Oncoytic Pressure in Solid Tumors Is Elevated\(^1\)

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ABSTRACT

Oncoytic and hydrostatic pressure differences control the movement of fluid and large molecules across the microvascular wall of normal and tumor tissues. Recent studies have shown that the interstitial fluid pressure in tumors is elevated and is approximately equal to the microvascular pressure. Oncoytic pressure in blood plasma of various species is known, no data are available on the oncotic pressure in the interstitial space of tumors. We hypothesize that because of the leaky nature of tumor vessels, oncoytic pressure in tumor interstitium should be close to that in plasma. To this end, we first developed a chronic wick method for the direct measurement of oncoytic pressures in the interstitial fluid of tumors grown in mice. We found interstitial oncoytic pressures in four human tumor xenografts to be higher than in s.c. tissue and comparable to that in plasma [rhabdomyosarcoma (RD), 24.2 ± 4.7; squamous cell carcinoma (FaDu), 19.9 ± 1.9; small cell lung carcinoma (54A), 21.1 ± 2.8; colon adenocarcinoma (LS174T), 16.7 ± 3.0 mm Hg; s.c. tissue, 8.2 ± 2.3; plasma, 20.0 ± 1.6 mm Hg]. These results support our hypothesis that the oncoytic pressure difference across the tumor microvascular wall is low. The high oncoytic pressure in tumors is consistent with the elevated interstitial fluid pressure, and it contributes to the suboptimal delivery of large therapeutic agents to neoplastic cells.

INTRODUCTION

Fluid movement across vessel walls is governed by the differences in hydrostatic and oncotic pressures across the vessel wall (1, 2). Our group and others have measured hydrostatic pressures in tumor vessels [microvascular pressure] and interstitium [IFP; Refs. 1 and 2]. The IFP of human tumors in situ is significantly elevated compared to normal tissues (3–5). In most normal tissues, the IFP is about 0 mm Hg, whereas for the different carcinoma types measured to date, the mean IFPs vary between 14 and 30 mm Hg. In general, the IFP increases with tumor size (2–5). However, in some studies, the IFP was found to be independent of the tumor volume (6, 7). Measurements in experimental tumors have demonstrated that (a) the IFP is uniform throughout the center of the tumors and drops steeply in the tumor periphery or in the normal tissue immediately surrounding the tumor (8, 9), and (b) the hydrostatic pressures in the vascular and interstitial spaces are nearly equal (10, 11). The latter is thought to be a major mechanism limiting the convective delivery of large therapeutic agents to solid tumors (12, 13).

The equilibration of hydrostatic pressures between the interstitial and microvascular spaces of tumors coupled with the leaky nature of tumor vessels suggests that the oncoytic pressure in both spaces should also be similar (1, 2, 10). Despite its importance in drug delivery, however, the oncoytic pressure of tumor interstitial fluid has not been measured to date. Estimates of oncoytic pressure in tumors are usually based on the protein content of interstitial fluid obtained from tumors (1, 2). Gullino et al. (14) sampled the interstitial fluid of tumors with a microprobe chamber and found the protein concentration to be ~60% of the plasma concentration. Sylven and Bois (15) obtained interstitial fluid with micropipettes and found the protein concentration to be higher (between 67 and 97% of plasma values). The data of Sylven and Bois (15) suggest that the proteins in plasma and in the tumor interstitial fluid are close to equilibrium, whereas the data of Gullino et al. (14) would suggest a significant gradient between the two spaces. The reasons for these differences between these two studies are not clear. Heterogeneity between tumor lines, and/or different techniques used to collect the interstitial fluid could explain this discrepancy. For example, the connective tissue layer formed around the chamber used by Gullino et al. (14) could potentially hinder protein accumulation in the chamber. Similarly, the suction applied by a micropipette could increase the filtration rate and dilute interstitial proteins. Thus, a technique is needed to overcome these potential artifacts.

