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RESEARCH ARTICLE

Heparanase prevents the development of type 1 diabetes in non-obese diabetic mice by regulating T-cell activation and cytokines production

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Abstract

Background Heparanase is an endo-β-D-glucuronidase that cleaves heparan sulfate saccharide chains. The enzyme promotes cell adhesion, migration and invasion, and was shown to play a significant role in cancer metastasis and angiogenesis.

Methods The present study focuses on the involvement of heparanase in autoimmunity, applying the murine non-obese diabetic (NOD) model, a T-cell-dependent disease often used to investigate the pathophysiology of type 1 diabetes.

Results It was found that intra-peritoneal administration of heparanase ameliorated the clinical signs of the disease. *In vitro* studies revealed that heparanase has an inhibitory effect on the activation of T-cells through modulation of their repertoire of cytokines indicated by a marked increase in the levels of IL-4 and IL-10, and a parallel decrease in IL-12, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ).

Conclusions We suggest that heparanase induces a shift from a Th1- to Th2-phenotype, resulting in inhibition of diabetes in NOD mice and possibly other autoimmune disorders. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords heparanase; non-obese diabetes; T-cells activation; cytokines

Introduction

Heparan sulfate proteoglycans(HSPGs) are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of cells [1–3]. The basic Heparan sulfate proteoglycan structure consists of a protein core to which several linear heparan sulfate (HS) chains are covalently O-linked [1]. HS chains, unique in their ability to bind a multitude of proteins, ensure that a wide variety of bioactive molecules bind to the cell surface and ECM and thereby, function in the control of diverse normal and pathological processes [1–3]. The majority of studies on cell interaction with the microenvironment focused, among other approaches, on proteolytic enzymes [4]. The involvement of glycosaminoglycan- (e.g. heparan sulfate) degrading enzymes (e.g. heparanase) was underestimated, primarily due to a lack of appropriate molecular probes to explore their causative role in cell-ECM interactions and related effects. A long-term research on the biology of the heparanase enzyme led to the cloning of a single gene encoding a HS-degrading endoglycosidase (heparanase), [5–7] which plays important roles in cancer metastasis, angiogenesis and inflammation [8–14].

Heparanase is synthesized as a 65-kDa non-active precursor that subsequently undergoes proteolytic cleavage, yielding 8- and 50-kDa protein sub-units that heterodimerize to form an active enzyme [15,16]. The enzyme has been identified in invasive normal and malignant cells, including activated cells of the immune system, cytotrophoblasts, keratinocytes, lymphoma, melanoma and carcinoma cells [8,11,12,14,17,18].

Extravasation of circulating hematopoietic and immune cells is accompanied by degradation of various components of the sub-endothelial ECM. Activated immune cells produce and secrete a variety of ECM-degrading enzymes, including heparanase [17,19]. Degradation of HS disintegrates the supramolecular structure of the sub-endothelial basal lamina, consequently facilitating trans-endothelial migration of neutrophils and activated lymphocytes, thereby mediating their extravasation during immune responses [17,20].

Autoimmune diseases are caused by failure of self-tolerance and subsequent immune responses against autologous antigens [21]. This process is an active dynamic state in which potentially pathogenic autoreactive cells are prevented from causing damage by regulatory mechanisms [22]. Type 1 diabetes mellitus results from autoimmune destruction of islet β-cells in the pancreas mediated by CD4+ and CD8+ T-cells. More specifically, type 1 diabetes-causing CD4+ and CD8+ T-cells are activated by antigens released from pancreatic islet cells that contain β-cells and initially presented in the pancreatic lymph nodes [23]. The disease process begins with infiltration of mononuclear cells around the islets and proceeds with destruction of insulin-producing β-cells to the point that an exogenous supply of insulin becomes mandatory to sustain life [24].

The non-obese diabetic (NOD) mouse is the best-characterized model for type 1 diabetes in humans [25]. It has become apparent that the destructive effector mechanisms of many systemic autoimmune diseases, including type 1 diabetes, are mediated by autoantigen-specific CD4+ T-cells. Controversy exists regarding the exact contribution of the different sub-types, Th1 and Th2 CD4+ T-cells to the destructive process. Diabetogenic CD4+ T-cell clones usually produce Th1 cytokines [25,26] and many Th1 cells derived from islet infiltration of NOD mice and reactive to insulin can transfer diabetes [27]. On the other hand, T-cells expressing Th2 phenotype may play a suppressive role in diabetes [28,29]. This phenotype includes cytokines like IL-4, IL-5 and IL-10. Moreover, pancreatic expression of IL-4 completely prevents diabetes in NOD mice [30], and treatment with IL-4 induces a switch from Th1-type to a Th2-type response and prevents the inflammatory arthritis in the respective model [31]. Another cytokine IL-10, included in the Th2 repertoire, has also been shown to have an anti-inflammatory role in a couple of autoimmune disease models [32,33]. In contrast, some immune modulation protocols report that diabetes protection may be attributed to preferential survival of both Th1 and Th2 cells [34,35]. Moreover, recent reports show that autoimmune processes are composed not only of autoaggressive T-cell responses but also of autoreactive regulatory components, and that IL-10 can be derived from Th1 cells as well [36–38].

