Extravascular Diffusion in Normal and Neoplastic Tissues

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ABSTRACT

Extravascular transport of fluorescein isothiocyanate-conjugated bovine serum albumin and a graded series of fluorescein isothiocyanate-dextran from M. 19,400 to 71,800 were studied in both normal tissue (granulation) and tumor (VX2 carcinoma) grown in a rabbit ear chamber. Sodium fluorescein was used as a representative small molecule. A one-dimensional diffusion model adequately described extravascular transport in both normal and tumor tissue. Measured diffusion coefficients showed a relationship with molecular size which progressively deviates from that of free diffusion in water, with values for albumin being significantly reduced from that for a dextran of equivalent size. Macromolecular transport in tumor tissue was hindered to a lesser extent than in normal tissue, which is consistent with reports of reduced contents of glycosaminoglycans, and markedly large interstitial space in tumors. Diffusion coefficients for dextran were found to vary with molecular weight according to the expression, D = a(M)^b, in both normal tissue (a = 10^6 and b = -2.96) and tumor (a = 2.51 × 10^-2 and b = -1.14).

INTRODUCTION

The efficacy of various methods of cancer detection and treatment is affected by the extravascular transport of molecules in normal and neoplastic tissues (21, 22, 30). Although the diffusion coefficients have been measured for solutes of a wide range of molecular weights in normal tissues (11, 25, 34), these measurements have been limited to only small molecular weight species (e.g., oxygen and glucose) in tumors (5, 15, 36).

The objective of this work was, therefore, to measure the diffusion coefficients of various test molecules in the extravascular space of normal (mature granulation) and neoplastic (VX2 carcinoma) tissues grown in a rabbit ear chamber preparation. The test molecules included a graded series of FITC-conjugated dextran (M, 19,400 to 71,800) and BSA (M, 67,000). Sodium fluorescein was used as a representative small molecule (M, 376). Transport of these molecules in the rabbit ear chamber was monitored using a photometric technique reported recently (26). The diffusion coefficients calculated using a one-dimensional diffusion model were related to the molecular radius, referred to as the Stokes-Einstein radius (aE), and the tissue matrix structure.

MATERIALS AND METHODS

Animals and Tumor. Male, New Zealand white rabbits (Three Springs Kennel Company, Zelienople, Pa.), 2 to 3 kg, were fed Purina Laboratory Chow (Ralston Purina Company, St. Louis, Mo.). The normal tissue studied was the mature granulation tissue, and the transplantable tumor studied was the VX2 carcinoma, both grown in the modified Sandison-Clark transparent ear chambers.

Normal and Tumor Tissue Preparation. Transparent chambers (One of a Kind, Limited, Lincoln Park, N. J.) were surgically implanted in the ears of male rabbits following the procedure described previously (27, 37). The chamber design allowed for the formation of a thin granulation tissue bed (thickness, 40 ± 5 μm; diameter, 5.4 mm). Granulation tissue grew in the chamber at an average of 8 days post-operation, and reached maturity at approximately 40 days postoperation (9). At this time, the chamber was used either for normal tissue studies or for tumor implantation. Morphometric and blood flow characteristics of this tissue (Fig. 1A) are described elsewhere (9, 37).

For tumor implantation, the animal was anesthetized (Nembutal, 25 mg/kg i.p.), and the cover glass which formed the top plate of the transparent chamber was carefully removed. VX2 carcinoma, excised from the flank of a tumor-bearing host, was minced and placed in 0.9% NaCl solution and then spread uniformly over the cover glass. The cover glass was placed back flush against the intact normal tissue. In the majority of cases, this procedure caused no apparent damage to the tissue as observed under the microscope. Damaged tissues were not used for experiment. Angiogenic response was observed 3 to 4 days postimplant. As seen in Fig. 1B, the vessels became dilated and tortuous, and their density increased significantly. Tumor preparation was used for diffusion studies about 10 days postimplant.

In a limited number of animals, transverse sections of the normal and neoplastic preparations were examined histologically. Fig. 2, A and B, shows histologies of the normal preparation, and Fig. 2, C and D shows histologies of the tumor preparation. Note that neoplastic cells mostly grow on the surface (Fig. 2C), and sometimes invade the stroma (Fig. 2D). Although the number of vessels in the stroma increased significantly due to the angiogenic effect of the neoplastic cells, it was not possible to ascertain a priori that enough tumor surrounded a vessel monitored under the microscope to make it a "tumor vessel." Despite this limitation of our technique, our results clearly demonstrate that the presence of neoplastic cells in a tissue preparation significantly lowers the extravascular resistance to molecular transport when compared to transport in preparations free of tumor (see "Results" and "Discussion").

