Heparan sulfate depletion amplifies TNF-α-induced protein leakage in an in vitro model of protein-losing enteropathy

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Bode, Lars, Erik A. Eklund, Simon Murch, and Hudson H. Freeze. Heparan sulfate depletion amplifies TNF-α-induced protein leakage in an in vitro model of protein-losing enteropathy. Am J Physiol Gastrointest Liver Physiol 288: G1015–G1023, 2005.—Protein-losing enteropathy (PLE), the excessive loss of plasma proteins through the intestine, often correlates with the episodic loss of heparan sulfate (HS) proteoglycans (HSPGs) from the basolateral surface of intestinal epithelial cells. PLE onset is often associated with a proinflammatory state. We investigated whether loss of HS or treatment with the proinflammatory cytokine TNF-α directly causes protein leakage and whether a combination of both exacerbates this process. We established the first in vitro model of PLE and measured the flux of albumin/FITC through a monolayer of intestinal HT29 or Caco-2 cells grown on transwells and determined the integrity by transepithelial electrical resistance (TER). Loss of HS from the basolateral surface, either by heparanase digestion or by inhibition of HS synthesis, increased albumin flux 1.58 ± 0.03-fold and reduced TER by 75.7 ± 4.7% but only slightly decreased HS content. The combined effects of HS loss and TNF-α treatment were not only additive, but synergistic, with a 7.00 ± 0.11-fold increase in albumin flux and an 83.9 ± 8.1% reduction of TER. Coincubation of TNF-α with soluble HS or heparin abolished these synergistic effects. Loss of basolateral HS directly causes protein leakage and amplifies the effects of the proinflammatory cytokine TNF-α. Our findings imply that loss of HSPGs renders patients more susceptible to PLE and offer a potential explanation for the favorable response some PLE patients have to heparin therapy.

intestinal protein loss; congenital disorders of glycosylation; Fontan surgery; heparin therapy

PROTEIN-LOSING ENTEROPATHY (PLE) is defined as the enteric loss of plasma proteins, which can exceed 20 g/day (1). PLE is not a disease itself but a symptom in several ostensibly unrelated diseases, including Crohn’s disease (1), congenital disorders of glycosylation (CDG) (36), and as a long-term complication of Fontan surgeries to correct congenital heart malformations (16). In CDG and post-Fontan patients, PLE is episodic and heparan sulfate (HS) proteoglycans (HSPGs) on the basolateral surface of intestinal epithelial cells are absent or mislocalized during these episodes (18, 19, 29, 36). In addition, PLE onset is often associated with a proinflammatory state (15, 36), suggesting that multiple factors combine to exceed critical thresholds. However, mechanisms that link PLE with loss of HSPG and a proinflammatory state are yet unknown.

HS is a large, highly sulfated glycosaminoglycan (GAG) composed of alternating units of α-N-acetylgalcosamine and β-glucuronic acid (12). These chains are assembled in the Golgi on specific core proteins. These molecules are well established as an important barrier against protein leakage from the kidney and are absent from the glomerulus in patients with nephrotic syndrome (10, 23). Murch et al. (19) first implicated HSPGs in PLE. Three infants who suffered severe PLE from birth showed an absence of enterocyte HSPG expression specifically from the basolateral surface of small intestinal epithelial cells. Overall, intestinal architecture and other matrix components including laminin, collagen-I, and proteoglycans synthesized by other cells within the lamina propria remained intact and normal (19).

Since then, absence of HSPGs from the basolateral surface of intestinal epithelial cells has been described in several other diseases associated with PLE. Histological assessment of small-bowel biopsies from an ALG6 deficient CDG-Ic patient with PLE was mostly normal, but HSPGs were completely absent from the basolateral surfaces of epithelial cells. A second biopsy, taken after PLE resolved, showed a considerably improved basolateral HSPG-staining pattern. All biopsies showed normal basolateral HSPG staining in the patient’s colon, stomach, and esophagus, suggesting that the pathology was restricted to the small intestine (36). Recently, we found that other CDG-Ia, Ib, and Ic patients (phosphomannomutase, phosphomannose isomerase, and hALG6 deficiency, respectively) with PLE also lack basolateral HSPGs (S. Murch, unpublished data). Also, mannos treatment of CDG-Ib patients relieves PLE (21) and improves HSPG staining (S. Murch, unpublished data). Moreover, duodenal biopsies from five of six post-Fontan patients with PLE revealed strikingly reduced HSPG expression on the basolateral surface of intestinal epithelial cells, whereas HSPG staining in lamina propria cells was normal (29).

