[CANCER RESEARCH 44, 238–244, January 1984]

Extravascular Diffusion in Normal and Neoplastic Tissues[1]

Lawrence J. Nugent and Rakesh K. Jain[2]

Department of Chemical Engineering, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15232

ABSTRACT

Extravascular transport of fluorescein isothiocyanate-conjugated bovine serum albumin and a graded series of fluorescein isothiocyanate-dextrans from M_r 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, $D = a(M_r)^b$, in both normal tissue ($a = 10^6$ and $b = -2.96$) and tumor ($a = 2.51 \times 10^{-2}$ 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[3]-conjugated dextrans (M_r 19,400 to 71,800) and BSA (M_r 67,000). Sodium fluorescein was used as a representative small molecule (M_r 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 (a_E), 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. 1*A*) 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. 1*B*, 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. 2*C*), and sometimes invade the stroma (Fig. 2*D*). 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 dextrans (FITC-dextrans) obtained from Sigma Chemical Company, St. Louis, Mo., and Pharmacia, AB, Uppsala, Sweden. The weight average molecular weight (M_r) and the number average molecular weight (M_n) 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 fluorescein television microscopy (27). The chamber was secured

[1] Financial assistance was furnished by grants from the American Cancer Society, the National Science Foundation, the R. K. Mellon Foundation, and a Research Career Development Award from the National Cancer Institute to Rakesh K. Jain. A preliminary report of this work has been presented at the 74th Annual Meeting of the American Institute of Chemical Engineers, November 1982, Los Angeles, Calif.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; GAG, glycosaminoglycans.

Received May 12, 1983; accepted September 23, 1983.

to the microscope stage with an aluminum adapter. The tissue preparation was brought in focus and locked in place for the duration of the experiment.

After the optical system was standardized, preinjection control images were recorded on a video tape recorder (Model AV-3650; Sony Inc.) to identify the region of interest. At this time, the test molecule was injected as a pulse into the auricular vein of the contralateral ear (10 to 25 mg/kg body weight as 2.5% solution in 1 to 3 ml 0.9% NaCl solution). The optics and peripheral instrumentation were designed so that the fluorescent emission reaching the detector was linear with the concentration of the test molecule. Therefore, the light intensity distribution over the video field after background subtraction was equivalent to the concentration distribution within the tissue.

The video images were later analyzed off-line to obtain concentration of test molecules as a function of time at selected points along a line perpendicular to vessels using the photometric method reported previously (27). The temporal and spatial concentration data were stored in a computer for later analysis.

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:

$$C(x, t) = 2(\pi)^{1/2} \int_{x/(2\sqrt{D})}^{\infty} v_0 f[t - x^2/(4D\tau^2)]e^{-\tau^2}d\tau \qquad (A)$$

where t is time (sec), x is position along a line perpendicular to a vessel (cm), τ 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.

Table 1

Physical parameters of test molecules

Test molecule	Source	Wt av. molecular wt (M_r)	No. av. molecular wt (M_n)	Polydispersity (M_r/M_n)
Na-F	Aldrich	376	376	1.0
FITC-D20	Sigma	20,500	17,900	1.21
	Pharmacia	19,400	17,400	1.11
FITC-D40	Sigma	44,200	36,600	1.21
	Pharmacia	39,000	32,000	1.22
FITC-D70	Sigma	71,800	62,100	1.16
	Pharmacia	62,000	51,500	1.20
FITC-BSA	Sigma	67,000	67,000	1.0

In these experiments, the origin ($x = 0$) was defined at a position just outside the capillary wall. The concentration values as a function of time at the origin ($x = 0$) were fitted to a polynomial, $f(t)$.

