Abstract

Purpose: Convective transport of macromolecules from the peritoneal cavity into tumor is determined by its hydraulic permeability and the pressure gradient. Previous studies showed that establishing a pressure gradient into the tumor failed to result in significant penetration. This study addresses the hypothesis that the extracellular matrix is the major resistance to the penetration of an i.p. injected antibody.

Experimental Design: Human ovarian tumors (SKOV-3 and OVCAR-3) were established in the abdominal wall of athymic rats. After anesthesia, the tumor serosal surface was treated for 2 hours with Krebs solution (control), collagenase (37.5 unit/mL), or hyaluronidase (10 unit/mL) followed by 3 hours of convective delivery of radiolabeled IgG. Transport of antibody into the tumor was measured with quantitative autoradiography along with the tumor interstitial pressure, concentration of collagen and hyaluronic acid, and IgG volume of distribution.

Results: Antibody was excluded from 42% to 53% of tumor extracellular volume. Exposure of tumors to hyaluronidase did not enhance IgG transport despite removal of 90% of the hyaluronan from the exposed tumor. In contrast, collagenase reduced collagen content, lowered tumor interstitial pressure, and markedly enhanced antibody penetration.

Conclusions: Reduction of collagen, but not hyaluronan, in the matrix of ovarian xenografts enhanced the transport of i.p. injected antibody. Although high interstitial pressure is a deterrent to convective transport of macromolecules into the tumor parenchyma, the structure of the interstitial matrix provides an inherent resistance, which must be overcome before effective delivery of an antibody.

Materials and Methods

Tumor-bearing animal model. The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. The study...
isolated by centrifugation of the tumor sections over nylon membrane (N20R CellMicroSieves, BioDesign, Inc., of New York, Carmel, NY) as described by Wiig et al. (18). The concentration ratio of the interstitial fluid to plasma was used to judge whether the interstitial water was diluted with intracellular water (18). The concentration ratio of the IgG interstitial fluid to plasma concentration was divided by that of the mannitol to provide an estimate of the proportion of interstitium available to the IgG (19):

$$\text{Available fraction} = \frac{C_{IgG}}{C_{IgG}} \cdot \frac{C_{\text{mannitol}}}{C_{\text{mannitol}}}$$

where $C_{IgG}$ is the IgG concentration in the interstitial fluid, $C_{\text{mannitol}}$ is the IgG concentration in plasma fluid, $C_{\text{mannitol}}$ is the mannitol concentration in the interstitial fluid, and $C_{\text{mannitol}}$ is the mannitol concentration in plasma.

**Modification of extracellular matrix components and transport studies.** The tissue content of collagen or hyaluronan was modified by local treatment of the tumor with collagenase (Sigma, St. Louis, MO) or hyaluronidase (Calbiochem, La Jolla, CA) for 2 hours. The tumor-bearing animal was anesthetized by i.m. injection of sodium pentobarbital (60 mg/kg). Catheters were placed in the femoral vein to maintain anesthesia with additional pentobarbital and in the femoral artery to monitor blood pressure. Temperature was maintained at 37°C and mean arterial blood pressure remained >80 mm Hg. The surface layer of the tumor was carefully exposed to ensure the effectiveness of the enzyme treatment. The interstitial pressure of the tumor was measured before the enzyme treatment and after the transport study using the wick-in-needle technique as described earlier (14, 20). As shown in Fig. 1, a plastic chamber was affixed to the exposed tumor (21) and one of the following solutions was placed in the chamber: Krebs-Ringer solution (10 mL) alone (control), 10 unit/mL hyaluronidase in Krebs-Ringer, or 37.5 units/mL collagenase in Krebs-Ringer. The composition of the Krebs-Ringer solution was as follows (mmol/L): 120 NaCl, 10 KCl, 2 CaCl2, 25 NaHCO3, 0.28 KH2PO4, and 1.2 MgSO4. The concentration used for each enzyme was based on previous studies (22, 23). The depth of solution in the chamber was 6 cm to facilitate hydrostatic pressure-driven transport of the enzyme into the tumor tissue. After 2-hour treatment, solution was carefully removed and the tumor surface was rinsed thrice with Krebs-Ringer solution. Approximately 10 mL Krebs-Ringer solution plus [125I]trastuzumab (purchased from University of Mississippi Medical

