Vascular Permeability in a Human Tumor Xenograft: Molecular Size Dependence and Cutoff Size

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Abstract

Molecular size is one of the key determinants of transvascular transport of therapeutic agents in tumors. However, there are no data in the literature on the molecular size dependence of microvascular permeability in tumors. Therefore, we measured microvascular permeability to various macromolecules in the human colon adenocarcinoma LS174T transplanted in dorsal skin chambers in severe combined immunodeficient mice. These molecules were fluorescently labeled and injected i.v. into mice. The microvascular permeability was calculated from the fluorescence intensity measured by the intravital fluorescence microscopy technique. The value of permeability varied approximately 2-fold in the range of molecular weight from 25,000 to 160,000. These data indicate that tumor vessels are less permselective than normal vessels, presumably due to large pores in the vessel wall. The transport of macromolecules appears to be limited by diffusion through these pores. The cutoff size of the pores was estimated by observations of transvascular transport of sterically stabilized liposomes of 100–600 nm in diameter. We found that tumor vessels in our model were permeable to liposomes of up to 400 nm in diameter, suggesting that the cutoff size of the pores is between 400 and 600 nm in diameter.

Introduction

Previous quantitative studies have demonstrated that the permeability of tumor vessels (Pv) is in general higher than that of normal vessels (1–6). The increase in permeability is hypothesized to be a necessary condition for angiogenesis in tumors or wound-healing process (7). Vascular permeability may depend on tumor type (5), (8) and increase with tumor size (9, 10) and growth rate (11). It may be higher in the periphery than in the central region of tumors (12–14). However, the regulation of tumor vascular permeability is not well understood. Certain brain tumors possess a tight blood-tumor barrier to the transport of molecules (5, 10, 15); angiogenesis in these tumors does not require the vessels to be hyperpermselective (5).

The present study was designed to address two critical questions regarding the transvascular transport in tumors: (a) how does molecular size influence transport? and (b) what is the maximum size of particles that can cross the tumor vessel wall? The size of molecules or particles is one of the key determinants of the microvascular permeability because it can vary by several orders of magnitude. Our hypothesis is that tumor vessels may lack permselectivity to macromolecules or even liposomes due to the existence of large pore structures (1). Experiments were performed in the human colon adenocarcinoma LS174T implanted in the dorsal skin fold chamber in SCID mice (16). The microvascular permeability to proteins with different molecular weight (25,000–160,000) was measured using an intravital fluorescence microscopy technique (3–5). The maximum size of pores in vessels of LS174T tumors was estimated by monitoring extravasation of i.v. injected liposomes of different sizes (100–600 nm in diameter).

Materials and Methods

Tracer Molecules and Liposomes. The microvascular permeability to seven macromolecules was measured (Table 1). The mouse IgG fragments [Fc, Fab, and F(ab')2] were produced by enzymatic digestion and labeled with indocarbocyanine Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The other three tracer molecules were labeled with tetramethylrhodamine (Molecular Probes, Eugene, OR). The SDS-PAGE analysis showed that the molecular weight of IgG fragments (Fc and Fab) were different from those reported in the literature (17). This discrepancy may be related to the method of preparation of these molecules using enzymes. There were two fluorescence bands of F(ab')2 in SDS-PAGE analysis (25,000 and 110,000), and they were separated by the size exclusion column (Econo-Pac 10DG; Bio-Rad Laboratories, Hercules, CA). The Stokes-Einstein radius of each molecule was calculated assuming that (a) the radius of albumin is 3.5 nm and (b) the diffusion coefficient is inversely proportional to the cube root of the molecular weight (18). The free fluorescent dye in the solution was removed by passing the solution through the size exclusion columns (Econo-Pac 10DG; Bio-Rad). The final solute concentrations were 1.3 mg/ml for IgG and its fragments and 6.5 mg/ml for the other three tracers.

