Correlating structure with solute and water transport in a chronic model of peritoneal inflammation

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Flessner, Michael F., Jaehwa Choi, Heather Vanpelt, Zhi He, Kimberly Credit, Jeffrey Henegar, and Michael Hughson. Correlating structure with solute and water transport in a chronic model of peritoneal inflammation. Am J Physiol Renal Physiol 290: F232–F240, 2006. First published August 23, 2005; doi:10.1152/ajprenal.00211.2005.—To study the process of chronic peritoneal inflammation from sterile solutions, we established an animal model to link structural changes with solute and water transport. Filtered solutions containing 4% N-acetylglucosamine (NAG) or 4% glucose (G) were injected intraperitoneally daily in 200- to 300-g rats and compared with controls (C). After 2 mo, each animal underwent transport studies using a chamber affixed to the parietal peritoneum to determine small-solute and protein mass transfer, osmotic filtration, and hydraulic flow. After euthanasia, parietal tissues were sampled for histological analysis, which demonstrated significant differences in peritoneal thickness (μm; C, 42.6 ± 7.5; G, 80.4 ± 22.3; NAG, 450 ± 104; P < 0.05). Staining for VEGF correlated with CD-31 vessel counts (no./mm2; C, 53.1 ± 16.1; G, 166 ± 32; NAG, 183 ± 32; P < 0.05). Tissue analysis showed treatment effects on tissue hyaluronan (μg/g; C, 962 ± 73; G, 1,169 ± 69; NAG, 1,428 ± 69; P < 0.05) and collagen (μg/g; C, 56.9 ± 12.0; G, 107 ± 12; NAG, 97.6 ± 11.4; P < 0.05) but not sulfated glycosaminoglycan. Transport experiments revealed no significant differences in mannitol transfer or osmotic flow. Changes were seen in hydrostatic pressure-driven flux (μl.min−1.cm−2; C, 0.676 ± 0.133; G, 0.317 ± 0.124; NAG, 0.284 ± 0.117; P < 0.05) and albumin transfer (μl.min−1.cm−2; C, 0.331 ± 0.028; G, 0.286 ± 0.026; NAG, 0.229 ± 0.025; P < 0.04). We conclude that alteration of the interstitial matrix correlates with diminished hydraulic conductivity and macromolecular transport.

THE PROCESS OF PERITONEAL inflammation, which results from exposure to sterile solutions during dialysis, is under intense scrutiny. This inflammation is evidenced by the appearance of various cytokines, proliferation of fibroblasts, increased thickness, and angiogenesis in the submesothelial tissue. The impetus for these studies stems from the observation that only ~30% of patients treated with peritoneal dialysis are able to continue the technique beyond 6 yr (14). Biopsy studies have verified that during this period of time, the submesothelium becomes markedly thickened with an acellular, avascular fibrotic layer, coupled with vascular changes in the underlying tissue (31). Several groups have developed animal models of chronic exposure to sterile solutions to investigate this process of inflammation (15, 20, 21). In nearly all of these studies, animals are injected daily via a catheter with a subcutaneous port. After 8–20 wk, transperitoneal transport is estimated with a 4-h, whole-cavity dialysis experiment, similar to the technique used in humans (26). This dialysis experiment is an observation of multiple, simultaneous processes (3, 4), which makes its individual correlation with structural changes problematic. In addition, the magnitude of alteration may vary in different parts of the peritoneum, (20, 31) and only 20–40% of the anatomic peritoneum is in contact with the dialysis fluid at any one time during the experiment (10, 11). Therefore, there is uncertainty about the actual tissues exposed during the dialysis experiment, their area of contact with the dialysis solution, and the residence time for fluid contact. These additional factors make the direct correlation of structure of individual tissues with the transport function of a varying portion of the peritoneum difficult.

Our hypothesis is that local measurement of transperitoneal transport function can be correlated directly with tissue histology and structure. In this paper, we describe methods designed to measure more precisely each of four different transport modes, which occur during peritoneal dialysis: small-solute diffusion, macromolecular transport, osmotically induced water flow, and hydrostatic pressure-driven water flow. Our technique eliminates surface area and the duration of fluid contact as variables during the transport experiment and permits measurements in specific peritoneal tissues. After euthanasia, these tissues are analyzed for structural alterations in interstitial matrix components and blood vessels, both of which have been shown to be the major components of the peritoneal barrier.