---

**Fig. 1.** Diagram of chamber apparatus used to treat tumors in vivo. After laparotomy, a 7-cm-high chamber was affixed to the peritoneal surface surrounding the tumor that was located in the abdominal wall. During the first 2 hours, the chamber was filled to a level of 6 cm with one of three solutions (control, collagenase, or hyaluronidase) to treat one element of the tumor interstitial matrix. Subsequently, a solution with labeled antibody was placed in the chamber to study the hydrostatic pressure-driven convection of the antibody into the tumor.
Center Pharmacy, labeled weekly and purified daily in accordance with our previously published procedure (14), and used in the OVCAR-3 control and collagenase-treated tumors or $[^{25}S]$IgG (a nonspecific antibody used in the interstitial space experiments above and in all transport experiments except for the OVCAR-3 tumors treated with collagenase; this antibody was previously shown to have identical transport characteristics of labeled trastuzumab; ref. 15) were placed in the chamber from a preweighed syringe-needle-catheter to exert a hydrostatic pressure of 6 cm H$_2$O and to determine the hydrostatic pressure–driven transport characteristics of the tumor. The chamber solution volume and labeled antibody concentration were determined hourly for 3 hours. At the end of the experiment, the animal was sacrificed, the chamber was removed, and the tumor tissue rapidly frozen with isopentane, cooled to −70°C, for subsequent slicing and quantitative autoradiography.

Quantitative autoradiography. The tissue distribution of $[^{25}S]$trastuzumab was determined as described previously (13, 14, 24). Briefly, whole-tumor sections of 20 µm were excised from the frozen specimen in a cryotome and heat-dried to prevent further transport of the labeled antibody. Sections were placed together with isotope standards against X-ray film to develop autoradiograms. These were later analyzed with a computerized densitometer (MCID Imaging Research, St. Catherine’s, Ontario, Canada). In the data analysis, antibody concentration profiles beyond 2 mm from the surface were <1% of the surface concentration and, therefore, were truncated in data plots.

Histology. Tumor tissues were collected and immediately frozen and were cut to 4 µm with a cryomicrotome (Leica CM3050S, Leica, Nussloch, Germany). Frozen tissue sections were stained for collagen using Gomori’s one-step trichrome staining method. The sections were incubated in Bouin’s solution (Sigma) for 1 hour in a 60°C oven. After staining, sections were stained with Weigert’s hematoxylin (Sigma), rinsed, and stained with trichrome (American Master*Tech Scientific, Inc., Lodi, CA).

Formalin-fixed tissue sections were also stained for hyaluronic acid using a biotinylated hyaluronic acid–binding protein (22, 25, 26). Deparaffinized and hydrated tissue sections were treated with 3% hydrogen peroxide in PBS for 20 minutes at room temperature. Antigen retrieval was done in citrate buffer (BioGenex, San Ramon, CA) for 30 minutes in a steamer. After blocking overnight with 3% bovine serum albumin in PBS at 37°C, the slides were rinsed with PBS. The sections were then incubated overnight at 4°C with biotinylated hyaluronic acid–binding protein (1 µg/mL in blocking solution). Color was developed with ABC Standard kit (Santa Cruz Biotechnology, Santa Cruz, CA) and 3,3′-diaminobenzidine kit (Vector Laboratories, Inc., Burlingame, CA). Controls for background and nonspecific binding were run on replicate sections without the hyaluronic acid–binding protein and after treatment of the section with hyaluronidase (50 unit/mL in PBS), respectively. Such controls were always negative, providing assurance that the staining was specific.

