Heparan Sulfate Plays a Central Role in a Dynamic in Vitro Model of Protein-losing Enteropathy*

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Protein-losing enteropathy (PLE), the loss of plasma proteins through the intestine, is a symptom in ostensibly unrelated diseases. Emerging commonalities indicate that genetic insufficiencies predispose for PLE and environmental insults, e.g. viral infections and inflammation, trigger PLE onset. The specific loss of heparan sulfate (HS) from the basolateral surface of intestinal epithelial cells only during episodes of PLE suggests a possible mechanistic link. In the first tissue culture model of PLE using a monolayer of intestinal epithelial HT29 cells, we proved that HS loss directly causes protein leakage and amplifies the effects of the proinflammatory cytokine tumor necrosis factor α (TNFα). Here, we extend our in vitro model to assess the individual and combined effects of HS loss, interferon γ (IFNγ), TNFα, and increased pressure, and find that HS plays a central role in the patho-mechanisms underlying PLE. Increased pressure, mimicking venous hypertension seen in post-Fontan PLE patients, substantially increased protein leakage, but HS loss, IFNγ, or TNFα alone had only minor effects. However, IFNγ up-regulated TNFR1 expression and amplified TNFα-induced protein leakage. IFNγ and TNFα compromised the integrity of the HT29 monolayer and made it more susceptible to increased pressure. HS loss itself compromises the integrity of the monolayer, amplifying the effects of pressure, but also amplifies the effects of both cytokines. In the absence of HS a combination of increased pressure, IFNγ, and TNFα caused maximum protein leakage. Soluble heparin fully compensated for HS loss, providing a reasonable explanation for patient favorable response to heparin therapy.

The intestinal epithelium blocks the entry of pathogens and their products into the body and also prevents the loss of plasma components into the intestinal lumen. In protein-losing enteropathy (PLE),1 where plasma proteins leak into the intestine, this essential barrier is impaired (1–4). PLE develops as a life-threatening complication of seemingly unrelated diseases, e.g. Crohn’s disease (1), Congenital Disorders of Glycosylation (CDG) (2), or after Fontan surgery to correct congenital univentricular hearts (3, 4). Emerging commonalities are beginning to suggest patho-mechanisms underlying PLE. It appears to involve a combination of genetic insufficiencies and environmental insults. The evidence for this is that patients do not suffer from PLE continuously; rather, PLE is episodic. Its onset is often associated with viral infections and a pro-inflammatory state (2, 5–8), indicating that multiple factors combine to trigger PLE. Most intriguing is the loss of heparan sulfate (HS) proteoglycans (HSPG) specifically from the basolateral surface of intestinal epithelial cells only during PLE episodes (1, 2, 9, 10) followed by its reappearance when PLE resolves (2).

The combination of multiple factors contributing to PLE is most apparent in post-Fontan patients. The Fontan procedure was developed to separate the systemic and the pulmonary circulation in patients with congenital heart malformations (11). Although the surgery vastly improves survival, it often elevates central venous pressure (11). Venous hypertension in post-Fontan patients is thought to be associated with intestinal protein leakage with the increased pressure from the basolateral side pressing plasma proteins into the intestinal lumen (11, 12). Because venous pressure increases immediately after the intervention and remains elevated for the rest of the patient’s life, there is no good explanation why PLE develops in only 3–10% of the patients months to years after the surgery (3, 4). The most likely explanation is that genetic factors and Fontan-induced venous hypertension predispose for PLE, which precipitates upon a series of sequential or simultaneous environmental insults. Consistent with this hypothesis, seven out of eight post-Fontan patients have been diagnosed with viral infections at the onset of PLE symptoms (5), indicating that this additional insult triggered PLE. Jejunal biopsies, taken during episodes with PLE, revealed an increased IFNγ concentration (7), most likely as a response to the viral infection and elevated levels of the pro-inflammatory cytokine TNFα (6). Both cytokines are known to impair the integrity of the intestinal epithelial barrier (13–18).