METHODS

Overview of Animal Model Design

The major innovation in this animal model is the method of functional transport measurement at the end of the chronic treatment period and the ability to correlate directly with structural changes in the peritoneal barrier. The exposure technique used in the rats is not different from that used in previously published papers (15, 20). The subcutaneous port implanted in the neck with the catheter tunneled subcutaneously to the peritoneal cavity is used as the delivery device for the daily injections. At the end of 8 wk of chronic injections, the animal is anesthetized and the abdominal cavity is opened, inspected, and a transport chamber is affixed to the abdominal wall. While chambers can be affixed to the abdominal wall, stomach, liver, or cecum (7), all experiments in this paper are performed on the abdominal wall. The four specific types of transport are then measured (see Transport Studies below). After the animal is euthanized, tissues under and around the transport chamber and from other sites (contralateral side of abdominal wall) are collected for histological or biochemical analysis. The chamber permits the control of the exposed...
tissue area, exposure time, and precise measurement of different types of transport: small solute diffusion, protein transport, osmotic pressure-driven water flow, and hydrostatic pressure-driven water flow. The resulting transport phenomena are then correlated with changes in the number of vessels in the tissue, the thickness and character of the submesothelium, the presence of a variety of cytokines, and quantitative measurements of interstitial collagen, hyaluronic acid (HA), and sulfated glycosaminoglycans (GAG).

**Animals and Implantation of Tunneled Catheters**

Sprague-Dawley female rats, 200–225 g (n = 29), were anesthetized with intramuscular injections of pentobarbital sodium (60 mg/kg). All procedures were performed in accordance with the University of Rochester Committee on the Use and Care of Animals or the Institutional Animal Care and Use Committee of the University of Rochester. Using an aseptic technique, an incision was made in the skin of the posterior neck and in the skin overlying the linea alba in the abdominal wall; blunt dissection of each area was performed. A Silastic catheter connected to a small subcutaneous chamber (Rat-O-Port CP6–9S, Access Technologies, Norfolk, VA) was tunneled from the neck under the skin to the abdomen. A trocar was used to penetrate the abdominal wall, and the catheter tip was inserted into the peritoneal cavity and secured with a purse stitch in the abdominal wall. The skin overlying the abdomen was closed with wound clips. The chamber in the neck was secured to the underlying muscle with absorbable suture. Before closure of the skin over the chamber, 1 ml of isotonic Krebs-Ringer bicarbonate (KRB) solution was placed in the chamber to the level of the skin over the subcutaneous port was carefully prepped with Betadine (12, 27). Before injection, the solutions were sterilized with a 0.2-

**Chronic Injections**

After 1 wk, wound clips were typically removed and daily 30- to 40-ml injections (varied depending on animal size) were administered for 2 mo via the SC port while the animal was under halothane or isoflurane gas anesthesia. The volumes were scaled on the basis of (body weight)\(^{0.7} \times 2,000 \text{ ml}) (5), which produces pressures that are the same as those observed in humans (12, 27). The sterile solutions were as follows. Controls (C) included J age-control animals receiving no treatment, no catheter; 2) catheter control animals, which had a catheter placed and weekly injection of 1 ml KRB with 10 U heparin; and 3) animals injected with KRB. These three subgroups had matched transport and tissue characteristics and were therefore lumped. A second group (G) was treated with a KRB solution containing 4% glucose (anhydrous dextrose, 1919.01, Baker, Phillipsburg, NJ). The third group (NAG) was injected with a KRB solution containing 4% N-acetylgalactosamine (A8625, Sigma, St. Louis, MO); the rationale for this solution was the potential for decreased inflammatory changes in the peritoneum (33). Solutions G and NAG had an osmolality of 480 ± 10 mosmol/kg\(H_2O\). All solutions were sterilized with a 0.2-µm filter and were handled within a laminar airflow hood during syringe filling. Before injection, the skin over the subcutaneous port was carefully prepped with Betadine or 70% alcohol. Other than the initial dose given at catheter placement, antibiotics were not administered prophylactically. Because the goal of these experiments was to test the hypothesis that local transport measurements would better correlate with local morphological studies, cultures of dialysate and the final peritoneum were not performed as in studies by others (19). Wound dehiscence near the port with an active infection, failure to thrive, catheter obstruction, or visceral-to-parietal peritoneal adhesions at laparotomy were, however, used as criteria for removing an animal from the study.

**Transport Studies**

**Surgical preparation and chamber placement.** After 2 mo of daily injections, each animal was anesthetized with pentobarbital sodium (60 mg/kg im). Intravenous and intra-arterial catheters were placed; a tracheostomy was performed; the animal’s temperature was maintained between 35 and 38°C; blood pressure was monitored to ensure that the mean was >80 mmHg. A laparotomy was performed, and the abdomen was carefully inspected for gross abnormalities such as lesions on the peritoneum or adhesions between the parietal and visceral peritoneum. A plastic chamber (diameter ~1.3 cm, height ~7–8 cm, manufactured from polystyrene; see Ref. 7 and Fig. 1 for details) was affixed to the serosa of the middle of the abdominal wall and lateral to the rectus abdominus muscle; in normal animals, we found consistent results in this position (7). The chamber constrains transport to a specific area of the tissue and eliminates peritoneal contact area as an unmeasurable variable in transport calculations. An isotonic Krebs-Ringer bicarbonate solution was placed in the chamber to a level of 1 cm for 30 min before transport experiments to allow tissue recovery from placement of the chamber.