Although the pathways are complex, there is convincing evidence that anti-inflammatory cytokines play essential roles in regulating the development of chronic Th1-mediated autoimmune responses in animals [39]. *In vitro*, IL-4 and IL-10 mediate their functions by inducing expression of the IL-1R antagonist [40,41], and down-regulate the production of IL-1 and tumour necrosis factor-alfa (TNF-α), known inflammatory cytokines, from monocytes [42].

Facing the challenge of type 1 diabetes and its pathogenesis, and the known and possible roles of heparanase in inflammation and immune responses, we decided to elucidate the potential involvement of heparanase in this autoimmune process.

Methods and materials

Mice

Female NOD/Ltj, 6- to 12-week-old mice weighing 20 g were obtained from the Jackson Laboratory or Harlan Laboratories. The animals were fed Purina chow and acidified water (pH 2.7) ad libitum. They were maintained in a specific pathogen free (SPF) animal facility at 21 °C with a 12 h cycling of light. All procedures were conducted using facilities and protocols approved by the Animal Care and Use Committee of the Hadassah-Hebrew University School of Medicine.

Experimental design

Female NOD 6-week-old mice were injected i.p. with several doses of heparanase. The control group received injections of saline only. Diabetes was monitored by testing urinary glucose with a test strip (Medi-Test, Combi 9, Macherey-Nagel, Duren, Germany) once or twice weekly and was considered positive after the appearance of glucosuria in at least two determinations. The intraperitoneal glucose tolerance test (IPGTT) was performed as follows: blood was drawn from the paraorbital plexus at 0 min and 60 min. after an i.p. injection of glucose (1 g/kg body weight). Plasma glucose levels were determined (as glucose mmole/L) with a Glucose Analyser 2 (Becknan Instruments). A glucose level above 15 mmole/L at the 60 min. time point was considered a positive IPGTT.

Heparanase

Active recombinant human heparanase was produced in insect cells and purified as described [16]. The construct encoding the 8- and 50-kDa heparanase sub-units was

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kindly provided by Dr E. McKenzie (Oxford Glycoscience Ltd., UK). Recombinant 65-kDa latent human heparanase was purified from the culture medium of heparanase-transfected HEK-293 cells, as described [43]. Experiments were done both with the active and the latent forms of the heparanase.

Histology

Tissue samples were fixed with 4% formaldehyde, embedded in paraffin and sectioned (5 μm). Sections were stained with hematoxyline and eosine, as described [44]. The sections were screened and scored by two uninformed observers according to the following criteria:

Score 0 – No cell infiltration.
Score 1 - infiltrates in small foci at the islet periphery.
Score 2 - infiltrates surrounding the islets (peri-insulitis).
Score 3 - intraislet infiltration <50% of the islet, without islet derangement.
Score 4 - extensive infiltration, = 50% of the islet, cell destruction and prominent cytoarchitectural derangement.
Score 5 - islet atrophy because of β-cell loss.

The sections were also stained for insulin and heparanase.

Immunohistochemistry

Immunohistochemistry was performed as previously described with minor modifications [44]. Briefly, 5 μm sections were deparaffinized and rehydrated. The tissue was then denatured for 3 min in a microwave oven in citrate buffer (0.01 M, pH 6.0). Blocking steps included successive incubations in 3% H_2O_2 in methanol and 5% goat serum. Tissue sections were incubated with anti-human insulin antibodies or with Dulbecco's modified eagle's medium (DMEM)-supplemented with 3.3% horse serum as control, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (Jackson Laboratories). Colour was developed using Zymed AEC substrate kit (Zymed, South San-Francisco, CA) for 10 min, followed by counter-staining with Mayer's hematoxylin [44].

T-cell activation

Concavalin A (ConA)
Mouse spleen cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% heat-inactivated human AB serum in flat-bottomed 96-well microtiter plates (Nunc) containing 0.5×10^6 cells/well/0.2 mL. Responses to 2 μg/mL concanvalin-A (Sigma, St. Louis, MO) were assessed by ^{3}H-thymidine incorporation, as described [45].

Mixed lymphocyte culture (MLC)
Mouse spleen cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated human AB serum in 96-well flat-bottomed microtiter plates (0.2 mL well, Nunc) containing 1×10^6 responding cells and 1×10^6 irradiated (3000 cGy) stimulating cells per well. Cells were cultured for 5 days in 5% CO_2 in air, in a humidified incubator. Twenty hours before harvesting, 1 μCi ^{3}H-thymidine was added to each well and thymidine incorporation was measured as described [45].

