Associations between Radiocurability and Interstitial Fluid Pressure in Human Tumor Xenografts without Hypoxic Tissue

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Abstract

**Purpose:** The interstitial fluid pressure (IFP) of the primary tumor is an independent prognostic parameter for cervical cancer patients treated with radiation therapy. The aim of this preclinical study was to investigate whether tumor radiocurability may be associated with IFP through hypoxia-independent mechanisms.

**Experimental Design:** Small A-07 and R-18 melanoma xenografts without hypoxic tissue were used as preclinical tumor models. IFP was measured by using the wick-in-needle method. Radiation dose resulting in 50% local tumor control (TCD50), cell density, cell tumorigenicity, plating efficiency in vitro, mitotic index, fraction of Ki67-positive cells, vascular endothelial growth factor-A (VEGF-A) concentration, and radiation-induced endothelial cell apoptosis were assessed in tumors with low and high IFP.

**Results:** TCD50 was found to be higher for tumors with high IFP than for tumors with low IFP by factors of 1.13 ± 0.03 (A-07; \( P < 0.0001 \)) and 1.10 ± 0.03 (R-18; \( P < 0.0001 \)). In the A-07 line, tumors with high IFP showed a larger number of clonogenic cells and a higher rate of cell proliferation than tumors with low IFP. In the R-18 line, tumors with high IFP showed a higher concentration of VEGF-A and a lower endothelial cell apoptotic index after irradiation than tumors with low IFP.

**Conclusions:** The radiation resistance of normoxic tumor tissue with highly elevated IFP may be an indirect consequence of increased tumor cell clonogenicity as well as increased VEGF-A expression, possibly caused by hypertension-induced modifications of signaling pathways regulating cell proliferation, cell survival, and/or angiogenesis. *Clin Cancer Res; 16(3); 936–45. ©2010 AACR.*

Clinical investigations have shown that the interstitial fluid pressure (IFP) is elevated in several tumor types, including lymphoma, breast carcinoma, head and neck carcinoma, cervical carcinoma, and cutaneous melanoma (1–3). Tumor tissues typically show IFP values within the range of 5 to 40 mmHg, whereas the IFP values of most normal tissues range from −3 to +3 mmHg. The IFP may be particularly high in malignant melanomas, where IFP values of up to 100 mmHg have been recorded (4). Only one large prospective clinical study of the association between IFP and patient outcome has been reported thus far (5), and the independent prognostic effect of IFP for recurrence and survival was strong, whereas the independent prognostic effect of tumor hypoxia was of borderline significance and was limited to patients without nodal metastases (6). Recently, the main findings reported by the Toronto group were confirmed in a smaller prospective study of patients with cervical carcinoma treated with radiation therapy alone at Chungnam National University Hospital in Daejeon (7).

Comprehensive studies of the mechanisms leading to elevated IFP in malignant tissues have been carried out in experimental tumor models (8–10). These studies have revealed that a high IFP in tumors is a consequence of severe microvascular, lymphatic, and interstitial abnormalities. Briefly, tumors develop elevated IFP primarily because they show increased transcapillary fluid flow and lack functioning lymphatic vessels. The microvascular hydrostatic pressure is the principal driving force for interstitial hypertension in tumors. Thus, fluid is forced from the microvasculature into the interstitium where it accumulates, distends the extracellular matrix, and causes an elevation of the IFP. Differences in IFP among tumors are
Translational Relevance

The outcome of cervical cancer patients treated with radiation therapy is associated with the interstitial fluid pressure (IFP) of the primary tumor. The mechanisms linking high IFP to increased probability of developing local and distant recurrences have not been revealed. In a recent preclinical study, we showed that radiation resistance may be associated with high IFP through hypoxia-dependent mechanisms. In the present communication, we report significant evidence that hypoxia-independent mechanisms also may be involved. Our study shows that poor radiocurability of tumors with highly elevated IFP may be an indirect consequence of an increased number of clonogenic tumor cells as well as increased expression of vascular endothelial growth factor-A. The preclinical data reported here provide a rationale for future clinical studies investigating the validity of IFP as a biomarker for individualizing cancer treatment.

primarily a consequence of differences in several physiologic parameters, including resistance to blood flow, microvascular hydrostatic pressure, resistance to transcapillary fluid flow, interstitial hydraulic conductivity, and interstitial matrix elasticity (10). One important consequence of interstitial hypertension in tumors is poor and heterogeneous uptake of macromolecular, nanoparticle, and cellular therapeutic agents (11).