Similar to other primary diseases associated with PLE, episodes of post-Fontan PLE are characterized by a loss of HSPG specifically from the basolateral surface of intestinal epithelial cells. HSPG expression in the lamina propria is normal (10).3 Overall intestinal architecture remains intact, and the expression of other matrix components is also normal (10). The reasons why HSPGs are lost during episodes of PLE are still unknown. Recently, we established the first in vitro model of PLE and proved a direct link between HS loss and protein leakage through a monolayer of HT29 cells, a human intestinal epithelial cell line. In addition, we showed that HS loss amplifies TNFα-induced protein leakage (19), providing the first reasonable explanation on how two of the factors associated with PLE onset synergize. Heparin compensates for the loss of cell-associated HS and abolishes the synergism between HS loss and TNFα (19). These results offer a potential explanation for the favorable response some PLE patients have to heparin treatment (20–23).

HS loss and elevated TNFα concentrations are just two factors thought to trigger PLE. Based on the strong correlation between PLE

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2 The abbreviations used are: PLE, protein-losing enteropathy; CDG, congenital disorder of glycosylation; HS, heparan sulfate; HSase, heparanase II; HSPG, HS proteoglycans; β-xylene, p-nitrophenyl-β-D-xylene; IFN, interferon; TNF, tumor necrosis factor; TNFR, TNF receptor; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycan.

3 S. Murch, unpublished data.
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onset and viral infections together with increased IFNγ concentrations, we hypothesized that IFNγ either directly or indirectly causes protein leakage. We further hypothesized that venous hypertension caused by the Fontan surgery predisposes for protein leakage. To test these hypotheses, we modified the in vitro model to mimic venous hypertension and assessed the individual and combined effects of increased pressure, HS loss, IFNγ, and TNFα on protein leakage. We find that HS is the central figure coordinating their synergistic effects.

MATERIALS AND METHODS

The human intestinal epithelial cell line HT29 (ATCC HTB-38) was grown in Dulbecco’s modified Eagle’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (HyClone, Logan, UT).

Albumin Flux—Cells were grown on a semipermeable membrane in Snapwell inserts (12-mm diameter, 3.0-µm pore size, Corning, NY) for 5 days until they reached confluence. Albumin flux was measured in cells treated with heparinase III (HSase), p-nitrophenyl-β-D-xylopyranoside (β-xyloside), heparin (Sigma), recombinant human IFNγ, or recombinant human TNFα (R&D, Minneapolis, MN) at final concentrations and time points as indicated for each experiment. Cells were washed twice with RPMI 1640 (with/without serum, with/without phenol red) (Invitrogen). The ring-supported membrane with the HT29 monolayer was mounted on a slider and inserted into the EasyMount Ussing Chamber System (Physiologic Instruments, San Diego, CA). Both sides of the HT29 monolayer were bathed in 4-ml Ringers solution (pH 7.4) gassed with 95% O₂, 5% CO₂. The chamber system was calibrated with a MRC 1024 SP Bio-Rad laser point scanning confocal microscope.

Confocal Microscopy—HT29 cells grown on the Snapwell membrane were fixed with paraformaldehyde and incubated with a mouse monoclonal antibody against dipeptidyl peptidase IV (clone 202–36, LabVision, Fremont CA). The secondary antibody was FITC-labeled (Sigma). Cell nuclei were stained with ToPro3 (Molecular Probes, Eugene, OR). The Snapwell membrane was carefully cut out of the ring support with a razor blade and placed on a microscope slide, and the cells were visualized with an MRC 1024 SP Bio-Rad laser point scanning confocal microscope.

Flow Cytometry—HT29 cells were harvested with EDTA and incubated with a mouse monoclonal antibody against TNFR1 (clone H-5, Santa Cruz Biotechnology, Santa Cruz, CA) or with an isotype-matched control antibody to determine nonspecific binding. The secondary antibody was FITC-labeled (Sigma). Flow cytometry was performed on a BD Biosciences FACSort and the CellQuest software. Data for 10,000 events were collected. Events were considered positive if their fluorescence intensity exceeded that of 98% of the isotype-matched control antibody. The median fluorescence intensity for TNFR1(FITC) in untreated controls is defined as 1.0. The Annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to quantitatively determine the percentage of cells undergoing apoptosis and cell death.

