Irradiation Reduces Interstitial Fluid Transport and Increases the Collagen Content in Tumors

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ABSTRACT

Purpose: We have shown that the interstitial diffusion of large molecules is significantly hindered in tumors with high collagen levels. Because large therapeutic agents (e.g., monoclonal antibodies and viral vectors) will be combined with radiation or chemotherapy, it is significant to determine how cytotoxic therapies modify the transport and composition of the interstitial space in tumors. To test the hypothesis that radiation alters tumor interstitial transport, we measured tumor hydraulic conductivity (K) and hyaluronan and collagen type I levels after irradiation.

Experimental Design: K and the quantification of interstitial matrix components were determined in sections of s.c. implants of the human colon adenocarcinoma LS174T. K was measured on days 1 and 5 after 10 Gy of irradiation or on day 5 after 30 Gy of irradiation.

Results: Compared with control tumors, K decreased by approximately 12-fold after 10 or 30 Gy of irradiation on day 5. At 24 h after irradiation with 10 Gy, the decrease in K was not significant. Five days after 10 and 30 Gy of irradiation, the decrease in K was associated with significantly higher levels of collagen type I. The collagen type I content was not changed 24 h after irradiation with 10 Gy.

Irradiation did not significantly increase hyaluronan levels in LS174T tumors.

Conclusions: After irradiation, the decrease in K and increase in collagen type I levels could significantly hinder the convective movement and diffusion of large therapeutic agents in tumors.

INTRODUCTION

In tumors, fluid flow and the movement of large molecules from the microcirculation to the interstitium and from one region to another in the interstitial space are significantly impeded. Because of the equilibrium in hydrostatic pressure between the microvascular and interstitial space in the tumor center and the steep drop in IFP7 in the periphery, bulk flow occurs mostly at the tumor edge and in the surrounding normal tissue (1, 2). Thus diffusion is the main mode of transport for large molecules in the center of tumors. We have recently shown that the diffusion of IgG, IgM, and liposomes is significantly hindered in tumors with high collagen levels (3, 4). Macromolecular diffusion and fluid flow can also be restricted by hyaluronan and sulfated proteoglycans (5, 6); however, in the study of Netti et al. (3), the low levels of sulfated or nonsulfated glycosaminoglycans in tumors did not correlate with the diffusion coefficients.

Tumor treatment with large agents such as viral vectors or antibodies can be combined with chemotherapeutic agents or radiation. Thus it is significant to understand how treatment with cytotoxic agents alters transport parameters and drug delivery. We have shown that Taxol increases the diameter of blood vessels and RBC velocity, suggesting that Taxol increases tumor blood flow and the vascular surface area for transvascular transport. Furthermore, Taxol can reduce IFP and increase fluid flow (K) in tumors (7). Similarly, radiation can enhance tumor blood flow and vascular surface area and reduce IFP (8–10). It could be speculated that radiation-induced apoptosis or necrosis could also lead to an increase in interstitial transport. However, radiation is also known to increase the production of collagen (11–14), which could reduce or prevent an improvement in interstitial transport. The main objective of this study was to determine the effects of radiation on tumor interstitial transport and relate the changes in fluid flow with the composition of the interstitial space. The flow chamber developed by Swabb et al. (15) was used to measure the K of tumor slices. The tumors were also analyzed by semiquantitative immunohistochemistry for changes in the content of hyaluronan and collagen type I.

7 The abbreviations used are: IFP, interstitial fluid pressure; K, hydraulic conductivity.
MATERIALS AND METHODS

Tumor Model. The human colon adenocarcinoma LS174T was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained as a monolayer in plastic flasks containing RPMI 1640 (Life Technologies, Inc., Gaithersburg MD) supplemented with 10% bovine calf serum (Life Technologies, Inc.), 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 µg/ml insulin, 100 µg/ml streptomycin, 100 units/ml penicillin, and 2.5 µg/ml gentamicin (all obtained from Sigma, St. Louis, MO). Both flanks of 6–8-week-old female athymic nude (BALB/c) mice (Harlan Sprague Dawley, Indianapolis, IN) received s.c. injection with 10^6 cells in 0.1 ml of HBSS. Tumors were 10–15 mm in diameter (approximately 10–14 days after implantation) when used.

