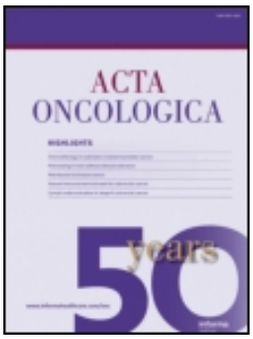

Acta Oncologica

ISSN: 0284-186X (Print) 1651-226X (Online) Journal homepage: informahealthcare.com/journals/ionc20

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To cite this article: Ingunn Tufto & Einar K. Rofstad (1998) Interstitial Fluid Pressure, Fraction of Necrotic Tumor Tissue, and Tumor Cell Density in Human Melanoma Xenografts, Acta Oncologica, 37:3, 291-297, DOI: 10.1080/028418698429603

To link to this article: https://doi.org/10.1080/028418698429603

Published online: 08 Jul 2009.

Interstitial Fluid Pressure, Fraction of Necrotic Tumor Tissue, and Tumor Cell Density in Human Melanoma Xenografts

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Acta Oncologica Vol. 37, No. 3, pp. 291–297, 1998

Interstitial fluid pressure (IFP) has been shown to differ substantially between individual tumors, but the tumor properties governing the intertumor heterogeneity in IFP have not been identified conclusively. The purpose of the work reported here was to investigate whether the fraction of necrotic tissue and the density of tumor cells are major determinants of the intertumor heterogeneity in IFP. The study was based on the hypothesis that the resistance against fluid flow in the tumor interstitium is influenced significantly by these parameters. Xenografted tumors of four human melanoma lines (A-07, D-12, R-18, U-25) were included in the study. Tumors showing large variation in necrotic fraction but similar cell densities (D-12, U-25) were used to study the influence of necrosis on IFP, whereas tumors showing no or insignificant necrosis but large variation in cell density (A-07, R-18) were used to search for correlations between IFP and cell density. IFP was recorded using the wick-in-needle technique. Necrotic fraction and cell density were measured by stereological analysis of histological sections using an image processing system. Significant correlations between IFP and necrotic fraction were not found, implying that the IFP of tumors is not influenced significantly by the development of necrosis. The R-18 tumors, which had a high cell density, showed a significantly higher IFP than the A-07 tumors, which had a low cell density. Significant correlations between IFP and cell density were not found when individual tumors of the same line were considered. These two observations suggest that the IFP of tumors depends on the cell density, but the cell density is probably not a major determinant of the IFP.

Received 20 October 1997
Accepted 4 March 1998

Most tumors show a higher interstitial fluid pressure (IFP) than normal tissues (1). The elevated IFP in tumors is proposed to be due to an abnormal vascular network (2) and the lack of a functional lymphatic system (3). Theoretical and experimental studies have suggested that in tumors growing as a single nodule, the IFP is relatively uniform throughout the tumor and drops precipitously to the normal tissue level at the tumor-normal tissue interface (4, 5). The microvascular hydrostatic pressure (MVP) is the principal driving force for the elevated IFP in tumors (6). The arteriovenous pressure difference drives blood through the capillaries, and owing to the high resistance against blood flow in the capillary network and the high permeability of the capillary wall (7, 8), fluid is forced from the capillaries into the interstitial compartment (1). It has been suggested that this fluid accumulates in the interstitium and leads to an elevated IFP unless the reabsorption by the vascular system is efficient and/or the efflux at the tumor periphery is high (9, 10). The magnitude of the IFP in tumors might therefore depend on the resistance against fluid flow in the interstitium as well as the resistance against fluid flow in the capillary network and across the capillary wall.