Statistical Analysis—Results are given as the means ± S.D. 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—Microscopic observation revealed that the HT29 cells had grown into a confluent monolayer 5 days after seeding on Snapwell inserts. We used confocal microscopy to determine monolayer polarity. The apical surface marker dipeptidyl peptidase IV was exclusively expressed on the cell membrane facing the media (data not shown), defining the apical surface. The cell membrane attached to the porous membrane of the Snapwell did not express dipeptidyl peptidase IV, therefore defining the basolateral surface. To prove that the untreated monolayer at day 5 provides a barrier to prevent basolateral-to-apical protein leakage, we added albumin-FITC to the basolateral side and determined albumin flux through the monolayer by measuring the concentration of albumin-FITC on the apical side over time. Although albumin-FITC concentrations on both sides of an empty insert (without cells) reached equilibrium in less than 4 h, the HT29 monolayer grown on the insert prevented protein leakage and allowed only 1.2% albumin-FITC to pass from the basolateral to the apical side even after 8 h. This percentage did not further decrease when cells were grown on the Snapwell inserts for more than 5 days, confirming that the cells reached confluence at day 5 (data not shown).

Effects of Individual Factors—HS loss, IFNγ, TNFα, and venous hypertension are thought to be involved in the molecular pathogenesis leading to protein leakage in patients with PLE. Our first aim was to evaluate the individual contributions of each of these four factors alone. To deplete cell-associated HS, we incubated the basolateral surface of the HT29 monolayer with HSase at 0.6 million units/ml for 1.5 h. We have reported earlier that these conditions cause maximum depletion of cell-associated HS (19). Here, albumin flux increased 2.6 ± 0.1-fold compared with untreated controls (p < 0.01) (Fig. 1A). Co-incubation with 100 µM β-xyloside, which competes out GAG chain synthesis on core proteins, did not further increase HSase-induced protein leakage (data not shown).

Incubating the cells with TNFα at 2 or 20 ng/ml for 12 h increased albumin flux 2.3 ± 0.4 (p < 0.01)- and 4.9 ± 0.5-fold (p < 0.001), respectively (Fig. 1A). These results with our Ussing chamber model are consistent with our previous data in a Transwell system showing that HS loss and TNFα cause protein leakage (19). IFNγ had no effect on protein leakage when applied in low (1 ng/ml) or moderate concentrations (10 ng/ml), but high concentrations (100 ng/ml) slightly increased albumin flux 1.5 ± 0.2-fold (p < 0.05) (Fig. 1A). TNFα or IFNγ at concentrations and incubation times used in these experiments did not alter the percentage of annexin V-positive cells or the uptake of propidium iodine (data not shown), and we concluded that neither TNFα nor IFNγ-induced protein leakage was caused by increased apoptosis or cell death.

To mimic venous hypertension, we raised the hydrostatic pressure on the basolateral side of the monolayer by 1.0, 2.5, and 5.0 mm H₂O. Albumin flux increased 4.1 ± 0.8-, 8.9 ± 1.1-, and 15.8 ± 0.7-fold, respectively (Fig. 1B). To assure that the hydrostatic pressure did not cause the cells to detach from the Snapwell membrane, we examined the monolayer by confocal microscopy after measuring albumin flux. The monolayer was still intact up to 5.0 mm H₂O, but the cells began to detach from the membrane at 10.0 mm H₂O (data not shown).

Effects of Paired Combinations; Synergy—After determining the effects on protein leakage for each of the four factors alone, we next did pairwise combinations. First, we combined HS loss and pressure since
we hypothesized that loss of HS from the basolateral surface compromises the integrity of the monolayer and makes the monolayer more susceptible to increased pressure. Results are shown in Fig. 2A. Adding the individual effects of HS loss and 2.5 mm H2O pressure, we predicted a 10.5-fold increase in albumin flux. In fact, we measured a 15.7-0.9-fold increase in albumin flux (p < 0.001), indicating that the combined effects of HS loss and increased pressure were not only additive, but synergistic.

Similarly, we hypothesized that TNFα also compromises the integrity of the monolayer and enhances the effects of increased pressure. We incubated the cells with TNFα (2 ng/ml) for 12 h, applied different amounts of hydrostatic pressure on the basolateral side, and determined protein leakage (Fig. 2B). The measured effects on albumin flux were higher than the predicted sum, suggesting synergistic effects of TNFα and increased pressure. In contrast, incubating the cells with IFNγ (10 ng/ml) instead of TNFα did not affect pressure-induced protein leakage (data not shown).

