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Involvement of Heparanase in Empyema: Implication for Novel Therapeutic Approaches

Moshe Lapidot[1], Uri Barash[2], Yaniv Zohar[3], Yuval Geffen[4], Inna Naroditsky[3], Neta Ilan[2], Lael Anson Best[1] and Israel Vlodavsky[2*]

[1]*Department of General Thoracic Surgery, Rambam Health Care Campus , Haifa, Israel*

[2]*Cancer and Vascular Biology Research Center, Rappaport Faculty of Medicine, Technion, Haifa, Israel*

[3]*Department of Pathology, Rambam Health Care Campus , Haifa, Israel*

[4]*Department of Microbiology, Rambam Health Care Campus , Haifa, Israel*

[*]**Corresponding author:** Israel Vlodavsky, Cancer and Vascular Biology Research Center, Rappaport Faculty of Medicine, Technion, P.O. Box 9649, Haifa 31096, Israel, Tel: 972-4-8295410; Fax: 972-4-8510445; E-mail: vlodavsk@mail.huji.ac.il

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Abstract

Pleural empyema is an inflammatory condition that progresses from acute to chronic, life-threatening, phase. The incidence of empyema has been increasing both in children and adults worldwide in the past decades, mainly in healthy young adults and in older patients. Despite continued advances in the management of this condition, morbidity and mortality have essentially remained static over the past decade. Better understanding of the disease and the development of new therapeutic approaches are thus critically needed. Heparanase is an endoglucuronidase that cleaves heparan sulfate chains of proteoglycans. These macromolecules are most abounded in the sub-endothelial and sub-epithelial basement membranes and their cleavage by heparanase leads to disassembly of the extracellular matrix that becomes more susceptible to extravasation and dissemination of metastatic and immune cells. Here, we provide evidence that heparanase expression and activity are markedly increased in empyema and pleural fluids, associating with disease progression. Similarly, heparanase expression is increased in a mouse model of empyema initiated by intranasal inoculation of S. pneumonia. Applying this model we show that transgenic mice over expressing heparanase are more resistant to the infection and survive longer.

Keywords: Heparanase; Empyema; Chronic inflammation; Transgenic mice

Introduction

Pleural empyema remains a significant medical problem due to substantial morbidity, prolonged hospitalization, and increased risk of death [1]. Invasion of the pleural space by pathogenic microorganisms initiates a cascade of orderly events that start with the recognition of the pathogen and lead to either resolution of the insult with restoration of the normal mesothelial barrier, or to pleural destruction and fibrosis. The development of inflammatory process in the pleural space may result in increased pleural vascular permeability, leading to the accumulation of fluid enriched in proteins, and the recruitment of cells into the pleural space [2].

Exudates due to parapneumonic pneumonia and empyema are characterized by neutrophilic pleocytosis, decreased pH, and increased lactate dehydrogenase (LDH) levels. Moreover, in contrast to transudates, exudates due to parapneumonic effusions and empyema show increased procoagulant and repressed fibrolytic activities, thus favoring fibrin deposition in the pleural space. As leucocytes begin to counteract bacterial infection, dying bacteria and leucocytes release more bacterial compounds, proteases, proteins and other factors, leading to greater inflammation [3]. However, since bacteria and bacterial degradation products cannot be drained, as in pneumonia, the infection-associated inflammation prevails and may further increase even under appropriate antibiotic treatment. Thus, early and sufficient drainage of pleural empyema is important in interrupting the vicious cycle and preventing long-term sequel. If therapeutic intervention is not initiated at this stage, transition to chronic empyema occurs, which involves proliferation of fibroblasts and deposition of fibrin [4]. Fibrosis eventually forms inelastic membranes promoting both lung entrapment and restriction of movement of the chest wall and diaphragm, leading to a shrunken, immobile hemithorax with crowding of the ribs and scoliosis [5].

Heparanase is an endo-β-glucuronidase that selectively cleaves heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPG) presumably at sites of low sulfation, releasing saccharide products with appreciable size (4-7 kDa) that can still associate with protein ligands and facilitate their biological potency. Enzymatic degradation of HS leads to disassembly of the extracellular matrix (ECM) and is therefore involved in fundamental biological phenomena related to tissue remodeling and cell invasion such as tumor metastasis, angiogenesis and inflammation by enhancing the egress of migrating cells from the blood stream [6-9].

