

Heparanase affects secretory granule homeostasis of murine mast cells through degrading heparin

Bo Wang, MD, PhD,^{a*} Juan Jia, MD, PhD,^{a*} Xiao Zhang, MD, PhD,^b Eyal Zcharia, PhD,^c Israel Vlodavsky, PhD,^c Gunnar Pejler, PhD,^d and Jin-Ping Li, MD, PhD^a Uppsala, Sweden, and Haifa, Israel

Background: Heparanase degradation of heparan sulfate plays important roles in a number of pathological processes, including inflammation. *In vitro* experiments show that heparanase is capable of degrading heparin, a polysaccharide present in mast cells (MCs), in which it has a key role in promoting the storage of secretory granule compounds.

Objective: We sought to investigate the functions of heparanase in MCs.

Methods: Primarily cultured fetal skin-derived mast cells (FSMCs) isolated from embryos and adult peritoneal MCs were analyzed for storage and release of granule molecules in response to MC activation.

Results: FSMCs from heparanase-overexpressing mice contained substantially shorter heparin chains and significantly less proteases than control cells. Conversely, FSMCs lacking heparanase contained heparin of larger size and more proteases than control cells. Correspondingly, heparanase-overexpressing adult MCs exhibited reduced release of heparin-bound proteases, a finding that could be attributed to spontaneous release of granular compounds. Heparanase was found to be upregulated in MCs on activation.

Conclusion: These findings reveal a novel function of heparanase in maintaining MC homeostasis through controlled degradation of heparin present in the MC secretory granules. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Mast cell, heparin, heparanase, protease, allergy

Mast cells (MCs) are found in most connective tissues and are characterized by their high content of secretory granules. In addition to their recognition as critical effector cells in allergic disorders and other IgE-dependent immune responses, recently,

Abbreviations used

CPA:	Carboxypeptidase A
CTMC:	Connective tissue-type mast cell
DAPI:	4',6-Diamidino-2-phenylindole
DNP:	2,4-Dinitrophenol
FACS:	Fluorescence-activated cell sorting
FITC:	Fluorescein isothiocyanate
FSMC:	Fetal skin-derived mast cell
GAG:	Glycosaminoglycan
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
<i>hpa</i> -KO:	Heparanase knockout
<i>hpa</i> -tg:	Heparanase transgenic
HS:	Heparan sulfate
MC:	Mast cell
MMC:	Mucosal mast cell
mMCP:	Murine mast cell protease
PMA:	Phorbol 12-myristate 13-acetate

MCs have been strongly implicated in autoimmune disease. Activation of MCs can trigger degranulation, resulting in release of various preformed compounds that are stored in the granules, including histamine, cytokines, heparin, and a range of MC-specific proteases.^{1,2} Rodent MCs are classified as being of either mucosal mast cells (MMCs) or connective tissue-type mast cells (CTMCs). MMCs are mainly located on mucosal surfaces, whereas CTMCs are present in, for example, the skin and peritoneal cavity.³ These 2 MC subclasses have distinct secretory granule contents, particularly with regard to the repertoire of MC proteases expressed and the types of proteoglycans synthesized.⁴⁻⁷ For example, MMCs predominantly produce highly sulfated chondroitin sulfate,⁸ whereas CTMCs mainly produce heparin.³

Heparin is a negatively charged glycosaminoglycan (GAG) composed of repeating disaccharide units of glucosamine and hexuronic acid (glucuronic or iduronic acid) that can be sulfated at various positions.⁹ The nascent heparin chain has an average molecular weight of 60 to 100 kd.¹⁰ However, heparin recovered from various organs is often a mixture of fragments with an average molecular weight of 5 to 30 kd, indicating *in vivo* degradation of macromolecular heparin. However, the mechanisms behind the *in vivo* processing of MC heparin and the functional consequences of this processing are not known. Although exogenously administered heparin has a wide clinical use as an anticoagulant, the fact that endogenous heparin is virtually undetectable in blood argues against a major physiological role of heparin in the regulation of blood coagulation. Instead, previous studies have pointed to a key role for heparin in regulating MC granule composition, as shown by severely impaired protease and histamine storage in MCs with defective heparin biosynthesis.^{11,12}

From the Departments of ^aMedical Biochemistry and Microbiology and ^bPublic Health and Caring Sciences, Uppsala University; ^cthe Cancer and Vascular Biology Research Center, Technion, Haifa; and ^dthe Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala.

*These authors contributed equally to this work.

Supported by the Swedish Medical Research Council (K2009-67X-21128-01-3), the Swedish Cancer Research Foundation (09 0717), the Polysackaridforskning Foundation (Uppsala, Sweden), and National Institutes of Health grant CA106456 (I.V.).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication July 15, 2010; revised April 3, 2011; accepted for publication April 5, 2011.

Reprint requests: Jin-Ping Li, MD, PhD, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden. E-mail: Jin-Ping.Li@imbim.uu.se. 0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2011.04.011

Early studies revealed the presence of a heparin-cleaving endoglucuronidase in a murine mastocytoma.¹³ Later, an enzyme degrading heparan sulfate (HS), a polysaccharide structurally related to heparin, was identified in platelets, but it was assumed that the heparin- and HS-degrading activities detected in mastocytoma and platelets, respectively, were due to distinct enzymes.¹⁴ However, recent work indicates that mammalian cells express primarily a single heparanase, and it has been shown that this enzyme can cleave both HS and heparin *in vitro*.¹⁵⁻¹⁸ Heparanase specifically cleaves glycosidic bonds between glucuronic acid and glucosamine in heparin and HS, yielding fragments of variable size.¹⁸⁻²⁰

Overexpression of heparanase in mice results in extensive degradation of HS, whereas inactivation of the heparanase gene leads to accumulation of nondegraded HS accompanied by diverse phenotypes that might be related to alterations in HS structure.²¹⁻²⁵ However, the function of heparanase in MCs has not been reported previously. In this study we investigated the effects of heparanase overexpression or absence on MCs. Our results point to a key role for heparanase in regulating the structure of MC heparin, indicating modulatory effects of heparanase on MC functionality, as evidenced by modification of protease storage, granular morphology, and the ability to respond to MC-activating agents. Based on this study, heparanase emerges as a novel regulator of MC function.

METHODS

Animals

Heparanase transgenic (*hpa-tg*) and knockout (*hpa-KO*) mice were generated as previously described^{21,24} and maintained in the animal facility at the Biomedical Center, Uppsala, Sweden. The experiments were conducted in accordance with local ethics regulations.

Preparation of fetal skin-derived MCs and isolation of peritoneal MCs

The fetal skin-derived mast cells (FSMCs) were prepared essentially as previously described²⁶ and used when they reached greater than 95% toluidine blue positivity. The peritoneal cells were collected from lavage fluid of *Hpa-tg* and control mice (12-18 weeks of age). Total cell numbers from each mouse were counted with a phase contrast microscope. The MCs were isolated from the peritoneal lavage fluid by means of fluorescence-activated cell sorting (FACS) after staining with fluorescein isothiocyanate (FITC)-conjugated anti- α -kit (CD117) antibody (see Methods in this article's Online Repository at www.jacionline.org). The control samples were stained with FITC-conjugated IgG. A minimum of 10,000 events (cells) per sample were counted (see Figs E1 and E2 in the article's Online Repository at www.jacionline.org).

Purification and analysis of heparin from FSMCs

Preparation of metabolically labeled heparin from FSMCs and structural analysis were performed as previously described.^{23,27}

Characterization of MCs

The MCs were characterized by staining with toluidine blue, May-Grünwald/Giemsa, anti- α -kit, anti-heparanase, and anti-heparin antibodies. The properties of the cells were examined by using different microscopic techniques.