PLE or increased intestinal permeability may be triggered by infection (15, 36) and is often associated with a proinflammatory state (2, 3, 7, 8, 24, 34). An elevated systemic concentration of the proinflammatory cytokine TNF-α (22) as well as increased intestinal levels of IFN-γ (32) have been associated with PLE.

One-half of the patients who develop PLE months to years after Fontan surgery have a fatal outcome. Therapeutic options for post-Fontan PLE are limited. Albumin infusions are used in severe cases (27). The inflammatory aspect of PLE is commonly treated with long-term, high doses of anti-inflammatory steroids, but serious undesirable side effects appear (35). Subcutaneous injections of high-molecular weight heparin reverse...
PLE in some patients (5, 11, 17, 28), especially when given early after onset, but the basis and mechanism of heparin improvement are yet unknown (4).

Patient susceptibility to PLE, its cause, the underlying cellular and molecular mechanisms, and a rational basis for treating this condition all suffer the absence of a fundamental understanding. On the basis of the observations that intestinal epithelial HSPGs are absent during episodes of PLE and that PLE onset is often associated with a proinflammatory state, we hypothesized that each of these factors contributes to PLE. To test this hypothesis, we established an in vitro tissue culture model of PLE and investigated the contributions of HSPGs and the proinflammatory cytokine TNF-α to protein leakage.

MATERIALS AND METHODS

The human intestinal epithelial cell lines HT29 (ATCC #HTB-38) and Caco-2 (ATCC #HTB-37) were grown in Dulbecco’s modified eagle’s medium and RPMI 1640 (Irvine Scientific, Santa Ana, CA), respectively. Both media were supplemented with 10% fetal calf serum (HyClone, Logan, UT), penicillin, streptomycin, and l-glutamine.

Albumin flux. Cells were grown on semipermeable inserts (1.0-μm pore size, PET, BD, Franklin Lakes, NJ) for 5 days until they reached confluence as determined by transepithelial electrical resistance (TER). Albumin flux was measured in cells treated with heparanase III (HSase), sodium chlorate, p-nitrophenyl-N-β-d-xylopyranoside (β-xylan- side), genistein, heparin (Sigma, St. Louis, MO), recombinant human TNF-α (R&D, Minneapolis, MN), chondroitinase ABC (Seikagaku, Tokyo, Japan), H-8 (Calbiochem, La Jolla, CA), GM6001 and AGN (kindly provided by Dr. A. Strongin, The Burnham Institute, La Jolla, CA), and/or soluble HS (sHS) or chondroitin sulfate (sCS; both courtesy of Dr. A. Malmström, Lund University, Lund, Sweden) at final concentrations and time points as indicated. The inserts and the wells were washed twice with RPMI 1640 (without serum, without phenol red; Invitrogen, Grand Island, NY), and 400 μg/ml albumin:FITC in RPMI (Sigma) were added to the inserts. The albumin:FITC concentration was measured in the well after 1 h using a spectrofluorometer (excitation: 485 nm; emission: 538 nm). Albumin flux through the untreated monolayer is defined as 1.0.

TER. We monitored the integrity of the monolayer before and after different interventions by measuring TER with an epithelial voltohmmeter and STX2 electrodes (WPI, Sarasota, FL). TER in the untreated monolayer is defined as 100% after correcting for the resistance of a filter in tissue culture media without cells.