Cytokine assay

In vivo *assay*
NOD mice were treated with saline or recombinant heparanase as described above. Peritoneal macrophages were then harvested, and aliquots of the culture medium were subjected to ELISA analysis of IL-4, IL-10, IL-12, interferon-gamma (IFN-γ) and TNF-α, as described [46].

In vitro *assay*
Blood was drawn from the paraorbital plexus at 12, 18 and 20 weeks of age. Spleen lymphocytes ($2.5–5.0 \times 10^6$ cells/mL) cultured in 75 mL flasks were stimulated for 24 h with rIL-2 (6000 IU/mL) in the absence and presence of heparanase in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The culture supernatant was then subjected to ELISA analysis of IL-4, IL-10, IL-12, IFN-γ and TNF-α, as described [46].

Statistics

Student's *t*-test was used for statistical analysis of the results.

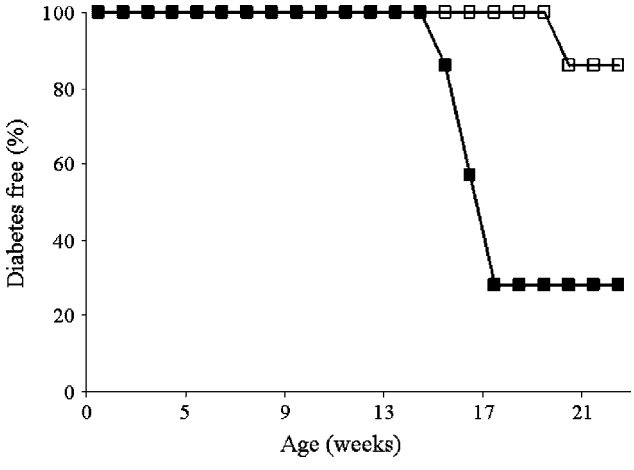

Figure 1. Effect of heparanase on clinical signs of diabetes. Female, NOD mice (n = 8 mice per group) were taken at 6 weeks of age. Heparanase was administered (i.p. 35 μg/day) twice weekly, for 8 weeks only. At the age of 12 weeks, clinical signs of diabetes were first observed in the control group receiving phosphate buffered saline (PBS) alone (■), and continued thereafter. In contrast, there were no signs of diabetes in the heparanase-treated mice (□)

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Results

Effect of heparanase on the development of diabetes in NOD mice

Treated mice were injected i.p. with heparanase 35 μg/mice/day twice weekly for 8 weeks. Age of the treated mice at the time of starting the treatment was 6 weeks old. Control group mice were the same age and were injected with normal saline for the same period of time. In control mice, overt glucosuria was detected at an age of 12–13 weeks, resembling the development of clinically signed diabetes. In contrast, a significant decline in the number of animals developing diabetic symptoms was observed in NOD mice that were treated with heparanase (Figure 1). This effect was sustained until the end of the experiment at an age of 22 weeks ($p < 0.003$).

Histopathology of pancreases from treated and untreated mice

Pancreatic tissue samples were taken and treated as described above. Hematoxyline/eosine staining revealed accumulation of the immune mononuclear cells in a crescent-like appearance at the edges of the islets, in sections obtained from heparanase-treated mice (Figure 2a). On the contrary, sections from control mice showed massive infiltration by inflammatory cells all over the islets (Figure 2b). In order to confirm the results, we stained both sections for the presence of insulin using specific anti-insulin antibody. Significant reduction in the evidence of islets and in the presence of insulin was detected in the sections obtained from control mice (Figure 2c and, f), in comparison with sections from the heparanase-treated mice. Many areas positive for insulin were detected in those sections, demonstrating almost a normal distribution of islets (Figure 2d–f). Applying the insulitis scoring system, it was demonstrated that heparanase-treated mice had a significantly lower score (3.38, $n = 41$) than the control group mice (1.07, $n = 41$), with $p < 0.0001$.

Regulatory mechanisms

Activation of T lymphocytes

Autoimmune diseases are characterized mainly by an attack of host tissues and organs by host T-cells. In the