**Experimental procedure for determination of small-solute transport and osmotic flux.** To study osmotic pressure-driven convection into the chamber and mannitol transport from the chamber into the tissue, a hypertonic solution (4% mannitol in Krebs-Ringer bicarbonate, ~500 mosmol/kg\(H_2O\)) containing \(^{14}C\) mannitol (0.3 µCi/ml) was placed in the chamber to the level of ~1 cm height to avoid hydrostatic pressure-driven convection, ~3-ml volume (12, 35) for 90 min, during which the volume \(V_{cham}(t)\) and tracer concentration \([C_{cham}(t)]\) were measured to determine the osmotic volume flux \(J_v\) (flow rate/chamber area) into the chamber and the mass of mannitol \(M(t) = C_{cham}(t)V_{cham}(t)\) remaining in the chamber vs. time. Volume was determined by weight by withdrawing the solution from the chamber with a preweighed syringe-needle catheter every 30 min; the tissue at the bottom of the chamber was always observed to be moist during the 30-s period of weighing. Fifty-microliter samples of chamber fluid and 40- to 50-µl samples of blood were withdrawn at 0, 15, 30, 45, 60, and 90 min; all of these sample volumes were carefully accounted for in the volume calculations (7). One gram of fluid weight was equated to 1 ml of fluid. After the 90-min experiment, the chamber solution was replaced by a 3-ml solution of isotonic KRB for 20–30 min.

To calculate a mass transfer coefficient for mannitol (MTC), the modified Powell algorithm with least squares was used to fit the

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[Image: Design of “localized” transport experiments across an area of peritoneum defined by the base of a chamber affixed to the tissue. P, hydrostatic pressure; J\(_p\), convective flux due to hydrostatic pressure; J\(_o\), either J\(_{o,\text{osm}}\) or J\(_{o,\text{alb}}\) (osmotic and albumin flux, respectively), mass transfer of mannitol is from the tissue to the chamber with a preweighed syringe-needle catheter every 30 min; the tissue at the bottom of the chamber was always observed to be moist during the 30-s period of weighing. Fifty-microliter samples of chamber fluid and 40- to 50-µl samples of blood were withdrawn at 0, 15, 30, 45, 60, and 90 min; all of these sample volumes were carefully accounted for in the volume calculations (7). One gram of fluid weight was equated to 1 ml of fluid. After the 90-min experiment, the chamber solution was replaced by a 3-ml solution of isotonic KRB for 20–30 min.]

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**Fig. 1.** Design of “localized” transport experiments across an area of peritoneum.
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experimental results (MicroMath, Salt Lake City, UT)

\[
\frac{dM}{dt} = \frac{d(C_{\text{cham}}V_{\text{cham}})}{dt} = -MTC\left(A_{\text{cham}}(C_{\text{cham}} - C_p)\right)
\]

where \(C_p\) is plasma concentration, and \(A_{\text{cham}}\) is the surface area of tissue exposed at the base of the chamber (see Fig. 1 and Ref. 7). \(C_p\) was measured and found to be negligible compared with \(C_{\text{cham}}\).

The following equation was used to calculate the osmotic volume flux \(J_{\text{osm}}\):

\[
J_{\text{osm}} = \frac{1}{A_{\text{cham}}} \int_0^t \frac{d(B_{\text{plasma}}V_{\text{cham}})}{dt} \, dt
\]

**Experimental procedure for determination of albumin flux.** With the KRB solution in the chamber at a height of \(\leq 1\) cm, FITC-albumin (~15 mg in 0.3 ml KRB) was administered intravenously, and the plasma fluorescence \((B_{\text{plasma}})\), chamber volume \((V_{\text{cham}})\), and chamber fluorescence \((B_{\text{cham}})\) were measured hourly over 180 min. From these measurements, the albumin flux \(J_{\text{alb}}\) (mass transfer rate per plasma concentration per chamber area) was calculated with the following equation:

\[
J_{\text{alb}} = \frac{1}{A_{\text{cham}}} \int_0^t \frac{B_{\text{plasma}}V_{\text{cham}}}{dt} \, dt
\]

**Experimental procedure for measuring hydrostatic pressure-driven flux into the tissue.** The chamber was washed with Krebs-Ringer solution and then it was filled to a depth of 6 cm with Krebs-Ringer solution containing 5% BSA, 0.05% Evans blue dye, and \(^{125}\)I-labeled IgG (~1 µCi/ml). IgG has previously been shown to be a marker for hydrostatic pressure-driven flow across the peritoneum (12). The volume of the chamber solution and the concentration of \(^{125}\)I-IgG \((C_{\text{IgG}})\) were measured every hour and averaged over a period of 3 h to calculate \(C_{\text{IgG}}\). At the end of experiment, the chamber was removed and the tissue under the chamber was identified from the staining with Evans blue dye. Tissue from this region was recovered, and its radioactive content \((M_{\text{IgG}})\) was measured to estimate transport of \(^{125}\)I-IgG into the tissue. The hydrostatic pressure driven volume flux \(J_{\text{p}}\) (total tissue IgG per duration per mean IgG chamber concentration per chamber area) was calculated from these measurements:

\[
J_{\text{p}} = \frac{M_{\text{IgG}}}{A_{\text{cham}}C_{\text{IgG}}Nd}\]

**Tissue Sampling and Analysis**

**Initial tissue collection.** At the end of the acute transport studies, the animal was euthanized with an overdose of pentobarbital sodium, and tissue samples were obtained. The abdominal wall, adjacent to the chamber and untouched by the chamber, was collected and frozen for analysis for HA, collagen, and GAGs. Portions of the contralateral abdominal wall corresponding to the position of the chamber, as well as uniform and positionally consistent samples of stomach, large and small intestine, and liver were collected and placed in 10% formalin for histological analysis. To correlate with the transport data, the results from the abdominal wall will be presented in this paper.