Experimental studies attempting to relate the outcome of cancer treatment to IFP are sparse. Thus, the mechanisms linking elevated IFP to poor disease-free and overall survival rates in patients treated with radiation therapy are not understood. Preclinical studies aiming at identifying these mechanisms are currently being done in our laboratory by using human melanoma xenografts as experimental tumor models. Studies carried out thus far have suggested that hypoxia-dependent as well as hypoxia-independent mechanisms may be involved (12–14). Thus, high IFP in the primary tumor is associated with increased incidence of pulmonary and lymph node metastases in A-07 melanomas without hypoxia (i.e., tumors with volumes of 150-200 mm$^3$), probably because strong mechanical forces may facilitate tumor cell intravasation into blood vessels and tumor-surrounding lymphatics in tumors with high IFP (13). Moreover, high IFP has been found to be associated with poor radiocurability [i.e., high radiation dose resulting in 50% local tumor control (TCD$_{50}$)] in A-07 melanomas with hypoxic regions (i.e., tumors with volumes of 250-350 mm$^3$). Because IFP was positively correlated to the fraction of acutely hypoxic cells, this association was primarily an indirect consequence of an association between tumor hypoxia and poor radiocurability (14). In the present communication, evidence is presented that elevated IFP also may be linked to poor radiocurability through hypoxia-independent mechanisms, and that these mechanisms may be tumor line dependent. Any confounding effects of hypoxia were avoided by using small A-07 and R-18 human melanoma xenografts as tumor models. A-07 and R-18 tumors are well vascularized and do not develop hypoxic regions at volumes of <200 mm$^3$ as shown previously by using pimonidazole as a hypoxia marker (13) and confirmed here by using a radiobiological assay.

Materials and Methods

Mice. Adult (8-10 wk of age) female BALB/c nu/nu mice, bred and maintained as described elsewhere (15), were used as host animals for xenografted tumors. The animal experiments were approved by the institutional committee on research animal care and were done according to the USPHS Policy on Humane Care and Use of Laboratory Animals.

Tumors. The A-07 and R-18 human melanoma cell lines were established in our laboratory as described earlier (15). The cells used in the present experiments were obtained from our frozen stock and were maintained in monolayer culture in RPMI 1640 (25 mmol/L HEPES and l-glutamine) supplemented with 13% bovine calf serum, 250 mg/L penicillin, and 50 mg/L streptomycin. Xenografted tumors were initiated by inoculating aliquots of $\sim3.5 \times 10^5$ cells intradermally (i.d.) into the left mouse flank (15). The tumors were included in experiments when they had grown to a volume of $\sim100 \text{mm}^3$.

Irradiation. A Siemens Stabilipan X-ray unit, operated at 220 kV, 19 to 20 mA, and with 0.5-mm Cu filtration, was used for irradiation. Monolayer cultures in exponential growth were irradiated under aerobic conditions at a dose rate of 3.4 Gy/min. Xenografted tumors were irradiated locally by using a radiation field of $15 \times 15 \text{mm}^2$ and a dose rate of 5.1 Gy/min (16).

Cell survival assay. Cell survival in irradiated monolayer cultures was measured by using a plastic surface colony assay (17). This assay was also used for measuring the plating efficiency of untreated tumors and cell survival in irradiated tumors. Briefly, single-cell suspensions were obtained by treating resected tumors with an enzyme solution (0.2% collagenase, 0.05% Pronase, and 0.02% DNase) at 37°C for 2 h. Trypan blue-negative cells were plated in 25-cm$^2$ tissue culture flasks and incubated at 37°C for 14 d. Cells giving rise to colonies $>50$ cells were scored as clonogenic. The cell surviving fraction of an irradiated tumor was calculated from the plating efficiency of untreated tumors and cell survival in irradiated tumors. Briefly, single-cell suspensions were obtained by treating resected tumors with an enzyme solution (0.2% collagenase, 0.05% Pronase, and 0.02% DNase) at 37°C for 2 h. Trypan blue-negative cells were plated in 25-cm$^2$ tissue culture flasks and incubated at 37°C for 14 d. Cells giving rise to colonies $>50$ cells were scored as clonogenic. The cell surviving fraction of an irradiated tumor was calculated from the plating efficiency of the cells of the tumor and the mean plating efficiency of the cells of six untreated control tumors. Plots of cell surviving fraction versus radiation dose were generated, and cellular radiation sensitivities (i.e., $D_0$ values) were determined by regression analysis.

