Contractile activity of lymphatic vessels is altered in the TNBS model of guinea pig ileitis

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THE LYMPHATIC SYSTEM is an essential component of the cardiovascular and the immune systems. Lymphatic vessels collect fluid, extracellular proteins, lipids, and antigenic substances from the interstitium and, through rhythmic phasic contractions of the smooth muscle in the vessel wall, propel this material along a network of collecting vessels through lymphoid tissue (e.g., lymph nodes) and back to the blood stream.

Lymphatic circulation is thus likely to play a crucial role during IBD, but the involvement of lymphatic contractile function in inflammatory diseases and its role during IBD has never been addressed. Although the complexity of human IBD disorders cannot be reproduced in any animal model, the TNBS model of guinea pig ileitis is a well-described model of intestinal inflammation, by videomicroscopy in vivo and in vitro. The present results demonstrate that lymphatic contractile function is altered in TNBS-induced ileitis and suggest a role for prostanoids in the lymphatic dysfunction.

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ileum was identified and exteriorized through a midline laparotomy. 2,4,6-Trinitrobenzenesulfonic acid (TNBS, Sigma-Aldrich) was injected intraluminally into the ileum (0.425 ml; 30 mg/ml in 30% ethanol) 10 cm proximal to the ileo-cecal junction. An equivalent volume of physiological saline (0.9% NaCl) was injected into the ileum lumen of the sham group to control for volume effects. The midline laparotomy was surgically closed, and the animals were maintained in a controlled environment for 1, 3, or 6 days.

Assessment of Inflammation

Macroscopic damage was assessed on ileal and duodenal tissues excised immediately after death by awarding a value of 0 (not evident), 1 (mild), or 2 (severe) to the following parameters: erythema, hemorrhage, edema, presence of strictures, presence of mucus, ulceration, and adhesions. Microscopic signs of inflammation were assessed from adjacent 5-μm ileal transverse sections fixed in buffered formalin and processed for routine histological hematoxylin-eosin staining. Scores of 0, 1, or 2 were assigned to three sections from three different slides for each animal by a blinded investigator to the following parameters: neutrophil infiltration, distance of neutrophil infiltration, submucosal thickness, hemorrhage, villi tip damage, villi surface damage, and dilation of the central lacteals. Scores for each parameter were added to give composite macroscopic and microscopic scores.

Assessment of Bowel Wall Edema

Ileum samples of 50–100 mg were harvested, thoroughly rinsed in sterile saline solution, blotted dry, and carefully weighed. Samples were allowed to dry to a constant weight for weeks and weighed again. The weight difference was calculated and taken as the weight of water in the sample: % water weight = [wet weight (mg) – dry weight (mg)]/[wet weight (mg)] × 100.

In Vitro Experiments

One, three, or six days after surgery, guinea pigs, fasted for 24 h and were euthanized by decapitation under halothane-induced anesthesia, and lymphatic vessels were prepared as previously described (12, 43). Briefly, the small intestine and associated mesentery were dissected out and placed in a physiological saline solution (PSS) of the following composition (in mM): 2.5 CaCl2, 5 KCl, 2 MgCl2, 120 NaCl, 25 NaHCO3, 1 NaH2PO4, and 11 glucose, with pH maintained at 7.4 by constant bubbling with a 95% O2–5% CO2 gas mixture. A section of mesentery, together with the associated arteries, veins, and collecting lymphatic vessels, was dissected and pinned onto the Sylgard-coated base of a 2-ml organ bath, which was placed on the stage of an inverted microscope (CK40, Olympus) and continuously superfused (3 ml/min) with warm (36–37°C) PSS. Selected collecting lymphatic vessels were cut open, and a fine glass cannula was inserted into their lumen and passed through at least one lymphatic valve to minimize back flow. Vessels were perfused with a low (0.3 mM) calcium solution to prevent precipitate obstructing the cannula (43). Lymphatic activity was observed on a television monitor connected to a video camera; the change in vessel diameter was monitored using a video-dimension analyzer (model V94; Living Systems Instrumentation, Burlington, VT) and recorded on a computer via an analog-to-digital converter and the “Chart” software (PowerLab/4SP; ADInstruments, Mountain View, CA).