The goals of the present study, therefore, are: (a) to develop a technique to measure directly the oncoytic pressure in the interstitial space of tumors, and (b) to test the hypothesis that the oncoytic pressures of the tumor interstitial fluid and plasma are similar. To collect interstitial fluid, multifilamentous wicks were implanted at the time of tumor inoculation (chronic wicks) or once the tumor had grown to a size of 8–10 mm (acute wicks). The oncoytic pressure of the collected wick fluid was measured with a colloid osmometer. To understand differences in oncoytic pressure values between normal and tumor tissues, total protein concentration and the molecular weight distributions of proteins in the wick fluid were characterized with a commercial kit and SDS-gel electrophoresis, respectively.

MATERIALS AND METHODS

Animals and Tumors. Four human tumor cell lines, the colon adenocarcinoma (LS174T), rhabdomyosarcoma (RD), human squamous cell carcinoma (FADu), and small cell lung carcinoma (54A), were transplanted s.c. under aseptic conditions in the right hind leg of 8-week-old athymic NCr/Sed nude (nu/nu) mice, weighing 22–25 g. The experiments were performed when the tumors reached a diameter of 8.5–10 mm.

Colloid Osmometer. A membrane colloid osmometer for measurements in samples as small as 5 \( \mu \)l was used (16). Amicon PM10 (Beverly, MA) membrane with a molecular weight cutoff of \( M_w \), 10,000 was inserted. The proteins in the sample chamber create hypotension in the reference fluid chamber, which is recorded via the pressure transducer (model P23XL, Spectramed Inc., Oxnard, CA) and is equivalent to the oncoytic pressure.

Plasma Oncoytic Pressure. To our knowledge, the oncoytic pressure of mice plasma has never been measured. Therefore, we characterized the plasma oncoytic pressure systematically and designed experiments to control for the influence of anesthesia and repeated blood sampling. Three experimental groups were compared. In group I (\( n = 5 \)), four blood samples were taken at \( t = 0, 0.5, 1, \) and 8 h from the venous sinus of the eye. From animals of group II (\( n = 4 \)), blood was taken at \( t = 0, h \), and mice were then anesthetized with a mixture of ketamine (80 mg/kg body weight) and xylazine (12 mg/kg body weight). Blood samples were obtained at \( t = 0.5, 1, \) and 8 h. Mice were waking up after 1 h and had completely recovered from anesthesia after 8 h. The animals of group III (\( n = 5 \)) were treated like those of group II with the only difference that there was no blood sampling before anesthesia; the first blood sample was obtained 30 min after anesthesia. Forty \( \mu \)l of blood were taken from the venous sinus of the eye using heparinized glass capillary tubes and...
transferred to 2 ml centrifuge tubes. After centrifugation for 5 min at 400 g, 5–10 μl of plasma were used for measurement of oncotic pressure.

**Interstitial Fluid Oncotic Pressure.** Oncotic pressure was determined in tumor interstitial fluid collected with a wick technique (17). Interstitial fluid was sampled with three-stranded nylon wicks (~1 mm in diameter; Enkaldon, Arnhem, the Netherlands) prewashed in acetone, ethanol, and distilled water. Saline-soaked acute wicks were inserted in the tumor tissue. Chronic wicks were implanted simultaneously with the tumors. At the end of the implantation period, the wicks ends along with any bloodstained portions were cut off, and the remaining sections were transferred to 2-ml centrifuge tubes provided with a funnel-shaped wick support (18). The wicks were centrifuged in an Eppendorf table centrifuge (model 5415 C; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) for 10 min at 16,000 g. The 5–10 μl of fluid obtained were used for an oncotic pressure measurement in the above-described colloid osmometer.

**Electrophoresis of Plasma and Wick Fluid.** Total protein concentration was determined by a micro protein determination kit (Sigma, St. Louis, MO). The molecular weight distribution of proteins was characterized after separation of proteins by SDS-gel electrophoresis. Gels were stained with Coomassie blue and scanned on a Bio-Rad densitometer (model GS-670; Bio-Rad Laboratories Inc., Richmond, CA).