The IFP differs substantially between individual experimental tumors of the same line (11, 12) and between individual human tumors of the same histological type (13, 14). This heterogeneity might be caused by intertumor differences in resistance against fluid flow in the capillaries, intertumor differences in resistance against fluid flow across the capillary wall, and/or intertumor differences in resistance against fluid flow in the interstitium. The flow resistance in the capillaries is governed mainly by the irregularity of the capillary network (2, 15) and the viscosity of the blood (16). The resistance against transcapillary flow is determined primarily by the structure of the capillary wall (17, 18). The transcapillary flow resistance is low in most tumors, as tumor capillaries usually have wide interendothelial junctions, large numbers of fenestrae and transendothelial channels, and a discontinuous or absent basement membrane (2, 17) and, as a result, the IFP is not

 ISSN 0284-186X

significantly different from the MVP (6, 19). The flow resistance in the interstitium is governed mainly by the fractional volume and the composition of the interstitial space (1). The interstitial compartment of tumors is generally larger than that of normal tissues and is thus characterized by a higher hydraulic conductivity (20).

The relative importance of intertumor differences in resistance against intracapillary fluid flow, intertumor differences in resistance against transcapillary fluid flow, and intertumor differences in resistance against interstitial fluid flow for the intertumor heterogeneity in IFP have not been explored. The objective of the study reported here was to search for a possible relationship between tumor IFP and flow resistance in the tumor interstitium. Adequate in vivo methods for measurement of the flow resistance in the interstitium of tumors have not been established. Two tumor parameters which are believed to have a strong influence on the interstitial flow resistance were therefore measured here: fraction of necrotic tumor tissue and tumor cell density. Tumor tissue with large necrotic regions or low cell density is hypothesized to have high hydraulic conductivity, low resistance against interstitial flow, and low IFP, as the interstitial matrix is demolished in necrotic tumor regions and the fractional volume of the interstitial space is high in tumors with low cell density.

The studies were performed using A-07, D-12, R-18, and U-25 human melanoma xenografted tumors. The resistance against transvascular fluid flow is negligible in these tumors, as the neovasculature shows incomplete endothelial lining, interrupted or absent basement membrane, and/or lack of pericytes (21). The D-12 and U-25 lines, which develop tumors showing large individual variation in necrotic fraction but similar cell densities, were selected in order to study the influence of necrosis on IFP. The A-07 and R-18 lines, which develop tumors showing no or insignificant necrosis but large individual variation in cell density, were selected in order to search for correlations between IFP and cell density.

MATERIAL AND METHODS

Mice and tumors

Adult Balb/c nu/nu mice (8–12 weeks old), bred at our research institute, were used as host animals for xenografted tumors. The mice were maintained under specific pathogen-free conditions at a constant temperature (24–26°C) and humidity (30–50%). Sterilized food and tap water were given ad libitum.

The experiments were performed using four human melanoma cell lines (A-07, D-12, R-18, U-25) (21). The cell lines were verified to be free from *Mycoplasma* contamination by using the Hoechst fluorescence and mycotrin methods. Fifteen xenografted tumors of each line were initiated from exponentially growing monolayer cultures in passages 75–100. Monolayer cells, cultured in RPMI-1640 medium (25 mM Hepes and L-glutamine) supplemented with 13% fetal calf serum, 250 mg/l penicillin, and 50 mg/l streptomycin, were detached by trypsinization (treatment with 0.05% trypsin/0.02% EDTA solution at 37°C for 2 min). Approximately 3.5×10^5 cells in 10 μl of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution were inoculated intradermally in the flanks of the mice by using a 100 μl Hamilton syringe (21). Tumors with wet weights ranging from 150 to 950 mg were first subjected to measurement of IFP and then to measurement of necrotic fraction or cell density.

Interstitial fluid pressure

Tumor IFP was measured by using the wick-in-needle technique (22). A 23-gauge needle (Microlance, Dublin, Ireland), filled with multifilamentous nylon thread, was connected to an Abbott Transpac II pressure transducer (Abbott Ireland Ltd., Sligo, Ireland) by a polyethylene tubing filled with sterile heparinized (70 units/ml) saline. The pressure transducer was connected to a model 13-6615-50 preamplifier and a model TA240 Easygraf dual-channel chart recorder (Gould Inc., Cleveland, OH, USA). The pressure of 30 cm of saline was maintained for 5 min to test for possible leaks in the system.