Analysis of protein and gene expression

Proteases and heparanase were determined by means of Western blotting after separation on 12% SDS-PAGE. The results were analyzed by means of quantification of the band intensity with Image J software (National Institutes

of Health, Bethesda, Md). For gene expression, quantitative PCR was applied by using the primers shown in Table E1 (available in this article's Online Repository at www.jacionline.org). Samples were normalized by using the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from each sample, and data are reported as the ratio of treated cells/untreated cells or expression fold.

Activation of MCs and analysis of degranulation

The MCs were activated by means of *in vitro* ionomycin plus phorbol 12-myristate 13-acetate (PMA) or anti-2,4-dinitrophenol (DNP)-IgE plus DNP-BSA stimulation or by means of *in vivo* stimulation through intravenous injection of anti-DNP-IgE plus DNP-BSA. β -Hexosaminidase activity was determined as previously described.²⁸ Substrate conversion was measured by means of absorbance at 405 nm (OD). Histamine was quantified with the Histamine Enzyme Immunoassay Kit (SPI-Bio, Montigny-le-Bretonneux, France), according to the manufacturer's instruction. The values were normalized to the total MC number in each mouse.

A detailed description of the methods is presented in the Methods section in this article's Online Repository.

Data analysis and statistics

Statistical differences were determined by using the unpaired *t* test.

RESULTS

Heparanase is responsible for degradation of heparin in MCs

FSMCs were established from E16.5 murine embryos and were allowed to differentiate *in vitro*. Western blot analysis of cell lysates confirmed overexpression of heparanase in *hpa-tg* FSMCs and null expression of heparanase in *hpa-KO* FSMCs (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). Cytospin slides prepared from *in vitro*-matured cells were stained with toluidine blue at pH 2.5, and microscopic analysis showed a typical metachromatic staining of control FSMCs (Fig 1, A). In comparison, *hpa-tg* FSMCs stained substantially weaker (Fig 1, B) and *hpa-KO* FSMCs showed more pronounced staining compared with control cells (Fig 1, C). Toluidine blue is a cationic dye that binds strongly to negatively charged GAGs, such as heparin. Hence the altered toluidine blue staining in heparanase-overexpressing and heparanase-deficient FSMCs indicates a role for heparanase in regulating the properties of the sulfated GAG contents of FSMCs. In contrast to the dramatic change in toluidine blue staining, 4'-6'-diamidino-2-phenylindole (DAPI) staining of nuclei did not reveal any alterations in *hpa-KO* or *hpa-tg* cells compared with control FSMCs (Fig 1, A-C, inserts).

FSMCs were cultured in the presence of ³⁵S-sulfate, followed by purification and characterization of metabolically labeled GAGs, to further elucidate the effects of heparanase on GAGs. Treatment of the GAGs with Chondroitinase ABC (Seikagaku, Tokyo, Japan) resulted in negligible degradation, indicating that FSMCs express low amounts of chondroitin sulfate. This is in accordance with our previous observations.²⁷ In contrast, the purified ³⁵S-labeled material was completely degraded by treatment with nitrous acid at pH 1.5, confirming that FSMCs predominantly produced heparin. Gel chromatographic analysis revealed substantially shorter heparin chains derived from *hpa-tg* FSMCs than from control cells, whereas *hpa-KO* FSMCs contained substantially longer heparin chains than material from control counterparts (Fig 1, D). Together, these findings demonstrate a role for

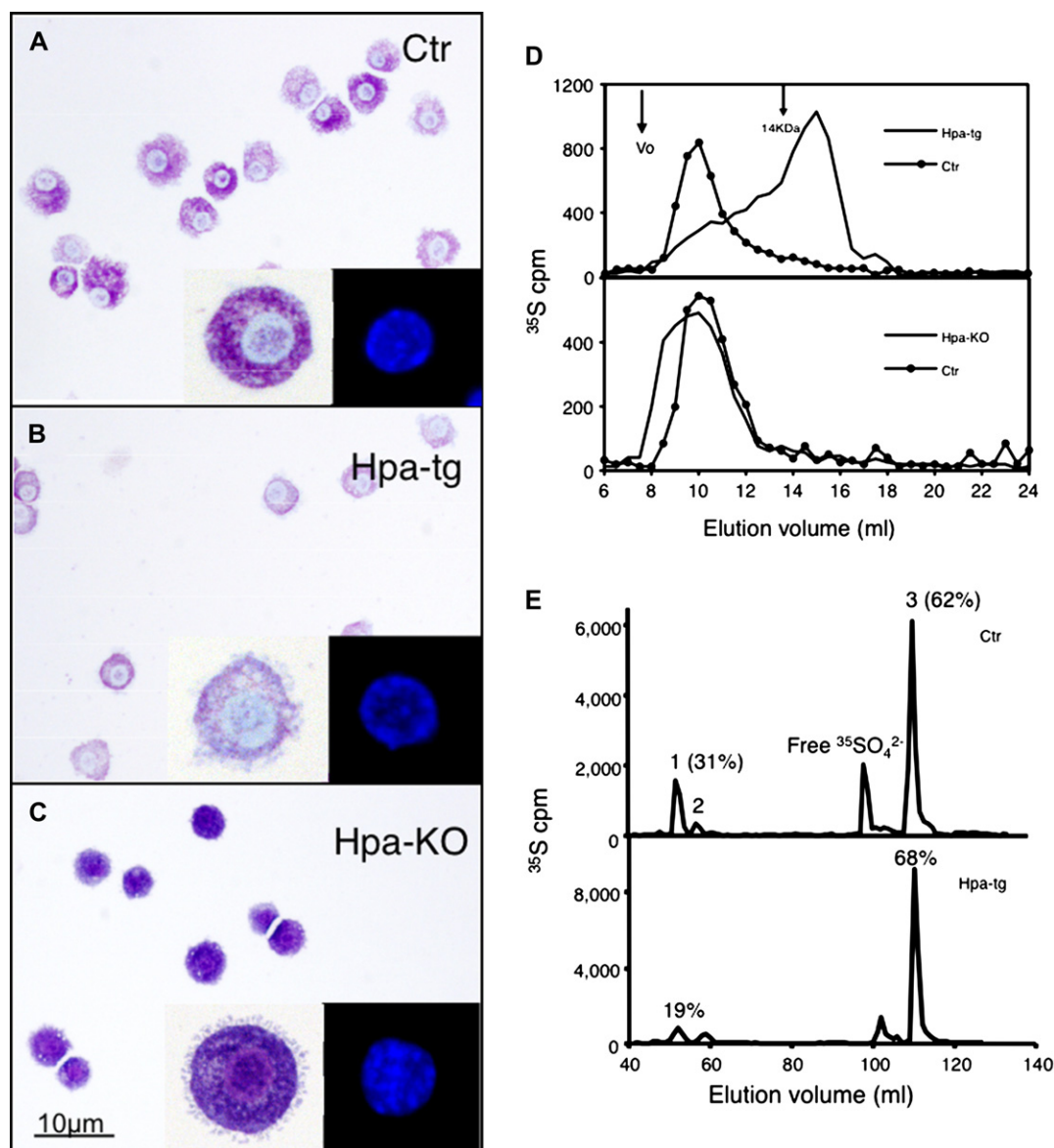


FIG 1. Characterization of FSMCs. *In vitro*-differentiated FSMCs from control (Ctr; **A**), *hpa-tg* (**B**), and *hpa-KO* (**C**) embryos were stained with toluidine blue (magnification $\times 40$). Inserts represent enlarged or DAPI-stained cells. **D**, Gel chromatographic analysis of metabolically ^{35}S -labeled heparin from FSMCs. Upper panel, Ctr versus *hpa-tg*; lower panel, Ctr versus *hpa-KO*. **E**, Analysis of disaccharides by means of anion-exchange HPLC. The numbered peaks represent the -GlcA-GlcNS6S- (1), -IdoA-GlcNS6S- (2), and -IdoA2S-GlcNS6S- (3) sequences of the intact heparin chains. The percentages of the respective components in total recovered disaccharides are indicated for peaks 1 and 3. Upper panel, Ctr; lower panel, *hpa-tg*.

heparanase in degrading and thereby controlling the size of heparin in MCs.