**Data Analysis.** All values are presented as mean ± SD. One-way ANOVA was performed to test whether groups differed more than by chance. If the ANOVA was significant (P < 0.05), Dunnett’s multiple comparison test was applied to determine the groups that were significantly different.

**RESULTS**

**Optimization of Blood Sampling for Measurement of Blood Plasma Oncotic Pressure.** Oncotic pressure in blood plasma (Table 1; Fig. 1) of awake mice was 20.9 ± 1.8 mm Hg (t = 0; n = 9; the data of groups I and II at t = 0 were combined). In group I, oncotic pressure of plasma decreased by 12%, 15%, and 14% after 30 min, 60 min, and 8 h, respectively. After anesthesia (group II), oncotic pressure of plasma decreased further by 17%, 23%, and 13% after 30 min, 60 min, and 8 h, respectively. In the last group (group III), there was a similar decrease of plasma oncotic pressure (24% after 30 min, 28% after 60 min, and 16% after 8 h). Thus, to minimize the changes in oncotic pressure induced by anesthesia and blood collection, the oncotic pressure data were obtained from only one puncture of the venous sinus of the eye of nonanesthetized mice. Oncotic pressure in blood plasma of mice without tumors was 20.9 ± 1.8 mm Hg (n = 9); oncotic pressure in blood plasma of tumor-bearing mice used for optimization of interstitial fluid sampling was 20.3 ± 1.5 mm Hg (n = 22); and oncotic pressure in blood plasma of mice with the different tumor types was 19.9 ± 1.7 mm Hg (n = 129). The mean of all these data is 20.0 ± 1.6 mm Hg (n = 160). Tumor-bearing animals had slightly reduced plasma oncotic pressures compared with mice without tumors, but the difference was not significant (two-tailed t test; P < 0.07).

**Optimization of Tumor Interstitial Fluid Sampling for Measurement of Interstitial Oncotic Pressure.** LS174T tumors were used to establish the optimal approach for sampling of tumor interstitial fluid with the wick technique. Oncotic pressure was determined after different sampling times, in tumors of dead mice, and from the fluid of chronic wicks, which were implanted at the same time with the tumors (Fig. 2). Oncotic pressures were high in tumor tissue of dead mice (21.6 ± 1.5 mm Hg; n = 5; 60-min sampling) and after short sampling times (after 10 min: 19.6 ± 3.2 mm Hg, n = 6; after 30 min: 21.4 ± 2.3 mm Hg, n = 6; after 60 min: 21.0 ± 4.7 mm Hg, n = 6) and decreased with longer sampling times (after 120 min: 17.9 ± 4.1 mm Hg, n = 5; after 24 h: 17.5 ± 1.1 mm Hg, n = 4). The detected differences were not significant, which is probably attributable to the small sample size. The lowest values were measured with chronic wicks [16.7 ± 3.0 mm Hg; n = 12; significantly different from 30-min sampled acute wicks and interstitial fluid collected from dead tumor tissue (P < 0.05), respectively; Fig. 2].

**Oncotic Pressure in Different Tumor Types.** Oncotic pressures in plasma and in interstitial fluid of the subcutis were 19.9 ± 1.7 mm Hg (n = 129) and 8.3 ± 2.5 mm Hg (n = 39), respectively. The oncotic pressure for tumor fluid sampled with chronic wicks is given in Fig. 3. Oncotic pressures in subcutis (P < 0.001) and LS174T (16.7 ± 3.0 mm Hg; n = 12; P < 0.05) were significantly lower than the oncotic pressure of plasma. However, the oncotic pressure of the three other tumor types and plasma were not significantly different: RD: 24.2 ± 4.7 mm Hg, n = 7; FADU: 19.9 ± 1.9 mm Hg, n = 13; 54 A: 21.1 ± 2.8 mm Hg, n = 20. The oncotic pressure was significantly lower in LS174T compared with RD (P < 0.001), FADU (P < 0.05), as well as 54 A (P < 0.001).