In this study, concentration-time measurements were made in 10 normal (granulation) and 5 tumor preparations. To estimate the extent of spatial variability, diffusion coefficients were determined in 3 to 4 separate regions in each granulation tissue, and in 6 to 7 separate regions in each tumor tissue. For a given region in granulation and tumor tissues, concentrations were measured at various uniformly spaced positions from the origin along a line perpendicular to the vessel. Chart 1 shows the cross-section of tissue in a given region, and the positions perpendicular to the vessel where concentrations were measured (*e.g.*, $x = 0$, x_1, and x_2 μm). In granulation tissues, concentrations were measured at 3 to 6 positions (*e.g.*, $x = 0$, 11, 21, 32, 42, 53, and 63 μm). In tumor tissues, due to the small intercapillary distances (approximately 40 μm), concentrations were measured only at 1 to 3 positions (*e.g.*, $x = 0$, 11, 16, and 21 μm). The diameter of vessels around which the concentration-time measurements were made ranged from 15 to 40 μm. The average diffusion coefficient in a given region (D_r) was calculated as an average of diffusion coefficients at these positions (D_x). The tissue average diffusion coefficient (D_t) was then calculated by averaging the regional diffusion coefficients (D_r).

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_o) and their effective molecular radius (r_E). The latter was calculated using the Stokes-Einstein relation (14):

$$r_E = kT/(6\pi\mu D_o) \qquad (B)$$

Here, k is the Boltzmann constant, T is the absolute temperature, and μ 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 2

Tissue diffusion coefficients in rabbit ear chamber

Test molecule	Wt av. molecular wt (M_r)	Effective molecular radius (r_E, Å)	Diffusion coefficient in water ($D_o \times 10^7$; sq cm/sec)	Tissue diffusion coefficients: Normal ($D \times 10^7$; sq cm/sec)	Tissue diffusion coefficients: Tumor ($D \times 10^7$; sq cm/sec)	Ratios: Normal (D/D_o)	Ratios: Tumor (D/D_o)
Na-F	376	4.8	70[a]	20		0.29	
				24	64	0.34	0.89
FITC-D20	20,500	32.0	10.26[b]	1.7		0.17	
	19,400	31.1	10.55	2.3	7.5	0.22	0.71
FITC-D40	44,200	46.2	7.11[b]	0.22		0.031	
	39,000	43.5	7.54	0.18	4.2	0.024	0.56
FITC-D70	71,800	57.9	5.67[b]	0.048		0.0085	
	62,000	53.9	6.09	0.061	1.9	0.010	0.31
FITC-BSA	67,000	35.5	9.3[c]	0.16		0.017	
				0.11	0.91	0.012	0.10

[a] Value for sucrose ($M_r = 342$; $r_E = 4.8$ Å) was used; from the data of Garlick and Renkin (12).
[b] From the data of Granath and Kvist (14), corrected to 37°.
[c] 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_r 376 to 71,800; effective radius range, 4.8 Å to 57.9 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. 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 Krebbs 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 (1.4×10^{-3}%) and Walker 256 carcinoma (0.64×10^{-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:

$$D = a\,(M_r)^b \text{ sqcm/sec} \qquad \text{(C)}$$

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, $2.75 \times 10^{-3}\,(M_r)^{-0.758}$ ($3{,}400 < M_r < 393{,}000$) 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 ($32 < M_r < 69{,}000$) and proposed the correlation, $1.778 \times 10^{-4}\,(M_r)^{-0.75}$. While this correlation represented the data adequately for small solutes ($M_r < 1000$), a large scatter was observed for macromolecules 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 mature granulation tissue and VX2 carcinoma led to the correlations (Chart 2), $10^6\,(M_r)^{-2.96}$ ($19{,}400 < M_r < 71{,}800$; $r^2 = 0.98$) 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

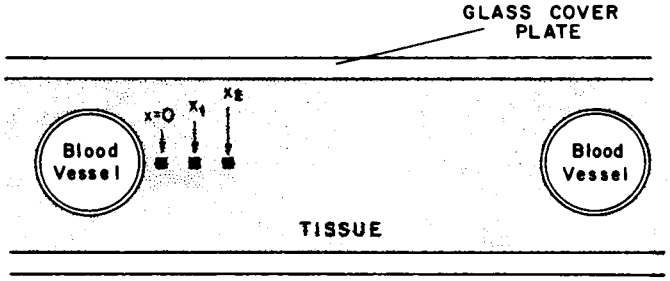

Chart 1. Chamber cross-section showing 2 blood vessels surrounded by the tissue sandwiched between 2 glass plates. Concentrations of the fluorescent molecules were measured at various positions along a line perpendicular to the blood vessel (*e.g.*, $x = 0$, x_1, and x_2 μm). The number of positions chosen for measurements depended upon the intercapillary distance, and ranged from 3 to 6 in granulation tissues and one to 3 in tumors.