The fine structure of the heparin was further assessed by means of analysis of its disaccharide composition. Deaminative cleavage (pH 1.5) of the ^{35}S -labeled heparin resulted in complete degradation. The resultant disaccharides were collected and separated by using anion-exchange HPLC (Partisil-10 SAX). The proportion of trisulfated disaccharide (peak 3), -IdoA2S-GlcNS6S-, did not show any appreciable difference when comparing *hpa-tg* and control heparin (68% and 62%, respectively). However, the amount of disaccharide units (peak 2) containing the heparanase cleavage sequence (ie, -GlcA-GlcNS6S) was significantly decreased in *hpa-tg* heparin, from 31% in control cells to 19%, confirming

substantial cleavage of heparin by heparanase (Fig 1, E). There was no difference in disaccharide composition between the heparin samples from control and *hpa-KO* FSMCs (data not shown).

Heparanase degradation of heparin impairs storage of proteases in FSMCs

Previous studies have shown that a major function of heparin is to promote the storage of MC-specific proteases within the MC secretory granules.^{11,12} It was therefore of interest to assess whether the effect of heparanase on heparin molecular structure could lead to alterations in the storage of these proteases in MCs. Indeed, Western blot analysis revealed a substantial

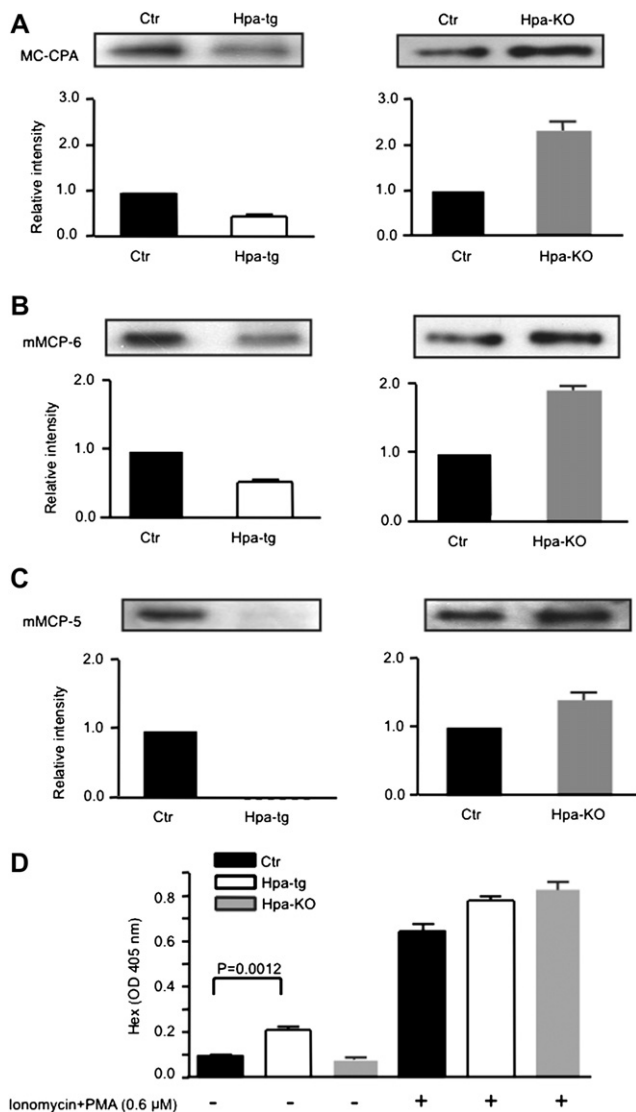


FIG 2. Protease storage and β -hexosaminidase release in FSMCs. Western blotting of MC-CPA (A), mMCP-6 (B), or mMCP-5 (C) in cell lysates. Data show the ratio of band intensity/total protein amount. The respective levels in control (Ctrl) cells are defined as 1. Total protein loaded for detection of the following: MC-CPA, 0.1 μ g; mMCP-5, 0.4 μ g; and mMCP-6, 0.2 μ g. D, β -Hexosaminidase (Hex) activity in the conditioned media of FSMCs cultured with or without ionomycin plus PMA stimulation. The data represent 3 independent experiments (means $\pm$ SDs).

decrease in the amount of stored MC carboxypeptidase A (MC-CPA), as well as in the content of the tryptase murine mast cell protease (mMCP) 6, and an almost complete loss of the chymase mMCP-5 in *hpa-tg* FSMCs in comparison with that seen in control counterparts (Fig 2, A-C, left panels). Conversely, increased storage of these proteases was observed in *hpa-KO* FSMCs (Fig 2, A-C, right panels).

The release of β -hexosaminidase in response to ionomycin plus PMA activation was assessed to investigate whether heparanase has a role in regulating FSMC degranulation events. Interestingly, a significantly higher basal release of β -hexosaminidase was observed in cultures of *hpa-tg* cells compared with that seen in their control counterparts (Fig 2, D), suggesting that overexpression of heparanase impaired the integrity of secretory

granules, leading to leakage of granule mediators into the extracellular space. Ionomycin plus PMA stimulation resulted in substantial release of β -hexosaminidase from control cells (7-fold increase over nonstimulated cells), and the *hpa-KO* cells responded even more vividly (9.4-fold increase). In contrast, *hpa-tg* FSMCs responded poorly to ionomycin plus PMA stimulation, with only a 2.7-fold increase of β -hexosaminidase released into the cell-culture medium on stimulation.

Overexpression of heparanase perturbs the morphology of peritoneal MCs

To verify that the findings in FSMCs were not an embryonic development-associated phenomenon, we extended the investigation to adult mice. Because peritoneal MCs are characterized as CTMCs and are known to produce heparin, we analyzed MCs from peritoneal lavage fluid of the transgenic mice along with control animals. May-Grünwald/Giemsa staining revealed the expected presence of strongly stained MCs in lavage fluid from control animals (indicated by arrows in Fig 3, A). In comparison, MCs present in peritoneal lavage fluid from *hpa-tg* mice stained substantially weaker (Fig 3, B, arrows; ie, resembling the difference observed when comparing *hpa-tg* and control FSMCs; see Fig 1, A and B). Moreover, *hpa-tg* MCs appeared smaller than control cells, as shown both with May-Grünwald/Giemsa staining (Fig 3, A and B) and staining for the MC cell-surface marker c-kit (Fig 3, C and D). As shown by means of Western blot analysis, the total level of c-kit was not affected by the overexpression of heparanase (see Fig E3, D). Further examination by means of transmission electron microscopy revealed striking morphologic effects of heparanase overexpression on granule ultrastructure, with MCs from *hpa-tg* mice containing smaller granules than the corresponding control cells. Moreover, whereas the granules in control cells were rounded, the granules in *hpa-tg* MCs were irregularly shaped (Fig 3, E and F). In contrast, the morphologies of control and *hpa-KO* peritoneal MCs were indistinguishable (data not shown).