**Oncotic Pressure in s.c. Tissue.** Interstitial oncotic pressure in s.c. tissue was measured as an additional control parameter for the method and the condition of animals. Oncotic pressure in subcutis of mice used for optimization of tumor interstitial fluid sampling was 8.2 ± 2.0 mm Hg (n = 22), and in subcutis of mice with tumors of different cell lines, it was 8.3 ± 2.5 mm Hg (n = 39). The mean of all these data is 8.2 ± 2.3 mm Hg (n = 61).

**Protein Fractions in Plasma and Interstitial Fluid of Tumor and s.c. Tissue.** According to their electrophoretic mobility, the proteins (concentration in g/100 ml) were categorized in four different molecular weight intervals (Table 2; Fig. 4). The larger proteins (> M, 75,000) are present in the highest concentration in blood plasma. As a consequence of their reduced penetration across the vascular wall and because of their high osmotic reflection coefficient at the microvascular wall, they are significantly lower in the subcutis. The gradient between plasma and tumor is smaller because of the high vascular permeability of tumor blood vessels. The same pattern is found in the next two fractions (M, 75,000–50,000 and 50,000–25,000), with a significant gradient for subcutis. With further reduction in protein size, the pattern is opposite; the concentration of small proteins is highest in tumor interstitial fluid.

There are also different protein patterns in the three tumor lines: LS174T (n = 6), 54A (n = 9), and RD (n = 2; Fig. 4B). The albumin-containing fraction (M, 75,000–50,000) of LS174T is lower than 54A (P < 0.01) and RD (P < 0.05). The small protein fraction (< M, 25,000), however, is similar between tumor types.

**DISCUSSION**

Before the present study, oncotic pressure in tumors was estimated using an empirical equation that relates oncotic pressure with protein concentration (1, 2, 19, 20). However, the proportions of various proteins vary from one species to another, so that equations that accurately predict plasma oncotic pressure for one animal species may be inaccurate for another species (20). In addition, there is also systematic variation in the proportion of proteins in plasma of animals of the same species (21). The relative proportions of albumin and globulin may also change as a consequence of diseases (22). In the
present study, the significantly higher levels of protein fractions $<M_r$ 25,000 in tumors as compared with plasma would increase the complexity and inaccuracy of oncotic pressure estimates based on protein concentrations. As a result, we chose to develop a method for direct measurement of oncotic pressure in tumors. In what follows, we will discuss the problems encountered and how we resolved them, as well as the results obtained and the underlying mechanisms.

Effect of Anesthesia and Repeated Bleeding on Oncotic Pressure. We measured the plasma oncotic pressure (Fig. 1) in awake mice without tumors to be $20.9 \pm 1.8$ mm Hg. In awake mice, plasma oncotic pressure decreased with repeated blood sampling (Fig. 1). After anesthesia, plasma oncotic pressure decreased further (Table 1; Fig. 1). The effect of anesthesia and blood volume withdrawal can be explained by a reduction in mean arterial blood pressure leading to an increase in fluid reabsorption across the microvascular wall. Therefore, repeated blood sampling and anesthesia should be avoided for the measurement of plasma oncotic pressure of mice using blood sampled from the venous sinus of the eye. As a result, plasma oncotic pressure was measured as a control parameter, in all tumor experiments, without anesthesia and with a single puncture of the venous sinus of the eye: $19.9 \pm 1.7$ mm Hg, $n = 129$ (Fig. 1). This value is similar to the plasma oncotic pressure of most species, which is typically around 20 mm Hg (23, 24).