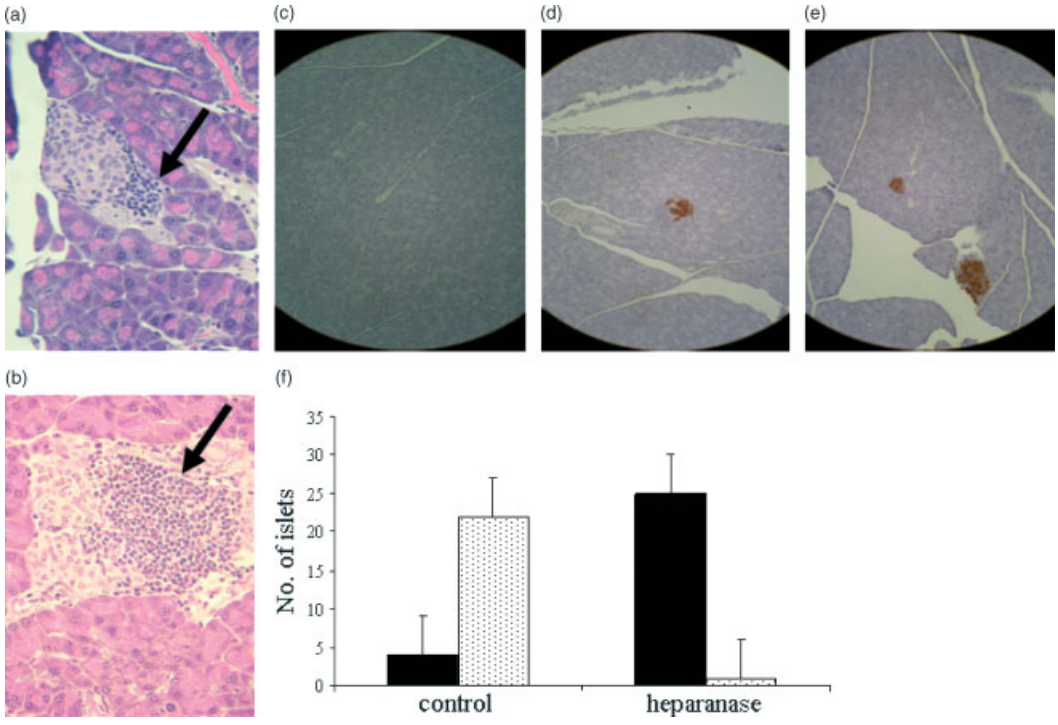

Figure 2. (a)–(b) Lymphocyte infiltration in islets of control *versus* heparanase-treated mice. Paraffin-embedded sections of pancreatic tissue were taken from heparanase (a) and control (b) treated mice, at the age of 16–20 weeks. The sections were stained with hematoxyline and eosine. A crescent-like appearance of mononuclear cells at the edges of the islets can be seen in the heparanase-treated section (black arrow, figure a), while massive infiltration by those cells are observed in sections obtained from control mice (black arrow, figure (b)) (c)–(e) Specific staining for insulin in pancreases from control *versus* heparanase-treated mice. Paraffin-embedded sections of pancreatic tissue were taken from control (c) and heparanase (d)–(e)-treated mice, at the age of 16–20 weeks. Significant reduction in positive staining for insulin was seen in the control mice as compared to many positive areas documented in the heparanase-treated mice, demonstrating the protective effect of heparanase against the autoimmune process (f) Number of destroyed/normal islets in pancreases from control *versus* heparanase-treated mice. β-islets were counted in heparanase-treated *versus* non-treated NOD mice. A significant elevation in the number of normal islets (■) and concomitant significant reduction in the number of the destroyed islets (▒) were observed in the pancreases obtained from heparanase-treated NOD mice as compared to the control non-treated NOD mice. This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

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case of NOD mice, $CD4^+$ cells are being polarized towards the Th1 phenotype and hence, mediate the inflammatory process through which diabetes occurs. To elucidate the involvement of heparanase in this process, we investigated its effect on isolated T-cells, emphasizing their pattern of cytokine production.

NOD mice-derived spleen cells were cultured (12 h, 37 °C) without or with increasing amounts of the heparanase (1 μg/mL and 5 μg/mL), and subjected to ConA-induced cell proliferation. As demonstrated in Figure 3a, addition of heparanase to the culture medium resulted in a significant, dose-dependent decrease in Con-A activation of the spleen cells ($p < 0.001$).

A similar decrease in ConA activation was obtained in the absence or presence of the heparanase inhibitor ST1514 (Figure 3b), suggesting that heparanase enzymatic activity is not required for its inhibition of T-cell activation. In order to verify this aspect, we utilized heparanase in which glutamic acid residues 225 and 343 that comprise the enzyme active site [47] were point mutated, yielding an inactive enzyme [48]. As shown in Figure 3b, the inactive enzyme inhibited the activation of T-cells by ConA to an extent comparable in magnitude to that of active (8 + 50 kDa) heparanase ($p < 0.002$), implying that enzymatic activity is not required for heparanase-mediated inhibition of ConA-induced T-cell activation.

In a subsequent experiment, spleen cells were taken from BALB/c and C57BL/6 mice. The cells were then activated against each other in one-way mixed lymphocyte culture (MLC) in the absence or presence of 5 μg/mL heparanase. As shown in Figure 3c, heparanase markedly inhibited the activation of spleen cells obtained from BALB/c mice reacting against C57BL/6 mice ($p < 0.001$).