Radiolabeling of GAGs. To determine the GAG turnover rate in HT29 cells, we labeled them with Na235SO4 (50 μCi/ml) for 4 h and then measured the amount of 35S-labeled GAGs in the tissue culture media and the cell layer at different times after labeling as described in Purification of 35S-labeled GAGs. To determine the effects of HSase and TNF-α on the amount of cell-associated GAGs, we labeled the HT29 cells with Na235SO4 (100 μCi/ml) for 8 h, incubated the washed cells with HSase and TNF-α for 2.5 and 8 h, respectively, and measured 35S-labeled GAGs in the tissue culture media and the cell layer as described below.

Purification of 35S-labeled GAGs. After we radiolabeled, we collected the tissue culture media and harvested the cells in 4 M guanidine HCl/50 mM acetic acid. Lysed cells were diluted with 50 volumes of 6 M urea/50 mM acetic acid. We purified the radiolabeled proteoglycans/GAGs on anion-exchange chromatography columns (DE53, Whatman, Maidstone, UK) (6). Briefly, we equilibrated the columns with 3 volumes of 6 M urea/50 mM acetic acid, applied the samples, and washed the columns with 3 × 20 volumes of 6 M urea/50 mM acetic acid and 6 volumes of 6 M urea 0.6 M acetic acid. Proteoglycans were eluted with 3 volumes of 4 M guanidine HCl/50 mM acetic acid. All buffers contained 10 mM EDTA. In addition, all buffers used for cell layer fractions contained 1% Triton X-100. Radioactivity was determined by liquid-scintillation counting.

Statistical analysis. Results are given as means ± SD from three independent experiments. Differences between interventions were tested by the two-tailed Student’s t-test. P < 0.05 is considered significant.

RESULTS

In vitro monolayer of intestinal epithelial cells. Five days after seeding the HT29 cells, TER was maximal (214.3 ± 1.4 Ω × cm², defined as 100%). We concluded that the cells had grown into a confluent monolayer and confirmed this by microscopic observation. To determine monolayer polarity, we used confocal microscopy to visualize the expression of the apical surface marker dipeptidylpeptidase IV (DPP IV) at day 5. DPP IV was only expressed on the cell surface facing the media, defining the apical side, but it was not expressed on the cell surface facing the porous membrane of the insert (data not shown), defining the basolateral surface. To prove that the untreated monolayer at day 5 does not leak protein, we added albumin:FITC to the basolateral surface and determined albumin flux through the monolayer by measuring the concentration of albumin:FITC on the opposite side. Even after 24 h, only 0.8% albumin:FITC had passed through the monolayer. In contrast, without cells the albumin:FITC concentrations on both sides of the porous membrane of the insert reached equilibrium in <12 h (data not shown).

Loss of HS causes protein leakage. Our first aim was to determine whether the loss of HS from the basolateral surface of intestinal epithelial cells caused protein leakage, as seen in patients with PLE. We incubated the basolateral surface of the HT29 monolayer with HSase (0.6 mU/ml, 2.5 h), which decreased the amount of cell-associated GAGs by 77.0 ± 1.0% (P < 0.001; Fig. 1A). In parallel, albumin flux increased 1.58 ± 0.09-fold (P < 0.01; Fig. 1B), and TER decreased by 23.4 ± 6.5% (P < 0.01; Fig. 1C). Albumin flux from the basolateral to the apical surface as well as from the apical to the basolateral surface was equally increased (data not shown). Similar HSase digestion of the apical surface only slightly increased albumin flux 1.24 ± 0.09-fold (P < 0.05) and decreased TER by only 6.0 ± 0.9% (P < 0.05). Combined apical and basolateral treatments did not significantly increase the effects of the basolateral treatment alone.

HSase effects are concentration and time dependent and are maximal at 0.6 μM for 1 h (Fig. 2, A–D). To verify that the observed effects are HSase specific, we coincubated the cells with sHS during basolateral HSase treatment. A predicted 2.2-fold excess (0.1 mg/ml) of soluble HSase substrate partially reduced the HSase-induced effects on albumin flux (Fig. 2E) and TER (Fig. 2F). A 22-fold excess (1 mg/ml) of soluble substrate completely blocked the HSase-induced effects on albumin flux and TER. In contrast, sCS (1 mg/ml), which is not a substrate for HSase, did not compete out the HSase-induced increased flux.