Effect of Sampling the Interstitial Fluid. The published results on interstitial fluid composition are controversial because of methodological problems as well as the heterogeneity of tumors (1, 2). Three methods have been used to collect fluid from the interstitial space: direct sampling using needle or micropipettes; implanted wicks; and a chronically implanted micropore chamber. Each method has its own limitations. The puncturing of tissue with the direct sampling method causes cellular and vascular damage; as a result, the collected fluid may be a mixture of vascular, cellular, and pericellular fluids. Furthermore, the applied suction may increase net capillary filtration and lower the concentration of interstitial proteins. The micropore chamber may influence the structure of the surrounding tissue; the chamber fluid may not represent interstitial fluid because of hindered transport across the micropore membrane or the surrounding connective tissue layer. Consequently, we decided to use the wick technique to collect fluid from the tumor interstitium. One problem of this method is that artificially high protein concentrations in the wick fluid can be caused by the wick insertion provoking bleeding or local inflammation, and thus increasing microvascular permeability to proteins. On the other hand, hyperemia with increased capillary pressure and without change of microvascular permeability increases filtration rate and produces lower interstitial protein concentrations than normal.

The influence of bleeding can be easily excluded by cutting the
A more microscopic technique is needed to address these two issues. Whether the total protein content in tumors is similar to plasma may not be equal to that in tumor microvessels. Furthermore, the heterogeneous in the oncotic pressure. Furthermore, the oncotic pressure of s.c. tissue, which is 41% of the plasma value. The oncotic pressure in chronic wicks of 16.7 mm Hg for LS174T is 82% of the total protein concentration in the interstitium of different tumors (chronic wick method; *, P < 0.05; ***, P < 0.001).

**Mechanisms of High Oncotic Pressure in Tumors.** The larger proteins (M_r > 75,000) are found in the highest concentration in blood plasma. As a consequence of their reduced penetration across the vascular wall, they are significantly lower in the subcutis. The gradient between plasma and tumor is smaller because of the high vascular permeability of tumor blood vessels and the absence of functional lymphatic vessels in tumors. The same pattern is found in the next two protein fractions (M_r 75,000–50,000 and M_r 50,000–25,000), with a significant gradient for the subcutis. In contrast, for the smaller molecular weight fraction, a higher concentration of protein was found in tumor interstitial fluid than in plasma. The smaller proteins could be breakdown products from necrotic areas or other tumor cell-derived proteins. Recent studies have shown that the presence of a tumor can induce antiangiogenic molecules, which are proteolytic fragments of larger proteins. The molecular size of known antiangiogenic molecules resulting from enzymatic fragmentation, like vascu-lostatin, prolactin, and endostatin, varies between M_r 13,000 and M_r 20,000 (25). Some of the matrix-degrading enzymes released by tumor cells are also < M_r 25,000. Cellular enzymes can be released into tumor interstitial fluid (15). Because the identity and molecular weight of different proteins in the tumor interstitium are not known, calculating oncotic pressure from the total protein concentration using blood-stained parts of the wicks. Two approaches were chosen to control for increased protein concentrations resulting from local inflammation: (a) comparison with dead tumor tissue, and (b) comparison of different sampling times. Oncotic pressures were elevated in dead tissue and after short sampling times. This could be attributable to the loss of cell membrane integrity with release of intracellular proteins in dead tissue and the occurrence of temporary inflammation after short sampling times. This problem is probably less significant or absent with longer sampling times or chronic wicks. The oncotic pressure in chronic wicks of 16.7 mm Hg for LS174T is 82% of the blood oncotic pressure, which is higher than the interstitial oncotic pressure of s.c. tissue, which is 41% of the plasma value.

The approach used here has two limitations: Protein distribution in the interstitium is heterogeneous, and, thus, may lead to microscopic heterogeneities in the oncotic pressure. Furthermore, the oncotic pressure of systemic blood may not be equal to that in tumor microvessels. A more microscopic technique is needed to address these two issues.