Th1 and Th2 phenotype analysis

Effect of heparanase on cytokine production in vitro. CD4+ T-cells with polarization towards the Th1 phenotype exhibit accelerated autoimmune activity against components of the pancreas causing the symptoms observed

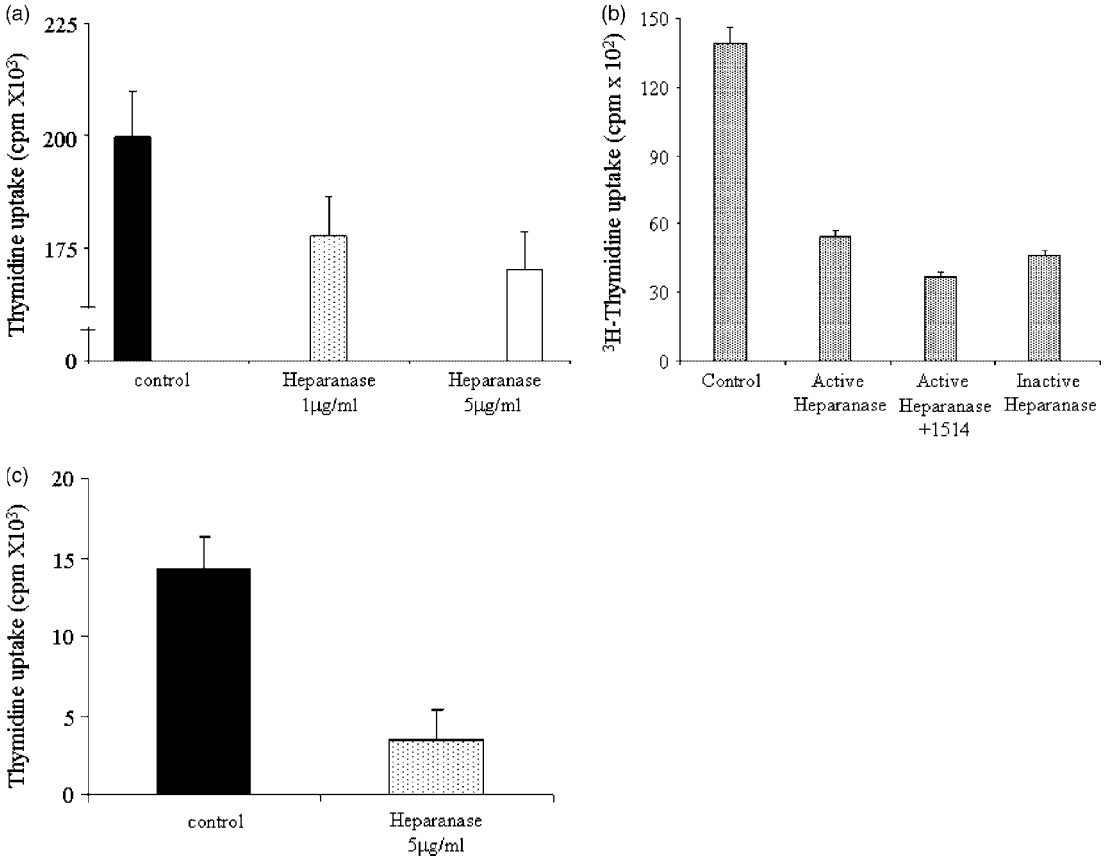

Figure 3. (a) Effect of heparanase on ConA activation of T-cells. Mouse spleen cells were isolated and subjected to activation with ConA in the absence (■) and presence of 1 (▒) and 5 (□) μg/mL heparanase, followed by measurements of ^{3}H- thymidine incorporation, as described in 'Materials and methods'. Thymidine incorporation was inhibited in response to heparanase in a dose-dependent manner. Each data point is the mean ± SD of triplicate wells (b) Reduced activation of T-cells is independent of heparanase enzymatic activity. Mouse spleen cells were isolated and subjected to activation with ConA in the absence (control) and presence of active heparanase, active heparanase plus glycol-split heparin (100 μg/mL, compound 1514), or inactive heparanase (point mutated in glutamic residues 225 and 343). Thymidine incorporation was inhibited to a similar effect in response to active heparanase, active heparanase + its inhibitor (compound 1514) or inactive heparanase (c) Effect of heparanase on MLC. Left bar: control MLC; Right bar: MLC in the presence of 5 μg/mL heparanase. Mixed lymphocyte culture (MLC) in the absence or presence of heparanase was performed as described in 'Materials and methods'. Significant reduction in the activity of BALB/c-derived lymphocytes against C57Bl-derived lymphocytes was documented in the heparanase-treated culture

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in the NOD mice model [26]. Increasing the cytokine profile characteristic of Th2 cells, and in parallel, decrease in the amount of Th1-type cytokines, ameliorated the signs and symptoms of diabetes [28,29]. The development of Th1 or Th2 cells from naïve CD4+ T-cells is determined by the cytokine milieu during the initial phase of the immune response. IL-12 has a well-known role in driving differentiation of uncommitted T-cells towards a Th1 phenotype [49]. Conversely, IL-4 and IL-10 are known to be produced by Th2 cells [50].