**Tissue analysis for content of collagen, HA, and GAGs.** Tissue analysis was performed on full-thickness samples of the abdominal wall, including the peritoneum and the underlying muscle. Because the solutes and the solution penetrate to ~1 mm (9), the effect of the test solution likely goes well beyond the peritoneum itself. Therefore, we believe that it is important to analyze the entire tissue, which is 1.5–2 mm thick. The tissue content of collagen was quantified by assessment of the hydroxyproline content (24). In brief, full-thickness samples of abdominal wall muscle, including the peritoneum and the altered submesothelial tissue were freeze-dried overnight. The dry weight was measured, and the tissue was digested overnight at 60°C with 125 µg/ml papain in a buffer of 0.2 M sodium acetate, 10 mM EDTA (pH 5.8), and 10 mM cysteine. The digestion was stopped by adding 0.5 M iodoacetic acid, and particulate matter was removed by centrifugation. Samples were then hydrolyzed with sodium hydroxide (2 M final concentration) for 20 min at 120°C. Chloramine-T was added to oxidize the samples for 25 min at room temperature. After neutralization with 0.17 M citric acid, Ehrlich’s aldehyde reagent was added. The absorbance was measured at 550 nm using a microplate reader ( Molecular Devices, Sunnyvale, CA).

HA content was determined using an enzyme-linked binding protein assay (Corgenix, Westminster, CO) according to the manufacturer’s instructions. Tissue digests and standards were added to microplates coated with HA binding protein and incubated at room temperature for 1 h. After being washed four times, horseradish peroxidase-conjugated HA binding protein solution was added and incubated for 30 min at room temperature. The wells were washed, and substrate solution was added. Following incubation at room temperature for 30 min, the stop solution was added. Absorbance was determined at 450 nm, with measurements at 650 nm serving as a reference.

Sulfated GAG (sGAG) content was assayed using the Blyscan method (Biocolor, Newtownabbey, UK). The Blyscan dye reagent was added to each sample and standard and mixed for 30 min. The insoluble pellet of sulfated GAGs was precipitated by centrifugation at 12,000 g for 10 min. Bound dye was released with the dissociation reagent, and the absorbance was measured at 650 nm.

**General Histology and Thickness Measurement**

Tissue was dehydrated, embedded in paraffin, and 5-µm sections were cut. Sections were stained with standard hematoxylin and eosin or Gomori’s one-step trichrome staining method. The sections were incubated in Bouin’s solution (Sigma) for 1 h in a 60°C oven. After washing, sections were stained with Weigert’s hematoxylin (Sigma), rinsed and stained with trichrome (American Master Tech Scientific, Lodi, CA). With regard to the analysis of peritoneal thickness, it is not clear how other groups select the portions of the tissue that are used for measurements (29). Peritoneal thickness was quantified in two ways: 1) by measuring the distance from an intact mesothelium to the muscle at five random locations on each slide and 2) by measuring the thickest span of peritoneum on each slide. These measurements were performed by three independent observers with a calibrated optical micrometer and light microscopy (Olympus BX-40, Melville, NY).

**Cytokeratin immunohistochemistry.** Sections were deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide to quench endogenous peroxidase. Sections were then incubated with a cocktail of cytokeratin antibodies (Pan-keratin, Ventana Medical Sciences, Tucson, AZ), washed, incubated with an appropriate secondary antibody conjugated with horseradish peroxidase, and developed with diaminobenzidine. All sections were then examined with a Nikon light microscope for presence of the mesothelium and surface coating.