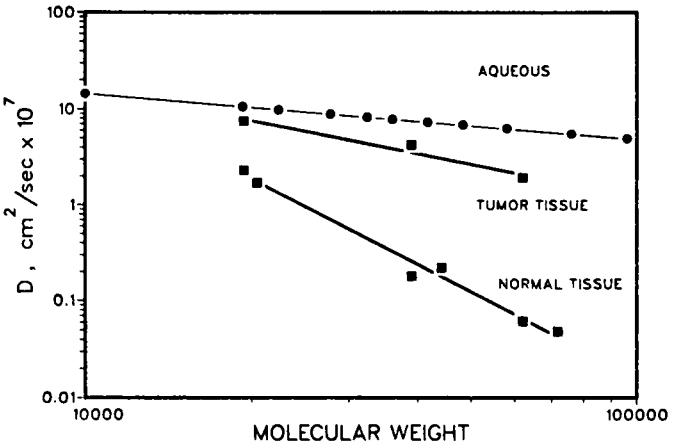

Chart 2. Dependence of dextran diffusion coefficients on molecular weight. *Upper curve* (●), data of Granath and Kvist (14) for aqueous diffusion corrected to 37°. *Middle and lower curves* (■), our data for VX2 carcinoma and mature granulation tissue, respectively; ——, best fits to the expression $a(M_r)^b$ (r^2 = 1.0, 0.96, and 0.98 for water, tumor, and granulation tissue, respectively).

proteins of comparable size (32). In support of our results on interstitial diffusion, Fox and Wayland (11) found diffusive transport of BSA in the rat mesentery to be hindered more than dextran of same molecular size.

The deviation of diffusion coefficients from a strict molecular size basis could be explained in terms of configuration, charge, and binding of the test molecule. The dextrans used in this study are linear polymers with a slight degree of branching (5%), and albumin molecule is loosely coiled in an ellipsoidal shape in aqueous solution (14). Various *in vitro* and *in vivo* studies have shown greater transport rates of linear molecules than that of globular molecules of equivalent Stokes-Einstein radius (8, 23).

Electrostatic repulsion of negatively charged albumin by negative charges of the interstitial matrix would lead to a smaller effective volume for diffusion. Works by various investigators on capillary permeability to charged proteins support this hypothesis as reviewed previously (32, 33).

Finally, binding of BSA to the tissue components would further reduce the diffusivity of albumin. The precise role of configuration, change, and binding in albumin diffusion could not be determined in our study.

Role of Interstitial Convection. Gullino *et al.* (6, 34) discovered and characterized the existence of interstitial convection in tumors. The excellent agreement between the measured concentrations and predictions based on a pure diffusion model (Equation A) suggest the absence of a substantial convective component in our experimental system of normal and neoplastic tissues. Further, the systematic variation of calculated diffusion coefficients with molecular weight (Chart 2) makes the presence of interstitial convection unlikely. The only explanation for the difference in our results and those of Gullino is the anatomical difference between the tissue preparations; 2-dimensional in the former *versus* 3-dimensional in the latter.

Implications for Cancer Therapy. The present study shows quantitatively that the interstitial resistance to the transport of molecules ($376 < M_r < 71{,}800$) in a tumor (VX2 carcinoma) is considerably lower than in mature granulation tissue. Since common anticancer agents have less than M_r 2000 (when not bound to proteins) and plasma protein-bound drugs of M_r 10,000 to 100,000, one might expect a greater localization of drugs and 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

We gratefully acknowledge the help of Leonard Gerlowski and Dr. Charles Cha in characterizing dextrans using gel permeation chromatography, Kimberly Ward-Hartley in histological studies, Sharon Strait for laboratory assistance and preparing this manuscript, Dr. Sudhir Shah in tumor transplantion, and Dr. Pietro Gullino, Dr. Shu Chien, and Dr. Herbert Lipowsky for many helpful discussions.