Medium of NOD mice-derived spleen lymphocytes incubated with or without heparanase *in vitro* as described above was subjected to ELISA analysis of IL-4, IL-10, IL-12, TNF-α and IFN-γ. As demonstrated in Figure 4a, the amount of the secreted Th2-type cytokine IL-4 was increased significantly by more than three-fold, ($p < 0.001$) and IL-10 (Figure 4a) was increased significantly by almost 100% ($p < 0.004$), following exposure to heparanase. In contrast, there was a marked decrease (almost three-fold) in the amount of IL-12 ($p < 0.005$), representing a Th1- associated cytokine, in cells that were treated *in vitro* with heparanase for 24 h (Figure 4b).

Cytokines analysis in macrophages derived from NOD mice treated with heparanase in vivo. In the same manner as described for T lymphocytes, but *in vivo*, we evaluated the cytokine repertoire obtained from macrophages taken from mice that were treated with i.p. injections of heparanase in comparison to untreated mice. The results confirm and augment those that were achieved *in vitro*. The amount of the secreted Th2-type cytokine IL-4 was increased significantly by more than three-fold ($p < 0.002$) (Figure 4c), and IL-10 (Figure 4d) was increased significantly by more than 60% ($p < 0.002$), following exposure to heparanase *in vivo*. In contrast, there was a marked decrease (six-fold) in the amount of IL-12 ($p < 0.03$), representing a Th1- associated cytokine, in cells that were derived from heparanase-treated mice (Figure 4d).

Tumour necrosis factor-alpha (TNF-α) and Interferon-gamma (IFN-γ). The main function of TNF-α is to stimulate inflammation by turning on gene transcription through the IKK/NFkappaB and JNK/AP-1 signalling cascades [51]. Neutralization of TNF-α is therefore, applied to suppress a broad spectrum of inflammatory

