under pressure with HOS. Nevertheless, it should be noted that after a period of pressurization, the HOS cells seemed to have adapted to this suppressive effect and approximated growth at 0 mmHg by 72 hours (Fig. 2A).

Figure 4 shows the results of the cisplatin and doxorubicin chemosensitivity assays under conventional normal pressure conditions. Whereas there is a statistically significant difference ($P < 0.05$) in the ED$_{50}$ between cells grown at atmospheric pressure and those grown under 20 mmHg pressure, the absolute difference is small with cisplatin and almost negligible with doxorubicin. When this experiment was done with cells exposed under pressure to serial dilutions of cisplatin or doxorubicin, there were marked differences in growth between the pressurized and nonpressurized systems at 0.16 and 1 g/mL (Fig. 5). This resembled the findings in the clinical study and underscored the importance of chemosensitivity testing under physiologically comparable conditions of pressure.

**DISCUSSION**

This paper is the first to establish that clinical human osteosarcomas generate interstitial fluid pressures in the order of 35.2 ± 18.6 mmHg (1–4). Good chemotherapy-associated necrosis was predictive of better survival as shown in most studies (16, 24). Interestingly, higher grades of chemotherapy-associated necrosis were correlated with higher pressure readings ($P < 0.05$) even in the relatively small cohort of 12 patients for whom necrosis data were generated (Fig. 1A). This suggests that increased interstitial fluid pressures did not prevent necrosis, and that adequate delivery of small and intermediate size chemotherapy molecules must have occurred. Evidently, other variables confound the relationship between interstitial fluid pressure, drug delivery, and drug efficacy (13).

We show that raised interstitial fluid pressure was not consistently associated with reduced tumor perfusion in human osteosarcomas. This has similarly been shown in xenograft models and spontaneously occurring tumors in animals (3, 12, 25, 26). We were unable to show any relationship between tumor interstitial fluid pressure and tumor size (Fig. 1C). Size has been an inconsistent predictor of tumor interstitial fluid pressure in the literature (2–4, 27, 28).

Variations in blood flow as measured by Doppler do not fully account for the extent of tumor necrosis observed in this series. The greater necrosis in the tumors with higher pressure could not be explained by reduced blood flow causing ischemic necrosis, nor can it be explained by increased flow-related drug delivery resulting in increased necrosis. Another mechanism that has not been excluded is the possible increased toxic effect of the high interstitial fluid pressure...
Physiologic Pressure and Osteosarcoma Chemosensitivity

itself on the cancer cells. This is unlikely given that these tumors are able to grow in the presence of raised pressure as shown in Fig. 2. The association between chemotherapy-associated necrosis and high pressure suggests that raised interstitial fluid pressure may potentiate the effects of chemotherapeutic agents. To investigate the effects of raised pressure at the cellular level, we did in vitro assessments of cells grown under pressure. It would have been interesting to grow patient-derived samples under pressures corresponding to in vivo measurements, but we were unable to perform this analysis for lack of cell lines for which pressure readings were available; however, this should be addressed in future work.

Among the important observations from the experiment described in Fig. 2 is that different cell lines have different susceptibility to pressure. Osteosarcoma cell lines grown under physiologic hydrostatic pressure yielded two distinct patterns of proliferation. With the HOS and SaOS cell lines, growth under gauge pressure of 20 and 50 mmHg resulted in relative suppression of proliferation. Despite this, there was an apparent adaptation to the pressure and increased proliferation of cells after 24 hours. With the U2OS cell line, the reverse pattern occurred with proliferation becoming relatively greater under pressure. To neutralize the potential influence of small variation in initial plating density of the cells, the results were normalized and three separate experiments were run. Each data point, therefore, represents the mean of three experiments. It should be noted that within individual experiments, growth under pressure could be shown to exceed growth at 0 mmHg in all cell lines. It has, therefore, been shown that tumor cells grown under replicaded physiologic tumor pressures is not only possible but may be optimal in the case of individual cell lines.

Cell proliferation was investigated using cell cytometry. The experiments suggest that after 48 hours of pressurization, the cells adopt a more proliferative phenotype with more cells in S phase compared with the unpressurized cell population. The finding that the E2F transcription factors were up-regulated suggests that pressure may induce the cells to undergo proliferation via a pressure-mediated response upstream of the cell cycle–related pathways.

Proliferating tumors are theoretically more sensitive to chemotherapy. Cell cycle active agents would interfere with processes leading to cell division and, hence, cells at a higher rate of division would be more profoundly affected. Cell cycle–independent agents like cisplatin directly platinate DNA and, hence, affect most functions of the cell. Therefore, these effects would also be more pronounced in proliferative cell types where cell activity is increased (29).

In this study, cells grown under physiologic tumor pressure were more sensitive to chemotherapeutic agents as shown in Figs. 4 and 5. This resembles the clinical finding of increased chemosensitivity being associated with higher tumor interstitial fluid pressures. Of interest, this effect was muted when cells were trypsinized, freed from excess pressure, and allowed to reattach over a 24-hour period before being tested again as shown in Fig. 4. This suggests that the pressure effect is a phenomenon that exists only while the cells remain pressurized as in the in vivo situation. In all experiments, chemosensitivity was significantly increased under elevated pressure in the clinically relevant range of 0.04 to 4 μg/mL (30). The other implication is that tumors that experience elevated pressures in their in vivo state propagate as relatively resistant lines in cultures at atmospheric pressure. This has bearing on investigations involving pathways and mechanisms that influence the proliferative state of cells. The findings suggest that chemotherapy testing in conventional systems may not adequately reflect the in vivo situation.

In conclusion, elevated interstitial fluid pressures are a characteristic of native human osteosarcomas and higher pressures correlated with greater necrosis after neoadjuvant chemotherapy. This study has shown that the incorporation of physiologic pressure as an additional variable in cell culture may result in significant changes to the cell under study. A paradox ensues—cells become more proliferative and consequently more sensitive to chemotherapeutic agents when subjected to elevated pressure. These findings suggest that pressure is a critical parameter that should be included in the design of cell culture systems that would better replicate the clinical situation.

REFERENCES


Elevated Physiologic Tumor Pressure Promotes Proliferation and Chemosensitivity in Human Osteosarcoma


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/6/2389

Cited articles
This article cites 26 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/6/2389.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/11/6/2389.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/11/6/2389.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.