Radiation. Animals were anesthetized with pentobarbital (50 mg/kg) and irradiated with a 6 MeV linear accelerator (Varian, Palo Alto, CA) at a dose rate of 2 Gy/min. Animals were placed on an acrylic sheet with one tumor-bearing limb taped to the acrylic sheet. A 1-cm bolus was placed on the tumor to focus the beam on the tumor, and the whole legs were exposed to irradiation. Tumors were irradiated with 30 Gy or 2 fractions of 5 Gy administered at 24-h intervals. Irradiation with 30 Gy was selected to induce a maximum effect on K and the content of interstitial matrix molecules, whereas the two exposures of 5 Gy separated by 24 h was closer to the radiation therapy of human tumors. Measurements were made on days 1 and 5 after irradiation with 10 Gy and on day 5 after irradiation with 30 Gy. The control tumors were the nonirradiated tumors from the contralateral leg of mice. The radiation source had a well-collimated beam, and there was only internally generated scatter of radiation to other sites.

Hydraulic Conductivity Measurements. K was measured with the in vitro technique developed by Swabb et al. (15). A tissue clamp was connected to a pressure head via polyethylene tubing filled with mouse serum (Taconic, Germantown, NY). The tissue clamp consisted of a clamp with a porous glass frit to support the tumor slice on the outlet side of the clamp. The glass frit and the inlet side of the clamp were of the tumor slices, calipers were not used to measure the tumor thickness. The tumor slices grossly free of necrosis and <2-mm thick were used. The selected tumor slice was measured visually for thickness with a ruler, which had a resolution of 0.25 mm. To avoid destroying the integrity of the tumor slices, calipers were not used to measure the tumor thickness. The glass frit and the inlet side of the clamp were covered with discs of nylon mesh, and the tumor slice was sandwiched between two discs of nylon mesh. The nylon mesh prevented the tumor from plugging the inlet and outlet of the clamp. To prevent leakage, an O-ring sealed the interior of the clamp. The clamp was tightened and placed in a water bath at 37°C. The ends of tubing were placed in water-filled beakers of a known pressure head measured by the difference in height of the water levels. Flow rate was measured by the velocity of a bubble’s meniscus inside a capillary tube with a precision bore inside diameter of 0.5 mm. The flow rate was measured for the incoming and outgoing streams, and inflow and outflow values were averaged. Measurements were usually completed in 1–2 h after removal of the tumor from the animal. For a few selected tumors whose measurements were completed quickly, the measurement was repeated for different pressure heads (range, 10–30 mm Hg) to ensure that K was independent of the pressure driving force.

Hydraulic conductivity was calculated from the pressure drop-bubble velocity data in a manner similar to that of Swabb et al. (15). Using Darcy’s law for one-dimensional flow through porous media, K can be calculated using the following equation:

$$K = \frac{v \cdot A_{oc} \cdot A_{cc}}{\Delta P / \Delta x}$$  \hspace{1cm} (Eq. A)

where v is the velocity of the bubble’s meniscus through the capillary tube, A_{oc} is the cross-sectional area of the capillary tube (π(inner diameter)^2)/4, A_{cc} is the cross-sectional area of the tissue in the tissue clamp, ΔP is the applied pressure drop, and Δx is the thickness of the tissue slice. All K values reported are corrected to flow of isotonic saline at 20°C.

Immunohistochemistry. Collagen type I and mouse decorin were detected with rabbit antiserums (16, 17). Both antibodies were provided by Dr. Larry Fisher (National Institute of Dental Research, Bethesda, MD). Hyaluronan was localized with a hyaluronan biotinylated proteoglycan fragment, a generous gift of DrVinata Lokeshwar (University of Miami, Miami, FL). The tumors were placed in a solution of 2% paraformaldehyde for 1 h, followed by immersion in a 30% sucrose solution overnight. The tumors were then frozen using Histofreeze (Fisher, Pittsburgh, PA) and stored in a −80°C freezer. Tissue sections were cut using a Microm Cryostat (Micromia). Sections were cut 10-μm thick and placed on Superfrost slides (Fisher). Sections were kept frozen until use. For immunostaining, sections were blocked with rabbit serum, incubated with the primary antibody overnight at 4°C, and then incubated with the secondary antibody conjugated to FITC. For hyaluronan labeling, the sections were incubated for 1 h with the biotinylated proteoglycan fragment diluted in 10% calf serum, and, after washing, the sections were incubated with streptavidin-FITC. The cell nuclei were stained with 4',6-diamidino-2-phenylindole.