The rate of luminal perfusion was initially set at 2.5 μl/min, which induces a consistent rhythmic constriction in control lymphatic vessels (12). Following a 30-min equilibration period, the average number of beats/min over a 5-min period served as the baseline control activity. The vessel was considered inactive if it did not exhibit contractions within 30 min of perfusion at 2.5 μl/min. Changes in lymphatic activity were assessed while intraluminal flow was increased from 0.5 to 10 μl/min in 2.5-μl/min increments for 10 min at each rate. After the perfusion rate reached 10 μl/min, indomethacin (nonselective COX inhibitor; Sigma-Aldrich), celecoxib (COX-2 selective inhibitor; NicOx, Sophia Antipolis, France), or SC-560 (COX-1 selective inhibitor; Boehringer-Ingeheim, Ingelheim, Germany) were added to the superfusion for 20 min, followed either by addition of a second antagonist for 20 min or by a wash-out period of 10 min in normal PSS. Alternatively, vessels were superfused with indomethacin, celecoxib, or SC-560 in PSS while increasing intraluminal flow. Following 10 min at 10 μl/min, the tissue was treated with a second antagonist for 20 min or washed with regular PSS.

A minimum of two tissue preparations were taken from each animal from an area as close to (proximal sample) the site of injection as permitted by the severity of the inflammation, use of the mesentery directly at the injection site being sometimes precluded by bowel wall adhesions. To assess lymphatic contractile function in an area potentially less affected by the inflammation, a section of duodenal mesentery was also collected at least 8 cm away (distal sample) from the injection site.

In Vivo Experiments

Intravital microscopy was used to examine lymphatic contractile function in vivo. Guinea pigs, fasted for 18–24 h prior to experimentation at 1, 3, and 6 days postsurgery, were anesthetized with intramuscular injection of ketamine (0.27 ml/kg of 100 mg/ml), xylazine (0.375 ml/kg of 20 mg/kg), and diazepam (0.375 ml/kg of 5 mg/ml). Half-dose supplements were administered as required. Following anesthesia, the carotid artery and trachea were cannulated with polyethylene tubing (0.8 and 1.8 mm inner diameter, respectively) to monitor arterial pressure (Harvard Apparatus) and ensure spontaneous respiration. Saline was continuously infused (0.03 ml/min) through the carotid artery to maintain adequate hydration of the animal.

A 2-cm incision was made in the lower abdomen, and a section of the small intestine and associated mesentery was externalized. The animal was transferred onto a holding plate, the externalized loop of ileum was placed onto a clear pedestal placed under a stereomicroscope (MZ 12.5, Leica) or an upright microscope (Axioscope, Zeiss), and lymphatics were visualized through a camera (Hyper HAD, Sony) and television monitor. Animal body temperature was monitored and maintained using a homeothermic blanket control unit (Harvard Apparatus). The exposed tissue was continuously superfused with PBS of the following composition (in mM): 140 NaCl, 7.5 Na2HPO4, and 2.5 NaH2PO4 (pH 7.4), supplemented with 1% BSA (Sigma-Aldrich) and maintained at 37°C. The protein-enriched PBS was used to prevent osmotic imbalance between the observation bath and tissue (1). Exposed bowel was covered with moist gauze to minimize dehydration of the tissue.

The preparation was allowed to stabilize for 30 min, after which a 10-min period was used to determine the baseline activity of lymphatic vessel constriction rate and diameter. A vessel that showed no constrictions within 1 min of visualization was defined as “inactive.” Responsiveness of lymphatic vessels to indomethacin was assessed after a 30-min superfusion with 10 μM indomethacin, followed by a 10-min wash-out period using regular PBS. Constriction frequency and changes in diameters were monitored by video microscopy.