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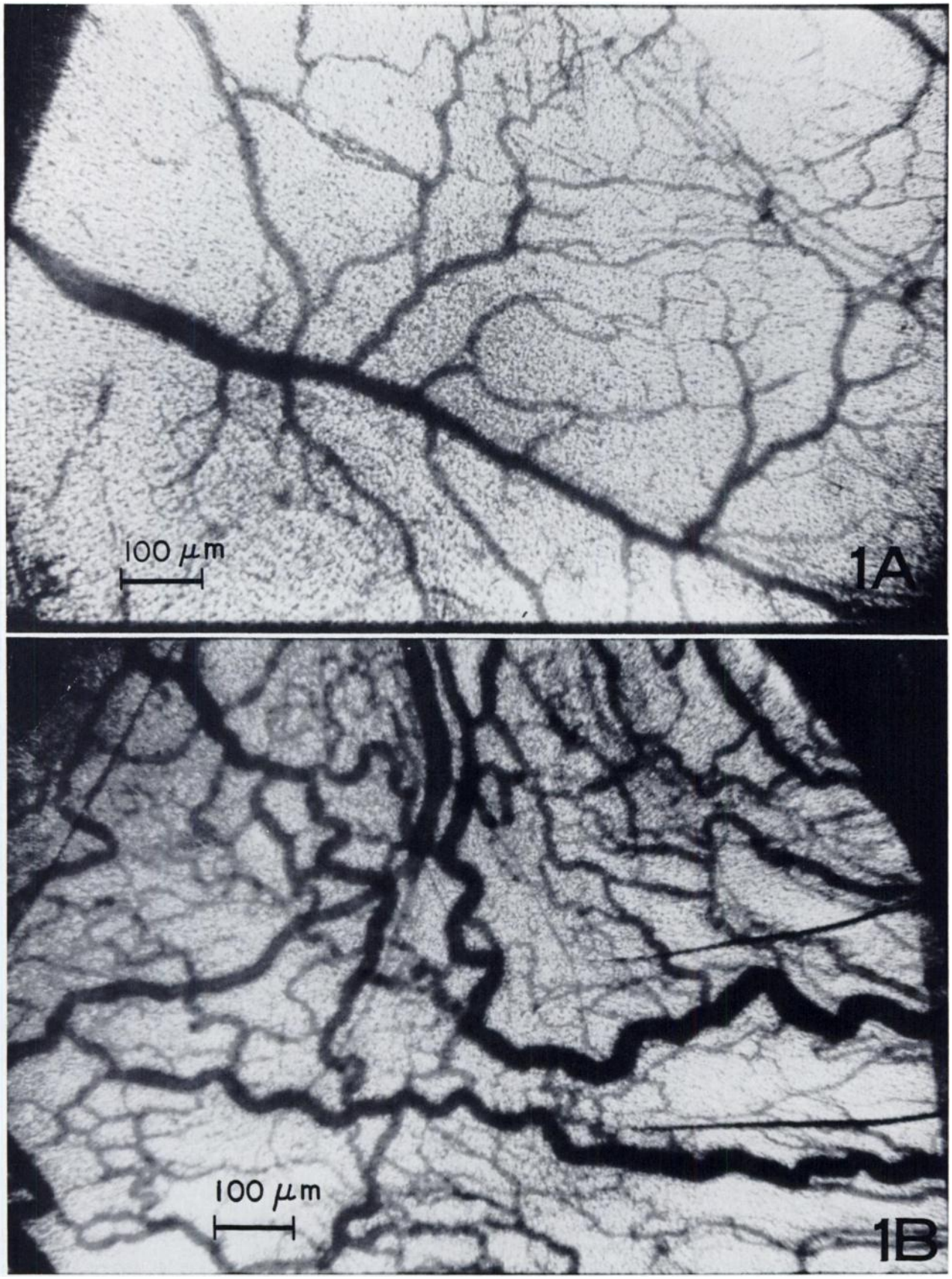

Fig. 1. Mature granulation tissue (*A*) and VX2 carcinoma (*B*) grown in the rabbit ear chamber. Vessels in the granulation tissue are relatively straight. Vessels in the tumor preparation are tortuous and dilated, and the number of blood vessels per unit area has increased due to the tumor angiogenic response.