A role of heparanase in empyema has not been so far investigated. Here, we provide evidence that heparanase expression and activity are markedly increased in empyema and pleural fluids, associating with disease progression. Similarly, heparanase expression is increased in a mouse model of empyema initiated by intranasal inoculation of S. pneumonia. Applying this model we show that transgenic mice over expressing heparanase are more resistant to the infection and survive longer.

Materials and Methods

Experimental design

Empyema fluids (chest-tube drainage) were freshly collected from forty patients that were diagnosed in the Department of General Thoracic Surgery, Rambam Health Care Campus, Haifa, Israel, and hospitalized due to empyema. Fluids were centrifuged (300g, 10 min), and the supernatants and cell pellets were recovered and evaluated for heparanase enzymatic activity and the levels of pro-inflammatory cytokines. The study also included archival specimens obtained from 46 patients with empyema (acute or chronic phases) for which paraffin blocks and clinical records were available. The clinical data of all patients was reviewed and correlated with heparanase activity levels and immunostaining intensity. The study protocol was approved by the Institutional Review Board.

Heparanase immunostaining

Staining of formalin-fixed, paraffin-embedded 5 micron sections for heparanase was performed essentially as described [10,11]. Briefly, sections of empyema specimens were deparaffinized, rehydrated and endogenous peroxidase activity was quenched (30 min) by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling (20 min) in 10 mM citrate buffer, pH 6. Slides were incubated with 10% normal goat serum (NGS) in phosphate buffered saline (PBS) for 60 min to block nonspecific binding and incubated (20h, 4°C) with anti heparanase 733 antibody diluted 1:100 in blocking solution. Antibody 733 was raised in rabbits against a 15 amino acid peptide (KKFKNSTYSRSSVDC) that maps at the C-terminus of the 50 kDa heparanase subunit, and preferentially recognizes the 50kDa active heparanase subunit vs. the 65 kDa latent pro-enzyme [11]. Slides were extensively washed with PBS containing 0.01% Triton X-100 and incubated with a secondary reagent (Envision kit) according to the manufacturer's (Dako, Glostrup, Denmark) instructions. Following additional washes, color was developed with the AEC reagent (Dako), sections were counterstained with hematoxylin and mounted, as described [11]. Immunostained specimens were examined by senior pathologist who was blind to clinical data of the patients, and were scored according to the intensity of staining (1-very weak, 2-weak; 3-moderate; 4-strong). Specimens that were similarly stained with pre-immune serum, or applying the above procedure but lacking the primary antibody, yielded no detectable staining. Immunofluorescent double staining of heparanase and macrophages applying anti-heparanase and anti-CD163 antibodies was carried out essentially as described [12,13].

Heparanase activity

Preparation of $Na_2{}^{35}SO_4$-labeled ECM-coated 35-mm dishes and determination of heparanase activity were performed essentially as described in detail elsewhere [11,14,15]. Briefly, freshly collected empyema fluids were centrifuged (10 min, 300 g) for 10 minutes and the resulting cell pellet and cleared supernatants were recovered. Cells (2×10^6) were lysed in phosphate/citrate buffer (pH 5.2) by three cycles of freeze/thaw. The cell lysates and the empyema fluid supernatants were incubated (18 h, 37°C) with 35S-labeled ECM. The incubation medium (1 ml) containing sulfate-labeled degradation fragments, was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity was counted in a β-scintillation counter. Degradation fragments of HS side chains produced by heparanase are eluted at 0.5<Kav<0.8 (peak II, fractions 12–22). Nearly intact HSPGs released from the ECM are eluted just after the Vo (Kav<0.2, peak I, fractions 3-12) [11,14,15]. These high molecular weight products are released by proteases that cleave the HSPG core protein.