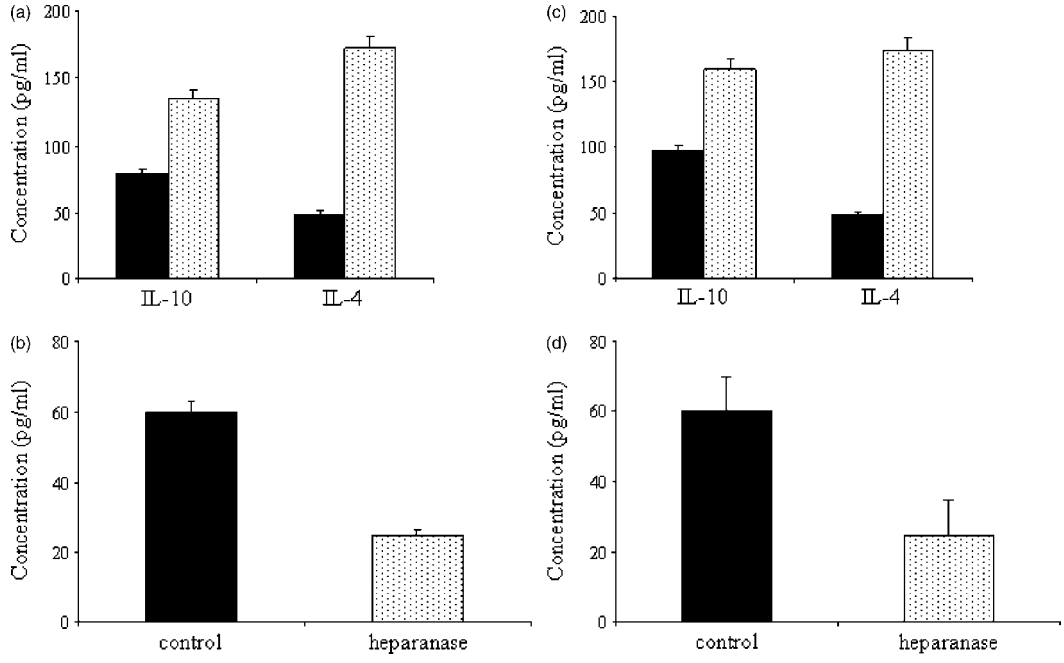

Figure 4. (a) Effect of heparanase on the level of Th2-type cytokines secreted by activated T-cells *in vitro*. Medium from NOD mice-derived spleen lymphocytes, isolated and incubated (24 h, 37 °C) with (▒) or without (■) heparanase, was analysed by ELISA for the amounts of IL-4 and IL-10, representing the Th2 repertoire of cytokines. A marked increase in the levels of IL-4 and IL-10 was noted when heparanase was included in the incubation medium. Each data point is the mean ± SD of triplicate wells (b) Effect of heparanase on the level of IL-12 secreted by activated T-cells *in vitro*. Medium from NOD mice spleen lymphocytes that were incubated (24 h, 37 °C) with (▒) or without (■) heparanase, was analysed for IL-12, representing a Th1 associated cytokine. A marked decrease (two-fold) in the level of IL-12 was noted when heparanase was included in the incubation medium. Bars represent mean ± SD of triplicate determinations (c) Effect of *in vivo* heparanase treatment on the level of Th2-type cytokines secreted by activated macrophages. NOD mice were subjected to a daily injection of active (8 + 50 kDa) heparanase (3 days, 5 μg/mouse/day) (▒) or saline (■) (control). Peritoneal macrophages were then harvested, and aliquots of the culture medium were subjected to ELISA analysis of IL-4, IL-10 and IL-12. A marked increase in the levels of macrophages-derived IL-4 and IL-10 was noted when heparanase was i.p. injected. Each data point is the mean ± SD of triplicate wells (d) Effect of *in vivo* heparanase treatment on the level of IL-12 secreted by activated macrophages. NOD mice were subjected to a daily injection of active (8 + 50 kDa) heparanase (3 days, 5 μg/mouse/day) (▒) or saline (■) (control). Peritoneal macrophages were then harvested, and aliquots of the culture medium were subjected to ELISA analysis of IL-12. A marked decrease (more than two-fold) in the level of IL-12 was noted when heparanase (30 μg/mL) was i.p. injected. Bars represent mean ± SD of triplicate determinations

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autoimmune diseases [52]. Likewise, IFN-γ is a known pro-inflammatory agent produced by Th1 cells [53,54]. We investigated the effect of *in vitro* heparanase treatment on the levels of TNF-α and IFN-γ secreted by T-cells, and its *in vivo* effect on the levels of TNF-α and IFN-γ secreted by macrophages taken from mice that were treated with heparanase injections. The amounts of TNF-α, produced by splenic lymphocytes (*in vitro* assay) (Figure 5a) and by macrophages (*in vivo* assay) (Figure 5b) and determined by ELISA, were three- to four-fold lower in cells that were exposed to heparanase, either *in vitro* (lymphocytes) ($p < 0.001$) or *in vivo* (macrophages) ($p < 0.002$), as compared to non-exposed cells. Evaluation of the IFN-γ response to heparanase revealed results in the same manner. The levels of IFN-γ were reduced by ~three-fold both *in vitro* (lymphocyte assay) ($p < 0.003$) (Figure 5a) and *in vivo* (macrophages assay) ($p < 0.001$) (Figure 5b). Thus, *in vivo* and *in vitro* treatments with heparanase revealed the same effect in terms of TNF-α and IFN-γ levels. Both caused significant reduction in the amount of these cytokines, resembling once more the anti-inflammatory influence of heparanase.

Discussion

As we recently discovered, heparanase plays a protective role in a mice model of multiple sclerosis – experimental autoimmune encephalitis (EAE) (Bitan *et al*. In submission). In order to evaluate whether this effect is unique to EAE or it is rather more generalized in autoimmune processes, we investigated, here, its function in another autoimmune model – the NOD mice model for diabetes. Heparanase prevented the development of diabetic symptoms almost completely as compared to the control untreated group. This phenomenon was even more dramatic than in the EAE model, since we injected the heparanase only twice weekly and not every day. Moreover, though the injections last for only 8 weeks, the protective effect continued until the end of the experiment, 8 weeks later, suggesting an irreversible mechanism. Histopathological sections revealed accumulation of the immune mononuclear cells in a crescent-like appearance at the edges of the islets. This is contrary to massive infiltration by inflammatory cells in sections obtained from control mice. In a recent study, we have demonstrated that the enzyme elicits a firm cell adhesion [47–52,55] and survival signals (i.e. Akt phosphotylation) [56], independent of its enzymatic activity, suggesting a possible involvement of heparanase in cell-ECM interaction and signal transduction. Moreover, it has been demonstrated that the enzymatic activity of heparanase is optimal in an acidic environment and as the pH goes up, the enzyme loses its activity. In normal body pH, heparanase is almost not active enzymatically, and in higher pH this phenomenon is even non-reversible. Therefore, it may be reasonable to assume that at normal pH, as it is in normal pancreatic tissue before the onset of symptoms, the influence that was found did not operate through the enzymatic pathway, but rather through another domain of the enzyme, involving, maybe, the adhesion-signalling influence, and causing the immune cells to be adherent and accumulate at the edges of the involved islets. Specific staining for insulin revealed an almost complete removal of insulin and β-cells in the control untreated mice. On the contrary, heparanase-treated mice showed a normal distribution of insulin β-cells and islets of pancrease, thus confirming the protective role of heparanase against the autoimmune process.