For quantification of the staining intensity and the fraction of tissue occupied by collagen type I and hyaluronan, images were taken with a custom-made two-photon microscope based on a MRC 600 platform (Bio-Rad; Ref.4). One advantage of two-photon microscopy is that the excitation wavelength can be fixed precisely. Sixteen fields per section were randomly selected. Using a constant 10 mW of 720-nm light through a 0.4 numerical aperture 20× objective lens image, image stacks of each field were generated, with 5–6 images (separated by 5 μm) per stack. To include the full thickness (~10 μm) of the section in the stacks, several images were obtained. A maximum intensity projection was performed on the image stacks to form a single image of the section, which selected the pixel of highest intensity for each x, y location. Using a macro constructed in Scion Image software (Scion Corp.), nuclei and holes (glandular structures) were removed from the image area via image subtraction and thresholding. The resulting image was divided into areas of background (nonspecific) staining, using information

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from negative control sections, and specific staining. The area of the resulting specific stain was measured, and the average intensity within that area was calculated. The fractional area of the collagen type I and hyaluronan pixels were determined in images of 100,925 μm². Collagen type I staining was either amorphous or appeared as dense bands. To determine the intensity of amorphous and bands of collagen type I, the appropriate areas were selected.

**Statistical Analysis.** All values are shown as mean ± SD. Normally distributed data were evaluated with Student’s t test or by ANOVA. The Mann-Whitney nonparametric U test was used for data that were not normally distributed.

**RESULTS**

**Hydraulic Conductivity.** For the tissue chamber used in the study, the mean flow rate of an empty (no tissue) clamp was $2.8 \pm 1.6 \times 10^{-3}$ cm³/min, and the mean pressure drop was $12.5 \pm 0.1$ mm Hg. The resistance due to the flow chamber was 2 orders of magnitude less than the resistance due to the tissue, so the resistance of the apparatus was considered negligible for this system. Typical flow rates (bubble position versus time) for slices of control and radiation-treated tumors are shown in Fig. 1. Flow rate values for estimating $K$ were established from the best-fit of the steady-state data.

$K$ was measured for different pressure drops in a few selected tumors to test for independence of $K$ from pressure drop. $K$ varied by <30% when the pressure drop was changed from 10 to 30 mm Hg. $K$ could not be measured for different pressure drops for all tumor slices. Because the flow rate was very low, a single measurement could take up to 1 h. After that time, we could not ensure that the tissue integrity would remain unchanged (15). Because $K$ was relatively independent of the pressure drop in the tumors tested, one pressure drop per tumor was deemed sufficient to determine $K$.

For nonirradiated tumors, the mean value of $K$ was $23.8 \pm 10.7$ cm²/mm Hg s⁻¹. On day 5, $K$ had decreased by approximately 12-fold in the tumor groups treated with 10 or 30 Gy of irradiation (Table 1). Twenty-four h after irradiation with 10 Gy, $K$ decreased by 2-fold; however, the difference was not significant (Table 1).

**Interstitial Matrix.** The interstitial space of LS174T tumors is formed of narrow and larger connective tissue septa, which are in continuity with the capsule surrounding the tumor. The narrow septa surround the glandular structures that form the parenchyma (Fig. 2A). Staining of LS174T sections with Masson trichrome showed that collagen was present at the tumor edge and in the large connective tissue septa. Five days after irradiation with 10 Gy (B), collagen occupies the large (+) and narrow connective tissue septa (arrows). Bar = 80 μm.

### Table 1 Radiation decreases hydraulic conductivity in LS174T

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>$K \times 10^8$ (cm²/mm Hg s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>$23.8 \pm 10.7$</td>
</tr>
<tr>
<td>10 Gy, 1 day</td>
<td>8</td>
<td>$12.5 \pm 8.6$</td>
</tr>
<tr>
<td>10 Gy, 5 days</td>
<td>9</td>
<td>$2.03 \pm 0.83^b$</td>
</tr>
<tr>
<td>30 Gy, 5 days</td>
<td>9</td>
<td>$1.92 \pm 0.45^b$</td>
</tr>
</tbody>
</table>

*Values are adjusted to saline at 20°C and are given as mean ± SE.

*Statistically significant change compared with control.
collagen was rarely detected in the narrow septa (Fig. 2A). Five days after 10 and 30 Gy of irradiation, there was a greater accumulation of collagen in both the narrow and larger connective tissue septa (Fig. 2B). Necrosis was evident in the center of untreated tumors. Five days after radiation, the proportion of necrotic areas increased, and necrosis was also present in the peripheral regions of the tumors.