Overexpression of heparanase in *hpa-tg* MCs was confirmed by means of Western blot analysis of the c-kit⁺ cells isolated by means of FACS (see Fig E3, C). Note that heparanase was less than the level of detection in control peritoneal MCs, as determined by using the method applied in agreement with our early notion that the enzyme is expressed at a low level in normal tissues.²³ In contrast to the surface staining of c-kit, immunocyto-staining with anti-heparanase antibodies resulted in strong granule-shaped intracellular signals in *hpa-tg* MCs (Fig 3, G); in comparison, control MCs had substantially weaker signals for heparanase staining, confirming the low level of the enzyme in normal MC cells (see Fig E4, A, in this article's Online Repository at www.jacionline.org).

Overexpression of heparanase results in distressed MC degranulation

To determine whether the morphologic disturbances of the granules in *hpa-tg* MCs have functional implications, we examined the degranulation properties of peritoneal lavage cells on activation. Analysis of β -hexosaminidase release revealed, similar to the findings in FSMCs, a higher baseline release of β -hexosaminidase in *hpa-tg* cells than in control cells (Fig 4, A and B). Moreover, in response to ionomycin plus PMA, but not in

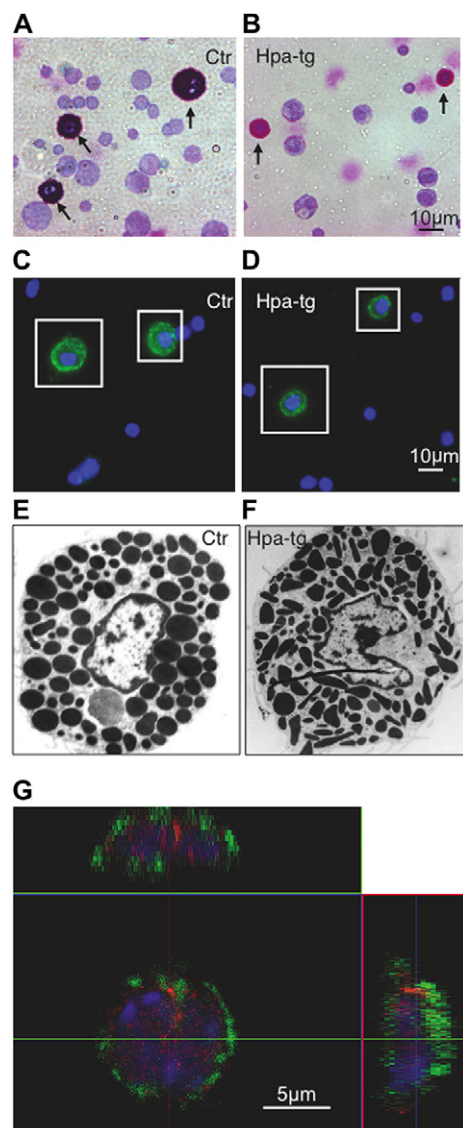


FIG 3. Morphologic examination of peritoneal MCs. Peritoneal cells were stained with May-Grünwald/Giemsa (A, control [Ctr]; B, *hpa-tg* [arrows point to MCs]) or with FITC-conjugated anti-c-kit antibody (C, Ctr; D, *hpa-tg*; MCs are indicated by squares; original magnification $\times 40$). E and F, Transmission electron microscopy analysis of Ctr (Fig 3, E) and *hpa-tg* (Fig 3, F) peritoneal MCs (original magnification $\times 3000$). G, *Hpa-tg* MCs double immunostained for c-kit (green) and heparanase (red) examined by means of confocal microscopy (z-scan). The nuclei were stained with DAPI (blue).

response to IgE-mediated activation, *hpa-tg* cells released more β -hexosaminidase than control cells.

To further examine the effect of heparanase overexpression on MC degranulation events, we measured the histamine release in response to IgE-mediated peritoneal cell activation. As shown in Fig 4, C, the baseline release of histamine was lower in *hpa-tg* cells compared with that seen in control cells. Moreover, the release of histamine in response to IgE-mediated activation was profoundly lower in *hpa-tg* cells compared with that seen in control cells. A likely explanation for the differential effects of heparanase overexpression on β -hexosaminidase versus histamine release might lie within their differential dependence on heparin for storage; whereas β -hexosaminidase is reported to be heparin

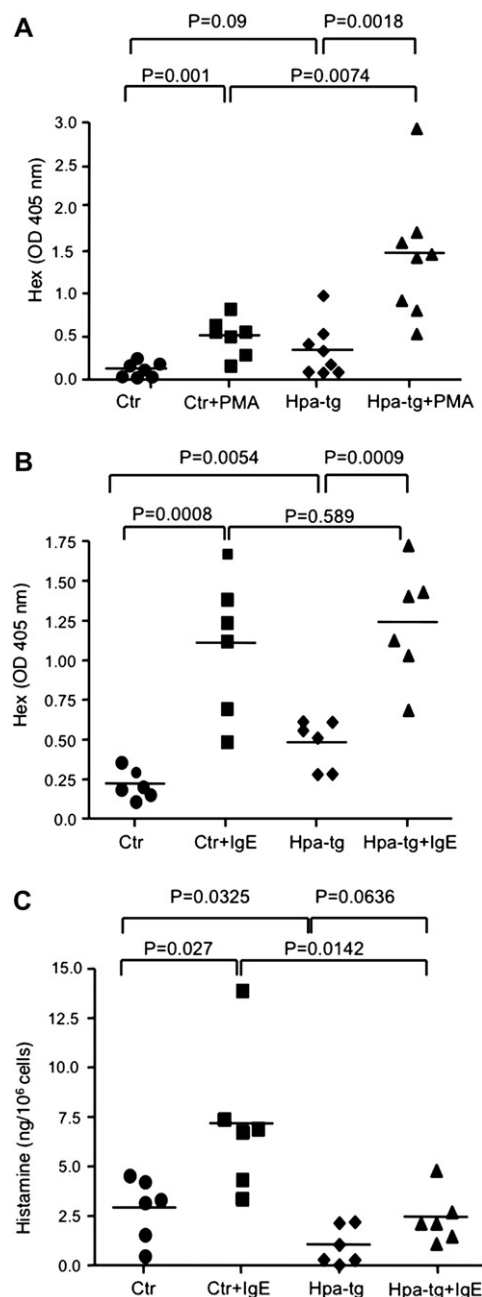


FIG 4. Effect of heparanase overexpression on MC degranulation. A and B, Release of β -hexosaminidase (Hex) by peritoneal cells on ionomycin plus PMA (Fig 4, A) or anti-DNP-IgE plus DNP-BSA (Fig 4, B) treatment. The data are expressed as OD_{405nm} values (β -hexosaminidase activity) in the medium before and after activation. C, Release of histamine by peritoneal cells on anti-DNP-IgE plus DNP-BSA treatment. Each symbol represents data from an individual animal.

independent,²⁹ histamine is known to be strongly dependent on heparin.³⁰

Next, the effect of heparanase overexpression on MC protease storage and release was assessed by means of Western blot analysis. Quantification of the band intensities normalized against the number of MCs showed a significantly reduced storage of MC-CPA in *hpa-tg* peritoneal cells compared with that seen in control cells; in comparison, the reduction of mMCP-5 levels