**VEGF immunohistochemistry.** Sections were deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide to quench endogenous peroxidase. After the treatment of antigen unmask with Antigen Retrieval Citra Plus (BioGenex, San Ramon, CA), sections were appropriately blocked for avidin, biotin, and serum (VectorStain Elite, Vector Labs, Burlingame, CA). Sections were incubated overnight at 4°C with VEGF antibody (Sc-7269, Santa Cruz Biotechnology, Santa Cruz, CA). On day 2, the slides were washed three times in PBS, treated for 30 min with biotinylated secondary antibody, and washed again. An avidin-biotinylated-horseradish peroxidase complex (30-min incubation) and 3,3-diaminobenzidine tetrahydrochloride (10 min) were used for detection. The sections were counterstained with Gill’s hematoxylin. Negative controls were run exactly as above with omission of the primary antibody.
CD31 immunohistochemistry. CD31, a vascular endothelial cell marker (13, 28), was assessed to determine the total vascular space in the peritoneum and subperitoneal tissue. Samples preserved in formalin were embedded in paraffin wax and cut into 4-μm sections for CD31 staining. The sections were rehydrated with xylene and graded concentrations of alcohol, then treated with Antigen Retrieval Citra Plus, for antigen unmask. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. Staining was performed at room temperature throughout. Nonspecific binding sites were blocked with 2.5% horse serum in PBS for 1 h. Sections were treated for 90 min with goat polyclonal anti-CD31 antibody (Santa Cruz Biotechnology) diluted 1:350 in blocking solution. The slides were washed three times in PBS, treated for 30 min with biotinylated secondary antibody, and washed again. An avidin-biotinylated-horseradish peroxidase complex (30-min incubation) and 3,3'-diaminobenzidine tetrahydrochloride (10 min) were used for detection. The sections were counterstained with Gill’s hematoxylin. Negative controls were run exactly as above with omission of the primary antibody.

Image analysis for CD31 staining. CD31-positive blood vessels were counted using a microscope with a 10 × 10 graticule in one eyepiece. The length and width defined by the graticule were calibrated with a stage micrometer. Any CD31-positive cell cluster that was distinguishable from adjacent cells and connective tissue elements was counted as a single microvessel. Three locations were randomly selected on each sample, and the total number of vessels was determined in each location within the altered peritoneum (mesothelium-to-muscle layer) and divided by the area as determined by the graticule. A second measurement at each of these locations was made of the number of vessels per length of mesothelium. Vessel numbers were also selectively determined in the highest density areas. Measurements represent the averages of three independent observers.

Statistics

Results are reported as means ± SE. Specific comparisons were tested with a one-way ANOVA or two-sided t-tests. In some cases, regression analysis was used to test the correlation of transport parameters with structure. Significance is indicated if the probability of a type I error is <0.05. NCSS 97 (Kaysville, UT) was used for statistical calculations.

RESULTS

Survival of Study Animals

Table 1 lists the animal characteristics from the study. All animals survived the entire 8 wk. Two of the control (1-Krebs and 1-catheter) and of the glucose group and one of the NAG group demonstrated adhesions. These were not used in the subsequent analysis. There were no significant differences among the groups in terms of initial weight, final weight, and weight change (g and % of initial weight). All of the animals gained weight over the experimental period.

Histology and Quantitative Measurements

Stains with hematoxylin and eosin and trichrome (not shown) demonstrated very significant changes in thickness of the mesothelium-to-muscle layer of peritoneum exposed to the glucose or NAG solutions. Figure 2 displays the quantitative measurements of thickness that were made in two ways: random measurements and maximum thickness in each section. Each treatment resulted in significant increases in both measures of thickness, with the NAG-treated animals having the greatest change.

The effects of the solutions were very significant in the induction of angiogenesis. Figure 3 demonstrates almost no staining in the control tissues, whereas the animals treated with glucose or NAG had markedly increased staining for VEGF. The presence of VEGF correlates directly with CD-31 staining for vessels in the submesothelium, as seen in Fig. 4. Figure 5 summarizes the measurements of vessel density (per mm or mm²) and demonstrates a marked increase in vessel numbers in the animals treated with glucose or NAG. No significant staining was observed in negative control sections (without primary antibody) run in parallel for each staining.

Alterations in Interstitial Matrix

Figure 6 displays the concentrations of collagen, HA, and sGAG in the anterior abdominal wall. There are no significant changes in the sGAG content. Both of the experimental groups demonstrated very significant increases in both collagen and HA content. The HA content of the tissue under the chamber increased from 962 ± 73 for controls to 1,169 ± 69 for glucose and to 1,428 ± 69 μg/g dry tissue for the NAG group. Collagen concentrations increased from 56.9 ± 12.0 for controls to 107.1 ± 12.0 for glucose and 97.6 ± 11.4 μg/g dry tissue for NAG.

Transport Studies

As displayed in Fig. 7, there were no significant differences among the groups for small-solute (mannitol) transport from...
the chamber to the tissue (MTC, cm/min; C, 0.00284 ± 0.00043; G, 0.00306 ± 0.00043; NAG, 0.00304 ± 0.00048; not significant). Hydrostatic pressure-driven volume flow was significantly reduced from controls (μL·min⁻¹·cm⁻²; C, 0.676 ± 0.133; G, 0.317 ± 0.124; NAG, 0.284 ± 0.117; P < 0.05).

Figure 8 displays the results for osmotic flux (μL·min⁻¹·cm⁻²; C, 1.116 ± 0.156; G, 0.900 ± 0.153; NAG, 0.850 ±
0.144), which showed some decrease that was not significantly different at $P = 0.46$. Albumin flux ($\mu l$·min$^{-1}$·cm$^{-2}$) decreased significantly from control for the two treatments groups (C, 0.331 ± 0.028; G, 0.286 ± 0.026; NAG, 0.229 ± 0.025; $P < 0.04$).