The mice were kept under anesthesia during the IFP measurements. Propanidid (Gedeon Richter Ltd., Budapest, Hungary), fentanyl/fluanisone (Janssen Pharmaceutika, Beerse, Belgium), and diazepam (Dumex, Copenhagen, Denmark) were administered intraperitoneally in doses of 400 mg/kg, 0.24/12 mg/kg, and 4 mg/kg, respectively. The body core temperature of the mice, measured with a rectal probe, was kept at 36–38°C by using a heating pad. The needle was inserted in the central region of the tumor for measurement of IFP. The IFP was recorded for at least 10 min. After a stable IFP value was reached, the fluid communication between the pressure transducer and the tumor was tested by compressing and decompressing the tubing between the needle and the transducer using a screw clamp. Measurements were discarded if the readings following these tests differed by more than 1 mmHg. Tumor IFP was determined by calculating the mean of the reading before the compression test, the reading after the compression test, and the reading after the decompression test. The IFP measured in normal tissue; i.e., subdermally in tumor-free dorsal skin or intramuscularly in the proximal portion of the lower extremity, served as an internal control.

Histological analysis

Tumors were fixed in phosphate-buffered 4% paraformaldehyde and cut into four pieces of approximately the same size. The four pieces of tumor were embedded in a single paraffin cast and histological sections, 5-μm-thick, were cut and mounted on glass slides using standard procedures. The sections were stained with

hematoxylin and eosin and subjected to stereological analysis (23). The stereological analysis was performed using the KS300 image processing system (Kontron Elektronik GmbH, Munich, Germany). Volume fraction of necrosis was determined by measuring the area fraction of necrosis (24). Cell density was measured according to the method of Brammer & Jung (25). Briefly, the numerical density of tumor cell nuclei was derived from the volumetric density and the nuclear volume. The volumetric density was determined by measuring the area density. The nuclear volume was determined by measuring nuclear chord lengths. The numerical cell density was set equal to the numerical nuclear density, assuming one nuclei per cell. Three sections were analyzed for each tumor. Five fields of view in non-necrotic tissue, corresponding to 500–600 cells, were selected for measurement of cell density.

Statistical analysis

Statistically significant correlations between IFP and necrotic fraction or cell density were searched for by linear regression analysis. Statistical comparisons of data were performed by non-parametric analysis using the Mann-Whitney U-test for single comparisons and the Kruskal-Wallis H-test for multiple comparisons. A significance criterion of $p < 0.05$ was used.

RESULTS

The IFP values measured subdermally in tumor-free dorsal skin or intramuscularly in the proximal portion of the lower extremity ranged from -1 to $+1$ mmHg. All tumors showed an elevated IFP relative to these normal tissues. The intertumor heterogeneity in IFP was considerable; the IFP ranged from 3 to 13 mmHg (A-07), 3 to 15 mmHg (D-12), 9 to 34 mmHg (R-18), and 6 to 30 mmHg (U-25). Repeated measurements in the same tumors showed that the IFP measurements were highly reproducible.

The fraction of necrotic tissue differed considerably between individual D-12 and U-25 tumors—ranging from 4 to 56% (D-12) and 14 to 56% (U-25). There was no correlation between necrotic fraction and tumor wet weight in any of the lines. The heterogeneity in necrotic fraction is illustrated in Fig. 1, which shows a D-12 tumor with a small necrotic fraction (Fig. 1a) and a U-25 tumor with a large necrotic fraction (Fig. 1b). The necrosis in the D-12 tumor is located in the center and close to the tumor periphery. The U-25 tumor shows focal necrosis scattered throughout the tumor. The pattern of necrosis was not different for D-12 and U-25 tumors. The tumors with a small necrotic fraction showed a pattern similar to that illustrated in Fig. 1a and most tumors with a large necrotic fraction showed a pattern similar to that illustrated in Fig. 1b. However, a few tumors with a large necrotic fraction showed massive central necrosis accompanied by focal peripheral necrosis. There was no correlation between IFP and necrotic fraction, either for D-12 tumors ($p > 0.05$) (Fig. 2a) or for U-25 tumors ($p > 0.05$) (Fig. 2b). Moreover, the IFP values of the D-12 and U-25 tumors, which showed significant necrotic fractions, were not significantly different from those of the A-07 and R-18 tumors, which did not show significant necrosis ($p > 0.05$).