Test Molecules. The test molecules used in this study were sodium fluorescein (Na-F), FITC-conjugated bovine serum albumin (FITC-BSA), and FITC-conjugated dextran (FITC-dextran) obtained from Sigma Chemical Company, St. Louis, Mo., and Pharmacia, AB, Upsalla, Sweden. The weight average molecular weight (Mw) and the number average molecular weight (Mn) of these molecules were determined by gel permeation chromatography following the method of Granath and Kvist (14) and are listed in Table 1.

Experimental Protocol. Once the normal or neoplastic tissue reached the desired stage of growth, the animal was anesthetized (Nembutal, 25 mg/kg i.p.) and placed in a dorsal recumbent position in a cradle which restricted head movement while still maintaining proper circulation to the chamber. The ear containing the chamber was extended horizontally to the specimen plane of an intravital microscope adapted for transmitted light fluorescence television microscopy (27). The chamber was secured...
Calculation of Diffusion Coefficients. The extravascular concentration data were analyzed using a one-dimensional diffusion equation to yield the extravascular diffusion coefficient. The validity and details of the model are discussed elsewhere (28). In brief, the diffusion coefficient, $D$, can be calculated by fitting the following to the concentration data:

$$C(x, t) = 2\pi \int_0^t f(t - x^2 / (4Dt)) e^{-t^2 / r^2} dt$$

where $t$ is time (sec), $x$ is position along a line perpendicular to a vessel (cm), $r$ is the integration variable, and $C(x, t)$ is the concentration of the test molecule as a function of time at distance $x$ from a specified origin.

### RESULTS

Shown in Table 2 are the tissue average diffusion coefficients in granulation and neoplastic tissues. The S.D. in granulation tissue values was 20% of the mean and, in tumors, 12%. No systematic correlation between diffusion coefficient and position was noted. The results were compared with the free diffusion coefficients of test molecules in water ($D_0$) and their effective molecular radius ($r_E$). The latter was calculated using the Stokes-Einstein relation (14):

$$r_E = kT/6\pi \mu D_0$$

where $k$ is the Boltzmann constant, $T$ is the absolute temperature, and $\mu$ is the viscosity of water.

It is clear from this table that the granulation tissue offers considerably greater resistance to extravascular transport than does tumor tissue, which in turn offers more resistance than does water. The diffusion coefficient decreases with increasing molecular radius, except for FITC-BSA which has a diffusion coefficient lower than one would expect from molecular size considerations alone.

### Table 1

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>Source</th>
<th>Wt av. molecular wt (M₁)</th>
<th>No. av. molecular wt (M₂)</th>
<th>Polydispersity (M₃/M₁)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-F</td>
<td>Aldrich</td>
<td>376</td>
<td>376</td>
<td>1.0</td>
</tr>
<tr>
<td>FITC-D20</td>
<td>Sigma</td>
<td>20,500</td>
<td>17,900</td>
<td>1.21</td>
</tr>
<tr>
<td>Pharmacia</td>
<td></td>
<td>19,400</td>
<td>17,400</td>
<td>1.11</td>
</tr>
<tr>
<td>FITC-D40</td>
<td>Sigma</td>
<td>44,200</td>
<td>36,600</td>
<td>1.22</td>
</tr>
<tr>
<td>Pharmacia</td>
<td></td>
<td>39,000</td>
<td>32,000</td>
<td>1.22</td>
</tr>
<tr>
<td>FITC-D70</td>
<td>Sigma</td>
<td>71,800</td>
<td>62,100</td>
<td>1.16</td>
</tr>
<tr>
<td>Pharmacia</td>
<td></td>
<td>62,000</td>
<td>51,500</td>
<td>1.20</td>
</tr>
<tr>
<td>FITC-BSA</td>
<td>Sigma</td>
<td>67,000</td>
<td>67,000</td>
<td>1.0</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>Wt av. molecular wt (M₁)</th>
<th>Effective molecular radius ($r_E$, Å)</th>
<th>Diffusion coefficient in water ($D_0$ x 10⁻⁷ cm²/μsec)</th>
<th>Tissue diffusion coefficients Normal (D x 10⁻⁷ cm²/μsec)</th>
<th>Tumor (D x 10⁻⁷ cm²/μsec)</th>
<th>Normal (D/D₀)</th>
<th>Tumor (D/D₀)</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-F</td>
<td>376</td>
<td>4.8</td>
<td>70²</td>
<td>20</td>
<td>24</td>
<td>64</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>FITC-D20</td>
<td>20,500</td>
<td>32.0</td>
<td>10.26²</td>
<td>1.7</td>
<td>2.3</td>
<td>7.5</td>
<td>0.17</td>
<td>0.22</td>
</tr>
<tr>
<td>FITC-D40</td>
<td>44,200</td>
<td>46.2</td>
<td>7.11²</td>
<td>0.22</td>
<td>0.16</td>
<td>4.2</td>
<td>0.031</td>
<td>0.24</td>
</tr>
<tr>
<td>FITC-D70</td>
<td>71,800</td>
<td>57.9</td>
<td>5.67²</td>
<td>0.048</td>
<td>0.061</td>
<td>1.9</td>
<td>0.0085</td>
<td>0.010</td>
</tr>
<tr>
<td>FITC-BSA</td>
<td>67,000</td>
<td>35.5</td>
<td>9.3²</td>
<td>0.16</td>
<td>0.11</td>
<td>0.91</td>
<td>0.017</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* Value for sucrose ($M_e = 342$; $r_E = 4.8$ Å) was used; from the data of Garlick and Renkin (12).