Assessment of tissue content of collagen and hyaluronic acid. The collagen content of the tumor tissue was assessed by an assay of hydroxyproline content (27). The tumor was carefully excised after the end of a transport study. Three half-thickness (~5 mm) samples of tissue (each ~100 mg) were collected from the portion of the tumor directly exposed to the enzyme-containing chamber solution. To counter bias in tissue sampling, three samples were randomly selected to represent ~30% to 50% of the exposed portion of the tumor. Each tissue was freeze-dried overnight and its dry weight was determined to normalize measurement of the matrix components to the dry weight of the total tissue sample. The tissue was then digested overnight at 60°C with 125 µg/mL papain in a buffer of 0.2 mol/L sodium acetate, 10 mmol/L EDTA (pH 5.8), and 10 mmol/L cysteine. The digestion was stopped by adding 0.5 mol/L iodoacetic acid and particulate matter was removed by centrifugation. Samples were then hydrolyzed with sodium hydroxide (2 mol/L final concentration) for 20 minutes at 120°C. Chloramine-T was added to oxidize the samples for 25 minutes at room temperature. After neutralization with 0.17 mol/L citric acid, color was developed by adding Ehrlich’s aldehyde reagent. The absorbance was measured at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Hyaluronic acid content was determined using an enzyme-linked binding protein assay (Corgenix, Inc., Westminster, CO) in accordance with the instructions of the manufacturer. Tissue digests (set of three from each tumor) and standards were added to microplates coated with hyaluronic acid–binding protein and incubated at room temperature for 1 hour. After washing four times, horseradish peroxidase–conjugated hyaluronic acid–binding protein solution was added and incubated for 30 minutes at room temperature. The microplate wells were washed and substrate solution was added. Following incubation at room temperature for 30 minutes, stop solution was added. Absorbance was determined at 450 nm, with additional measurements at 650 nm serving as a reference.

Data analysis. Data are presented as the mean ± SE. Statistical analysis was done with Student’s t test. Significance was judged to be attained if the chance of a type 1 error was <5% ($P < 0.05$). NCSS 97 (Kaysville, UT) was used for statistical calculations.

### Results

**Tumor interstitial exclusion volume.** Table 1 displays the measurements of the relative interstitial space. The mannitol fractions for each tumor are very close to 1; a fraction of 1 means that the plasma-interstitial equilibrium for mannitol had been reached and that there was no dilution by intracellular water during the centrifugation procedure. According to the IgG fraction data, approximately half of the extracellular space of each tumor type is available to the antibody. When these fractions were multiplied by the previously measured values for the extracellular space for each tumor (14), the estimated space within each tumor is ~0.28 mL/g, which translates to an excluded volume of 0.72 mL/g for these two ovarian xenografts.

**Alteration of tumor interstitial matrix.** To identify the structural components of tumor tissue that might act as a barrier to the transport of antibody, the tumor tissue was examined after histologic staining. The primary focus was on the distribution of collagen and glycosaminoglycan, specifically.

<table>
<thead>
<tr>
<th>Tumors</th>
<th>$n$</th>
<th>Mannitol fraction</th>
<th>IgG fraction</th>
<th>Extracellular space (mL/g)*</th>
<th>IgG space (mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR-3</td>
<td>4</td>
<td>1.073 ± 0.087</td>
<td>0.467 ± 0.053</td>
<td>0.61 ± 0.03</td>
<td>0.28</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>6</td>
<td>0.973 ± 0.082</td>
<td>0.519 ± 0.010</td>
<td>0.54 ± 0.11</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Data from ref. 14.
hyaluronic acid, as these are the major components of the interstitium in tumor tissue (23). Both OVCAR-3 and SKOV-3 tumors in this rat model for peritoneal metastases typically have a lobular structure with the lobules ranging in diameters from 0.5 to 5 mm (see examples in reference ref. 14). Staining with hyaluronic acid–binding protein showed that hyaluronic acid was distributed predominantly on the outer layer of each lobule enveloping the tumor parenchyma in both tumors. After treatment with hyaluronidase, the staining seemed to be decreased ~1 mm into the tumor. The distribution of collagen in these tumors was similar based on the pattern of trichrome staining. After treatment with collagenase, tissue staining showed a slight decrease in the Trichrome staining at the edge, but the quality of the frozen sections did not permit conclusive assessment.