Assessment of cell tumorigenicity. Serial dilutions of cell suspensions, prepared from tumors as described above, were inoculated i.d. into the flanks of recipient mice for tumor formation (18). The mice were examined twice...
weekly for up to 90 d after the inoculations, and an inoculation was scored as positive if a tumor with a longest diameter of at least 8 mm was observed. The percentage of positive inoculations was plotted versus cell number, and the cell number resulting in 50% positive inoculations (TD25 value) was assessed by probability regression analysis (18, 19).

**TCD50 assay.** Irradiated tumors were examined twice weekly and scored as locally controlled if regrowth was not observed within 90 d after the radiation treatment. The percentage of locally controlled tumors was plotted versus radiation dose, and the TCD50 value was determined by probability regression analysis (19).

**Assessment of cell density and mitotic index.** Tumor cell density and mitotic index were assessed by quantitative analysis of histologic sections prepared from tumor tissue fixed in phosphate-buffered 4% paraformaldehyde. The sections were stained with hematoxylin to render the cell nuclei clearly visible. Cell density was determined by stereological analysis as described by Brammer and Jung (20). Briefly, grids were superimposed on histologic images shown on a monitor at a magnification of ×1,250. The fractional volume occupied by cell nuclei was determined by differential point counting using a grid with 117 test points. From the frequency of hits on a grid consisting of nine horizontal lines. From the measurements of chord lengths (i.e., lengths of each line segment running inside a nuclear profile), d was calculated as $d = \frac{3l}{2}$, where $l$ is mean chord length. The nuclear density ($N_v$), calculated as $N_v = 6V_v/\pi d^2$, was used as a measure of cell density. Mitotic index, calculated as the number of mitotic figures divided by the total number of melanoma cells, was assessed by examining histologic sections at a magnification of ×400. Three central sections, ~0.5 mm apart, were analyzed for each tumor.

**Detection of Ki67-positive cells.** Cells staining positive for the proliferation marker Ki67 were detected in immunohistochemical sections prepared from tumor tissue fixed in phosphate-buffered 4% paraformaldehyde by using a peroxidase-based indirect staining method (17). An anti-human Ki67 mouse monoclonal antibody (MIB-1; DAKO) was used as primary antibody. Diaminobenzidine was used as chromogen, and hematoxylin was used for counterstaining. Quantitative studies were based on three central sections, ~0.5 mm apart, for each tumor.

**Detection of apoptotic endothelial cells.** The apoptotic frequency of tumor-associated endothelial cells was assessed by examination of histologic sections prepared from tumor tissue snap frozen in liquid nitrogen (21). The sections were immunostained for either endothelial cells or apoptotic cells. Apoptotic cells were visualized by using an *in situ* apoptosis detection kit (Apopag; Oncor) as described by the manufacturer. Endothelial cells were visualized by using an avidin-biotin immunoperoxidase method. Anti-mouse CD31 rat monoclonal antibody (MEC 13.3; Research Diagnostics) was used as a primary antibody. Immunostained sections were counterstained with hematoxylin. Adjacent sections, one stained for apoptotic cells and the other for endothelial cells, were examined for calculation endothelial cell apoptotic frequency. Five section pairs, ~0.5 mm apart, were analyzed for each tumor.

**Measurement of IFP.** IFP was measured in the center of tumors by using the wick-in-needle method (13). Before measurements, the host mice were anesthetized with 0.63 mg/kg fentanyl citrate (Janssen Pharmaceutica), 20 mg/kg fluanisone (Janssen Pharmaceutica), and 10 mg/kg midazolam (Hoffmann-La Roche).

**Assessment of vascular endothelial growth factor-A concentration.** Tumors were frozen in liquid nitrogen immediately after resection, and the frozen tissue was pulverized with a homogenizer and was lysed in radioimmunoprecipitation assay buffer. The samples were centrifuged, and the supernatants were stored at ~80°C until analysis. Total protein concentrations were determined by using a standard method (BCA assay; Perbio). A commercial ELISA kit (Quantikine; R&D Systems) was used according to the manufacturer’s protocol to measure vascular endothelial growth factor-A (VEGF-A) concentrations. Resulting VEGF-A concentrations were normalized by the total protein concentration.