Mouse model of empyema

Heparanase transgenic (Hpa-Tg) mice carrying human heparanase under the beta actin promoter have been described [16,17]. Hpa-Tg mice were generated as mixed genetic background (C57Bl/6 × Balb/C) and were crossed for 10 generations with Balb/C mice to produce pure genetic background [18], thus eliminating the mistrust often associated with mixed genetic background. We utilized established protocols for the initiation of pleural empyema in mice [19,20]. Briefly, mice were inoculated intra-nasally with 2×10^8 CFU of S. pneumonia (strain D39). The existence of the pathogen in the pleural cavity was approved by microbiologic examination. Control mice were inoculated with equal volume of saline. Mice were sacrificed three days after inoculation and pleural fluids were collected and cleared by centrifugation. The supernatant was frozen at -80°C for subsequent determination of cytokine (i.e., IL-8, TNFα) levels by ELISA (R&D systems). The cell pellet was quantified for cell type and number by FACS analysis; Lung tissue was harvested, fixed, embedded in paraffin and subjected to pathological evaluation and immunohistochemical analysis.

Statistical analysis

Univariate association between heparanase parameters (activity; intensity of staining) and empyema stage were analyzed using Chi Square tests (Pearson, Fisher exact test). Multivariable logistic regression was performed to detect independent parameters that may affect patients` status and to estimate relevant Odd's ratio (OR) with 95% confidence interval (CI). Univariate association with survival and cause specific survival was evaluated by Kaplan Meier curves, and tested using Log-Rank test.

Results

Heparanase expression is increased in empyema patients

In order to examine the possible involvement of heparanase in pleural empyema we subjected archival paraffin sections from the acute and chronic phases of the disease to immunostaining applying anti-heparanase antibody. Specimens from forty-six patients were included (27 males and 19 females), ages 0.8-59 years (median-23.7, average-24.5). Eighteen patients (39%) were diagnosed with acute empyema, 15 patients (38%) were diagnosed with chronic empyema and 13 patients (27%) exhibited characteristics of chronic and acute empyema. Most cases (43/46; 93%) resulted from pneumonia. Positive staining for heparanase was noted in 90% of the cases, but the intensity of staining appeared stronger in the chronic (Figures 1B-1D) vs. acute (Figure 1A) phase. Thus, while all cases that were scored as negative staining (0) were diagnosed with acute empyema, cases with strong heparanase staining (+4) were mainly diagnosed with more advanced, chronic empyema (Figure1E). The association between heparanase staining intensity and the progression of empyema from the acute to the more severe chronic phase of the disease is statistically highly significant (p=0.007).