Data Analysis and Statistics

Constriction frequency, systolic and diastolic diameters, and amplitude changes of lymphatic vessels were assessed in vitro from the Chart software traces and in vivo by directly measuring the parameters from video images on the monitor screen. Indices for stroke volume and volume flow of lymph through a section of a mesenteric lymphatic vessel were calculated according to Benoit et al. (3). These calculations give the unit stroke volume as the difference between systolic and diastolic unit volumes and multiplying this by constriction frequency to give volume flow (cf. cardiac output = stroke
volume of the heart × heart rate). In vivo measurements were obtained from at least 3 vessels in 3 or more separate fields of view in the mesentery of each animal.

Statistical significance was assessed using either the Student’s t-test or one- and two-way repeated measures ANOVA, followed by appropriate post hoc tests. P values <0.05 were considered statistically significant.

RESULTS
Assessment of Inflammation
Administration of TNBS caused significant macroscopic evidences of inflammation at days 1, 3, and 6 compared with sham-treated animals. Microscopic damage score was significantly higher 1 and 3 days after TNBS administration, returning to sham-treated values at day 6 (Fig. 1).

Assessment of Bowel Wall Edema
To evaluate edema, the submucosal thickness, one of the parameters included in the microscopic evaluation of inflammation, was considered. In the TNBS-treated animals, submucosal thickness was increased to 162 ± 16% (P < 0.05) and to 135 ± 16% of sham values at days 3 and 6, respectively. The percent water weight was also measured in ileum samples. In the sham-treated animals, it was 78.5 ± 0.7% (n = 8) 1 day after surgery and did not change significantly at days 3 and 6 (n = 10). In contrast, the percent water weight in the TNBS-treated animals was significantly increased at day 3 (80.5 ± 0.4%, n = 10, P < 0.01) and day 6 postsurgery (81.7 ± 0.7%, n = 10, P < 0.01).

Lymphatic Vessel Contractile Function in TNBS-Treated Guinea pigs: In Vitro Data
Vessel diameter. In vitro lymphatic diastolic diameter increased with the severity of inflammation in the TNBS-treated group, reaching a maximum at damage scores between 5 and 8 (Fig. 2A) on day 3. There was variation in the degree of
inflammation in this group, and consequently, it was subdivided into a “low-damage” subgroup (macroscopic damage score of 0–4) and a “high-damage” subgroup (damage score over 5). In the high-damage subgroup, the mean diastolic vessel diameter was significantly greater than in sham-treated vessels for tissue taken from an area of the mesentery proximal to the TNBS injection site but not different in vessels taken more distally (Fig. 2B). Six days after induction of the ileitis, vessel diameters of the TNBS-treated group were not significantly different from their sham-treated counterparts, which were not significantly different from control (untreated) vessels (Fig. 2B).

Constriction frequency. In control and sham-treated animals, the frequency of lymphatic vessel constrictions increased as perfusion rate increased from 0.5 μl/min, reaching a maximum at 5.0–7.5 μl/min. Data from the day 3 group was subdivided according to macroscopic damage scores (see Vessel diameter above). Constriction frequency of vessels in the high-damage subgroup remained significantly lower than in sham-treated animals over the whole range of perfusion rates (Fig. 3). Vessels of the high-damage subgroup taken distal (≥8 cm) to the TNBS injection site showed weak contractile activity that remained significantly lower than sham-treated and control animals at all perfusion rates. In lymphatic vessels from the low-damage subgroup, constrictions could be stimulated at all perfusion rates, even in tissues that were taken directly from the injection site, albeit at lower frequencies than their respective sham controls. Lymphatic contractile activity in TNBS-treated animals 6 days following the induction of inflammation was not significantly different from control or sham values at all perfusion rates. Lymphatic vessels from a distal origin exhibited higher constriction frequencies at lower perfusion rates than that of vessels taken proximal to the injection site at all perfusion rates (Fig. 3B).