**Statistical analysis.** Experimental data are presented as arithmetic means ± SEM unless otherwise stated. Statistical comparisons of data were carried out by using the Student’s *t* test when the data complied with the conditions of normality and equal variance and otherwise by non-parametric analysis using the Mann-Whitney rank-sum test. Probability values of *P* < 0.05, determined from two-sided tests, were considered significant. The statistical analysis was carried out by using the SigmaStat statistical software (Jandel Scientific).

**Results**

The radiocurability of A-07 and R-18 tumors is associated with IFP. To investigate whether tumor radiocurability may be associated with IFP, TCD50 was determined for A-07 tumors with low IFP (IFP < 8 mmHg), A-07 tumors with high IFP (IFP > 12 mmHg), R-18 tumors with low IFP (IFP < 14 mmHg), and R-18 tumors with high IFP (IFP > 18 mmHg). The threshold values for low and high IFP were determined before the experiments were initiated, and based on previous experience, they were expected to correspond to the 40th and 60th percentiles of the IFP distributions of ~100-mm³ tumors. IFP was measured when a tumor reached a volume of ~100 mm³, and if the IFP was below the lower or above the higher threshold value, the tumor was exposed to irradiation according to a predetermined protocol. Tumors with IFP values between the lower and the higher threshold values were not irradiated. Threshold values for IFP were introduced to make it possible to compare two distinctly different tumor groups. Because both IFP and TCD50 depend on tumor volume,
confounding effects of volume variations were avoided by only including tumors with a volume of \( \sim 100 \text{ mm}^3 \). The 40th and 60th percentiles of the IFP distributions were found to be 8.2 and 12.1 mmHg for the A-07 tumors (Fig. 1A) and 14.2 and 18.3 mmHg for the R-18 tumors (Fig. 1B), close to the predetermined threshold values. TCD50 was found to be higher for the tumors with high IFP than for the tumors with low IFP by a factor of 1.13 \( \pm \) 0.03 for the A-07 line (Fig. 1C) and by a factor of 1.10 \( \pm \) 0.03 for the R-18 line (Fig. 1D). The numerical values of TCD50 [mean (95% confidence interval)] were 16.8 Gy (16.0-17.5 Gy) and 18.9 Gy (18.4-19.5 Gy) for the two groups of A-07 tumors and 20.5 Gy (19.9-21.0 Gy) and 22.6 Gy (21.9-23.3 Gy) for the two groups of R-18 tumors.

**The cellular radiation sensitivity of A-07 and R-18 tumors does not depend on IFP.** To investigate whether the differences in radiocurability between tumors with high IFP and tumors with low IFP were a consequence of differences in cellular radiation sensitivity, cell survival curves were established for A-07 tumors with low IFP, A-07 tumors with high IFP, R-18 tumors with low IFP, and R-18 tumors with high IFP. IFP was measured immediately before the tumors were exposed to single-dose irradiation in vivo. The cellular radiation sensitivity was not significantly different for A-07 tumors with high and low IFP (Fig. 2A) or for R-18 tumors with high and low IFP (Fig. 2B). The numerical values of \( D_0 \) were 0.90 \( \pm \) 0.07 Gy (A-07, low IFP), 0.87 \( \pm \) 0.08 Gy (A-07, high IFP), 1.04 \( \pm \) 0.08 Gy (R-18, low IFP), and 1.03 \( \pm \) 0.09 Gy for A-07 and R-18 tumors with low and high IFP, respectively.
(R-18, high IFP). Furthermore, for comparison, survival curves were established for A-07 and R-18 cells irradiated under aerobic conditions in vitro. The \( D_0 \) values were found to be 0.89 ± 0.07 Gy (A-07) and 1.06 ± 0.09 Gy (R-18). The cellular radiation sensitivity was not significantly different for ∼100-mm³ A-07 tumors and aerobic A-07 monolayer cultures (Fig. 2A) or for ∼100-mm³ R-18 tumors and aerobic R-18 monolayer cultures (Fig. 2B), consistent with the assumption that small A-07 and R-18 tumors do not have hypoxic cells.