Sterically protected long-circulating PEG liposomes were prepared by the detergent (octyl glycoside) dialysis method or by ultrasonication method and fluorescently labeled with membrane-incorporated Rho-PE (Molecular Probes). The molar ratio of egg phosphatidylcholine:cholesterol:PEG-DSPE:Rho-PE was 10:5:0.8:0.1. Lipid mixture was argon-dried from chloroform, vacuumed, solubilized with octyl glycoside in HBSS (pH 7.4) with a final total lipid concentration of 20 mg/ml, and dialyzed overnight against HBSS at 4°C. Alternatively, dried lipid mixture was supplemented with HBSS, hydrated for 30 min, and briefly sonicated in a bath-type ultrasonicator. Liposomes obtained were sized by multiple passing through the polycarbonate filters (Poretics, Livermore, CA) with pore diameters of 0.6, 0.4, 0.2, and 0.1 μm depending on the target size. The liposome size in the final preparation was determined with a Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics, Hialeah, FL). All liposome preparations had a narrow size distribution (95% of liposomes within a ±15-nm interval for 100- and 200-nm liposomes and a ±25-nm interval for 400- and 600-nm liposomes). In addition, surface-modified long-circulating polystyrene latex beads (Sigma Chemical Co., St. Louis, MO) with similar diameters as liposomes were prepared. To modify the beads, PEG-DSPE and Rho-PE were mixed in chloroform, argon-dried, vacuumed, and supplemented with latex suspension in HBSS. The weight ratio of latex:PEG-DSPE:Rho-PE was 1:10:0.1. The mixture was briefly sonicated and then stirred overnight at the room temperature. Surface-modified beads were separated from micelles of free PEG-DSPE and Rho-PE by centrifugation and washing with HBSS.

Measurement of Molecular Weight and Charge. The molecular weight and charge of proteins used were analyzed by SDS-PAGE (Mini-Protein II
were sandwiched between the host skin tissue (bottom) and a glass
value (Fig. 1B) obtained from Yuan et al. (4) was comparable with
(17). was prepared with or without reducing agent (3-mercaptoethanol because
permeability; D, diffusion coefficient in water.
vascular permeability, as described previously (3—5). The time constant of
skin chamber in SCID mice following the procedure described in Ref. 16.
concentration decay in the plasma was determined within 30 min after tracer
injections (4). Extravasation of liposomes was observed 24 h after injection of
liposomes.
Electrophoresis Cell; Bio-Rad) and isoelectric focusing electrophoresis (Model
111 Mini IEF Cell; Bio-Rad), respectively. The sample buffer for SDS-PAGE
was prepared with or without reducing agent β-mercaptoethanol because
β-mercaptoethanol may break the disulfide bonds in IgG and its fragments
(17).

Experimental Procedure. The LS174T tumor was grown in the dorsal
skin chamber in SCID mice following the procedure described in Ref. 16.
After injection of tracer molecules (0.1 ml/25 g body weight), the fluorescence
intensity of the tumor tissue was measured and used to calculate the tumor
vascular permeability, as described previously (3—5). The time constant of
concentration decay in the plasma was determined within 30 min after tracer
injections (4). Extravasation of liposomes was observed 24 h after injection of
liposomes. Mann-Whitney U test was used to compare the differences in permeability
between two groups. Kruskal-Wallis test was used when more than two groups
needed to be compared. The correlation between permeability and molecular
weight was checked using the Spearman Rank test.

Results and Discussion

Molecular Weight Dependence. Fig. 1A shows the molecular
weight dependence of tumor microvascular permeability, where the
molecular size is given as the Stokes-Einstein radius (Table 1). The value of permeability varied approximately 2-fold in the molecular
weight range from 25,000 to 160,000. Spearman Rank test indicated that there was no correlation between permeability and molecular
weight (P = 0.11).

To study the influence of diffusion on the transvascular transport,
we normalized the permeability by the diffusion coefficient (D) of the
macromolecules in water (Fig. 1B), where D was estimated as
$3.6 \times 10^{-3} \times (M_r)^{-0.34}$ (cm²/s) (18). The value of $P_J/D$ was compared
among all molecules, and the $P$ values are given in Table 2. The Spearman Rank test indicated that there was no correlation between permeability and molecular weight ($P = 0.11$).

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$\frac{P_J}{D}$ of various macromolecules and sterically stabilized liposome (Lipo). Note that the value of permeability varied approximately 2-fold in the molecular weight range from 25,000 to 160,000. Spearman Rank test indicated that there was no correlation between permeability and molecular weight ($P = 0.11$). $B$, ratio of permeability to diffusion coefficient ($P_J/D$) versus the Stokes-
Einstein radius of the same macromolecules and liposome. No statistical difference
between $P_J/D$ values of Fc, Fab, concanavalin A (Con A), and IgG was observed by
Kruskal-Wallis test ($P = 0.18$). The $P_J/D$ value of liposome was comparable with that of
BSA ($P = 0.47$). Points, medians; bars, ranges. Oval, ovalbumin.