Amplitude of constrictions. Many lymphatic vessels were quiescent in the day 3 high-damage subgroup; when constrictions occurred, their amplitude was significantly lower than that of sham-treated vessels. In these vessels, constrictions only increased at high flow rates (Fig. 4A), in contrast to the sham group, the day 3 low-damage subgroup (Fig. 4A), and the TNBS-treated vessels at day 6 (n = 8, data not shown), where the amplitude of constrictions were similar and slightly decreased with increasing intraluminal flow.
Calculated indices for stroke volume and lymph flow. Stroke volumes in the day 3 high-damage subgroup were low at low perfusion rates, increasing steadily with increase in intraluminal flow and reaching the same values observed in vessels from sham-treated animals (which remained almost constant across the flow range; Fig. 4B). The same trends were observed in lymph flow rate (Fig. 4C). Stroke volumes and lymph flow rate values in the day 3 low-damage subgroup and in TNBS-treated vessels at day 6 (n = 8, data not shown) were not significantly different from that in sham-treated animals or in animals 6 days after induction of inflammation (Fig. 5B). In vessels that were constricting, the frequency distribution followed a normal gaussian curve with the same mean (8.8 ± 0.5 and 8.2 ± 0.4 constrictions/min) and median (8.0) value in all observed TNBS- and sham-treated lymphatic vessels. There was no significant difference in the frequency of constrictions between treatment group, day, and distance from the injection site.

**Effects of COX Inhibition on the TNBS-Induced Decrease in Lymphatic Contractility**

The role of prostanoids in the TNBS-induced inhibition of lymphatic contractile function was assessed first in vitro using COX inhibitors. In the presence of the nonselective COX inhibitor indomethacin (10 μM) applied at the end of the in vitro increased perfusion rate protocol, lymphatic vessels from the high-damage subgroup of day 3 TNBS-treated animals had a significantly higher constriction frequency than when superfused with PSS alone, although the mean constriction frequency was still significantly less than in sham-treated animals (Fig. 6A). Indomethacin had no effect on constriction frequencies of lymphatic vessels from sham-treated and day 6 TNBS-treated animals, which were already close to control values.

The effect of increasing perfusion rate on constriction frequency of lymphatic vessels of the day 3 high-damage subgroup was enhanced by indomethacin, but constriction frequency was still significantly lower than that measured in vessels from the sham-treated (Fig. 6B) and control groups (data not shown). Constriction frequency of vessels from the low-damage subgroup was not significantly affected by indomethacin (n = 3, data not shown). Addition of indomethacin to the superfusate of vessels from day 6 animals slightly increased (without reaching significance) constriction frequency at all perfusion rates (n = 7, data not shown); constriction frequency in these vessels was not different from sham control vessels.

Lymphatic vessels were defined as inactive if they did not show any contractile activity at any of the perfusion rates tested during the in vitro experiments. In 73% of these inactive vessels from TNBS animals, a 10–15 min (mean ± SE; 11.9 ± 0.6 min) of superfusion with 10 μM indomethacin was able to initiate constrictions (Fig. 7A). The indomethacin-stimulated constriction frequency was irregular, alternating periods of activity (20.7 ± 4.7 beats/min for 2.0 ± 4.7 min), with periods of inactivity of 2.5 ± 0.5 min.