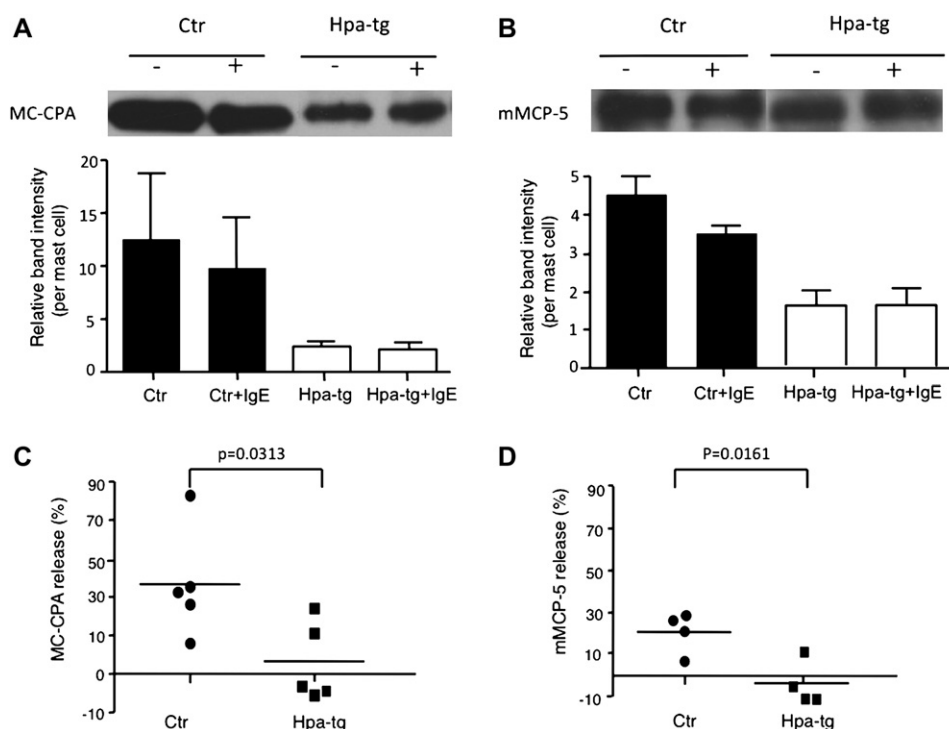


FIG 5. Detection of proteases in the peritoneal MCs. Representative Western blotting for MC-CPA (**A**) and mMCP-5 (**B**) in nontreated (–) or IgE-activated (+) peritoneal MCs is shown. The bands were quantified by using Image J software and normalized to the MC number in each mouse ($n = 4$ –5); the average relative band intensity is shown. Release of MC-CPA (**C**) and mMCP-5 (**D**) in response to IgE cross-linking is calculated as follows: (Nonactivated – Activated)/Nonactivated $\times 100\%$.

was modest (Fig 5, A and B). On IgE-mediated activation, on average, about 20% of the total MC-CPA and mMCP-5 was lost (released) from control cells. In contrast, IgE-activated *hpa*-tg cells released, on average, about 3% of the total MC-CPA and had essentially no release of mMCP-5. The reduced storage and release of proteases in *hpa*-tg cells were not due to a reduced expression of the proteases; on the contrary, expression of these proteases at the mRNA level was upregulated in *hpa*-tg MCs (see Fig E5 in this article's Online Repository at www.jacionline.org).

MC activation induces heparanase expression

The finding that heparanase modulates MC function prompted us to examine whether heparanase expression is altered in MCs in response to activation. First, FSMCs isolated from control embryos were stimulated with ionomycin plus PMA, followed by total RNA extraction and analysis of heparanase expression with quantitative real-time RT-PCR. Indeed, MC activation caused a robust upregulation of heparanase expression, particularly at early time points after MC activation (Fig 6, A). Next, we examined the heparanase expression in MCs after IgE-mediated activation *in vivo*. Adult mice sensitized with anti-DNP-IgE were challenged by means of intravenous injection of DNP-BSA conjugate. Total peritoneal cells were collected from treated and nontreated mice ($n = 7$). The *c-kit*⁺ cells (MCs) were collected by means of FACS for preparation of total RNA. Quantitative real-time RT-PCR revealed that *in vivo* IgE-mediated activation caused a minor upregulation of heparanase expression in peritoneal MCs (Fig 6, B).

DISCUSSION

Heparanase has been shown to specifically cleave the glycosidic bond between glucuronic acid and glucosamine residues in HS. Recognized for its upregulated expression in some pathological conditions, such as inflammatory reactions and metastatic tumors, heparanase has recently attracted much attention in the context of modulation of HS structure and function.^{23,25,31–33} However, although recombinant heparanase has been shown to cleave both HS and heparin *in vitro*¹⁹ and is able to degrade macromolecular heparin isolated from rat skin,¹⁸ direct evidence showing that heparanase can process MC heparin *in vivo* has not been reported. Moreover, the potential functional implications of MC heparin processing by heparanase have not been addressed previously.

The generation of transgenic mice overexpressing or lacking heparanase^{21,24} has provided excellent models to examine heparanase function *in vivo*. Because FSMCs have been shown to resemble CTMCs and produce heparin,²⁷ we used FSMCs to study the effect of heparanase on heparin processing and MC function. As expected, *in vitro*-matured control and *hpa*-KO embryonic FSMCs stained positively with toluidine blue; in comparison, *hpa*-tg FSMCs stained considerably weaker (Fig 1, A–C). Because toluidine blue stains negatively charged GAGs, the weaker staining of *hpa*-tg FSMCs reflects an alteration in the GAG content. Indeed, analysis of metabolically ³⁵S-labeled heparin showed an accumulation of dramatically shortened chains in *hpa*-tg cells (Fig 1, D), indicating an extensive fragmentation of the polysaccharide by overexpressed heparanase. Conversely, the heparin chains in *hpa*-KO FSMCs were nondegraded.

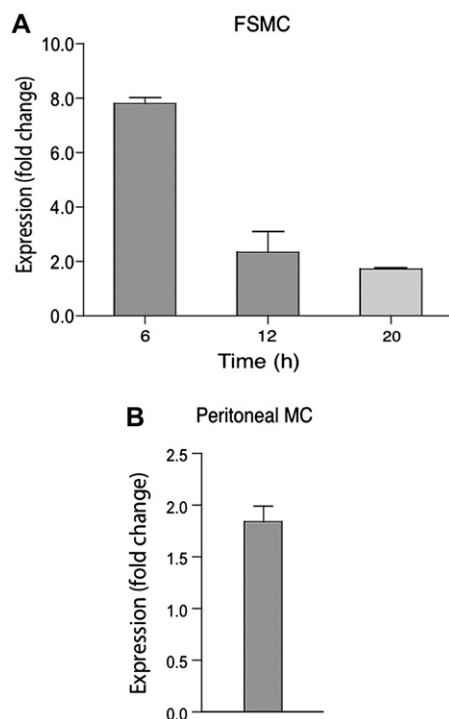


FIG 6. Effect of MC activation on heparanase expression. **A**, Quantitative RT-PCR analysis of heparanase expression in FSMCs (from 6 embryos) at the time points indicated after activation with ionomycin plus PMA. **B**, MCs were activated *in vivo* (7 mice per group) by anti-DNP-IgE plus DNP-BSA treatment. The quantitative PCR signals of heparanase were standardized against that of GAPDH (defined as 1). The error bars in Fig 6, A, represent the average of duplicate samples, and those in Fig 6, B, indicate the SD from 3 independent experiments performed in triplicate.