The negative charges of the sulfates on HS molecules are thought to prevent protein leakage (18, 19, 36). To address this hypothesis, we inhibited sulfation with sodium chlorate and measured the effects on albumin flux and TER. First, we used 35SO42− pulse-chase labeling to determine GAG chain turnover rate in HT29 cells (Fig. 3A). The amount of cell-associated 35S-labeled GAG chains decreased over time and plateaued after...
24 h. In parallel, the amount of $^{35}$S-labeled GAG chains in the tissue culture media increased and also plateaued after 24 h ($T_{1/2} = 4$ h). We therefore incubated the cells with chlorate (25 mM) for 24 h, which increased albumin flux 1.43 ± 0.02-fold ($P < 0.001$; Fig. 3B) and decreased TER 13.8 ± 2.8% ($P < 0.001$; Fig. 3C). Treatment with $\beta$-xyloside (p-nitrophenyl-$\beta$-D-xylopyranoside, 100 $\mu$M, 24 h), which competes out GAG chain synthesis on core proteins, increased albumin flux 1.71 ± 0.11-fold ($P < 0.001$; Fig. 3B) and decreased TER 11.9 ± 2.8% ($P < 0.001$; Fig. 3C), which was similar to the effects observed after HSase treatment. However, the effects on albumin flux were more pronounced after treatment with either $\beta$-xyloside or HSase than after treatment with chlorate ($P < 0.01$).

Treatment with chondroitinase ABC (0.6 mU/ml) to specifically cleave chondroitin sulfate only slightly increased protein leakage 1.21 ± 0.11-fold ($P < 0.05$) and decreased TER 9.9 ± 4.5% ($P < 0.05$), which was significantly less pronounced than the effects seen after HSase treatment ($P < 0.001$). The combined effects of HSase and chondroitinase did not exceed the effects of HSase alone (data not shown).

**TNF-α causes protein leakage.** PLE is often associated with a proinflammatory state (2, 3, 7, 8, 22, 24, 32, 34). We therefore investigated whether the proinflammatory cytokine...
TNF-α affects protein leakage and TER in our tissue culture model. After the basolateral surface was treated with 20 ng/ml TNF-α for 8 h, albumin flux increased 4.04 ± 0.03-fold \((P < 0.001)\) and TER decreased by 75.7 ± 4.7\% \((P < 0.001)\). The effects were concentration and time dependent, with a maximum after 8 h (Fig. 4, A-D). To study whether a brief TNF-α exposure later triggers albumin leakage or continuous TNF-α exposure is needed, we treated the cells with TNF-α for 15 or 30 min or 1, 2, 4, 6, or 8 h, washed the cells, incubated them without TNF-α, and measured albumin flux after a total of 8 h. Even a 15-min treatment increased albumin flux 1.83 ± 0.05-fold \((P < 0.001)\) and decreased TER 25.7 ± 3.6\% \((P < 0.01)\). However, at least 4 h of continuous TNF-α exposure were needed for maximal protein leakage (Fig. 4, E-F).

After the apical surface with TNF-α was treated for 8 h, albumin flux only slightly increased 1.24 ± 0.04-fold \((P < 0.05)\) (Fig. 4G) and TER did not change significantly (Fig. 4H). We used confocal microscopy to confirm the polarized expression of TNF-α receptor-1 (TNFR1) only on the basolateral and not on the apical surface (data not shown).

TNF-α treatment of the basolateral surface released a small but significant \((P < 0.05)\) amount of 35S-labeled GAG chains, PGs, and GAG synthesis. The effects were concentration and time dependent, with a maximum after 8 h (Fig. 4, A-D). To study whether a brief TNF-α exposure later triggers albumin leakage or continuous TNF-α exposure is needed, we treated the cells with TNF-α for 15 or 30 min or 1, 2, 4, 6, or 8 h, washed the cells, incubated them without TNF-α, and measured albumin flux after a total of 8 h. Even a 15-min treatment increased albumin flux 1.83 ± 0.05-fold \((P < 0.001)\) and decreased TER 25.7 ± 3.6\% \((P < 0.01)\). However, at least 4 h of continuous TNF-α exposure were needed for maximal protein leakage (Fig. 4, E-F).