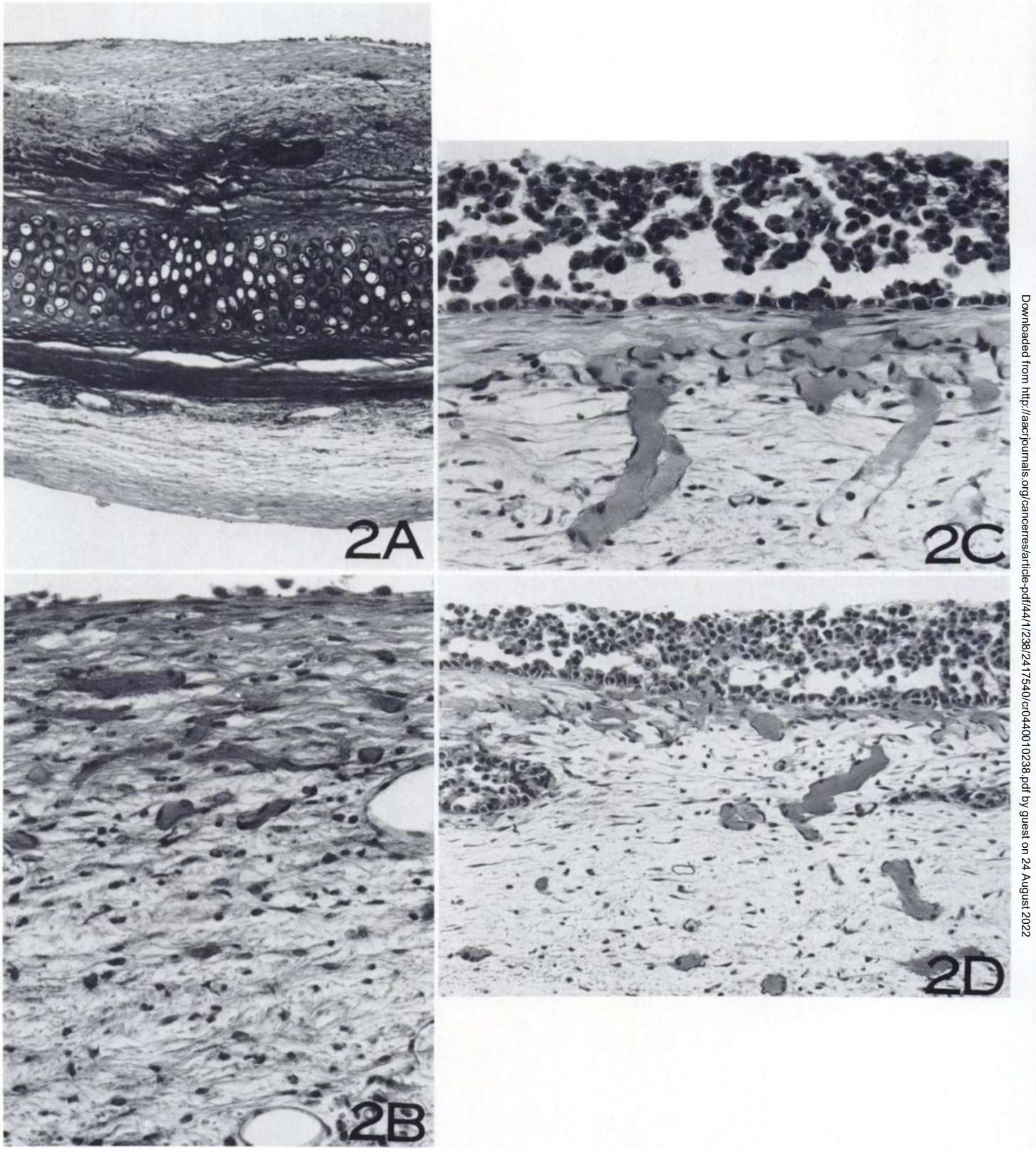

Fig. 2. Histological studies of the transverse sections of the tissues grown in the chamber. *A*, an overall view of the normal (mature granulation tissue) preparation. Note the cartilage (*center*) and the connective tissue layers on both surfaces of the cartilage (× 80). *B*, connective tissue of the normal preparation showing the presence of capillaries and precapillary vessels (× 330). *C*, neoplastic cells growing on the surface of the collagen stroma and numerous capillaries newly formed in the underlying stroma (× 330). *D*, another area of the preparation where the tumor grows mostly on the surface and invasive prongs are visible in the stroma (× 220).