In control tumors, the collagen type I staining was mainly localized at the tumor edge. In the tumor center, short bands of collagen type I were occasionally observed (Fig. 3A). In contrast, 5 days after irradiation with 10 or 30 Gy, collagen type I staining was frequently observed as long connective tissue bands of high intensity that separated or lined glandular structures (Fig. 3B). Amorphous collagen type I staining was seen lining glandular structures and separating small clusters of tumor cells. Quantitative measurements demonstrated that 5 days after 10 or 30 Gy of irradiation, there was a significantly greater fraction of the tumor that was occupied by collagen type I (Table 2). The staining intensity of the collagen bands was also significantly enhanced (Table 2). There was a small, insignificant increase in the intensity of amorphous collagen. On day 1 after 10 Gy of irradiation, the collagen type I immunostaining was variable, with intense staining in some tumors; in other tumors, there was no quantitative difference with most of the tumors in the control group. Immunostaining for the small proteoglycan decorin was found in the connective tissue of the capsule (data not shown). Decorin staining was not detected in the narrow and large connective tissue septa in the center of LS174T tumors.

Hyaluronan staining was intense in the connective tissue at the tumor edge and decreased significantly in the center (Fig. 4A). Glandular structures were sporadically lined by the hyaluronan staining. One day after 10 Gy of irradiation, the fraction of the tumor tissue occupied by hyaluronan was similar to that of the control group (Table 3). Five days after 10 and 30 Gy of irradiation, the hyaluronan staining varied from one tumor to another. In some tumors, similar to the control group, hyaluronan was found mostly at the edge, whereas in other tumors, hyaluronan staining separated or lined glandular structures. Hyaluronan staining also surrounded tumor cell clusters and was occasionally observed between neoplastic cells (Fig. 4B). Five days after 30 Gy of irradiation, there was a 2-fold increase in the fraction of tumor tissue occupied by hyaluronan; however, because of the large variability in hyaluronan staining between tumors, the difference was not significant (Table 3).

DISCUSSION

The major goal of this study was to determine the effects of irradiation on tumor interstitial transport and K in tumors. The in vitro values of K for slices of LS174T were similar to our previous in vivo values obtained with a micropipette technique (18). For our nonirradiated samples, K was only 30% different from the in vivo values of LS174T, which were known to be at steady state. K decreased to 9% of its original level for the two groups that were measured on day 5 after irradiation. Thus the in vitro clamp method provides values of K similar to those provided by in vivo measurements with micropipettes and can detect changes in K induced by radiation.

Cellular membranes have a low hydraulic permeability to water (5), which is 6–7 orders of magnitude lower than the K values that have been measured in tumor tissue (3, 7, 18). Levick (5) has shown that K is inversely correlated with the levels of collagen or glycosaminoglycans in tissues. The higher intensity of the collagen type I bands and the larger fraction of the tumor volume occupied by collagen could explain in part the reduction in K after radiation (Table 1). Based on measurements

### Table 2 Fraction and intensity of collagen type I staining

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor fraction (%)</th>
<th>Intensity (pixel intensity/μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dense bands</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 2.5</td>
<td>55.5 ± 25.0</td>
</tr>
<tr>
<td>10 Gy 1 day</td>
<td>3.5 ± 3.0</td>
<td>72.0 ± 33.0</td>
</tr>
<tr>
<td>10 Gy 5 days</td>
<td>6.5 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.5 ± 21.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 Gy 5 days</td>
<td>7.2 ± 5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>132.0 ± 46.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Statistically significant compared with control.
Irradiation Reduces Interstitial Fluid Transport in Tumors

Changes in the levels of other interstitial matrix molecules could also play a partial role in reducing K. For example, radiation enhances the production of collagen type III in several normal tissues (20–22) and the levels of dermatan and heparan sulfate in skeletal muscle (23). In LS174T, the enhanced accumulation of collagen type I in the interstitial space may be responsible, in part, for the significant reduction in K 5 days after radiation.

Because of radiation-induced necrosis (rupture of cellular membranes and the vasculature), an increase in surface area for fluid transport could increase K. Five days after radiation doses of 10 and 30 Gy, the fraction of tissue occupied by necrosis was severalfold greater than the volume occupied by collagen type I or hyaluronan. However, K did not increase. It is possible that cellular debris, the liberation of cellular proteins and mucus, prevented the increase in K. Additional studies are needed to elucidate the effect of necrosis on K.