The cell density differed between fields of view within individual A-07 and R-18 tumors by factors of 1.2–2.0 (A-07) and 1.1–1.9 (R-18). In spite of this intratumor heterogeneity, the individual tumors differed significantly in cell density. The mean values of the individual tumors,

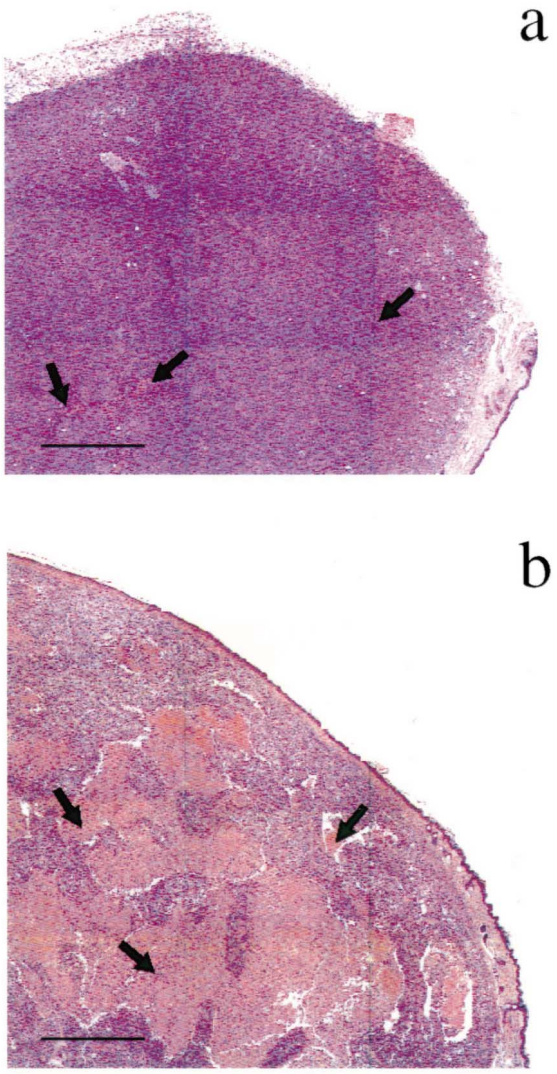

Fig. 1. Photomicrographs of histological sections from human melanoma xenografted tumors illustrating intertumor heterogeneity in necrosis: (a) D-12 tumor with a small necrotic fraction. (b) U-25 tumor with a large necrotic fraction. The photomicrographs represent approximately 25% of the central tumor cross-sections. Arrows indicate necrosis. Bars: 1000 μm.

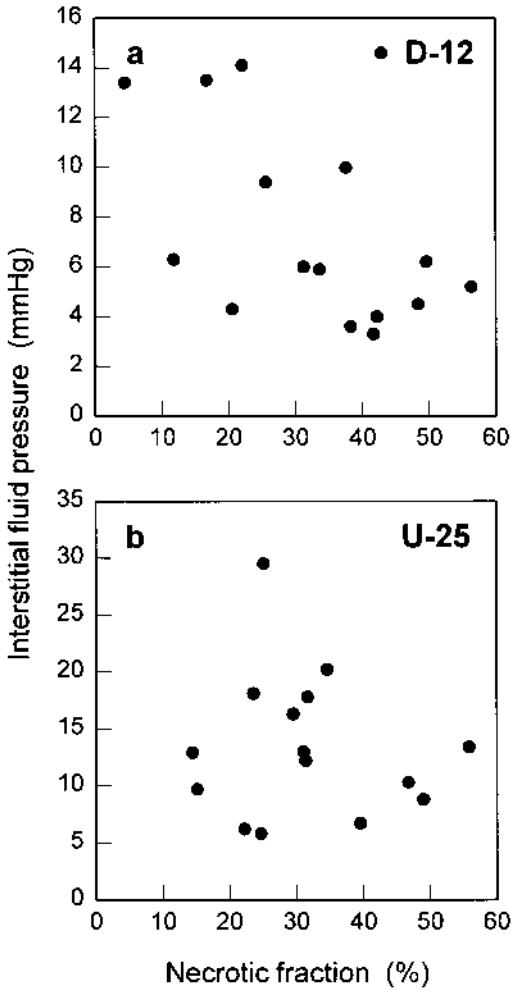

Fig. 2. Interstitial fluid pressure versus necrotic fraction for human melanoma xenografted tumors: (a) D-12 tumors. (b) U-25 tumors. Points represent individual tumors.