**The cell density, TD50, and plating efficiency of A-07 tumors are associated with IFP.** To investigate whether the differences in radiocurability between tumors with high IFP and tumors with low IFP were associated with differences in the number of clonogenic tumor cells, cell density, TD50, and plating efficiency in vitro were measured for A-07 tumors with low IFP, A-07 tumors with high IFP, R-18 tumors with low IFP, and R-18 tumors with high IFP. A-07 tumors with high IFP showed higher cell density (\( P = 0.014; \) Fig. 3A), lower TD50 (\( P = 0.0051; \) Fig. 3B), and higher plating efficiency (\( P = 0.040; \) Fig. 3C) than A-07 tumors with low IFP, implying that the number of clonogenic cells was higher for tumors with high IFP than for tumors with low IFP. On the other hand, R-18 tumors with high and low IFP did not differ significantly in cell density (\( P > 0.05; \) Fig. 3A), TD50 (\( P > 0.05; \) Fig. 3B), or plating efficiency (\( P > 0.05; \) Fig. 3C).

**The mitotic index and fraction of Ki67-positive cells of A-07 tumors are associated with IFP.** To investigate whether the differences in radiocurability between tumors with high IFP and tumors with low IFP were associated with differences in the rate of cell proliferation, mitotic index and the fraction of Ki67-positive cells were assessed for A-07 tumors with low IFP, A-07 tumors with high IFP, R-18 tumors with low IFP, and R-18 tumors with high IFP. In the A-07 line, tumors with high IFP showed a higher mitotic index (\( P = 0.022; \) Fig. 4A) and a higher fraction of Ki67-positive cells (\( P = 0.028; \) Fig. 4B) than tumors with low IFP. In the R-18 line, on the other hand, tumors with high and low IFP did not differ significantly in mitotic index (\( P > 0.05; \) Fig. 4A) or fraction of Ki67-positive cells (\( P > 0.05; \) Fig. 4B).

**The endothelial cell apoptotic index of irradiated R-18 tumors is associated with IFP.** To investigate whether the differences in radiocurability between tumors with high IFP and tumors with low IFP were associated with differences in the radiation sensitivity of the tumor-associated endothelial cells, A-07 tumors with low IFP, A-07 tumors with high IFP, R-18 tumors with low IFP, and R-18 tumors with high IFP were irradiated with 15 Gy, resected 16 h after the irradiation, and prepared for immunohistochemical assessment of endothelial cell apoptotic index. In irradiated A-07 tumors, the endothelial cell apoptotic index was low (i.e., similar to that in unirradiated control tumors) and did not differ significantly between tumors with high and low IFP (\( P > 0.05; \) Fig. 5A). In the R-18 line, on the other hand, the endothelial cell apoptotic index was higher for irradiated than for unirradiated tumors, regardless of whether the tumors had low (\( P = 0.0082) \) or high (\( P = 0.029) \) IFP (Fig. 5B). Furthermore, in irradiated R-18 tumors, the endothelial cell apoptotic index was higher for tumors with low IFP than for tumors with high IFP (\( P = 0.033; \) Fig. 5B), implying that the radiation sensitivity of the tumor-associated endothelial cells was higher for tumors with low IFP than for tumors with high IFP.

**The VEGF-A concentration of R-18 tumors is associated with IFP.** To investigate whether the differences in radiocurability between tumors with high IFP and tumors with low IFP were associated with differences in the tissue concentration of VEGF-A, A-07 tumors with low IFP, A-07 tumors with high IFP, R-18 tumors with low IFP, and R-18 tumors with high IFP were assessed for A-07 tumors with low IFP, A-07 tumors with high IFP, R-18 tumors with low IFP, and R-18 tumors with high IFP. The VEGF-A concentration of R-18 tumors is associated with IFP. A-07 tumors with high IFP showed a higher VEGF-A concentration (\( P = 0.014; \) Fig. 4A) and a higher fraction of Ki67-positive cells (\( P = 0.028; \) Fig. 4B) than tumors with low IFP. In the R-18 line, on the other hand, tumors with high and low IFP did not differ significantly in mitotic index (\( P > 0.05; \) Fig. 4A) or fraction of Ki67-positive cells (\( P > 0.05; \) Fig. 4B).