Moreover, costaining of heparanase with heparin in connective tissue (intestine) MCs of *hpa-tg* mice (Fig E4, B) provides additional evidence for the intracellular degradation of heparin by heparanase. Together, these findings clearly demonstrate that heparanase is responsible for processing of heparin within MC. Moreover, the decreased proportion of -GlcA-GlcNS6S- disaccharide units in heparin isolated from *hpa-tg* FSMCs provided additional molecular evidence for heparanase-catalyzed heparin cleavage (see Fig 1, E). It should be noted that a reduction of this disaccharide unit was also observed in HS isolated from tissues overexpressing heparanase,²³ suggesting that heparanase has a dual role in regulating the size of both heparin and HS *in vivo*. This notion is also in agreement with our earlier observations that heparanase can degrade heparin *in vitro*.¹⁸

Various proteases and other types of preformed granule compounds might account for up to 50% of the total weight of a mature MC.² Murine MCs express the exopeptidase MC-CPA and several MC-specific serine proteases, and large amounts of these proteases can be released when MC degranulation is induced.^{2,7,34} Recent investigations involving the assessment of murine strains lacking individual MC proteases in different animal models of disease have shown that these enzymes might account for many of the biological functions attributed to MCs.² Hence mechanisms that are involved in the regulation of MC protease storage are likely to have profound effects on MC function *in vivo*.

To examine the effect of heparanase on storage and release of MC proteases, we determined the cellular contents of selected

proteases before and after MC activation. Interestingly, the storage of 3 main CTMC proteases (ie, MC-CPA, mMCP-5, and mMCP-6) was reduced in *hpa-tg* FSMCs, indicating that optimal protease storage cannot occur if the granule heparin is subject to substantial degradation by heparanase. This notion was further substantiated by the finding that heparanase-deficient FSMCs contained higher amounts of these proteases than control cells, suggesting that MCs containing “oversized” heparin chains can accommodate super-optimal levels of MC proteases (Fig 2, A-C). Thus the storage of MC proteases might be regulated at the level of heparanase expression/activity, with induction of heparanase leading to reduced protease storage, in turn resulting in attenuated MC responses. Conversely, reduced heparanase expression might lead to super-optimal protease storage, possibly leading to exaggerated MC responses. An effect of heparanase overexpression was also seen in adult peritoneal MCs, which showed striking morphologic alterations (Fig 3) accompanied by reduced ability to store MC proteases (Fig 5) and to release histamine on activation (Fig 4, C). Interestingly, elimination of heparanase appeared to have minimal effects on adult peritoneal MCs (data not shown). A possible explanation for this finding might be that heparanase has profound effects on MCs during their developmental stages but less of an effect on normal mature cells. In line with this notion, heparanase protein is detectable in FSMCs but is undetectable in peritoneal MCs from adult mice (see Fig E3, A-C).

Intriguingly, whereas the storage of MC-CPA and mMCP-6 was affected to a relatively modest extent by the absence or overexpression of heparanase in FSMCs, mMCP-5 storage was dramatically reduced in *hpa-tg* cells. Although we cannot with certainty explain the mechanism behind this finding, it is notable that mMCP-5, but not MC-CPA or mMCP-6, contains 2 distinct patches of positively charged regions exposed on the protein surface.³⁵ Possibly optimal storage of mMCP-5 requires that both of these regions are bound to the same heparin chain and thus that optimal mMCP-5 storage requires long-chain heparin, whereas the heparin chain length is less critical in promotion of MC-CPA and mMCP-6 storage. Indeed, previous studies have shown that optimal interaction of MC chymase with heparin requires a high heparin chain length.³⁶

Together, the findings presented here suggest that heparanase, by regulating the size of MC heparin, can have an important indirect role in the regulation of MC protease storage and in control of MC degranulation processes. Considering that MC proteases have important functions in association with a number of pathological settings, including allergic reactions,^{2,37-39} heparanase-mediated regulation of MC protease storage/release might thus have profound consequences in various types of disease. Conceivably, induction or repression of heparanase expression in the context of a given pathological stimulation could lead to effects on the storage and release of the MC secretory granule mediators. Clearly, this modulation leads to altered MC-driven responses.

We thank Professor Ulf Lindahl (Uppsala University) for valuable discussions in the process of this study.

Clinical implications: A possible upregulation of heparanase activity in patients during allergic inflammation might affect treatment with heparin. Inhibition of heparanase degradation of heparin has the potential to attenuate MC activity in allergic reaction conditions.

REFERENCES

1. Lundequist A, Pejler G. Biological implications of preformed mast cell mediators. *Cell Mol Life Sci* 2011;68:965-75.
2. Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood* 2010;115:4981-90.
3. Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev* 1997;77:1033-79.
4. Stevens RL. Mast cell proteoglycans. *Prog Clin Biol Res* 1989;297:131-44.
5. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *J Leukoc Biol* 1997;61:233-45.
6. Miller HR, Pemberton AD. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology* 2002;105:375-90.
7. Pejler G, Abrink M, Ringvall M, Wernersson S. Mast cell proteases. *Adv Immunol* 2007;95:167-255.
8. Enerbäck L, Kolset SO, Kusche M, Hjerpe A, Lindahl U. Glycosaminoglycans in rat mucosal mast cells. *Biochem J* 1985;227:661-8.
9. Lindahl U. Biosynthesis of Heparin and related polysaccharides. In: Lane DA, Lindahl U, editors. *Heparin chemical and biological properties, clinical applications*. 1st ed London: Edward Arnold; 1989. p. 159-89.
10. Horner AA. Macromolecular heparin from rat skin. Isolation, characterization, and depolymerization with ascorbate. *J Biol Chem* 1971;246:231-9.
11. Humphries DE, Wong GW, Friend DS, Gurish MF, Qiu WT, Huang C, et al. Heparin is essential for the storage of specific granule proteases in mast cells. *Nature* 1999;400:769-72.
12. Forsberg E, Pejler G, Ringvall M, Lunderius C, Tomasini-Johansson B, Kusche-Gullberg M, et al. Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. *Nature* 1999;400:773-6.
13. Ogren S, Lindahl U. Cleavage of macromolecular heparin by an enzyme from mouse mastocytoma. *J Biol Chem* 1975;250:2690-7.
14. Thunberg L, Backstrom G, Wasteson A, Robinson HC, Ogren S, Lindahl U. Enzymatic depolymerization of heparin-related polysaccharides. Substrate specificities of mouse mastocytoma and human platelet endo- beta-D-glucuronidases. *J Biol Chem* 1982;257:10278-82.
15. Hulett MD, Freeman C, Hamdorf BJ, Baker RT, Harris MJ, Parish CR. Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. *Nat Med* 1999;5:803-9.
16. Toyoshima M, Nakajima M. Human heparanase. Purification, characterization, cloning, and expression. *J Biol Chem* 1999;274:24153-60.
17. Vlodavsky I, Friedmann Y, Elkin M, Aingorn H, Atzmon R, Ishai-Michaeli R, et al. Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis [see comments]. *Nat Med* 1999;5:793-802.
18. Gong F, Jemth P, Escobar Galvis ML, Vlodavsky I, Horner A, Lindahl U, et al. Processing of macromolecular heparin by heparanase. *J Biol Chem* 2003;278:35152-8.
19. Pikas DS, Li JP, Vlodavsky I, Lindahl U. Substrate specificity of heparanases from human hepatoma and platelets. *J Biol Chem* 1998;273:18770-7.
20. Peterson SB, Liu J. Unraveling the specificity of heparanase utilizing synthetic substrates. *J Biol Chem* 2010;285:14504-13.
21. Zcharia E, Metzger S, Chajek-Shaul T, Aingorn H, Elkin M, Friedmann Y, et al. Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. *FASEB J* 2004;18:252-63.
22. Zcharia E, Zilka R, Yaar A, Yacoby-Zeevi O, Zetser A, Metzger S, et al. Heparanase accelerates wound angiogenesis and wound healing in mouse and rat models. *FASEB J* 2005;19:211-21.
23. Escobar Galvis ML, Jia J, Zhang X, Jastrebova N, Spillmann D, Gottfridsson E, et al. Transgenic or tumor-induced expression of heparanase upregulates sulfation of heparan sulfate. *Nat Chem Biol* 2007;3:773-8.
24. Zcharia E, Jia J, Zhang X, Baraz L, Lindahl U, Peretz T, et al. Newly generated heparanase knock-out mice unravel co-regulation of heparanase and matrix metalloproteinases. *PLoS ONE* 2009;4:e5181.
25. Waern I, Jia J, Pejler G, Zcharia E, Vlodavsky I, Li JP, et al. Accumulation of Ym1 and formation of intracellular crystalline bodies in alveolar macrophages lacking heparanase. *Mol Immunol* 2010;47:1467-75.
26. Feyerabend TB, Hausser H, Tietz A, Blum C, Hellman L, Straus AH, et al. Loss of histochemical identity in mast cells lacking carboxypeptidase A. *Mol Cell Biol* 2005;25:6199-210.
27. Feyerabend TB, Li JP, Lindahl U, Rodewald HR. Heparan sulfate C5-epimerase is essential for heparin biosynthesis in mast cells. *Nat Chem Biol* 2006;2:195-6.
28. Schwartz LB, Austen KF, Wasserman SI. Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *J Immunol* 1979;123:1445-50.
29. Henningsson F, Hergeth S, Cortelius R, Abrink M, Pejler G. A role for serglycin proteoglycan in granular retention and processing of mast cell secretory granule components. *FEBS J* 2006;273:4901-12.
30. Ringvall M, Ronnberg E, Wernersson S, Duelli A, Henningsson F, Abrink M, et al. Serotonin and histamine storage in mast cell secretory granules is dependent on serglycin proteoglycan. *J Allergy Clin Immunol* 2008;121:1020-6.
31. McKenzie EA. Heparanase: a target for drug discovery in cancer and inflammation. *Br J Pharmacol* 2007;151:1-14.
32. Spiegel A, Zcharia E, Vagima Y, Itkin T, Kalinkovich A, Dar A, et al. Heparanase regulates retention and proliferation of primitive Sca-1+/c-Kit+/Lin- cells via modulation of the bone marrow microenvironment. *Blood* 2008;111:4934-43.
33. Massena S, Christoffersson G, Hjertstrom E, Zcharia E, Vlodavsky I, Ausmees N, et al. A chemotactic gradient sequestered on endothelial heparan sulfate induces directional intraluminal crawling of neutrophils. *Blood* 2010;116:1924-31.
34. Stevens RL, Adachi R. Protease-proteoglycan complexes of mouse and human mast cells and importance of their beta-tryptase-heparin complexes in inflammation and innate immunity. *Immunol Rev* 2007;217:155-67.
35. Sali A, Matsumoto R, McNeil HP, Karplus M, Stevens RL. Three-dimensional models of four mouse mast cell chymases. Identification of proteoglycan binding regions and protease-specific antigenic epitopes. *J Biol Chem* 1993;268:9023-34.
36. Pejler G, Maccarana M. Interaction of heparin with rat mast cell protease 1. *J Biol Chem* 1994;269:14451-6.
37. Marshall JS. Mast-cell responses to pathogens. *Nat Rev Immunol* 2004;4:787-99.
38. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008;454:445-54.
39. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol* 2010;10:440-52.