calculated from the values of the individual fields of view, ranged from 1.2×10^5 to 3.0×10^5 cells/mm^3 (A-07) and 2.7×10^5 to 6.4×10^5 cells/mm^3 (R-18). Moreover, the cell density was significantly higher in R-18 tumors than in A-07 tumors ($p < 0.05$). The mean values $\pm$ standard errors of the tumor lines, calculated from the mean values of the individual tumors, were $(2.4 \pm 0.5) \times 10^5$ cells/mm^3 (A-07) and $(4.2 \pm 1.1) \times 10^5$ cells/mm^3 (R-18). The heterogeneity in cell density is illustrated in Fig. 3, which shows an A-07 tumor with a low cell density (Fig. 3a) and an R-18 tumor with a high cell density (Fig. 3b). There was no correlation between IFP and cell density, either for A-07 tumors ($p > 0.05$) (Fig. 4a) or for R-18 tumors ($p > 0.05$) (Fig. 4b). However, the IFP values of the R-18 tumors, which showed a high cell density, were significantly higher than those of the A-07 tumors, which showed a low cell density ($p < 0.05$).

DISCUSSION

High IFP in tumors might be indicative of resistance to some treatment modalities. Thus, inverse relationships have been demonstrated between IFP and oxygen tension in some experimental and human tumors (26, 27). Moreover, the elevated IFP in tumors has been shown to restrict the access of macromolecular therapeutic agents to the neoplastic cells (1, 18). A thorough understanding of the causes of the elevated IFP in tumors might lead to methods to decrease the IFP and hence to improved outcome of therapy. Thus, a possible relationship between IFP and resistance against fluid flow in the tumor interstitium was sought in the present work.

The A-07, D-12, R-18, and U-25 tumors have biological properties rendering them suitable for a study aiming at detecting such a relationship, if it exists. Electron microscopy studies have shown that the capillaries in these tumors have an incomplete endothelial lining and a discontinuous basement membrane (21). Magnetic resonance imaging studies have shown that 20-kD macromolecules readily can traverse the tumor capillary wall (unpublished data). The transcapillary flow resistance is therefore low and can probably be disregarded as a variable in the studies reported here. Moreover, the IFP probably does

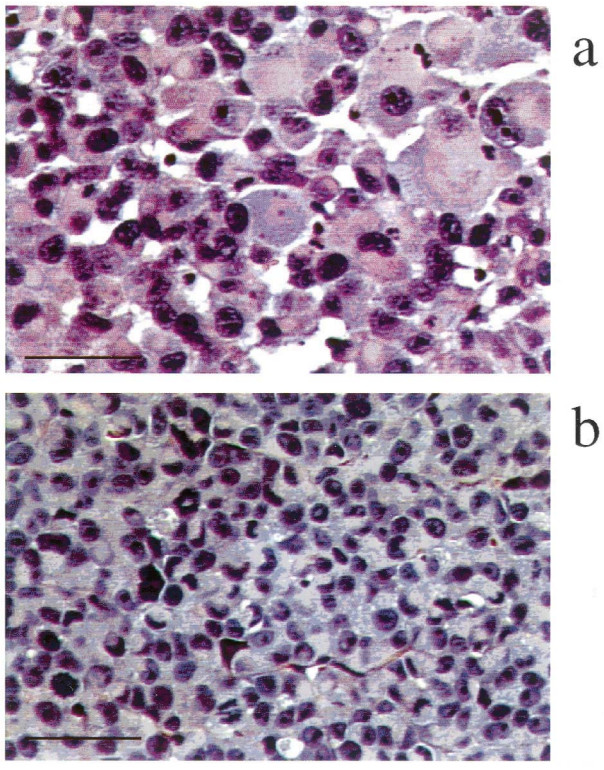

Fig. 3. Photomicrographs of histological sections from human melanoma xenografted tumors illustrating intertumor heterogeneity in cell density: (a) A-07 tumor with a low cell density. (b) R-18 tumor with a high cell density. Bars: 50 μm.