As described in the previous section, lymphatic vessels that were still rhythmically constricting in vivo in animals with highly (macroscopic score of 5 or above) inflamed ileums had a range of constriction frequencies not significantly different from that of sham-treated animals. To evaluate the effect of indomethacin on the contractile activity of lymphatic vessels in vivo, we performed experiments on vessels from the day 3 high-damage subgroup that display a low contractile activity (range of 1–8 constrictions/min) similar to that recorded in vitro at a high perfusion rate (as illustrated in Fig. 6). After being superfused with indomethacin (10 μM) for 5–7 min, the constriction frequency of these vessels increased to values that were not significantly different from that of sham-treated vessels (Fig. 7B).
To determine whether inhibition of COX-1 or COX-2 was specifically responsible for the indomethacin-induced increase in constriction frequency, response to increase in perfusion rate was investigated in vitro in vessels belonging to the day 3 high-damage subgroup in the presence of the selective COX-1 and COX-2 inhibitors SC-560 or celecoxib, respectively. The application of celecoxib (10 μM) did not change the constriction frequency response to increases in perfusion rate in these vessels compared with PSS superfusion (data not shown and Fig. 8Aa). However, when SC-560 (1 μM) was added to the celecoxib-containing PSS at the highest (10 μl/min) perfusion rate, constriction frequency was significantly increased (Fig. 8Aa). Similarly, constriction frequency was not increased by SC-560 alone at any perfusion rate (data not shown and Fig. 8Ab) but was significantly augmented after additional application of celecoxib at a perfusion rate of 10 μl/min (Fig. 8Ab). No difference was observed with any combination of the COX inhibitors in the day 3 low-damage subgroup (Fig. 8B) or in the day 6 group (data not shown).

**DISCUSSION**

Morphological and histological changes occur in intestinal lymphatic vessels during IBD (16, 20, 22). Pathologists have noted for decades that marked submucosal edema and engorgement of capillaries and lymphatics are one of the first and most consistent morphological changes that occur in intestinal inflammation (25, 36). In the present study, we showed significant macroscopic damage, microscopically evident edema, and increased tissue fluid in TNBS-treated animals at day 3, the latter continuing into day 6 after TNBS treatment. Typically, edema would result in increased lymphatic contractility and...
obstruction was observed in human patients who were undergoing surgery for Crohn’s disease (16), and lesions similar to those in IBD have been reported in animals with experimentally obstructed mesenteric lymphatic vessels (21, 22, 34), although not always (8, 16). Lymphatic vessel obstruction was not directly assessed in the present study. However, we observed no major lymph nodes or obvious sites of obstruction between vessels that were proximal or distal to the TNBS-affected ileum (unpublished observations). Since the distal vessels were largely unaffected by the TNBS treatment (i.e., their behavior did not seem to be affected by any downstream obstruction), the impaired lymph movement in vessels proximal to the TNBS-affected ileum must be due to impaired lymphatic contractile function. Furthermore, vessels from inflamed animals (day 3, high-damage subgroup) had larger diameters that were maintained in vitro, where vessels were removed from the influence of any possible obstruction. This impairment was accompanied by constrictions of reduced frequencies and amplitudes, leading to stroke-volume values and flow-rate indices that reached control values only at the highest in vitro perfusion rate. The contractile function was partially restored in vivo and in vitro by the topical application of indomethacin, which is unlikely to have “unblocked” downstream lymphatics. Taken together, these findings suggest that TNBS-induced ileitis results in impairment of lymphatic contractile function, which was likely to decrease drainage of lymph from the inflamed ileum. It is possible that edema consequent to severe inflammation leads to an increase in fluid load and lymphatic intraluminal pressure sufficient to overpower lymphatic contractile ability and convert the lymphatics to passive conduits. However, we often observed that small particles and cells that would usually be flowing down normal lymphatic vessels remained stagnant in vessels proximal to the TNBS-affected ileum (unpublished observation), suggestive of lymph stasis. Moreover, the active pump of mesenteric lymphatics has been described as able to sustain constrictions even at high imposed flow rates (13, 24). This robust contractile activity was confirmed in our in vitro experiments, where vessels from sham-treated animals were able to increase their constriction frequency with increasing perfusion rates before reaching a plateau. By contrast, frequency and amplitude of these constrictions were significantly lower in vessels isolated from animals with severe inflammation (day 3, high-damage subgroup), and they were less responsive to increasing perfusion rates. In summary, the inhibition of lymphatic contractility in TNBS-inflamed ileum must include direct effects of inflammation on the lymphatic vessel itself and is likely to contribute to reduced lymphatic drainage from the inflamed bowel.