The pathogenesis of most autoimmune diseases is mediated mainly through effector T-cells penetrating the affected tissue and causing a specific damage [57]. To better understand the mode of action of heparanase, we investigated its effect on T-cells *in vitro*. Applying the ConA and MLC lymphocyte activation assays, we demonstrated a direct inhibitory effect of heparanase, either active (8 + 50 kDa) or latent (65 kDa) on T-cell

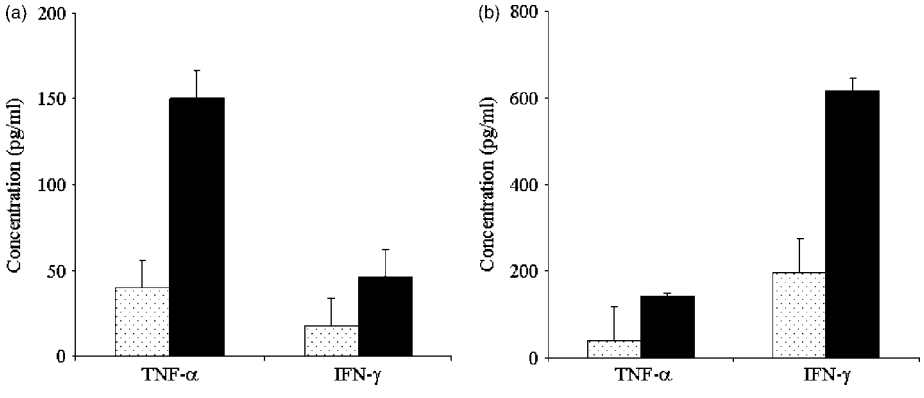

Figure 5. (a) Effect of heparanase on TNF-α and IFN-γ secretion *in vitro*. TNF-α and IFN-γ levels in the medium of ConA-activated NOD mice spleen lymphocytes incubated with (▒) or without (■) 30 μg/mL 65-kDa heparanase were determined by ELISA. A marked decrease in TNF-α and IFN-γ was noted in the presence of heparanase. Bars represent mean ± SD of triplicate determinations (b) Effect of *in vivo* heparanase treatment on TNF-α and IFN-γ secretion by macrophages. NOD mice were subjected to a daily injection of active (8 + 50 kDa) heparanase (3 days, 5 μg/mouse/day) (▒) or saline (■) (control). Peritoneal macrophages were then harvested, and aliquots of the culture medium were subjected to ELISA analysis of TNF-α and IFN-γ. A marked decrease in TNF-α and IFN-γ was noted when heparanase was i.p. injected. Bars represent mean ± SD of triplicate determinations

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activation and proliferation. Moreover, T-cell activation by ConA was inhibited also by an inactive enzyme in which the active site proton donor (Glu-225) and nucleophil (Glu-343) were replaced by inert residues, further supporting the notion that heparanase enzymatic activity is not involved in its ability to ameliorate the clinical signs of diabetes. ConA and MLC are non-specific immune responses and may not reflect the exact type of immune response that is activated in the heparanase-induced immune protection in the NOD mice model. Interestingly, applying a more specific immune reaction like delayed-type-hypersensitivity (DTH) revealed an enzymatic activity of heparanase in this inflammatory-immune process, promoting the inflammation [58]. Thus, it cannot explain the method of protection by heparanase seen in our study since it is suggested to be caused by a non-enzymatic process. Further studies will have to explore the exact method by which heparanase defend β-islands from the autoimmune destruction. Future study may also evaluate the specific T-cell sub-group, which is influenced by heparanase.

In addition to T-cells inactivation, stimulated T-cells were shifted towards increased production of IL-4, and IL-10, and a concomitant decrease in IL-12 in the medium of the activated cells. This cytokine-production shifting of lymphocytes is known to be associated with ameliorating of disease signs in the NOD mice model [28,29]. Thus, we confirmed the key mechanism by which heparanase inhibits the harmful activity of T-cells not only in the EAE model, but also in the NOD mice model, making this phenomenon more generalized in the autoimmune processes. Interestingly, pancreatic expression of IL-4 completely prevents diabetes in NOD mice [30], and treatment with IL-4 induces a switch from Th1-type to a Th2-type response and prevents inflammatory arthritis in the respective model [31]. Nevertheless, in some protozoal infections, IL-10 was shown to be produced and released by Th1 cells rather than by Th2 cells [37,38], demonstrating the complexity of the immune response in the diverse aspects of inflammation. As we have demonstrated here, heparanase increases the production of IL-4. Therefore, it may be assumed that the influence of heparanase can be achieved by one or more pathways. This could be either a direct effect on the process, or by increasing the IL-4 levels by which the diabetes prevention cascade occurs.

In conclusion, we demonstrated a protective effect of heparanase against the emerging of the autoimmune-type of diabetes. This study widens the perspective of influences of heparanase in the spectrum of autoimmune diseases, making it more generalized though affected by the same mechanisms. Evaluating the role of a newly-characterized heparan-sulfate-degrading enzyme in such fundamental processes as we elucidated here, will shed light on mechanisms underlying autoimmunity towards a better understanding and design of innovative potential future strategies.

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Conflict of interest

None declared.

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