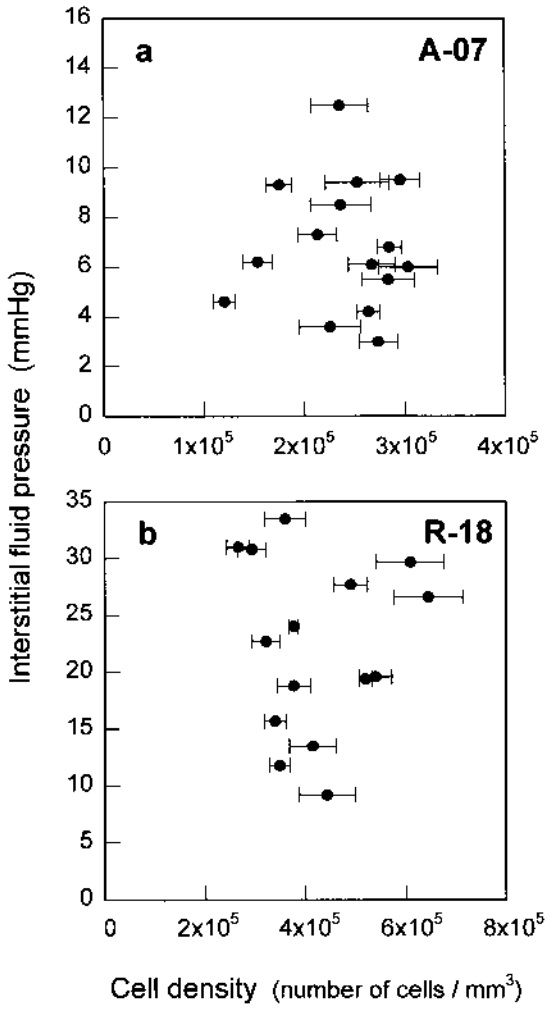

Fig. 4. Interstitial fluid pressure versus cell density for human melanoma xenografted tumors: (a) A-07 tumors. (b) R-18 tumors. Points and bars represent mean values and standard errors of individual tumors, calculated from the values measured in individual fields of view.

not differ from the MVP owing to the high permeability of the capillary wall to fluid and plasma proteins, an assumption which is in agreement with experimental data on rodent tumors with an immature capillary network (6, 19).

Furthermore, the A-07, D-12, R-18, and U-25 tumors show considerable heterogeneity in the biological parameters measured here. Thus, tumor IFP ranged from 3 to 34 mmHg, a range which is similar to those reported for rodent tumors (5, 28) and melanomas in man (11, 14). The fraction of necrotic tissue differed between individual D-12 and U-25 tumors from 4 to 56% and the density of tumor cells differed between individual A-07 and R-18 tumors from 1.2×10^5 to 6.4×10^5 cells/mm^3. D-12 and U-25 tumors show similar cell densities and A-07 and R-18 tumors show no or insignificant necrotic fractions (21). Consequently, D-12 and U-25 tumors are suitable for studying the influence of necrosis on IFP by avoiding confounding effects of heterogeneity in cell density, and A-07 and R-18 tumors are suitable for studying the influence of cell density on IFP in the absence of confounding effects of necrosis.

However, it should be noted that xenografted tumors of only four human melanoma lines were included in the present study and that all tumors were initiated by intradermal cell inoculation. The results presented here are therefore not necessarily generalizable to other tumor models and other sites of tumor implantation.