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which was only ~16% of that released by HSase digestion (Fig. 5).

We further investigated whether blocking of signal-transduction pathways inhibits TNF-α-induced effects by coincubating the cells with either the tyrosine kinase inhibitor genistein (185 μM) or the protein kinase A inhibitor H-8 (50 μM). Both kinase inhibitors almost completely blocked the TNF-α-induced increase in albumin flux \((P < 0.001)\) and decrease in TER \((P > 0.001)\) (Fig. 6B). Addition of either inhibitor alone, without TNF-α, had no effect. Neither genistein nor H-8 had an effect on the HSase-induced increase in albumin flux (Fig. 6C) or decrease in TER (Fig. 6D).

Because TNF-α is a known inducer of matrix metalloproteases (MMPs), we next addressed the question whether MMPs are involved in the TNF-α-induced effects on albumin leakage and TER. We coincubated the cells with TNF-α and either GM6001 (50 μM) or AGN (30 μM), two broad-range hydroxamate inhibitors of MMP activity. Both MMP inhibitors partially blocked the TNF-α-induced increase in albumin flux \((P < 0.001; \text{Fig. 6A})\) and decrease in TER \((P < 0.001; \text{Fig. 6B})\). Even a fivefold higher concentration of the MMP inhibitors \((250 \mu M \text{ for GM6001 and } 150 \mu M \text{ for AGN})\) did not further inhibit the TNF-α-induced effects on albumin flux and TER. The inhibitors alone, without TNF-α, had no effect. Again, these inhibitors did not affect the HSase-induced increase in albumin flux (Fig. 6C) and decrease in TER (Fig. 6D).

Even after 24 h, TNF-α did not alter the percentage of Annexin V-positive cells or the uptake of propidium iodine (data not shown) showing that TNF-α-induced protein leakage does not result from increased apoptosis or cell death.

Loss of HS and TNF-α act synergistically. Next, we combined both interventions. First, we digested the HT29 cells with TNF-α and either HSase, heparin (20 ng/ml), heparin (30 μM), respectively. Heparin had to be present together with TNF-α and different HS concentrations \((0.1–100 \mu M)\). Increasing HS concentrations \((k_i = -2.5 \mu M)\) did alleviate the synergistic effects on albumin flux (Fig. 8A) and TER (data not shown), and 6.25 μg/ml completely abolished it. Higher HS concentrations reduced the effects on albumin flux even below the level seen after TNF-α treatment when cell-associated HS is present.

Polymeric HS is required because digesting it to disaccharides with heparin had almost no effect on albumin flux and TER. The same concentration of heparin had a more pronounced effect than HS, whereas scs had lesser effects (Fig. 8, A and B). Addition of heparin also alleviates TNF-α-induced protein leakage when cell-associated HS is present on the cell surface (Fig. 8A).

The mitigating effects of heparin are diminished when added hours after TNF-α is added (Fig. 8C). Coincubation with TNF-α and heparin (25 μg/ml) for the full 8 h increased albumin flux only 2.24 ± 0.20-fold. In contrast, albumin flux is increased 3.78 ± 0.19 and 6.02 ± 0.30-fold when heparin is present only during the last 6 h and 4 h of TNF-α treatment, respectively. Heparin had to be present together with TNF-α to alleviate TNF-α-induced protein leakage. When we treated the cells with TNF-α for 4 h, washed the cells with media without TNF-α, and added heparin for 4 h, heparin had no mitigating effect anymore.

Studies with Caco-2 cells. To verify that our data is not specific for HT29, we repeated key experiments (similar to

Fig. 5. TNF-α induces loss of GAG chains from the cell layer. Amount of \(^{35}S\)-labeled GAGs \((\text{cpm/100} \mu \text{g protein})\) associated with the cell layer \((A)\) or present in the media \((B)\) before \((t = 0)\), open bars) and after TNF-α treatment \((20 \text{ ng/ml}, t = 8 \text{ h})\) (dark gray bars) compared with untreated controls \((t = 8 \text{ h}; \text{light gray bars})\).
Figs. 1, 2, 4, 7, and 8) with Caco-2, another frequently used intestinal epithelial cell line and got similar results (data not shown).