Exposure of the tumor surface for 2 hours to a solution containing hyaluronidase caused a substantial reduction in the hyaluronic acid content of the tumor. Tissues within 5 mm of the surface in both OVCAR-3 and SKOV-3 tumors showed a decrease of >90% in hyaluronic acid content (Fig. 2A). There seemed to be a much smaller decline in collagen content when the tumor was exposed to a solution containing collagenase (Fig. 2B). The difference in effect of the different enzymes may be due to their relative size and the difficulty of penetrating the tumor interstitial matrix. The hyaluronidase has a molecular weight of 55,000 Da, whereas the collagenase ranges from 68,000 to 125,000. In previous work in normal abdominal wall (24, 28), we observed marked differences in the penetration of labeled albumin (~55-60,000 Da) and IgG (150,000 Da). Because we did not have labels on the enzymes, we do not know how deep they actually penetrated. We suspect that a longer treatment time would result in alterations to deeper portions of the tumor.

Penetration of trastuzumab: effect of enzyme treatments. Following the 2-hour treatment to selectively decrease one portion of the interstitial matrix, transport of I25I-IgG was examined over 3 hours with quantitative autoradiography. In untreated tumors of both types, penetration of the IgG is limited to a few hundred micrometers of tumor tissue from the peritoneum (Fig. 3). In contrast, the untreated adjacent muscle tissue shows far greater antibody penetration throughout its thickness (Fig. 3).

Treatment of both tumors with hyaluronidase had minimal effect on the transport of IgG into the tumor (Fig. 3). The concentration of IgG at the tumor surface seemed to be slightly greater but the mean penetration distance was no greater than in the untreated tumors. In contrast, penetration of antibody was markedly enhanced after treatment of the tumor tissue with collagenase (Fig. 4). The concentration of IgG at the tumor surface was ~2-fold greater after collagenase treatment than in the untreated tumors. More notably, the mean penetration distance increased ~4-fold when the tumor surface was treated with collagenase. The concentration profiles for antibody were similar in the OVCAR-3 and SKOV-3 tumors although the transport studies in the OVCAR-3 tumors were done with labeled trastuzumab, whereas those in the SKOV-3 tumors were conducted with an IgG with no receptors on the tumor cells. Previously, we showed that these antibodies have similar transport characteristics in these tumors (15).
Effect of enzyme treatment on tumor interstitial pressure.

Tumor interstitial pressure was assessed before and after treatment with each enzyme to determine whether the treatment altered the interstitial pressure. A limited number of control measurements were also made with the treatment solution consisting of Krebs-Ringer-bicarbonate. Tumors treated with hyaluronidase did not show any significant changes in interstitial pressure after the enzyme treatment (mm Hg, mean ± SE, before to after: OVCAR3, 8.0 ± 1.3 to 7.9 ± 0.7, n = 7, P = 0.4; SKOV3, 10.7 ± 2.9 to 10.0 ± 4.1, n = 6, P = 0.4). About half of the tumors treated with collagenase, however, did show a substantive reduction in tumor pressure compared with the pressure before enzyme treatment but the differences in the means were not significant (mm Hg, mean ± SE, before to after: OVCAR3, 12.9 ± 2.4 to 9.1 ± 1.8, n = 9, P = 0.08; SKOV3, 12.9 ± 1.7 to 9.4 ± 2.8, n = 7, P = 0.06). In a limited number of animals (n = 3 each tumor type), a second tumor was treated with Krebs alone and these also showed a reduction in pressure: (mm Hg, mean ± SE, before to after: OVCAR3, 18.5 ± 0.8 to 11.5 ± 1.0, n = 3, P = 0.03; SKOV3, 11.3 ± 0.9 to 9.0 ± 0.6, n = 3, P = 0.01). There was no indication of a greater penetration of IgG into the tumors that showed such a reduction in interstitial pressure than into the other collagenase-treated tumors. It should be noted, however, that the wick-in-needle technique, as used in these experiments, cannot determine the pressure within 2 mm of the tumor surface, where the effect of the enzyme treatment is likely to be most significant.