**Fig. 2.** Cell surviving fraction versus radiation dose for A-07 monolayer cultures (○), A-07 tumors with an IFP of <8 mmHg (△), and A-07 tumors with an IFP of >12 mmHg (●); and for R-18 monolayer cultures (△), R-18 tumors with an IFP of <14 mmHg (●), and R-18 tumors with an IFP of >18 mmHg (●). Points, geometric means of six cultures or six tumors; bars, SEM. Solid curves, curves fitted to the data by regression analysis.
tumors with high IFP were irradiated with 15 Gy, resected 16 h after the irradiation, and prepared for measurement of VEGF-A concentration. Untreated control tumors of the A-07 line showed 5- to 15-fold higher VEGF-A concentrations than untreated control tumors of the R-18 line (Fig. 6). Irradiated tumors showed higher VEGF-A concentrations than unirradiated tumors, regardless of whether A-07 tumors with low IFP \((P = 0.046; \text{Fig. 6A})\), A-07 tumors with high IFP \((P = 0.017; \text{Fig. 6A})\), R-18 tumors with low IFP \((P = 0.019; \text{Fig. 6B})\), or R-18 tumors with high IFP \((P = 0.0009; \text{Fig. 6B})\) were considered. In the A-07 line, the concentration of VEGF-A did not differ between untreated tumors with low and high IFP \((P > 0.05; \text{Fig. 6A})\) or between irradiated tumors with low and high IFP \((P > 0.05; \text{Fig. 6A})\). In the R-18 line, on the other hand, tumors with high IFP showed higher VEGF-A concentrations than tumors with low IFP, regardless of whether unirradiated \((P = 0.011; \text{Fig. 6B})\) or irradiated \((P = 0.0017; \text{Fig. 6B})\) tumors were considered.

**Discussion**

In a previous study in our laboratory, the radiocurability of large A-07 melanoma xenografts with hypoxic regions was found to be associated with IFP (14). This association was primarily an indirect consequence of an association between radiocurability and tumor hypoxia because IFP showed a strong positive correlation to the fraction of acutely hypoxic cells in the tumors. The present work suggests that tumor radiocurability also may be linked to IFP through hypoxia-independent mechanisms. Thus, experiments with small A-07 and R-18 melanoma xenografts without hypoxic tissue showed that TCD_{50} was significantly higher for tumors with high IFP than for tumors with low IFP. Previous studies have shown that small A-07 and R-18 tumors do not stain positive for the hypoxia marker pimonidazole, suggesting that they do not have hypoxic cells (13). This was confirmed in the present work, which showed that the cellular dose-response curves

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**Fig. 3.** Cell density (A), TDM_{50} (B), and plating efficiency (C) for A-07 tumors with an IFP of <8 mmHg (low) and >12 mmHg (high), and for R-18 tumors with an IFP of <14 mmHg (low) and >18 mmHg (high). Columns, means of 8 (A), 6 (B), or 20 (C) tumors. Bars, SEM.

**Fig. 4.** Mitotic index (A) and the fraction of Ki67-positive cells (B) for A-07 tumors with an IFP of <8 mmHg (low) and >12 mmHg (high), and for R-18 tumors with an IFP of <14 mmHg (low) and >18 mmHg (high). Columns, means of eight tumors; bars, SEM.
for small A-07 and R-18 tumors irradiated in vivo were similar to those for the corresponding monolayer cell cultures irradiated under aerobic conditions in vitro.

The radiocurability of tumors depends primarily on four classic biological parameters: cellular radiation sensitivity, the fraction of hypoxic cells, the rate of cell proliferation, and the number of clonogenic cells (22). It is unlikely that the differences in radiocurability between tumors with high IFP and tumors with low IFP reported here were a consequence of differences in cellular radiation sensitivity or differences in fraction of hypoxic cells. This follows from the observations that the tumors did not contain radioresistant hypoxic cells and that the cellular dose-response curve did not differ between tumors with high IFP and tumors with low IFP. Moreover, because the tumors were given single-dose irradiation, the differences in radiocurability were not a direct consequence of differences in rate of cell proliferation either. On the other hand, there is significant evidence that the differences in radiocurability were related to differences in tumor cell clonogenicity and that this relation involved different mechanisms for A-07 and R-18 tumors.