<table>
<thead>
<tr>
<th>Name</th>
<th>$M_r$ (kDa)</th>
<th>$t$ (nm)$^a$</th>
<th>$p_l$</th>
<th>$S/V$ (mm²/mm³)</th>
<th>Median $K$ (range) (10⁻² cm/s)</th>
<th>Median $P_J$ (range) (10⁻⁷ cm/s)</th>
<th>Median $P_J/D$ (range) (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Fc fragment</td>
<td>25,000</td>
<td>2.54</td>
<td>4.8</td>
<td>269 (148-376)</td>
<td>15.8 (13.2-16.0)</td>
<td>3.74 (2.65-6.80)</td>
<td>0.33 (0.23-0.59)</td>
</tr>
<tr>
<td>Mouse Fab fragment</td>
<td>25,000</td>
<td>2.54</td>
<td>4.8</td>
<td>198 (147-262)</td>
<td>19.0 (18.1-19.3)</td>
<td>4.61 (3.6-6.13)</td>
<td>0.40 (0.23-0.53)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
<td>3.08</td>
<td>4.2-5.2</td>
<td>255 (220-303)</td>
<td>6.4 (6.2-8.7)</td>
<td>5.77 (4.36-7.07)</td>
<td>0.61 (0.46-0.75)</td>
</tr>
<tr>
<td>BSA</td>
<td>66,000</td>
<td>3.50</td>
<td>4.5</td>
<td>285 (203-392)</td>
<td>80.5 (77.4-130)</td>
<td>1.61 (0.65-1.93)</td>
<td>0.19 (0.08-0.23)</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>104,000</td>
<td>4.07</td>
<td>7.4-8.4</td>
<td>235 (202-278)</td>
<td>23.3 (22.6-23.4)</td>
<td>1.53 (1.34-3.04)</td>
<td>0.22 (0.19-0.43)</td>
</tr>
<tr>
<td>Mouse F(ab')₂ fragment</td>
<td>110,000</td>
<td>4.15</td>
<td>4.8-5.8</td>
<td>218 (171-275)</td>
<td>60.7 (46.5-105)</td>
<td>1.51 (0.99-1.57)</td>
<td>0.22 (0.14-0.23)</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>160,000</td>
<td>4.70</td>
<td>6.0</td>
<td>170 (122-254)</td>
<td>50.0 (33.7-82.2)</td>
<td>2.82 (1.47-4.07)</td>
<td>0.46 (0.24-0.67)</td>
</tr>
</tbody>
</table>

$^a$ $t$, Stokes-Einstein radius; $p_l$, isoelectric point; $S/V$, vascular surface area per unit vascular volume; $K$, time constant of concentration decay in the plasma; $P_J$, microvascular permeability; $D$, diffusion coefficient in water.

Convective transport across the vessel wall of tumors used in our
studies was likely to be small for the following reasons. These tumors
were sandwiched between the host skin tissue (bottom) and a glass
coverslip (top) (16). Because no fluid could leak out at the top surface
tumors, we would not expect the interstitial fluid pressure and
oncotic pressure there to be significantly different from that in the
central region of tumors, where these two pressures in interstitium are
similar to those in microvessels (19, 20).3 Thus, the convective
transport may be negligible in the top and central regions. In the

3 M. Stohrer, Y. Boucher, and R. K. Jain, unpublished data.
channels in vascular endothelium are reduced in these vessels. In limited cases, liposomes might be phagocytized by endothelial cells in tumors (28). However, there is no evidence showing that liposomes are transported across endothelial cells by large vacuoles.

To estimate the maximum pore size in the LS174T tumor, we injected sterically stabilized liposomes of different sizes. We found that the maximum diameter of liposomes that can escape from the lumen of tumor vessels was between 400 and 600 nm because the liposomes of 400 nm in diameter could penetrate into tumor interstitium, whereas liposomes of 600 nm in diameter were excluded from extravascular space (Fig. 2). The maximum pore size of the tumor vessel wall was assumed to be the maximum size of liposomes, which was around 500 nm as determined above. The validity of this estimation was based on the study of normal liver tissue, where Scherphof et al. (29) demonstrate that the liver sinusoids are permeable to liposomes of 100 nm in diameter, the size that is almost the same as the median diameter of pores (106 nm) in the sinusoids revealed by the electron microscopy. Larger liposomes (e.g., 500 nm in diameter) cannot cross these vessels (29). The cutoff size of the tumor vessel wall was also confirmed by latex beads coated with phospholipids (data not shown). Latex beads are more rigid; they are unlikely to deform when crossing the vessel wall.