**DISCUSSION**

**Design of the Animal Model**

Specific parameters were examined and selected in the design of the animal model. The rat was chosen as the model species because of ease of anesthesia, surgical manipulation, hardiness of the species, and cost. All animals have normal kidney function, which simplifies surgical preparation and eliminates the complications of uremia. However, the few studies on uremic animals have clearly shown some differences in response (23, 36) to inflammation; the current effort is considered a step toward a uremic chronic exposure model.

Injections in the reported experiments were made through an implanted subcutaneous port connected to a catheter tunneled to the peritoneal cavity (15). This allows injections only and does not permit withdrawal from the cavity as in normal dialysis. While this does not simulate dialysis, it does foster higher concentrations of cytokines in the cavity because they are not being diluted and removed by fresh dialysis solutions.

Other modes of injection are via transcutaneous catheters, which require prophylactic antibiotics to be added (23) or direct needle injection (22).

The volume injected in our experiments is much larger than that used by other groups, who utilized 10 ml twice a day in female Wistar rats with an initial weight of 213 g (19) or 10 ml twice daily in male Sprague-Dawley rats with initial weights of 276 g (22). Our group has used a scaling factor of ($body\ weight)^{0.7}$ to account for the relatively larger surface area of the peritoneum in rats and to maximize the peritoneal surface area exposed to the daily-injected solution. Scaling from 2,000 ml in a 70-kg human to a 275-g rat, the volume would be 41.4 ml, which produces similar intraperitoneal (ip) pressures to that observed in humans (5, 12, 27). The volume is completely absorbed in 18–24 h. At no time after injection did the animal display any signs of distress with the use of this volume.

The method of solution delivery was designed to maximize sterility and to minimize stress in the animal. Prepared in a laminar airflow hood, injections were carried out within 4–6 min of full anesthesia to minimize trauma to the animals and to ensure sterility of solution delivery. However, even with careful handling of sterile solutions and multiple cultures, a tran-

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**Fig. 6.** Parietal peritoneal matrix concentrations after 8 wk of daily treatments with sterile solutions. Values are means ± SE. Filled bar, no. of vessels/mm$^2$; open bar, no. of vessels/mm. *$P < 0.05$.

**Fig. 7.** Transperitoneal transport of small solutes (mannitol) or volume flow driven by hydrostatic pressure. There are no significant effects of peritoneal treatment on the mass transfer coefficient for mannitol. Comparison of the volume flux induced by hydrostatic pressure with the controls demonstrated significant decreases in the hydrostatic pressure-driven flow in both treated groups. *$P < 0.05$.

**Fig. 8.** Transperitoneal transport of albumin and water due to osmotic pressure in chamber. Volume fluxes were lower but not significantly different at $P < 0.1$. Albumin fluxes were significantly different at *$P < 0.05$. 
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remains unknown in whole-cavity experiments. It is typically separated from the intrinsic permeability of the barrier and properties of the peritoneum, which may be altered by the demonstrated the quantitative importance of the surface contact microvasculature. Indeed, animal and clinical studies have then there can be no mass or fluid transport from the underlying there is no contact of the dialysis fluid with the peritoneum, as carried out in each animal to measure transport function. This function. After 8–20 wk of exposure to solutions, a procedure demonstrated the general uniformity in testing of transport analyses. In preliminary studies, we observed that this closed-cavity experiment, the implicit assumption of the submesothelium was primarily avascular (not observed in these 8-wk studies; see Figs. 3 and 4), then that portion of the peritoneum would assume more significance if in contact with the dialysate. The altered interstitium would likely affect the osmotic filtration and small-solute transport, and Eq. 6, which assumes that the microvasculature is uniformly distributed, would have to be modified to reflect the primary process of tissue diffusion without uptake by a distributed microvasculature. The use of the transport chamber permits the estimation of the intrinsic permeability of the peritoneal barrier (MTC), which through separate experiments can be related to \( D_{\text{eff}} \) and (\( \rho \)) (8).

Choice of Tissue for Transport Studies

Although the transport chamber can be used with a variety of tissues (7), the abdominal wall was chosen as the tissue for transport studies. In preliminary studies, we observed that this tissue displayed the greatest changes; human biopsy studies have confirmed this (31). Mice treated with similar techniques also show the greatest changes in the abdominal wall (Robert B, personal communication). This may be due to a greater exposure of the abdominal wall to fluid, because the position of the animal in the prone or standing position promotes fluid pooling at the lowest point of the cavity. Indeed, we observed this with the study of the surface contact area in mice and rats (10, 11).