Next, we combined HS loss and TNFα. We incubated the cells with HSase for 1.5 h to digest cell-associated HS. At the same time, we added 100 μM β-xylol to inhibit GAG synthesis on newly synthesized core proteins and, thus, prevented the reappearance of cell-associated HS (19). Afterward, we incubated the cells with different concentrations of TNFα for 12 h and determined protein leakage. The effects of HS loss and TNFα were not only additive, but synergistic (Fig. 2C). For example, although TNFα (2 ng/ml) alone increased albumin flux 2.3 ± 0.4-fold and HS loss alone increased albumin flux 2.6 ± 0.1-fold, the combination of these two factors increased albumin flux 5.8 ± 0.9-fold (p < 0.01) instead of the predicted 2.9-fold. This observation is consistent with our previously reported data showing that HS depletion amplifies TNFα-induced protein leakage (19), especially at low cytokine concentrations.

We also combined HS loss with IFNγ. We either digested the cell-associated HS with HSase 1.5 h before IFNγ treatment or incubated the cells with IFNγ first and then digested with HSase. In both cases, IFNγ had no effect on albumin flux beyond that of HS loss alone (data not shown).

To combine the two cytokines, we preincubated the cells with IFNγ (10 ng/ml) for 12 h and then added TNFα (2 ng/ml) for another 12 h before measuring albumin flux. Although IFNγ alone had no effect on protein leakage and TNFα increased albumin flux only 2.3 ± 0.4-fold, incubation with IFNγ before the addition of TNFα increased albumin flux 4.4 ± 0.7-fold (p < 0.01), indicating that IFNγ amplifies TNFα-induced protein leakage (Fig. 2D). In summary, results from the two-factor systems revealed synergistic effects between HS loss and pressure, TNFα and pressure, HS loss and TNFα, and IFNγ and TNFα but not between HS loss and IFNγ or IFNγ and pressure.
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Effects of Triple Assaults; Enhanced Synergy—Next we tested combinations of three factors. Fig. 3A shows the results of combining HS loss, IFN\textgamma, and TNF\alpha treatments. We incubated the cells with IFN\textgamma (10 ng/ml) for 12 h and added TNF\alpha (2 ng/ml) for another 12 h. We depleted cell-associated HS before the addition of each cytokine. The individual effects of HS loss, IFN\textgamma, and TNF\alpha predict a 3.0-fold increase in albumin flux, but we actually measured a 11.2 ± 1.8-fold increase (p < 0.001).

Depleting cell-associated HS before the addition of IFN\textgamma but not before TNF\alpha still increased albumin flux 6.8 ± 0.8-fold (p < 0.01), which was higher than predicted but significantly lower compared with HS depletion before both the addition of IFN\textgamma and the addition of TNF\alpha (p < 0.01). We also depleted cell-associated HS before TNF\alpha addition but not before IFN\textgamma addition, which increased albumin flux 8.1 ± 0.9-fold (p < 0.01). This effect was again higher than predicted but significantly lower than the effect of HS depletion before both IFN\textgamma and TNF\alpha addition (p < 0.05). These results show that HS loss amplified the effects of both IFN\textgamma and TNF\alpha.

To investigate the combined effects of HS loss, TNF\alpha, and pressure, we depleted cell-associated HS, incubated the cells with TNF\alpha (2 ng/ml) for 12 h, and applied different amounts of hydrostatic pressure (Fig. 3B). With 2.5 mm H$_2$O pressure, the predicted increase is 11.8-fold, but we measured a 16.5 ± 0.8-fold increase in albumin flux.

Finally, we measured the combined effects of IFN\textgamma, TNF\alpha, and pressure. We incubated the cells with IFN\textgamma for 12 h and with TNF\alpha for another 12 h, applied different amounts of hydrostatic pressure, and measured albumin flux (Fig. 3C). Instead of the predicted 10.3-fold increase in albumin flux at 2.5 mm H$_2$O pressure, we measured a 14.9 ± 1.4-fold increase (p < 0.001).