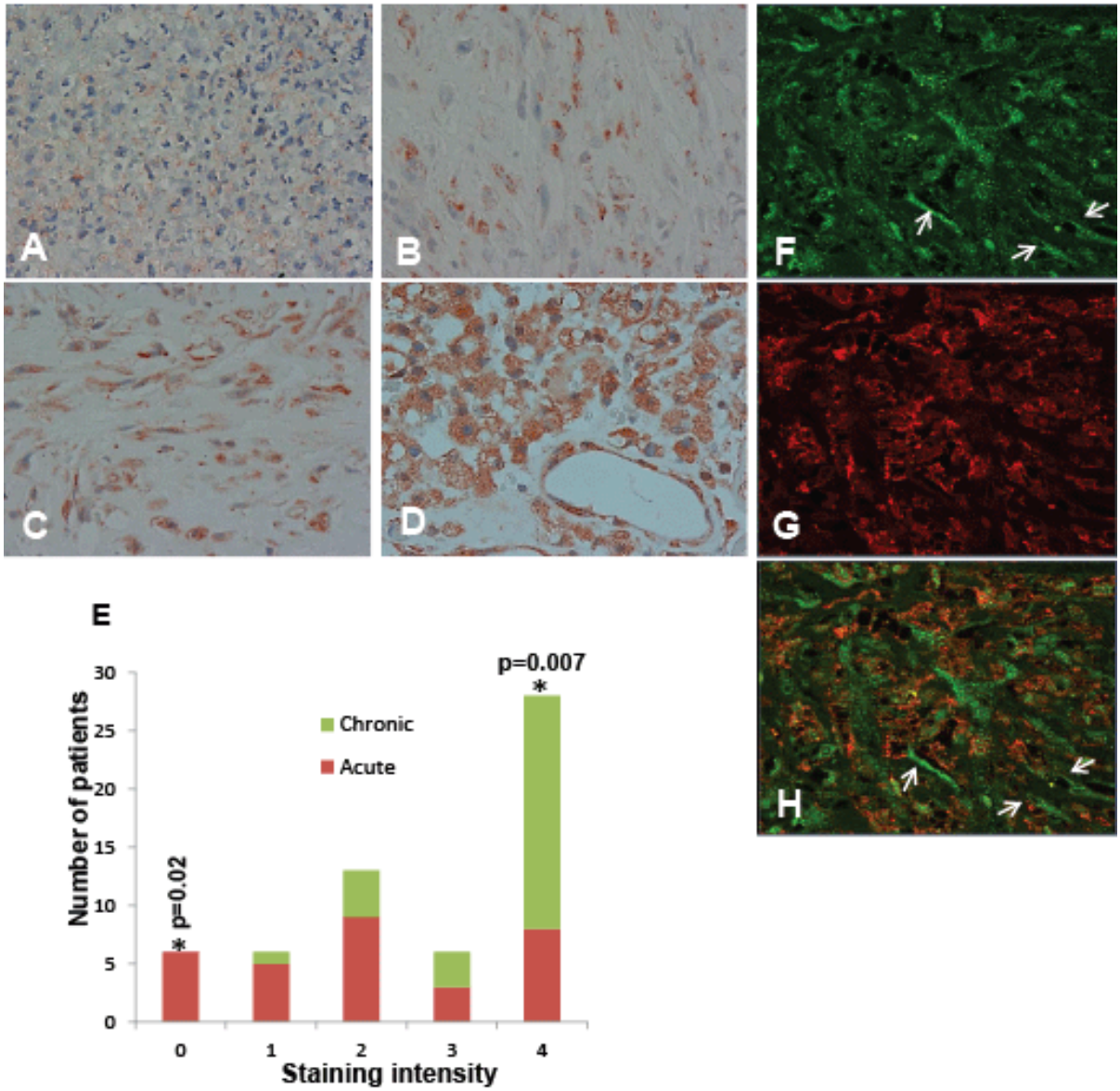

Figure 1: Heparanase elevation in pleural empyema. A-D: Immunostaining. Specimens from 46 empyema patients were subjected to immunostaining applying anti-heparanase antibody. Shown are representative photomicrographs of biopsies exhibiting very weak (+1; A), weak (+2; B), moderate (+3; C) and strong (+4; D) staining of heparanase. Very weak staining (+1) was most often observed in acute empyema while chronic empyema is associated with higher levels of heparanase (B-D). This association is shown graphically in (E). Original magnification: A-D x40. F-H: Immunofluorescent staining. Specimens of chronic empyema were subjected to immunofluorescent staining applying anti heparanase (F) and anti CD163 (human macrophage marker; G) antibodies. Merged image is shown in (H). (arrows). Shown are representative photomicrographs; Original magnification: x40. Note that heparanase staining is cytoplasmic whereas CD163 labels a membrane determinant, and that heparanase also labels CD163-negative cells that are suspected (by morphology) to be endothelial cells lining lumen-containing structures (arrows).

Pathological examination revealed that neutrophils are the main immune cells populating acute empyema lesions whereas macrophages and endothelial cells that populate chronic empyema (Figures 1B-1D) are stained positively for heparanase (Figure 1D). Immunofluorescent staining confirmed the abundant presence of macrophages in chronic empyema (Figure 1G; CD163) which are stained positive for heparanase (Figure 1F). Staining of heparanase is also observed in elongated, lumen-containing CD163-negative structures, most likely blood vessels (Figures 1F and 1H), in agreement with previous reports showing heparanase expression by these cell types [21-23].