Discussion

Regional pharmacotherapy depends on adequate delivery of the agent to the target. The theoretical rationale for i.p. delivery of antineoplastic agents for the treatment of malignancies located in the peritoneal cavity is well established (29). I.p. administration of low molecular weight drugs (MW <1,000 Da) permits the degree of transport observed in the untreated muscle. Points, mean; bars, SE.

**Fig. 4.** Effect of collagenase on the transport of IgG into tumor tissue. Antibody concentration profiles (ratio of antibody concentration in tumor to the mean concentration in the chamber) for SKOV-3 tumors (●) and OVCAR-3 tumors (□) exposed to collagenase and untreated adjacent muscle (●). Open symbols, antibody concentration profiles for the control tumors (○ and △). Treatment with collagenase over 2 hours markedly enhanced the transport of an antibody but did not permit the degree of transport observed in the untreated muscle. Points, mean; bars, SE.

Our histologic observations indicate similar patterns of distribution of collagen and hyaluronic acid in the experimental tumors in this rat model. Hyaluronic acid is a nonsulfated glycosaminoglycan composed of repeating disaccharide units of N-acetyl-d-glucosamine and d-glucuronic acid with a high molecular mass (600-1,000 kDa). Hyaluronic acid is a polyanionic molecule (thus called hyaluronan) that has a flexible chain structure and forms a hydrated network with collagen fibers (35). The glycoprotein collagen comprises long peptide chains (>1,000 amino acids), which assemble into the linear triple helix structure of a collagen fiber and constitute the primary network of the interstitial matrix. Both hyaluronan and collagen were located mainly at the periphery in the layer of proliferating cells surrounding the tumor cell mass. An analogous configuration for the interstitial structure has been reported in human ovarian tumors that have metastasized to the abdominal wall (36). The finding from the present study that collagen but not hyaluronic acid contributes resistance to the transport of antibody suggests that the nature of a potential transport barrier is as important as its structural location.

Previous studies have shown that the interstitial exclusion volume (the space that is not accessible) for a protein depends on the net charge of that protein, suggesting that this volume is governed by electrostatic repulsion between negative charges on the glycosaminoglycans and the protein in skin and muscle (16, 37) as well as in tumors (38).
However, our recent data imply that this charge effect makes little, if any, contribution to barrier function of the interstitial matrix as similar transport profiles were found for two different antibodies with different net charge (a nonspecific IgG isoelectric point of 7.6; ref. 38) and the monoclonal antibody trastuzumab (isoelectric point, 9.2; ref. 15). Table 1 shows that there is a large portion of the tumor extracellular space that is available to the antibody, in agreement with recent data for trastuzumab and IgG in 7,12-dimethylbenz(a)anthracene–induced rat mammary tumors (38).

Although the hyaluronic acid chain itself is flexible, it can be stiffened by hydrogen bonds and expand through internal electrostatic repulsion creating a very large and roughly spherical solvation domain of radius 100 to 200 nm (39). At high molecular weights (~10^6 Da), adjacent molecular domains overlap, resulting in chain-to-chain entanglement and a large network of loosely linked polymer chains (40). Hyaluronidase treatment shortens the glycosaminoglycan chains so that each domain occupies a smaller volume; hyaluronidase treatment has been shown to significantly enhance self-diffusion of hyaluronan in vitro (41). Therefore, treatment with hyaluronidase in the present study presumably reduced the negative charges and the excluded volume in the interstitium through cleavage of hyaluronic acid and further increased the space available to macromolecules. Yet, it resulted in only a small enhancement of antibody penetration in SKOV3 tumors and no apparent increase in antibody transport for OVCAR3 as illustrated in Fig. 3.

Our observation that such treatment did not improve the transport of antibody correlates with the finding that the space available to IgG in these tumors is quite large compared with normal muscle (11). The interstitial space available to IgG is thus unlikely to be the limiting factor for antibody transport so a further increase in this space after hyaluronidase treatment does not result in an enhancement of antibody transport. Hyaluronidase treatment of human osteosarcoma xenograft, however, has been reported to increase the transport of a specific antibody into the tumor (42). In that study, hyaluronidase was injected directly into the tumor at 3-day intervals over a period of 9 days and the treatment resulted in a reduction of tumor interstitial fluid pressure. Another study by the same group showed that transport of IgG in orthotopic osteosarcoma was correlated with the tumor interstitial pressure and the tumor volume but not with the content of any of the extracellular matrix constituents (43).