Four Factors Produce Maximum Synergy—Finally, we combined all four factors. We depleted cell-associated HS from the basolateral surface, incubated the cells with IFN\textgamma and TNF\alpha, applied different amounts of hydrostatic pressure, and determined protein leakage (Fig. 4). Although the addition of the individual effects of the four factors predicted a 7.1-fold increase in albumin flux for a hydrostatic pressure of 1.0 mm H$_2$O and a 11.9-fold increase in albumin flux for 2.5 mm H$_2$O, we actually measured a 18.7 ± 2.1- and 26.3 ± 1.4-fold increase, respectively. Notably, the effect of only 1.0 mm H$_2$O pressure in combination with the other three factors (18.7 ± 2.1-fold) is higher than the effect of 5.0 mm H$_2$O pressure alone (15.8 ± 0.7-fold). In fact, combining a hydrostatic pressure of 5.0 mm H$_2$O with the other three factors caused the cells to detach from the Snapwell membrane, which pressure alone or in combination with HS loss or TNF\alpha cannot do. These results indicate that the combined effects of all four factors were highly synergistic.
incubated the HT29 monolayer with IFNγ (10 ng/ml) for 12 and 24 h, harvested the cells, and determined TNFR1 expression by fluorescence-activated cell sorter analysis (Fig. 5A). Incubating the cells with IFNγ for 12 h increased TNFR1 expression 1.9-fold compared with untreated controls. In parallel, TNFα-induced albumin flux increased 2.0-fold when cells were preincubated with IFNγ 12 h before the addition of TNFα (Fig. 5B).

Because IFNγ binds to and may be inactivated by HS (25, 26), we further hypothesized that loss of cell-associated HS increases the amount of active IFNγ and amplifies IFNγ-mediated up-regulation of TNFR1 expression. To test this hypothesis, we digested cell-associated HS with Hsase, washed the cells, and incubated them with IFNγ for 12 or 24 h. That treatment up-regulated TNFR1 expression 2.7-fold (Fig. 5A) and, in parallel, increased TNFα-induced protein leakage 3.1-fold (Fig. 5B). These results support our hypothesis that IFNγ up-regulates TNFR1 expression and, therefore, enhances TNFα-induced protein leakage, which is further amplified in the absence of cell-associated HS. This provides a mechanistic explanation on how HS loss, IFNγ, and TNFα synergize.

**Heparin Abolishes Synergism between HS Loss, IFNγ, and TNFα**—In our previous study we showed that soluble HS and heparin compensate for the loss of cell-associated HS and abolish synergism between HS loss and TNFα (19). We now asked whether heparin also abolishes the synergistic effects between IFNγ and TNFα based on the report that binding to heparin inactivates IFNγ (25, 26). To answer these questions, we incubated the cells with IFNγ (10 ng/ml) for 12 h, washed them, added TNFα (2 ng/ml) for another 12 h, and measured albumin flux. We depleted cell-associated HS 1.5 h before both IFNγ addition and TNFα addition (Fig. 6A). This combination of HS loss, IFNγ, and TNFα increased albumin flux 11.4 ± 1.3-fold. To determine the effect of heparin on IFNγ, we used the same incubation scheme as before but co-incubated IFNγ with heparin (2.5 μg/ml). Now, albumin flux was increased only 8.5 ± 1.3-fold, which was significantly lower than the effects without heparin (p < 0.05). Similarly, co-incubating TNFα with heparin also reduced albumin flux, which confirmed our previous results. Finally, we co-incubated both IFNγ and TNFα with heparin, and albumin flux increased only 3.7 ± 0.9-fold. Notably, albumin flux after co-incubation of both IFNγ and TNFα with heparin in the absence of cell-associated HS is not significantly different from albumin flux after incubation with IFNγ and TNFα without heparin but in the presence of HS (4.4 ± 0.7-fold increase). Heparin at 10-fold higher concentrations reduced albumin flux even below these levels (only a 2.6 ± 0.3-fold increase). These results indicate that a low concentration of heparin fully compensates for HS loss. At a higher concentration heparin even quenches more cytokine activity than cell-associated HS would. However, heparin had no effect on pressure-induced protein leakage (Fig. 6B), suggesting that these soluble glycans cannot compensate for the mechanical barrier function of cell-associated HS.

**Albumin Flux Is Bidirectional**—So far we only determined the effects on basolateral-to-apical protein leakage. However, we hypothesized that the interventions not only cause leakage in one direction but impair barrier function in general and also induce apical-to-basolateral leakage. To test this hypothesis, we repeated key experiments and now added albumin-FITC to the apical side and determined apical-to-basolateral albumin flux by measuring the concentration of albumin-FITC on the basolateral side. There was no difference between basolateral-to-apical and apical-to-basolateral albumin flux in any of the interventions tested (Fig. 7, A and B).