Enhanced heparanase enzymatic activity in freshly collected empyema fluids and cells

In order to substantiate the association between heparanase levels and the progression of empyema, we evaluated heparanase activity in pleural fluids freshly collected (chest-tube drainage) from 40 empyema patients. The demographic and clinical characteristics of the patients are shown in Table 1. Pneumonia was the most common etiology for pleural fluid accumulation (35 patients; 87.5%), 3 patients (7.5%) were infected by empyema due to abdominal surgical fistula through the diaphragm, and 2 patients (5%) developed empyema after thoracic trauma. 23 patients (57.5%) demonstrated acute and chronic empyema, 10 (25%) had typical parapneumonic effusion and 7 (17.5%) had more advanced stages of parapneumonic effusion, in accordance to light criteria (Table 2).

Study Cohort	Number	% of Total
Gender		
Male	27	67.5
Female	13	32.5
Age		
<18	10	25
>18	30	75
Etiology		
Parapneumonic	35	87.5
Effusion		
Surgery	3	7.5
Trauma	2	5

Table 1: Demographic and clinical description of empyema patients subjected to chest-tube drainage.

Stage of Disease	Number of cases (%)	Heparanase activity in pleural fluids (% of cases)	Heparanase activity in cells (% of cases)
Typical	10 (25)	40	50
Simple complicated	4 (10)	75	67
Complex complicated	3 (7.5)	100	70
Acute+chronic empyema	23 (57.5)	95	100

Table 2: Heparanase activity correlates with empyema progression.

Following centrifugation of the fluids, samples of the cell pellet and the clear supernatant were applied onto sulfate labelled ECM and the release of HS degradation fragments by heparanase was quantified as described under 'Materials and Methods'. Heparanase activity was significantly higher in the pleural fluids (Figure 2A) and cells (Figure 2B) collected from patients with chronic vs. acute phase empyema, in agreement with the immunostaining results (Figures 1A-1E). The increase in heparanase activity as empyema progresses (disease stage 1 vs. 4) is statistically highly significant (p=0.0001 and p=0.001 for activity in the pleural fluids and cell pellet, respectively; Table 2). Furthermore, the increase in heparanase activity was associated with elevated levels of pro-inflammatory cytokines such as TNFα (p=0.03) and IL-8 (p=0.02) in the same patient samples.

Mouse model of empyema

In order to further explore the role of heparanase in empyema we established an in vivo model system. Intranasal inoculation of *S. pneumonia* into mice resulted in severe pneumonia followed by pleural empyema. Histological examination revealed typical strong inflammatory reaction in the lung (Figures 3A and 3B) and pleural space (Figures 3C and 3D) that is stained positive for heparanase (Figures 3E and 3F). We have next utilized this mouse model to reveal empyema severity in transgenic mice over expressing heparanase (Hpa-Tg; n=9; Figure 4B, left lower panel) vs. wild type Balb/C mice (Con; n=8; Figure 4B, left upper panel). Notably, survival of Hpa-Tg mice was significantly improved; only 2 out of 9 (22%) Hpa-Tg mice died 10 days after the inoculation of *S. pneumonia* compared with 6 out of 8 (75%) similarly treated wild type mice (Figure 4A), differences that are statistically significant (p=0.018). Importantly, while neutrophils were recruited to the lungs of wild type and Hpa-Tg mice to a comparable extent (Figure 4B, middle panels), inflammation in the pleural space took place only in the wild type mice (Figure 4B, right panels). This may suggest that heparanase, once present at high levels before the onset of the inflammatory insult decreases its severity. However, heparanase may exert the opposite effect once induced in the course of the inflammatory reaction and disease progression.