DISCUSSION

PLE occurs in seemingly unrelated diseases (1, 16, 36), and the only known common histological feature to date is loss of HS from the basolateral surface of intestinal epithelial cells (18, 19, 29, 36). Whether this is cause or effect is unknown. To address this question, we established the first in vitro PLE-like model of protein leakage using a confluent, polarized monolayer of HT29 cells, an intestinal epithelial cell line often used to study intestinal permeability (20, 25, 31, 33). In this model, removal of HS directly causes protein leakage and decreases the integrity of the monolayer (Figs. 1 and 2). HSase specifically degraded HS (as confirmed by competition with sHS, but not sCS), reducing cell-associated GAGs by 77%. Only treatment of the basolateral, but not the apical, surface induces protein leakage. We used confocal microscopy to confirm that HS is predominantly expressed on the basolateral surface of the HT29 monolayer (data not shown), which is in accordance with in vivo staining patterns of intestinal biopsies (18, 19, 36).

Loss of HS causes bidirectional protein leakage, indicating that it might not only be important for inside-out protein leakage in the context of PLE, but also for impaired barrier function for outside-in traffic.

The sulfate groups on HS are important to prevent protein leakage as shown by increased albumin flux after chlorate treatment. However, other portions of the HS molecule, e.g., the uronic acids, help prevent protein loss, because complete loss of HS chains after treatment with HSase or β-xylosidase increases protein leakage even more (Fig. 3).

PLE or increased permeability is often associated with a proinflammatory state (2, 3, 7, 8, 15, 24, 34, 36). One of the predominant proinflammatory cytokines, TNF-α, compromises epithelial barrier function, e.g., by the disruption of tight junctions (31). We have indeed shown that TNF-α induces protein leakage and reduces the integrity of the monolayer in our in vitro model (Fig. 4). These effects are partially mediated by MMPs (Fig. 6), but HSPGs are only slightly shed after TNF-α-treatment (Fig. 5). This result suggests that other MMP targets are involved, and it also implies that TNF-α and HS loss increase protein leakage by different mechanisms. We confirmed that TNF-α does not induce cytotoxic or apoptotic mechanisms in HT29 cells at concentrations and incubation times used in this study (31).

TNF-α was ineffective when added to the apical side, which is in accordance with previous reports and can be explained by the polarized expression of TNFR1 on the basolateral surface (31), as we confirmed by confocal microscopy.

TNF-α increases protein leakage even more than loss of HS, and the underlying mechanisms are obviously different. The combined effects of HS loss and TNF-α are not only additive but synergistic (Fig. 7). Remarkably, the increase in albumin flux caused by TNF-α (2 ng/ml) in the absence of cell-associated HS (4.60 ± 0.02-fold) is even more pronounced than the effect of a 10-fold higher TNF-α concentration (20 ng/ml) when HS is present (4.04 ± 0.03-fold increase in albumin flux). We hypothesize that cell-associated HS interferes with TNF-α-receptor binding or TNF-α-induced signal-
ing pathways responsible for protein leakage. Loss of cell-associated HS amplifies the TNF-α/H9251 response, whereas the addition of soluble HS alleviates it (Fig. 8). The addition of soluble heparin instead of soluble HS reduces the TNF-α/H9251-induced effects even more, providing an explanation for patients’ favorable response to heparin treatment. Although heparin is used as an anticoagulant, other mechanisms are probably involved here. Proteoglycans are known modulators of growth factor activities (26), e.g., high-affinity binding of growth factors, such as basic fibroblast growth factor (bFGF), employ HSPG as coreceptors (13, 37). Others speculate that loss of HSPG during episodic PLE may impair bFGF binding and reduce the rate of ulcer healing. Heparin might restore high affinity-receptor binding of bFGF and, as a consequence, increase mucosal recovery (4). In the case of bFGF binding, heparin would intervene at advanced steps during PLE pathogenesis involving mucosal recovery and healing. Our results provide the first evidence that heparin also alleviates the TNF-α-induced onset at the earliest stages of protein leakage. This is concordant with clinical observations that heparin injections reverse PLE, especially when given early after its onset (5, 11, 17, 28).