METHODS

Preparation of FSMCs

Day 16.5 trunk skin of embryos was collected and digested in PBS containing 1 mg/mL collagenase, 0.5 mg/mL DNase I, and 5% FCS. After washing 3 times in PBS fortified with 5% FCS, cells were resuspended and cultured in Dulbecco modified Eagle medium/F12 (Sigma, St Louis, Mo) medium supplemented with 10% heat-inactivated FCS, 1% penicillin-streptomycin liquid (Invitrogen, Carlsbad, Calif), IL-3 (20 ng/mL), stem cell factor (50 ng/mL), and 2-mercaptoethanol (50 μ mol/L) at 37°C. During the first 15 days of culture, half of the medium was replaced twice per week; after 15 days, nonadherent and loosely adherent cells were harvested by means of gentle shaking and transferring to new flasks. This transfer from the original wells was repeated 2 to 3 times at an interval of 3 to 4 days, and the suspension of MCs was matured for another 2 to 4 weeks before use in the experiments. The criteria for maturation of the FSMCs are detection of dense granules in the cells, as previously described.^{E1} The cells were used when they reached greater than 95% toluidine blue positivity.

Purification and analysis of heparin from FSMCs

The matured FSMCs were cultured in T75 flasks, as described above, until 80% confluence and were transferred to sulfate-free medium supplemented with 2 mmol/L glutamine, 1% Pest, 10% FCS, and 200 μ Ci/mL Na₂³⁵SO₄ (specific activity, 1500 Ci/mmol; PerkinElmer, Waltham, Mass). After culturing for 24 hours, the cells were collected and washed twice with PBS containing 5% FCS. The cell pellet was suspended in extraction buffer (50 mmol/L Tris-HCl, 2 mol/L KCl, and 1% TritonX-100 [pH 7.4]) and incubated for 3 hours. The cell lysate was diluted in 50 mmol/L Tris-HCl, 1 mmol/L CaCl₂, and 1% Triton X-100 (pH 8.0) containing 0.8 mg of protease for digestion of proteins at 55°C overnight. For elimination of nucleic acids, Benzonase (60 U; Merck, Whitehouse Station, NJ) and MgCl₂ (2 mmol/L) were added to the lysate, followed by incubation for an additional 2 hours at 37°C. The products were then applied to a 1.5-mL DEAE-Sephacel column (Sigma-Aldrich). After extensive washing with equilibration buffer (50 mmol/L Tris-HCl and 0.3 mol/L NaCl, pH 7.4), bound materials were eluted with 50 mmol/L Tris-HCl and 2 mol/L NaCl (pH 7.4). The eluted fractions were desalted by means of dialysis and lyophilized. After dissolving the material in a small volume, the products were treated with Chondroitinase ABC (25 mU, Seikagaku) and reapplied to the DEAE-Sephacel column to recover heparin.

The purified ³⁵S-labeled heparin was analyzed on a Superose-12 column (GE Healthcare Biosciences, Fairfield, Conn) in a buffer of 50 mmol/L Tris-HCl (pH 7.4), 1 mol/L NaCl, and 0.1% Triton-X 100. For the disaccharide compositional analysis, samples were subjected to cleavage at *N*-sulfated glucosamine (GlcNS) residues by means of treatment with nitrous acid (pH 1.5)^{E2} and reduced with NaBH₄. The resulting oligosaccharides were separated on a Superdex-30 column (GE Healthcare Biosciences) in 0.5 mol/L NH₄HCO₃ at a flow rate of 1 mL/min. The disaccharide fraction was collected and analyzed on a Partisil-10 SAX column, as previously described.^{E3}

Collection of peritoneal cells

Hpa-tg and control mice (12–18 weeks) were killed by means of CO₂ inhalation. After peritoneal injection of 10 mL of cold PBS (pH 7.4), the peritoneal lavage cells were collected by means of centrifugation. The collected cells were washed twice with PBS and suspended in PBS for the experiments described below. Total cell numbers from each mouse were counted with a phase contrast microscope.