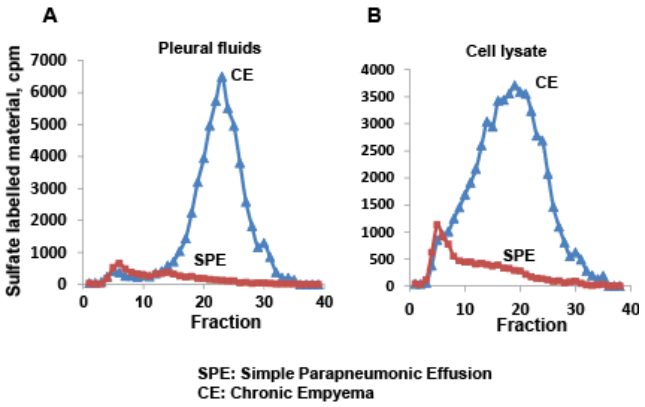

Figure 2: Heparanase activity. Fluids were freshly collected from chronic empyema (CE) patients or patients with simple paraneumonic effusion (SPE); Fluids were centrifuged (300 g, 10 min), and the supernatants and cell pellets were recovered and evaluated for heparanase enzymatic activity as described under 'Materials and Methods'. Shown are typical heparanase activities (i.e., release of HS degradation fragments from sulfate labeled ECM) in the clear fluids (A) and cell lysates (B).

Discussion

In analogy to the mobilization of metastatic cancer cells, remodeling of the ECM by heparanase is thought to facilitate transmigration of inflammatory cells towards the infected site [8,24]. In line with this notion, heparanase up-regulation was observed in different inflammatory conditions [25-28] and is thought to promote

inflammation. Indeed, heparanase gene silencing resulted in decreased delayed-type hypersensitivity reaction [25], and heparanase knockout mice showed reduced airway and acute lung injury responses in models of allergy and sepsis [29,30]. Furthermore, transgenic mice over expressing heparanase are endowed with increased colon (colitis) and skin (psoriasis-like) inflammation [26,31], collectively implying that heparanase is an important player in the inflammatory reaction [32-35]. The results presented here indicate that heparanase is also involved in the pathogenesis of pleural empyema, an inflammatory condition that progresses from acute to chronic, life-threatening phase. Notably, heparanase expression and activity are markedly increased in patients with chronic vs. acute pleural empyema (Figures 1 and 2) and in a mouse model of empyema (Figure 3). In empyema patients, heparanase elevation was associated with increased TNFα and IL-8 levels. The association between heparanase and TNFα has been observed previously in a number of studies, exhibiting a self-feeding loop in which heparanase enhances TNFα expression which in turn up-regulates heparanase gene transcription [21,26,30,31,36]. Elevated levels of TNFα further recruit and activate inflammatory cells such as neutrophils and macrophages [21], and amplify the inflammatory condition that may progress to tumor initiation [26], diabetic nephropathy [37] and atherosclerosis [21]. An association between the levels of heparanase and IL-8 has not been so far reported, and is joining an increasing number of cytokines being connected with heparanase levels [38].

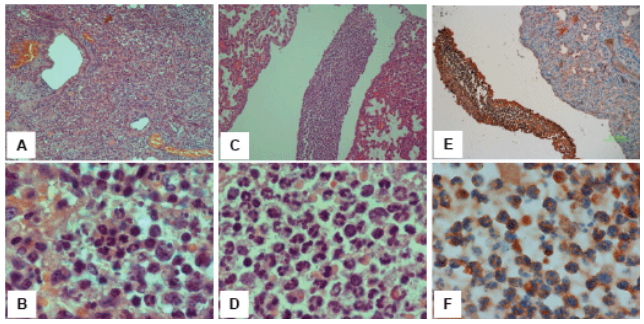

Figure 3: Mouse model of empyema. Mice were inoculated intranasally with 2×10^8 CFU of *S. pneumonia* (strain D39). Control mice were inoculated with equal volume of saline. Mice were sacrificed 3 days after inoculation and pleural fluid was collected and cleared by centrifugation; Lung tissue was harvested, fixed, embedded in paraffin and subjected to pathological evaluation and immunohistochemical analysis. Shown are representative H&E staining of the inflamed lung (A, B), and pleural space (C, D). Inflammatory cells in the pleural space are stained positive for heparanase (E, F). Original magnification: A, C, E x10; B, D, F x100.