In the A-07 line, tumors with high IFP showed a higher cell density, a lower TD50, and a higher plating efficiency in vitro than tumors with low IFP, suggesting that tumors with high IFP had a larger total number of clonogenic cells and, hence, showed a higher TCD50 value than tumors with low IFP. Moreover, tumors with high IFP showed a higher mitotic index and a higher fraction of Ki67-positive cells than tumors with low IFP, suggesting an increased rate of cell proliferation in tumors with high IFP. Increased cell proliferation within a confined space may cause increased cell density and, consequently, the increased proliferation rate in tumors with high IFP may have enhanced the TCD50 of these tumors indirectly by increasing the cell density. By culturing tumor cells in vitro in a hydrostatically pressurized system, Nathan et al. (23) have provided significant evidence that interstitial hypertension may promote tumor cell proliferation and survival by altering signaling pathways involved in cell cycle regulation and apoptosis. It is tempting to speculate that high IFP also may

Fig. 5. The apoptotic index of the tumor-associated endothelial cells in A-07 tumors with an IFP of <8 mmHg (low) and >12 mmHg (high; A), and in R-18 tumors with an IFP of <14 mmHg (low) and >18 mmHg (high; B). White columns, mean of eight unirradiated control tumors. Black columns, mean of eight tumors irradiated with 15.0 Gy. Bars, SEM.

Fig. 6. VEGF-A concentration for A-07 tumors with an IFP of <8 mmHg (low) and >12 mmHg (high; A), and for R-18 tumors with an IFP of <14 mmHg (low) and >18 mmHg (high; B). White columns, mean of eight unirradiated control tumors. Black columns, mean of eight tumors irradiated with 15.0 Gy. Bars, SEM.
modulate these signaling pathways in A-07 tumors, resulting in associations between IFP on the one hand and TCD$_{50}$, plating efficiency in vitro, and rate of cell proliferation in vivo on the other. If our speculation is correct, the association between radiocurability and IFP in A-07 tumors may have appeared as an indirect consequence of hypertension-induced alterations in the expression of transcription factors regulating cell proliferation and survival.

In the R-18 line, the apoptotic index of the tumor-associated endothelial cells was higher for irradiated than for unirradiated tumors, suggesting that treatment with radiation doses close to the TCD$_{50}$ value induced damage to the microvasculature. It is thus possible that a significant fraction of the clonogenic tumor cells that were not inactivated by the radiation treatment per se lost their clonogenicity because of inadequate supply of oxygen and nutrients. Because tumors with low IFP showed a higher endothelial cell radiation sensitivity than tumors with high IFP, this effect may have been largest for tumors with low IFP, resulting in a higher TCD$_{50}$ value for tumors with high IFP than for tumors with low IFP. Studies of experimental tumors have shown that VEGF-A may be upregulated by radiation treatment and that high expression of VEGF-A may protect tumor-associated endothelial cells from radiation-induced apoptosis and thus cause tumor resistance to radiation treatment (24, 25). The data reported here are consistent with these studies. Thus, the R-18 tumors showed substantially lower VEGF-A concentrations than the A-07 tumors, and tumors with high IFP showed a higher VEGF-A concentration and a lower endothelial cell radiation sensitivity than tumors with low IFP. Moreover, it is possible that the TCD$_{50}$ value was high for R-18 tumors with high IFP also because the high VEGF-A concentration promoted neovascularization and thus facilitated tumor regrowth after irradiation. The higher VEGF-A concentration in the tumors with high IFP may have been a consequence of hypertension-induced VEGF-A upregulation because the concentration of VEGF-A was found to be associated with IFP also in unirradiated tumors. This hypothesis is consistent with studies of cells in culture, which have shown that high hydrostatic pressure may cause upregulation of VEGF-A in some cell lines (26). If our hypothesis is valid, the association between radiocurability and IFP in R-18 tumors may have appeared as an indirect consequence of hypertension-induced alterations in endothelial cell radiation sensitivity and/or tumor angiogenesis mediated by VEGF-A.

The differences between the mean TCD$_{50}$ values for tumors with high and tumors with low IFP were 2.1 Gy for both lines, and because the D$_{0}$ values were ~0.90 Gy (A-07) and ~1.05 Gy (R-18), these differences suggest that the number of clonogenic cells was higher in the tumors with high IFP than in the tumors with low IFP by factors of ~10 (A-07) and ~7.5 (R-18). In the A-07 line, the differences in cell density (a factor of ~2) and cell clonogenicity (a factor of ~3.5 in vivo and a factor of ~1.5 in vitro) were probably not sufficiently large to account for the whole difference in TCD$_{50}$, suggesting that other, yet unidentified, mechanisms also were involved. In irradiated R-18 tumors, the endothelial cell apoptotic index was lower by a factor of ~2 and the VEGF-A concentration was higher by a factor of ~2.5 in the tumors with high IFP than in the tumors with low IFP. It is difficult to establish how these differences may translate into differences in tumor cell clonogenicity and, hence, whether the whole difference in TCD$_{50}$ can be accounted for by the mechanisms suggested here. Taken together, the present observations show that the relationship between tumor radiocurability and IFP is complex and suggest that preclinical studies involving a large number of tumor lines with highly different biological properties are needed to establish a universal explanation of this relationship.