Histological Studies and Quantification of Angiogenesis and Peritoneal Thickness

Analysis of hematoxylin and eosin, trichrome, and cytokerin stains of the abdominal wall demonstrated peritoneal thickening with exposure to the two test solutions, which has been observed in many other studies. Review of the work of others (18, 22, 29, 30) did not reveal how specific areas of tissues are selected for measurements or how specific areas

Advantages of Local Measurement of Transport

A recent review of chronic animal models (20) outlined the different methods of treatment with different solutions but also demonstrated the general uniformity in testing of transport function. After 8–20 wk of exposure to solutions, a procedure similar to the clinical Peritoneal Equilibration Test (26) is carried out in each animal to measure transport function. This single experiment does not separate diffusion from convection or osmotic filtration from hydrostatic pressure-driven flow and therefore limits the correlation of structural changes in the peritoneal barrier with specific transport phenomena. The use of the chamber technique in our animal model precisely determines the four major types of transport, which can be correlated to changes in the same tissue used in the experiment and offers advantages in quantitative assessment of chronic alterations in the peritoneal barrier.

The closed-cavity dialysis experiment used by all other groups presents a major disadvantage in data interpretation, in that it cannot eliminate the effects a variable area of peritoneal contact with the dialysis solution. Some would argue that the only area of importance is the perfused vascular area (16) and that peritoneal contact is unimportant. Most other models of transperitoneal transport are single-membrane models, such as the three-pore model (25), which does not include the interstitium or the peritoneal contact area. We would argue that if there is no contact of the dialysis fluid with the peritoneum, then there can be no mass or fluid transport from the underlying microvasculature. Indeed, animal and clinical studies have demonstrated the quantitative importance of the surface contact area (1, 10) to mass transfer. The contact area (10, 11) depends on the volume instilled, the size, position, and the intrinsic properties of the peritoneum, which may be altered by the experimental treatment. Thus the effect of the area cannot be separated from the intrinsic permeability of the barrier and remains unknown in whole-cavity experiments. It is typically included in the mass transfer area coefficient in the following equation

\[
\frac{dM}{dt} = MTAC(C_{\text{plasma}} - C_{\text{peritoneal}}) \tag{5}
\]

where the variables are similar to those of Eq. 1, in which the MTAC = \( MTC \cdot A_{\text{contact}} \). With the chamber experiment, the MTC can be related to the effective tissue diffusivity of the solute in tissue (\( D_{\text{eff}} \)) and the microvascular permeability (\( \rho \))-area density (\( a \)) of underlying tissue space by the “distributed” model (2)

\[
MTC = \sqrt{D_{\text{eff}} \cdot (\rho)} \tag{6}
\]

Careful quantitative assessments of the interstitial density, measurements of interstitial matrix components, and assessment of vascular density can be related to \( D_{\text{eff}} \) or (\( \rho \)) via the MTC. On the other hand, the MTAC (= \( MTC \cdot A_{\text{contact}} \)) cannot be related to changes in the tissue, unless the \( A_{\text{contact}} \) is known. In the same manner, water flow (\( Q_i = J_i \cdot A_{\text{contact}} \)) or albumin mass transport rate (\( F_{\text{alb}} = J_{\text{alb}} \cdot A_{\text{contact}} \)) all depend on \( A_{\text{contact}} \). In the typical closed-cavity experiment, the implicit assumption is that \( A_{\text{contact}} \) is equivalent among all experimental groups. In a badly scarred peritoneum, the distribution of fluid and the contact area are likely abnormal. Indeed, if the expansion of the submesothelium was primarily avascular (not observed in these 8-wk studies; see Figs. 3 and 4), then that portion of the peritoneum would assume more significance if in contact with the dialysate. The altered interstitium would likely affect the osmotic filtration and small-solute transport, and Eq. 6, which assumes that the microvasculature is uniformly distributed, would have to be modified to reflect the primary process of tissue diffusion without uptake by a distributed microvasculature. The use of the transport chamber permits the estimation of the intrinsic permeability of the peritoneal barrier (MTC), which through separate experiments can be related to \( D_{\text{eff}} \) and (\( \rho \)) (8).

Choice of Tissue for Transport Studies

Although the transport chamber can be used with a variety of tissues (7), the abdominal wall was chosen as the tissue for transport studies. In preliminary studies, we observed that this tissue displayed the greatest changes; human biopsy studies have confirmed this (31). Mice treated with similar techniques also show the greatest changes in the abdominal wall (Robert B, personal communication). This may be due to a greater exposure of the abdominal wall to fluid, because the position of the animal in the prone or standing position promotes fluid pooling at the lowest point of the cavity. Indeed, we observed this with the study of the surface contact area in mice and rats (10, 11).
within sections are analyzed. In our study, we measured sections both randomly selected and taken from obvious areas of inflammation. Despite relatively large variations in the NAG measurements, our results in Fig. 2 showed significant increases in the thickness of the submesothelium, as measured from the mesothelial cells to the muscle cells of the abdominal wall. This implies a proliferation of fibroblasts and other interstitial cells that produce hyaluronan and collagen. The data in Fig. 6 confirm that the interstitial content of collagen and hyaluronan in the glucose- and NAG-treated animals did indeed increase significantly. Thus the quantitative determination of interstitial components correlates with the qualitative observations in the histology.