Our data suggest direct interactions between heparin and TNF-α or its receptors. Previous reports (9, 14) show that heparin binds to TNF-α but has no affinity for TNF-α-binding protein I, a soluble fragment of TNFR1 (14). Heparin blocks TNF-α-induced protein leakage only if they are coincubated, consistent with formation of a complex. Adding heparin to cells anytime during the 8-h TNF-α exposure dampens protein leakage (Fig. 8 C), consistent with an ongoing need for avail-

Fig. 7. TNF-α and loss of HS act synergistically. A: effects of different TNF-α concentrations on albumin flux when cell-associated HS is present (●) or absent (○). The greatest synergy was found at a TNF-α concentration of 2.0 ng/ml. B–C: effects of different incubation times with TNF-α at 2 (B) or 20 ng/ml (C) when cell-associated HS is present (●) or removed with HSase (○) or a combination of HSase and β-xyl. (●).

Fig. 8. Synergistic effects can be abolished by the addition soluble HS and heparin. A: coincubation with TNF-α (20 ng/ml, 8 h) and increasing heparin concentrations alleviates the TNF-α-induced albumin flux when cell-associated HS is present. In the absence of cell-associated HS (after treatment with HSase/β-xyl.), increasing sHS or heparin concentrations also abolish the synergistic effects of HS loss and TNF-α. B: at the same concentration (2.5 g/ml), heparin was more effective than sHS, whereas sCS was less effective. sHS does not alleviate the synergistic effects when it is predigested with HSase [dark gray bar: relative albumin flux in cells lacking cell-associated HS (HSase/β-xyl.) after TNF-α treatment without any coincubation]. C: to study whether heparin has to be present at the initial time of TNF-α treatment or can be added hours later, we increased the interval between TNF-α and heparin additions. We treated cells lacking cell-associated HS (after treatment with HSase/β-xyloside) with TNF-α (20 ng/ml), added heparin (used at 0.25 or 25 g/ml) either together with TNF-α (t = 0) or 2, 4, 6, or 8 h after the addition of TNF-α, and measured albumin flux after a total of 8 h.
able TNF-α (Fig. 4, E and F). Similar to cell-associated HSPG, soluble heparin may buffer the impact of TNF-α on these cells or protect the host against damaging systemic effects (14).

We show that loss of HS directly causes protein leakage and amplifies the TNF-α-induced increase in albumin flux. However, reasons and mechanisms behind the specific loss of HSPGs from the basolateral surface of small intestine epithelial cells in PLE patients remain obscure. We postulate that this location-specific effect stems from the normally high turnover rate of these cells, which increases even more during inflammation and infections (30). The underlying cause of HSPG loss may vary between different disorders. In CDG patients, the genetically insufficient N-glycosylation might predisperse HSPG loss because HSPG carry N-glycans, which can be important for folding and intracellular trafficking. HSPG mislocalization may then precipitate on environmental stress and induce PLE. This hypothesis is in accordance with immunohistochemical data from CDG-Ic patients showing punctate intracellular GAG staining, indicating a disruption of HSPG trafficking during episodes of PLE (36).

This in vitro model focuses on the early events leading to PLE. In vivo, they may activate more robust responses of protein leakage. Mice with localized impaired HSPG synthesis may be the ideal models to assess the role of these glycans in preventing PLE.

PLE is often associated with loss of HSPG, and heparin reverses PLE in some post-Fontan patients. We have now shown that loss of HS directly causes protein leakage, and it also synergizes with TNF-α. Because heparin mitigates these effects, our in vitro findings warrant further in vivo studies to identify patients at risk to develop PLE and provide a rationale for beginning early heparin treatments to prevent PLE.

REFERENCES