* From the data of Granath and Kvasil (14), corrected to 37°.

* From the data of Garlick and Renkin (12).
DISCUSSION

The objective of this work was to characterize extravascular diffusion in normal and neoplastic tissues. Our technique has 2 limitations: (a) how well a mature granulation tissue represents various types of normal tissues is not known (9, 37); and (b) although numerous new capillaries are formed due to the presence of tumor in the ear chamber preparation, the extent of tumor invasion into the stroma of the granulation tissue cannot be determined a priori. Despite these limitations, our results clearly demonstrate that the diffusion coefficients of various test molecules \((M, 376 \text{ to } 71,800; \text{ effective radius range, } 4.8 \text{ Å to } 57.9 \text{ Å})\) are smaller in granulation tissue than in neoplastic (VX2 carcinoma) tissue. Diffusion coefficients in both types of tissue showed a dependence on molecular weight which progressively deviated from free diffusion in water (Table 2). The spatial variation in diffusivities (20% in normal and 12% in neoplastic tissues) was comparable to the limit of accuracy of methods. No systematic correlation between diffusion coefficient and spatial coordinates in the tissue could be established.

Diffusion coefficient of a molecule in a tissue matrix is influenced by the interstitial space, the tissue glycosaminoglycan content, and the size, configuration, charge, and binding of the molecule. Our results on the difference between granulation and neoplastic tissue diffusion coefficients are consistent with these physiochemical characteristics of the solute molecules and of the extravascular space of these tissues.

**Interstitial Space.** Published data suggest that the interstitial space of tumors is significantly larger than that of the normal tissue of similar origin, allowing more space in tumors for transport. Gullino et al. (18) found the interstitial space of a large number of transplantable rat tumors (Walker 256 carcinoma, fibrosarcoma, hepatoma 5123, hepatoma 3683, and Novikoff hepatoma) to be between 32 and 60%, and that of livers to be 20.5% of tissue water. Bauen et al. (31) measured the extracellular space of DS carcinosarcoma in the rat to be 38%. Bakay (2), using electron microscopy, determined the extracellular space in human gliomas (20 to 40%), meningiomas (13 to 15%), and normal brain tissue (6 to 7%). Fiszer-Szafarz and Cullino (10) determined the extracellular space of DS carcinosarcoma in the rat to be 38%. Cullino ef al. (17), based on an extensive number of transplantable rat tumors (Walker 256 carcinoma, fibrosarcoma, hepatoma 5123, hepatoma 3683, and Novikoff hepatoma) to be between 32 and 60%, and that of livers to be 20.5% of tissue water. Rauen et al. (31) measured the extracellular space of DS carcinosarcoma in the rat to be 38%. Bakay (2), using electron microscopy, determined the extracellular space in human gliomas (20 to 40%), meningiomas (13 to 15%), and normal brain tissue (6 to 7%). Appelgren et al. (1) reported a noticeably large extravascular space in 20 methylcholanthrene-induced sarcoma (40%) and in benzpyrene-induced sarcoma (50%) in rats when compared in muscle (13%). Therefore, the relatively large interstitial space in tumors could, in part, account for the lower diffusional resistance offered by tumors.

**Interstitial Matrix Structure.** The interstitial matrix of a tissue is composed of a collagen and elastin fiber network. Dispersed within this cross-linked structure are fluid and macromolecular constituents (glycosaminoglycans and glycoproteins) which form a hydrophilic gel phase. Various in vivo and in vitro studies have shown that the stabilized polysaccharides network (glycosaminoglycans and hyaluronic acid) offers considerable resistance to transport. Grabowiska (13) found the collagen content of s.c. tissue (11.24%) to be significantly higher than that of Guerin rat sarcoma (0.76%). Gullino et al. (17), based on an extensive study, concluded that various lines of hepatomas contained more collagen than normal liver in rats.