Several reports have described the effects of irradiation on the composition of interstitial matrix molecules in normal tissues. To our knowledge, the present study is the first to describe changes in the interstitial matrix of tumors after radiation. In LS174T tumors, the levels of collagen type I increased significantly 5 days after radiation; there was no change 1 day after irradiation (Table 1). The collagen type III protein and its mRNA have been shown to increase within 24 h after radiation of the mammary fat pad and then to decrease (11). The mRNA levels of collagen type I, III, and IV were enhanced 24 h after lung radiation and continued to increase for up to 14 days (13).

In a recent study, Giannopoulou et al. (14) demonstrated that the levels of the collagen type I protein did not always match the level of mRNA expression in the chick chorioallantoic membrane. Levels of the collagen type I protein decreased or did not change at 24 and 48 h after irradiation, whereas the mRNA expression did not change at 24 h and increased by 3-fold at 48 h.

Radiation did not significantly affect the hyaluronan levels in LS174T tumors. It is possible that in some limited parts of the tumor, hyaluronan levels increased after radiation. For example, in the capsule of both control and radiation-treated tumors, hyaluronan staining was significant. Because of the random sampling approach used to take the images throughout the sections, it is highly unlikely that changes in hyaluronan levels that were not uniformly distributed and confined to a small fraction of the tumor could have been detected.

**Clinical Implications.** To improve the efficacy of anticancer therapies, the combination of external beam irradiation with radiolabeled antibodies is being tested in preclinical models. The combined treatment can improve antibody uptake and the antitumor effects (24, 25). It is not clear whether the increased antibody uptake is responsible for the antitumor effects, especially if there is no improvement in antibody distribution throughout the tumor (26). The reduction in K and the increase in collagen levels after radiation could significantly restrict the convective and diffusive movement, as well as the distribution of antibodies and large molecules in tumors. In tumors or in collagen gels in vitro, the diffusion of IgG or IgM is inversely proportional to the collagen content (3, 4, 19). In vitro, K is also inversely related to the levels of collagen type I gels (19).

### Table 3 Fraction of hyaluronan staining

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean tumor fraction (%)</th>
<th>Median and range Tumor fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 ± 0.9</td>
<td>1.2 (0.3–2.3)</td>
</tr>
<tr>
<td>10 Gy, 1 day</td>
<td>1.7 ± 1.5</td>
<td>0.8 (0.6–1.0)</td>
</tr>
<tr>
<td>10 Gy, 5 days</td>
<td>2.9 ± 1.8</td>
<td>1.8 (0.4–4.7)</td>
</tr>
<tr>
<td>30 Gy, 5 days</td>
<td>4.1 ± 2.8</td>
<td>2.5 (0.4–5.6)</td>
</tr>
</tbody>
</table>
Disrupting the collagen network or inhibiting collagen synthesis could improve the diffusion of large therapeutic agents in tumors. Collagenase can increase the diffusion of large molecules in tumors (3). The small hormone relaxin, which inhibits the synthesis of collagen and increases the expression of matrix-degrading enzymes, increased the transport of macromolecules by 2–3-fold in tumors with a high collagen content (27).

The scheduling of external beam irradiation could significantly influence interstitial transport of large molecules. Five days after irradiation, K decreased by >10-fold, which could restrict the convective flow and diffusion of large molecules in tumors. Radiation can also influence the extravasation and uptake of antibodies in a time-dependent manner. Antibody accumulation is enhanced when administered at the same time or within 24 h of external beam irradiation. Longer delays (3–10 days) between irradiation and antibody injection reduced or did not change the uptake of antibodies (25, 28). Thus, to increase the concentration and favor the movement and distribution of large therapeutic agents in the interstitial space of tumors, it would be preferable to administer large therapeutic agents 1 day before or at the same time as external beam irradiation.

In conclusion, the present results demonstrate that radiation enhances the production of collagen type I and decreases K in tumors. The enhanced accumulation of collagen type I could be responsible, in part, for the decrease in K after irradiation. Our results suggest that the modifications in the permeability and composition of the interstitial matrix induced by radiation will reduce the convective and diffusive transport of large therapeutic agents in tumors.

REFERENCES

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