Tumor IFP can be measured easily and correctly by using the wick-in-needle technique (5, 6). The IFP was recorded in a single location in the central region of the tumors in the present work. This procedure is justified by theoretical and experimental studies, which suggest that in tumors growing as a single nodule, the IFP is uniform throughout the tumors up to the tumor-normal tissue interface, where it drops precipitously to the level of the surrounding normal tissue (4, 5). Thus, IFP measurements are probably not influenced significantly by the tumor histology just around the needle tip as long as the needle is not inserted in the tumor periphery. Moreover, IFP measurements using the wick-in-needle technique give highly reproducible results, as was shown by performing repeated measurements in the same tumors.

Morphological parameters of tumors are quantitated with high accuracy by analyzing histological sections according to stereological principles (23). Stringent stereological principles were applied in the present study. Thus, the tissue sections subjected to analysis had different, randomly selected orientations in the tumors. The histological preparations were thin compared with the diameters of the cell nuclei. The fields of view were selected randomly, and the number of fields of view subjected to analysis was slightly higher than strictly required to obtain accurate values. Moreover, repeated analyses by the same observer showed that the measurements were highly reproducible. All measurements were performed by the same investigator since minor inter-observer differences have been detected in the measurement of cell density. The cell densities were not corrected for tissue shrinkage during tumor fixation. Although the numeric values for cell density determined here might deviate somewhat from the true values, our analysis gives accurate relative values. Consequently, the numeric values for cell density are valid for use in correlation analyses.

The present study suggests that the IFP in tumors is not influenced significantly by the development of necrosis. A significant correlation between IFP and fraction of necrotic tissue was not found, either for D-12 tumors or for U-25 tumors. Moreover, a significant difference in IFP between D-12 and U-25 tumors on the one hand and A-07 and R-18 tumors on the other was not found, despite the D-12 and U-25 tumors showing significant necrotic fractions and the A-07 and R-18 tumors showing no or

insignificant necrosis. Necrotic and viable tumor tissue differs in structure and composition, and an increased osmotic pressure is associated with necrotic tissue (29). However, despite these differences, necrotic tissue probably does not differ sufficiently from viable tissue in resistance against fluid flow to influence tumor IFP significantly.

R-18 tumors showed a significantly higher cell density and a significantly higher IFP than A-07 tumors, suggesting that tumor IFP is influenced by the cell density. However, the cell density is probably not a major determinant of tumor IFP since significant correlations between IFP and cell density were not found when individual A-07 or R-18 tumors were considered. A-07 and R-18 tumors show comparable cell volume distributions. The differences in cell density between A-07 and R-18 tumors thus mainly reflect differences in the fractional volume of the interstitial space. The hydraulic conductivity of a tissue depends on the interstitial space volume fraction (1), and hence, differences between tumors in the interstitial space volume fraction should cause differences in the resistance against interstitial fluid flow. However, these resistance differences are probably not large enough for the intertumor heterogeneity in interstitial space volume fraction to be a principal cause of intertumor heterogeneity in IFP.

The resistance against fluid flow in the interstitium of tumors might be influenced significantly by parameters other than those studied here. The tumor interstitium consists of a colloid-poor free-fluid space and a colloid-rich gel space composed predominantly of polysaccharides enmeshed in a network of collagen and elastic fibers. The hydraulic conductivity of a tissue is assumed to depend on the structure and composition of the interstitial space (1), parameters which have been shown to differ substantially between individual tumors of the same histological type (30). Detailed studies of possible relationships between tumor IFP and the structure and composition of the tumor interstitium are therefore needed.

It is also possible that the intertumor heterogeneity in IFP is governed mainly by intertumor differences in resistance against intracapillary fluid flow rather than by intertumor differences in resistance against interstitial fluid flow. The intracapillary flow resistance in tumors is determined mainly by the irregularity of the vascular network and the viscosity of the blood (15, 16). The intertumor heterogeneity in these parameters has been shown to be considerable (2, 8), suggesting that attempts to identify tumor parameters of major importance for the intertumor heterogeneity in IFP should also include studies of vascular and rheological parameters.

ACKNOWLEDGEMENTS

Financial support was received from The Norwegian Cancer Society.

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