Comparison with Other Studies. Despite its clinical importance, there are few data quantifying the relationship between the microvascular permeability and the molecular size of drugs in solid tumors (1). Only the PS has been estimated for selected molecules with different sizes (30, 31). However, if the surface area of tumor vessels is assumed to be the same for different molecules, PS is equivalent to PV when the relative values are compared. Peterson and Appelgren (30) demonstrate that there is no significant difference in PS for albumin and IgG in chemical-induced sarcomas. Imoto et al. (31) have measured the extravascular concentration of various radiolabeled macromolecules [dextran molecules (M, 10,000 and 70,000), inulin (M, 5,200), and BSA] in isolated Walker 256 carcinoma implanted in rat ovarian tissue. The concentration of these tracer molecules in vessels was maintained at a constant level by continuous perfusion. They find that the ratios of the interstitial concentrations between small and large dextran molecules and between inulin and BSA are 1.6 and 2.0, respectively. Therefore, our results are qualitatively in agreement with these studies. In contrast to tumor vessels, the permeability of normal vessels depends significantly on the molecular size (24–26). Similarly, the ratio of lymph to plasma concentrations of various molecules in skeletal muscle drops significantly (5-fold) when the molecular radius increases from 2 to 4 nm, whereas the same ratio decreases only 2-fold in the liver (32). Sinusoids in the liver are known to be hyperpermeable to macromolecules due to the discontinuity in endothelial junctions and the lack of continuous basement membrane. This structure may be similar to that of tumor vessel wall (1). In light of the liver data, our data support the notion that tumor vascular endothelium is less selective for different macromolecules compared with most normal host vascular endothelium.

As stated in “Introduction,” tumor microvascular permeability may depend on tumor size and growth rate, and is in general higher than permeability of normal vessels. However, exceptions have also been reported in the literature. The discrepancy among different studies may be tumor related (i.e., each tumor line may have unique mechanisms for regulating its microvascular permeability). Alternatively, common mechanisms of regulation may exist only at cellular or molecular levels. For instance, VPF/VEGF has been identified, purified, and sequenced (7). It is expressed by several tumor and normal cells, and can function as a mitogen to endothelial cells to induce angiogenesis or as a factor to increase vascular permeability both in tumors and in normal tissue (7). In addition to VPF/VEGF, other tissue environmental factors may change the structure of the vessel wall because the vascular permeability of some tumors depends on the

### Table 2 Statistical comparison between PV or between P/D (in parentheses) of different molecules

<table>
<thead>
<tr>
<th>Molecule</th>
<th>PV or P/D (in parentheses)</th>
<th>F(ab’)2</th>
<th>Concanavalin A</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc</td>
<td>0.465* (0.028)</td>
<td>0.009</td>
<td>0.016</td>
<td>0.117 (0.028)</td>
</tr>
<tr>
<td>Fab</td>
<td>0.117 (0.016)</td>
<td>0.009</td>
<td>0.009</td>
<td>0.09 (0.076)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.006</td>
<td>0.009</td>
<td>0.009</td>
<td>0.09 (0.076)</td>
</tr>
<tr>
<td>BSA</td>
<td>0.648 (0.784)</td>
<td>0.465</td>
<td>0.144</td>
<td>0.006 (0.06)</td>
</tr>
<tr>
<td>F(ab’)2</td>
<td>0.347 (0.465)</td>
<td>0.060</td>
<td></td>
<td>0.009 (0.076)</td>
</tr>
</tbody>
</table>

* P values of Mann-Whitney U test.
VASCULAR PERMEABILITY IN A HUMAN TUMOR XENOGRAGT

Fig. 2. Extravasation of sterically stabilized liposomes in tumor tissue 24 h after injection. A, mean diameter of liposomes = 400 nm. B, mean diameter of liposomes = 600 nm. Both photos have the same magnification. Bar, 100 μm. Note that the liposomes of 400 nm in diameter could penetrate into tumor interstitium as indicated by the bright fluorescence spots, whereas liposomes of 600 nm in diameter were excluded from extravascular space.

location of tumor transplantation (13). In our previous study, we found that microvessels of a human glioblastoma HGL21 transplanted into the cranial window in SCID mice are almost impermeable to Lissamin green (M, 577) (5), but they become highly leaky to this dye when the tumors grow s.c., although the angiogenesis/vascular density is similar at two sites. Our current working hypothesis is that VPF/VEGF secretion by HGL21 cells may be reduced in the brain compared to that in s.c. tissue. This hypothesis is supported by our preliminary observation that the permeability of HGL21 in the cranial window increases significantly after superfusion with VPF/VEGF.

In summary, tumor vessels in general are more leaky and less permselective than normal vessels. The vascular permeability in our study is likely governed by diffusion across the vessel wall. Large pores may exist in the tumor vessel wall that allow the penetration of liposomes up to the size of 400 nm in diameter. Other physical and physicochemical properties such as charge, hydrophobicity, and configuration of therapeutic agents may also influence the permeability (1). The role of these molecular properties needs to be studied in the future with an approach similar to that used here.

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References


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