In some experimental settings, however, heparanase was noted to inhibit, rather than promote, inflammation. For example, heparanase was shown to have a protective effect in models of sepsis, graft versus host disease, and experimental autoimmune encephalomyelitis (EAE) once administrated before applying the insult [39-41]. Moreover, in models of hyperalgesia and neuroinflammation, recruitment and activation of neutrophils was attenuated in transgenic mice that constitutively over express heparanase [42,43]. This was reportedly due to cleavage of HS on the endothelial cell surface and the resulting disruption of chemokine gradient(s) which is critical for directional migration of immune cells and infection resolution [44]. Over expression of heparanase in Hpa-Tg mice results in structurally modified and significantly shorter HS side chains endowed with reduced ability for ligand binding [17,44]. Consequently, neutrophils crawling toward chemokine-releasing gel was absent in Hpa-Tg mice; Instead, Hpa-Tg neutrophils exhibited random crawling, ultimately leading to severely reduced ability to clear bacterial infection [44].

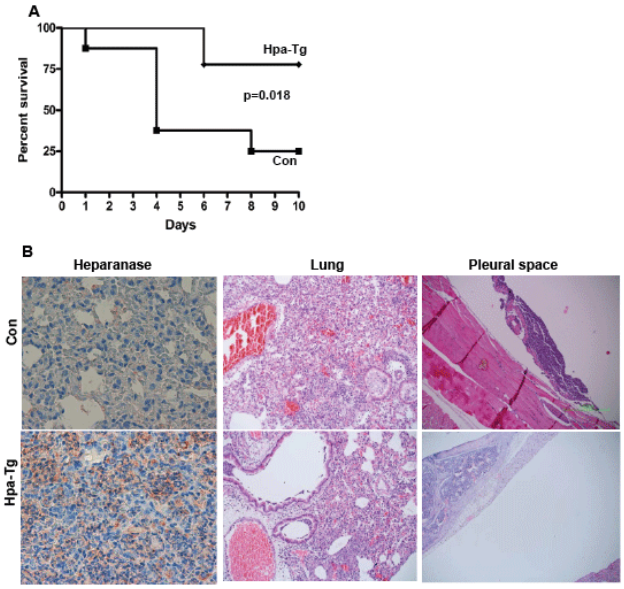

Figure 4: Heparanase over-expressing transgenic mice exhibit prolonged survival following induction of empyema. Control Balb/C (Con) and heparanase transgenic (Hpa-Tg) mice were inoculated with 2×10^8 CFU of *S. pneumonia* bacteria and survival of the mice was recorded (A). Lung tissue was collected from surviving mice and postmortem, fixed in formalin and embedded in paraffin. Shown are representative H&E staining of lung specimens (B, middle panels) and pleural space (B, right panels). Specimens were also subjected to immunostaining applying anti-heparanase antibody (left panels), depicting over expression of heparanase in lung tissue of Hpa-Tg mice. Note the lack of inflammation in the pleural space and prolonged survival of Hpa-Tg vs. control wild type mice. Original magnification: left panels x40; middle and right panels x10.

The absence of neutrophils in the pleural space of Hpa-Tg mice exposed to empyema and their prolonged survival (Figure 4) support the occurrence of a similar anti-inflammatory protective mechanism in this model. Thus, the net effect of heparanase on the recruitment of immune cells is balanced by the removal of glycocalyx, enabling acute immune cells adhesion to the vascular endothelium on one hand [30], and the disturbance of chemokine gradients at the endothelial cell surface on the other hand [30,32,44].

While the above examples illustrates the complexity and duality of heparanase function in inflammatory conditions, it should also be kept in mind that administration of heparanase or its expression at high levels prior to the immunogenic insult does not mimic the clinical onset of a disease. Increased heparanase levels in the course of human empyema, Crohn's disease and ulcerative colitis [10] and arthritis [28],

and the reduced severity of sepsis [30] and diabetic nephropathy [45] in heparanase knockout mice confer, among other results, more confidence that heparanase does play a role in inflammation and autoimmunity, and that heparanase inhibitors may prove beneficial in these conditions. The ability of the heparanase inhibitor PG545 to restrain the mobilization of macrophages to pancreatic and skin tumors [18,46] provides hope that this and others heparanase inhibitors will restrain the expanding variety of inflammation-based disorders [37,47]. Clearly, more research is critically required to validate this aspect.

Acknowledgments

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Conflict of Interest

The authors confirm that there are no conflicts of interest.

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