**DISCUSSION**

The Fontan procedure vastly improves survival of patients born with univentricular hearts but elevates venous pressure (11). We hypothesized that venous hypertension is one of the key players involved in the patho-mechanisms underlying PLE as a late complication of the Fontan surgery. To mimic venous hypertension in vitro, we established a tissue culture model of PLE using modified Ussing chambers that allow us to apply hydrostatic pressure to the basolateral surface of intestinal epithelial cells. The Fontan procedure elevates right atrial pressure by several mm Hg (mean, 10.9 mm Hg; normal, <3–5 mm Hg) (12), but the exact pressure in subepithelial capillaries is hard to assess. In our in vitro model, an increased hydrostatic pressure in the magnitude of a few mm H2O (9/3 mm Hg) already causes significant protein leakage (Fig. 1B). The cells even begin to detach from the Snapwell membrane at 10.0 mm H2O pressure, which is less than 1 mm Hg. The epithelial monolayer grown on the semipermeable Snapwell membrane in our in vitro model lacks a stable extracellular matrix and the context of subepithelial cells, which makes it more susceptible to increased pressure and limits the ability to directly translate in vivo venous pressure to the amounts of hydrostatic pressure applied in our in vitro model. However, results with mouse mucosal explants stripped of seromuscular layers show that increasing basolateral pressure to 5.0 mm H2O causes similar protein leakage ex vivo compared with our in vitro model (27).

Venous pressure is elevated immediately after the Fontan procedure, and post-Fontan patients already show a subtle increase in enteric protein loss determined by fecal α1-antitrypsin concentrations (12). Heart transplantation decreases pressure and normalizes protein loss (28), indicating a direct correlation between increased pressure and protein leakage. However, PLE becomes manifest only months to years after the surgery (3, 4), suggesting that multiple sequential or simultaneous factors finally trigger PLE. We now identify increased pressure together with HS loss, IFNγ, and TNFα as
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key players in the pathogenesis of post-Fontan PLE, evaluate their individual contributions and their combined effects, and provide mechanistic explanations on how they synergize.

We confirm previous results (19) showing that both HS loss and TNFα alone increase protein leakage (Fig. 1A) and that they act synergistically (Fig. 2C). In addition, we show that HS loss and TNFα make the HT29 monolayer more susceptible to increased pressure (Figs. 2, A and B, and 3B), presumably through different mechanisms. While cell-associated HS connects the cell to the extracellular matrix and to neighboring cells (29), TNFα regulates tight junctions (30) and shedding of membrane proteins that connect to the extracellular matrix (31). Results from our previous study in HT29 cells show that TNFα-induced protein leakage is mediated by tyrosine kinases and proteinase A and partially involves matrix metalloproteases (19). HS loss and TNFα may, therefore, impair the mechanical integrity of the monolayer and increase its susceptibility to increased pressure.

Onset of post-Fontan PLE is often associated with viral infections (5) and one of the predominant cytokines responding to viral infections is IFNγ, which is indeed elevated in post-Fontan PLE patients (7). Incubating T84 cells, another human intestinal epithelial cell line, with IFNγ (100 ng/ml) reduces their barrier function after 72 h but not after 24 or 48 h (18, 32). Although we did not test the long term effects of IFNγ, the results after 24 h of incubation are consistent with our observations in HT29 cells where IFNγ induced only minor albumin flux (Fig. 1A). However, in concert with the other three factors IFNγ is highly deleterious in our model. IFNγ enhances the expression of TNFR1 (Fig. 5A) to the same extent as it increases TNFα-induced protein leakage (Fig. 5B). IFNγ-mediated up-regulation of TNFR1 provides a reasonable explanation for the mechanisms of IFNγ and TNFα synergy.

Loss of cell-associated HS further amplifies the effects of both IFNγ and TNFα (Fig. 3A). HS binds to both IFNγ (25, 33) and TNFα (34, 35). In most cases, cytokines as well as growth factors employ cell-associated HS to enhance their signal, as previously shown for TNFα (34). In contrast, we report that binding to HS seems to lower the concentration of available TNFα as well as of IFNγ, whereas loss of cell-associated HS enhances their activities. Why cell-associated HS inactivates IFNγ in some cases but activates it in others is unknown. We speculate that these cell- and tissue-specific differences stem from the differential expression (36, 37) and Golgi organization (38) of HS-modifying enzymes such as sulfotransferases or sulfatases, which generate specific HS epitopes that bind cytokines and change their conformation to an active or inactive state (25).

The loss of cell-associated HS can be fully compensated by the addi-
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4. L. Bode, unpublished observations.

4 these mice are more susceptible to viral infections, inflammation, and increased venous pressure and lead to PLE.

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