The difference in GAG contents between normal and neoplastic tissues has been reported by several investigators. Pearce (29) and Boas (3) found GAG content in mouse s.c. tissues to be 0.1023% and 0.1855%, respectively. Brada (4) measured it to be 0.0274% and 0.0267% in mouse Ehrlich and Krebs tumors, respectively. Sylven (35) and Choi et al. (7) measured relatively high concentrations of GAG in various types of sarcomas (0.1 to 1.5%). Fiszer-Szafarz and Gullino (10) determined the GAG content in the interstitial fluids of s.c. tissue (1.4 \times 10^{-3}%) and Walker 256 carcinoma (0.64 \times 10^{-3}%) in rats. The markedly low contents of GAG in tumors support our results on tumor diffusion coefficients.

**Molecular Weight Dependence.** Diffusion coefficients of dextrans in water and normal tissues have been reported by various investigators and can be described by the following power law expression:

\[
D = a (M_r)^b \text{ sqcm/sec}
\]

The data of Granath and Kvist (14) on aqueous diffusions \((10,000 < M_r < 147,000)\) follow the relation, \(1.26 \times 10^{-4} (M_r)^{-0.478}\). In vitro measurements of dextran diffusion in human articular cartilage by the method of Maroudas (24) can be described by, \(6.17 \times 10^{-2} (M_r)^{-1.34}\) for 5,000 \(< M_r < 40,000\). In vivo measurements of dextrans in the mesenteries of cat and rat, respectively, by Wayland et al. (11, 25) can be fitted to the expression, \(2.75 \times 10^{-2} (M_r)^{-0.75}\) \((3,400 < M_r < 393,000)\) and \(5.5 \times 10^{-3} (M_r)^{-1.09}\) \((3,450 < M_r < 41,200)\).

Swabb et al. (34) compiled from the literature the diffusion coefficients data for normal tissues for various solutes \((M_r < 1000)\), a large scatter was observed for macro-molecules and values of "b" from -0.65 to -0.81 were needed to envelope the data. Due to data scatter and paucity of data, these investigators were unable to determine a dependency of \(D\) on the physical-chemical characteristics of the tissue-solute system.

Our data on dextran diffusion in normal granulation tissue and VX2 carcinoma led to the correlations (Chart 2), \(10^6 (M_r)^{-3.96}\) \((19,400 < M_r < 71,800; r^2 = 0.98)\) and \(2.51 \times 10^{-2} (M_r)^{-1.14}\) \((19,400 < M_r < 62,000; r^2 = 0.96)\), respectively. This distinct dependence of \(D\) on molecular weight in normal versus neoplastic tissue has been reported here for the first time.

**Dependence on Configuration, Charge, and Binding.** In both normal and neoplastic tissues, BSA diffusion is significantly reduced from that of a dextran of equivalent Stoke-Einstein radius (Table 2). This effect has been observed in several normal tissues; however, no effects of this nature have been reported for diffusion in tumors. It has been reported that dextran molecules penetrate more easily into the glomerular filtrate than...
Diffusion in Normal and Neoplastic Tissues

Various macromolecules in tumors. However, most tumor drug uptake studies suggest the contrary (19–21). The reason for this apparent contradiction is the fact that most solid tumors, especially carcinomas, have poor blood flow compared to that of the normal host tissue (16, 21). In addition, the blood flow rate of a tumor decreases as it grows larger (16, 21, 22). As a result, intracellular uptake of blood-borne substances in tumors is limited by blood flow and not by extravascular diffusion. In case of highly perfused tumors, e.g., sarcomas and lymphomas, the rate-limiting step is normally the transport across the cell membrane (21). In some tumors, the vascular permeability to the anticancer agents may be the rate-limiting step (19–21). Therefore, increasing the tumor perfusion rate and vascular and membrane permeabilities selectively by pharmacological agents may have beneficial effects on cancer chemotherapy and immunotherapy.

ACKNOWLEDGMENTS

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REFERENCES

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Fig. 1. Mature granulation tissue (A) and VX2 carcinoma (B) grown in the rabbit ear chamber. Vessels in the granulation tissue are relatively straight. Vessels in the tumor preparation are tortuous and dilated, and the number of blood vessels per unit area has increased due to the tumor angiogenic response.
Fig. 2. Histological studies of the transverse sections of the tissues grown in the chamber. A, an overall view of the normal (mature granulation tissue) preparation. Note the cartilage (center) and the connective tissue layers on both surfaces of the cartilage (x 80). B, connective tissue of the normal preparation showing the presence of capillaries and precapillary vessels (x 330). C, neoplastic cells growing on the surface of the collagen stroma and numerous capillaries newly formed in the underlying stroma (x 330). D, another area of the preparation where the tumor grows mostly on the surface and invasive prongs are visible in the stroma (x 220).
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