The marked increase in VEGF (Fig. 3) correlates with the proliferation of new vessels (angiogenesis) in the treated animals, as shown in Fig. 4. Determination of the vessel density with two techniques (per mm and mm²) revealed the same trends and demonstrates that either method may be useful in reporting the number of vessels. The number divided by the length of peritoneum provides a measure of total new vessels in a certain region and correlates with thicker layers of proliferation (see NAG vs. glucose in Figs. 2 and 5). The number of vessels per unit area of submesothelial tissue may be more useful in mathematical simulations of transport across such a layer.

**Linking Structure with Transport**

Increases in peritoneal thickness resulted in alterations in albumin transport from the blood to the chamber and in hydrostatic pressure-driven water flow into the tissue. The increased concentrations of collagen and HA (Fig. 7) and the marked increase in thickness in both the glucose and NAG groups is likely responsible for the reductions in the hydraulic conductivity and transport of protein (17, 34).

On the other hand, despite marked increases in angiogenic vessels in the submesothelium (Fig. 5), there was no change in the transport of mannitol (Fig. 7) or the osmotic water flux from the tissue to the chamber. Because of the pathological nature of the model and the variation noted in Fig. 2, there was some concern that the model may have some inherent bias or variation, which would not allow differentiation of the groups. However, all tests for normality of the data, including skewness and kurtosis, supported the validity of ANOVA. In a further effort to test the correlation of MTC_{mannitol} and J_{osm} with angiogenesis, all of these data were lumped into one group and were examined in regression analyses against the corresponding measures of tissue thickness and angiogenesis. No significant correlations resulted. Therefore, we conclude that, despite the observed variation in the model, these data support the conclusion that the structural changes in the tissue do not change mannitol transport or osmotic filtration.

If it is assumed that all of the angiogenic vessels were perfused and that this resulted in an increase in the perfused area of three to four times, one might anticipate an increase in the MTC in accordance with Eq. 6 (see Fig. 7) of 1.7-2 times

\[
\text{as derived from}\ \frac{a_{\text{treated}}}{a_{\text{control}}} = \sqrt[3]{3} - \sqrt[4]{3} \tag{7}
\]

This lack of correlation could be due to several factors: 1) not all of the angiogenic vessels marked by the CD31 stain are perfused; 2) the vessels are perfused, but there are blood flow limitations in the delivery of solute and fluid to this angiogenic microvasculature; 3) the vessels have markedly different permeability characteristics; or 4) there is a corresponding decrease in \(D_{\text{eff}}\) due to the marked changes in the interstitium that mitigates any increases in \(pa\) and results in a similar MTC (see Eq. 6). More detailed experiments, which couple the chamber with measurements of the perfused vascular and the local blood flow through these vessels, will have to be performed to sort this out (8). In the same way, the osmotic flow of water depends on the vascular density, the vascular hydraulic permeability, and the hydraulic conductivity of the tissue. Opposing changes in decreased tissue hydraulic conductivity or a lack of perfused vessels with a decreased permeability in the altered tissue layer could account for the apparent discrepancy between the observed tissue structure and transport. At this time, there are no data to support or refute any of these possibilities.

The effect of the chronic exposure to NAG demonstrates the usefulness of our approach to linking structure and function. Another group of investigators exposed rats over 8 wk to solutions containing glucose or NAG (50 mmol/l) and carried out the typical whole-cavity PET (in which they assume an equivalent \(A_{\text{contact}}\) to check transport function (33). After death, the peritoneum was stained with Alcian blue (stains all GAGs) over the liver and the abdominal wall, and increases in peritoneal thickness were measured in both regions with both solutions. Protein losses were significantly less with the NAG than the glucose group. Volumes recovered were not different in either group; however, injection of 30 ml into 324- to 447-g rats was likely insufficient to bring ip pressures to the typical 4–6 cmH₂O, which is observed in patients (27) and used in our experiments to test for hydraulic flow. Glucose concentration, on the other hand, dissipated more rapidly in the NAG-treated animals during the first hour of experimental dialysis. These findings were interpreted as positive for the peritoneal barrier function: lower protein loss and a denser submesothelial compact zone with NAG treatment. As we have observed in our study, 8-wk NAG treatment increased peritoneal thickness and matrix concentrations of both collagen and HA. The observed decreases in protein transport and hydraulic permeability and tendency for decreased osmotic filtration in our data would, in a whole-cavity transport study, demonstrate the same observations that were observed by the other group of investigators. However, the linkage of marked fibrosis and angiogenesis (Figs. 3 and 4) to these changes (Figs. 5 and 6) produces a very different conclusion concerning the potential long-term consequences of using NAG as an osmotic agent. Thus the local transport data with elimination of variables from the transport calculations allows a direct link between observed structural changes and function.

In summary, we have developed a model of chronic exposure to peritoneal dialysis solutions that permits direct correlation of transport function with structural changes. Precise measurements are made of the four different types of transport phenomena with a plastic chamber affixed to the peritoneum. The chamber eliminates area and duration of exposure to the test dialysate as variables. Histological and immunohistochemical analysis and extraction and chemical analysis of the interstitial matrix in the same tissue on which the chamber...
resides permit one-to-one comparison of physiological function with structural alterations.

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