Our results may differ from these findings because of the experimental design. In contrast to intratumoral injections, each enzyme in our experimental protocols was administered from one side of the tumor via pressure-driven convection. This likely limited the penetration of hyaluronidase (~55 kDa) or collagenase (68-125 kDa) to 1 to 2 mm from the surface of the tumor. Coupled to this were the interstitial pressure measurements that reflected pressures deeper within the tumor. The needles, which were used to perform the wick-in-needle measurements, required insertion to 3 mm, and therefore could not measure interstitial pressure close to the surface. Our result with hyaluronidase is consistent with the previous observation that the diffusion of IgG in tumors does not correlate with their total content of glycosaminoglycans or hyaluronic acid (23). We therefore conclude that the effects on hyaluronan within the interstitium of ovarian xenografts are not equivalent to those studied after direct injection of hyaluronidase into osteosarcomas and that the interaction of other structures in the interstitium limits the penetration of IgG into the tumor.

In contrast to the results with hyaluronidase, direct application of collagenase to the peritoneal side of either OVCAR3 or SKOV3 xenografts significantly enhanced the tissue penetration of the antibody in the first 1 to 2 mm of tumor over a period of 3 hours. Our results concur with those of Netti et al. (23), who found that collagen was the controlling interstitial element in several types of xenograft. In osteosarcoma xenografts, systemic treatment with collagenase resulted in reduction of tumor interstitial pressure and enhanced delivery of i.v. injected antibody (44). In our experiments, despite the very significant effect of the collagenase treatment on transport (Fig. 4), there was not a significant decrement in the apparent collagen concentration in the tumors (Fig. 2B). This is likely due to the measurement technique that determines the hydroxyproline content in the tissue as a surrogate for collagen (27, 45). The disruption of the collagen matrix may not result in the loss of significant amounts of hydroxyproline. In addition, the collagenase enzyme, which was likely still present in the tumor tissue during the transport phase of the experiment, has a hydroxyproline content of 2.75 mg/g protein (45). Therefore, the assay, as used in the experimental protocol, may underestimate the extent to which the collagenase has disrupted the collagen structure in these tumors.

The current study was designed to identify the molecular component of the tumor microenvironment that contributes most to the resistance to transport of an antibody into the tumor. Our findings indicate that collagen is the major contributor to such resistance. In contrast, hyaluronic acid seems to play little role. These observations, together with those of our previous study (15), support the concept that while the high interstitial pressure is a deterrent to convective penetration of macromolecules into the tumor parenchyma, there is also an inherent resistance in the structure of the interstitial matrix that must be overcome before effective delivery of an antibody. This hypothesis that the tumor interstitium acts as a barrier to antibody transport has been proposed earlier in a study of locally administered agents (23). The current study provides direct evidence that modulation of an interstitial component can facilitate transport of antibody into a tumor from regional administration. The physical presence of the collagen microfibrillar network could thus be the primary barrier to transport. Another explanation could be that there is a synergistic effect of both removing this physical barrier and lowering the interstitial pressure by disrupting collagen-integrin binding.

Injection of collagenase into the cavity during i.p. application of anticancer therapeutics is not a clinically viable approach because it may damage the visceral organs and could increase metastasis by degrading the tissue targets of the tumor cells (44). These concerns notwithstanding, the present studies provide proof-of-principle evidence that modulation of the interstitium can enhance the transport of antibody into a tumor. These studies are thus an incentive for a further characterization of the barrier to antibody transport and for the design of suitable methods to overcome this barrier.

www.aacrjournals.org Clin Cancer Res 2006;12(6) March 15, 2006 1911


References

Intraperitoneal Immunotherapy for Metastatic Ovarian Carcinoma: Resistance of Intratumoral Collagen to Antibody Penetration

Jaehwa Choi, Kimberly Credit, Karla Henderson, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/6/1906

Cited articles
This article cites 37 articles, 12 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/6/1906.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/12/6/1906.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.