Factors affecting lymphatic contractile function include soluble factors released by the microvascular endothelia and hormonal, humoral, or neural factors. Significant among these are the prostaglandins, the production of some of which has been shown to increase during IBD (26, 39) as a consequence of inducible COX-2 upregulation (17, 37). Most studies to date show that acute administration of COX metabolites exert a cytoprotective action on the mucosa, whereas other investigations (4, 11, 38, 45) demonstrate an exacerbation of inflammation in animals with experimental colitis and humans upon long-term administration of NSAIDs. To date, there has been no investigation of the effect of prostaglandins on lymphatics during intestinal inflammation, but we know that prostaglan-
dins, among the most important modulators of lymphatic function, can either enhance or inhibit lymphatic contractility (18, 42). Our present results clearly indicate that COXs might play a role in the dysfunction observed in lymphatic vessels of TNBS-treated guinea pigs, as inhibition of COX-1 and COX-2 with indomethacin was able to partly restore in vitro, and augment in vivo, rhythmical contractile activity. With respect to the metabolites that could be responsible for these effects, PGE\(_2\) and prostacyclin are plausible candidates, as they have been shown to potently dilate rat iliac lymphatics (31) and inhibit contractility in bovine as well as guinea pig mesenteric vessels (6, 15, 19). Studies involving deletion of prostaglandin receptors and neutralizing antibodies to PGE\(_2\) have reported (33) PGE\(_2\) and PGI\(_2\) as the primary prostanoids involved in inflammation. A study by Brock et al. (5) demonstrated that induction of COX-2 with LPS resulted in the preferential production of PGE\(_2\) and prostacyclin (accounting for \(~86%\) of the total prostaglandin produced) rather than simply increasing total prostaglandin production. TNBS-induced intestinal inflammation triggered production of COX-2 (35). It is possible that, as a consequence of our experimental conditions, the profile of prostaglandin synthesis was shifted towards the inhibitory metabolites, leading to the inhibition of lymphatic contractile activity and its partial restoration under COX inhibition with indomethacin. If indeed the production of prostacyclin and PGE\(_2\) from COX-2 resulted in the suppression of the total prostaglandin produced) rather than simply increasing production of PGE\(_2\) and prostacyclin (accounting for \(~86%\) of the total prostaglandin produced), the suppression of COX-2 would yield results similar to general COX inhibition alone to produce a significant effect on constriction frequency; the suppression of the activity of both isoforms of COX was required to produce an effect. One possible explanation for this could be that a small amount of inhibitory prostaglandins can be produced from COX-1. Brock et al. (5) showed that prostacyclin and PGE\(_2\) accounted for \(~26%\) of total COX-1 derived metabolites. Another explanation for the inefficacy of COX-2 inhibition alone to produce a significant effect on constriction frequency could be that the production of the inhibitory prostaglandins is due to upregulation of the selective prostaglandin E synthase (7), an enzyme downstream from COX in the metabolism of arachidonic acid, rather than the preferential production inhibitory prostaglandins by COX-2. Clearly, the role of COX metabolites in lymphatic function during inflammation needs further investigations.

We have demonstrated dilatation and inhibition of contractile function in mesenteric lymphatic vessels of guinea pigs with TNBS-induced ileum inflammation. The evidence presented strongly suggests a decrease in drainage during experimental ileitis, as lymphatic pumping is known to be the main driver of lymph flow (9, 27, 28). Our study gives new information on the effect of intestinal inflammation on the lymphatic system and its capability to drain interstitial fluid and resolve edema. We believe the lymphatic drainage system is a significant contributor to the development of intestinal inflammation.

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REFERENCES

ALTERED LYMPHATIC FUNCTION IN EXPERIMENTAL ILEITIS