Clinical investigations have provided significant evidence that the IFP of the primary tumor may be a strong prognostic parameter for patients treated with radiation therapy (3–7). The present study together with previous preclinical studies in our laboratory suggest that several mechanisms may be responsible for the poor prognosis of patients having tumors with high IFP. Thus, high IFP in A-07 tumors is associated with increased metastatic dissemination to lungs and lymph nodes through hypoxia-independent mechanisms (13) and with decreased radiocurability through hypoxia-dependent mechanisms (14). The present study suggests that high IFP also may be associated with decreased radiocurability through hypoxia-independent mechanisms. The increased metastasis may be a direct consequence of high IFP in the primary tumor, whereas the decreased radiocurability may be an indirect consequence of associations between IFP and the fraction of acutely hypoxic tumor cells, IFP and the number of clonogenic tumor cells, and/or IFP and the concentration of VEGF-A in the tumor tissue.

Patients with primary tumors showing highly elevated IFP may benefit from particularly aggressive radiation treatment owing to the poor prognosis. Because several mechanisms may link high IFP to poor treatment outcome, the design of a treatment protocol that can be expected to be beneficial for the majority of the patients that have tumors with significant interstitial hypertension may involve a great challenge. However, strategies combining radiation therapy with treatments aiming at inhibiting VEGF-A–induced signaling are interesting for several reasons. First, anti-VEGF-A treatment may normalize the microvasculature of tumors, leading to decreased IFP and increased oxygen tension in the tissue (27). Second, anti-VEGF-A treatment may reduce the transmural permeability of tumor vessels and thus inhibit tumor cell intravasation and metastatic spread (28). Third, anti-VEGF-A treatment may enhance the radiation sensitivity of microvascular endothelial cells and inhibit tumor neovascularization after irradiation and thus increase tumor radiocurability (24, 25).

However, significant progress in the treatment of patients having developed tumors with substantial interstitial hypertension cannot be expected until a noninvasive method for assessing the IFP in malignant tissues has been...
developed. Several strategies are currently under investigation, and approaches based on magnetic resonance imaging (MRI) seem to be particularly promising. Thus, by using a protocol based on the slow infusion of gadolinium-diethylene triamine pentaacetic acid, Hassid et al. (29) were able to show that parametric images of the steady-state concentration of gadolinium-diethylene triamine pentaacetic acid reflected the spatial distribution of IFP in non-small cell carcinoma and breast carcinoma xenografts. Haider et al. (30) subjected patients with cervical cancer to gadolinium-diethylene triamine pentaacetic acid–based dynamic contrast-enhanced MRI and measurement of IFP, and found weak but significant correlations between global parameters derived from the dynamic contrast-enhanced MRI series and IFP. In our laboratory, combined dynamic contrast-enhanced MRI and IFP studies of A-07 and R-18 melanoma xenografts have shown that high-resolution parametric images of tumor blood perfusion and extracellular volume fraction may provide significant information on the IFP of tumors without necrotic regions (12).

In summary, a previous preclinical study in our laboratory has suggested that poor radiocurability of tumors may be associated with high IFP through hypoxia-dependent mechanisms (14). In the present study, we present significant evidence that tumor radiocurability also may be linked to IFP through hypoxia-independent mechanisms, and that different hypoxia-independent mechanisms may be involved. Thus, associations between poor radiocurability and high IFP may be an indirect consequence of an increased number of clonogenic cells in tumors with significant interstitial hypertension, possibly caused by hypertension-induced modifications of signaling pathways regulating cell proliferation and survival, as well as an indirect consequence of hypertension-induced upregulation of VEGF-A.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Kristin Henriksen, Kristin Kindem, Liv Nordli, and Mai Nguyen for excellent technical assistance.

**Grant Support**

Norwegian Cancer Society.

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Received 10/10/09; revised 11/17/09; accepted 11/25/09; published OnlineFirst 1/26/10.

**References**