**Protein Distribution in the Tumor Interstitial Fluid.** Sylven and Bois (15) and Gullino et al. (14) found a significantly increased protein concentration in tumor interstitial fluid compared with normal tissue. The data of Sylven and Bois (Ref. 15; 4.9 g/100 ml in Ehrlich-Landschütz carcinoma and 5.5 g/100 ml in a mammary carcinoma) are closer to the present study (5.3 g/100 ml) than the data of Gullino et al. (Ref. 14; 3.2 g/100 ml, mean of four tumor types). However, in the data of Gullino et al. (14), the albumin concentrations in the fibrosarcoma 4956 and the Novikoff hepatoma were 1.55 and 1.65 g/100 ml, respectively, which are similar to our value of 1.54 g/100 ml in the LS174T tumor. The total protein content of the three tumors included in our study was similar to plasma. For the LS174T tumors, the total protein content was 12% lower and for RD, 20% higher than in plasma. This higher total protein content could result from difference in small proteins with < M_r 25,000, which were found to be ~2- to 4-fold higher in the interstitial fluid of tumors than in plasma (Fig. 4A).

**Fig. 4.** A, protein fractions in blood plasma and interstitial fluid of tumor and s.c. tissue. Significant differences with respect to plasma concentration: *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, protein fractions in the tumor lines LS174T, 54A, and RD. *, P < 0.05; **, P < 0.01. *, **, and *** indicate significant difference based on Dunnett’s Multiple Comparisons Test.

Table 2: Protein concentrations (g/100 ml) in different molecular weight intervals in tumor interstitium (n = 17), plasma (n = 19), and subcutaneous interstitial space (n = 6).

<table>
<thead>
<tr>
<th>Protein Distribution in the Tumor Interstitial Fluid.</th>
<th>M_r &gt; 75,000</th>
<th>75,000–50,000</th>
<th>50,000–25,000</th>
<th>&lt; 25,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>M_r</td>
<td>M_r</td>
<td>M_r</td>
<td>M_r</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.64 ± 0.24</td>
<td>2.04 ± 0.60</td>
<td>1.03 ± 0.36</td>
<td>1.58 ± 0.72</td>
</tr>
<tr>
<td>Subcutis</td>
<td>0.47 ± 0.21</td>
<td>1.48 ± 0.41</td>
<td>0.69 ± 0.13</td>
<td>0.84 ± 0.44</td>
</tr>
</tbody>
</table>

* P < 0.05; indicates significant difference from plasma; based on Dunnett’s Multiple Comparisons Test.

* P < 0.01; indicates significant difference from plasma; based on Dunnett’s Multiple Comparisons Test.

* P < 0.001; indicates significant difference from plasma; based on Dunnett’s Multiple Comparisons Test.
The difference between the measured oncotic pressure and the physiologically relevant oncotic pressure difference across the vascular wall must also be considered. Using the oncometer with a molecular cutoff size of $M_c$ 10,000, all proteins with a molecular weight $>M_c$ 10,000 contribute to the measured oncotic pressure independently of their individual size. But the physiologically relevant oncotic pressure difference across vascular wall is dependent on the molecular weight of each protein. Because small macromolecules can permeate the vascular wall more easily and quickly, their contribution to the effective oncotic pressure is less as compared with larger macromolecules (1). Interestingly, the difference in oncotic pressure between plasma and interstitial fluid in LS174T is in agreement with the significant hydrostatic pressure difference (2.0 mm Hg) between the microvascular and interstitial space in that tumor (11). In two other tumor types, the microvascular and IFPs were similar (10, 11). Thus, it is likely that the oncotic pressure measured in LS174T is close to the effective oncotic pressure.

**Conclusion.** This is the first study describing a method of direct measurement of oncotic pressures in tumor interstitium. The results indicate that long sampling times are required for acute wicks; when chronic wicks are used, the risk of artifacts attributable to cellular damage or bleeding during wick introduction is avoided. The high oncotic pressure is consistent with the elevated vascular permeability and IFP in tumors. This equilibration of oncotic and hydrostatic pressures across tumor vessels may reduce the convective delivery of macromolecules in large regions of solid tumors (12).

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