Characterization of MCs

The isolated FSMCs from *hpa*-tg and control mice were cultured in the medium specified above. The matured MCs (20 $\times$ 10³) were centrifuged onto cytospin slides, as previously described. The cytospin slides were fixed in methanol for 3 minutes and stained with toluidine blue at pH 2.5 for 5 minutes. After washing 3 times in distilled water, the slides were mounted with Vecta-Mount mounting medium (Vector laboratories, Burlingame, Calif). The nuclei were stained with DAPI (Sigma). For adult MCs, 30,000 cells from the total

peritoneal population were centrifuged onto cytospin slides and stained with May-Grünwald/Giemsa. For immunocytochemistry, cells were stained with FITC-conjugated anti-c-kit (CD117) antibody (BD PharMingen, San Jose, Calif) diluted 1:100 in PBS containing 5% FCS. For immunocytochemistry, total peritoneal cells (3 $\times$ 10⁵) were seeded on cover slips coated with fibronectin (50 g/mL) and cultured overnight. Then the cells were washed in PBS and fixed in 99% ethanol on ice for 5 minutes. Cells were permeabilized in 0.4% Triton X-100 at room temperature for 5 minutes, followed by blocking with Rodent Block M (Biocare Medical, Concord, Calif) at room temperature for 30 minutes. FITC rat anti-mouse CD117 (BD PharMingen) and anti-heparanase antibody^{E3} incubation was carried out overnight at 4°C in a humid chamber. After washing with PBS, cells were treated with Fluorescence Enhancement Probe (Biocare Medical) for 30 minutes at room temperature, followed by incubation with DyLight goat anti-rabbit 594 conjugate (Biocare Medical) for 30 minutes at room temperature. After washing with PBS and H₂O, cells were mounted with mounting medium containing DAPI for counterstaining of nuclei. For immunohistochemistry, the intestine was dissected from *hpa*-tg and control mice. Paraffin-embedded sections were stained with anti-heparin (10E4) and anti-heparanase (733) antibodies.^{E3} The images were acquired by using a Zeiss immunofluorescence microscope at $\times$ 40 magnification or confocal microscopy with a Z-scan.

Transmission electron microscopy

Mature FSMCs (1 $\times$ 10⁶) were fixed for 2 hours in 2% glutaraldehyde in a 0.1 mol/L sodium cacodylate buffer supplemented with 0.1 mol/L sucrose. After dehydration, the cells were embedded, and ultrathin sections were placed on copper grids that were covered with a film of polyvinyl formal plastic and contrasted with uranyl acetate and lead citrate. Electron micrographs were taken with a Hitachi electron microscope (Hitachi Ltd, Tokyo, Japan).

Immunoblot analysis

The MCs, FSMCs or MCs isolated from peritoneal lavage, as well as the total cells from peritoneal lavage, were washed twice with PBS followed by lysis in SDS sample buffer (2% SDS, 80 mmol/L Tris-HCl [pH 6.8], and 10% glycerol). The supernatant was collected for total protein determination with BCA reagent (Roche, Mannheim, Germany). After denaturing by boiling at 95°C for 5 minutes, the samples were separated on 12% SDS-PAGE. The proteins were subsequently blotted onto polyvinylidene difluoride membranes that were blocked and probed with antibodies, as indicated in the respective figures and legends. The signals were developed by using an ECL reagent (ECL plus, GE Healthcare Biosciences) and exposed to a film. The results were analyzed by means of quantification of the band intensity with Image J software.

Real-time PCR

FSMCs were matured as described above. Peritoneal MCs were isolated from total lavage fluid and sorted after immunostaining with anti-c-kit antibody. Total RNA was isolated with the RNeasy Micro Kit (Qiagen, Hilden, Germany). Synthesis of cDNA was carried out by means of reverse transcription of the total RNA by using SuperScript III First-Strand Synthesis System (Invitrogen) with random primers. The cDNA (50 ng) was used for quantitative real-time PCR with Evagreen dye on a MiniOpticon thermal cycler (Bio-Rad Laboratories, Hercules, Calif). The primers are summarized in Table E1. All reactions were performed in triplicate for 39 cycles. The data were evaluated by using the 2^{- $\Delta\Delta$ CT} method.^{E4} Samples were normalized with the mRNA level of GAPDH from each sample, and data are reported as the ratio of treated cells/untreated cells or expression fold.

Fluorescence-activated cell sorting (FACS)

Total peritoneal cells were washed twice in cold PBS and incubated on ice for 40 minutes with FITC-conjugated anti-c-kit (CD117) antibody (5 μ g/mL) or with FITC-conjugated isotype IgG at the same concentration. After staining, the cells were washed twice in cold PBS and diluted in PBS containing 2% FCS. Cells (3 $\times$ 10⁴) were analyzed on an LSRII instrument (BD Biosciences, Mississauga, Ontario, Canada). Fluorescence emission

signals for FITC were plotted against forward scatter light. The control samples stained with FITC-conjugated IgG were plotted by using side scatter light against forward scatter light. A minimum of 10,000 events (cells) per sample were counted.

For isolation of MCs, total peritoneal cells were stained with FITC-conjugated anti-c-kit antibody followed by sorting with a FACS Aria Cell Sorter (BD Biosciences). c-kit⁺ cells were collected for preparation of RNA (PCR) and cell lysate (Western blotting).

Activation of MCs

In vitro ionomycin plus PMA treatment. FSMCs (10,000) or total peritoneal lavage cells (1×10^6) were incubated in 60 μ L of Tyrode buffer (10 mmol/L HEPES [pH 7.4], 130 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, and 0.1% BSA) in the absence or presence of ionomycin and 20 ng/mL PMA for 30 minutes at 37°C during gentle rotation. After centrifugation, the media were collected for analysis.

In vitro treatment with anti-DNP-IgE. Total peritoneal cells suspended in PBS from each mouse were divided into 2 equal parts, one of which was sensitized in medium containing 0.5 μ g/mL anti-DNP-IgE (clone SPE-7, Sigma-Aldrich) for 1 hour at 37°C. After washing twice with Tyrode buffer to eliminate excess antibody, cells were activated with 0.5 μ g/mL DNP-conjugated BSA (DNP-BSA; Invitrogen) by means of incubation for 1 hour at 37°C.

In vivo activation. Adult control mice (12 weeks) were injected intravenously with 5 μ g per mouse (100 μ L) of anti-DNP-IgE mAb or PBS through the tail vein. The animals were kept with free access to water and food. After 24 hours, the mice were challenged by means of intravenous injection of

200 μ g of DNP-BSA dissolved in 100 μ L of PBS. The animals were killed 30 minutes after the challenge by means of inhalation of CO₂, and peritoneal cells were collected in the same way as described above.

Analysis of degranulation

β -Hexosaminidase activity from the media was determined as previously described.^{E5} Briefly, the samples were incubated with *p*-nitrophenyl-N-acetyl- β -D-glucosamine (1 mmol/L in 0.1 mol/L sodium citrate, pH 4.5) at 37°C for 90 minutes. The reaction was stopped by means of addition of 50 μ L of 0.4 mol/L glycine (pH 10.7). Substrate conversion was measured based on absorbance at 405 nm (OD). Histamine was quantified with the Histamine Enzyme Immunoassay Kit (SPI-Bio), according to the manufacturer's instructions. The values were normalized to the total MC number in each mouse.

REFERENCES

- E1. Feyerabend TB, Hausser H, Tietz A, Blum C, Hellman L, Straus AH, et al. Loss of histochemical identity in mast cells lacking carboxypeptidase A. *Mol Cell Biol* 2005;25:6199-210.
- E2. Guo Y, Conrad HE. The disaccharide composition of heparins and heparan sulfates. *Anal Biochem* 1989;176:96-104.
- E3. Escobar Galvis ML, Jia J, Zhang X, Jastrebova N, Spillmann D, Gottfridsson E, et al. Transgenic or tumor-induced expression of heparanase upregulates sulfation of heparan sulfate. *Nat Chem Biol* 2007;3:773-8.
- E4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402-8.
- E5. Schwartz LB, Austen KF, Wasserman SI. Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *J Immunol* 1979;123:1445-50.

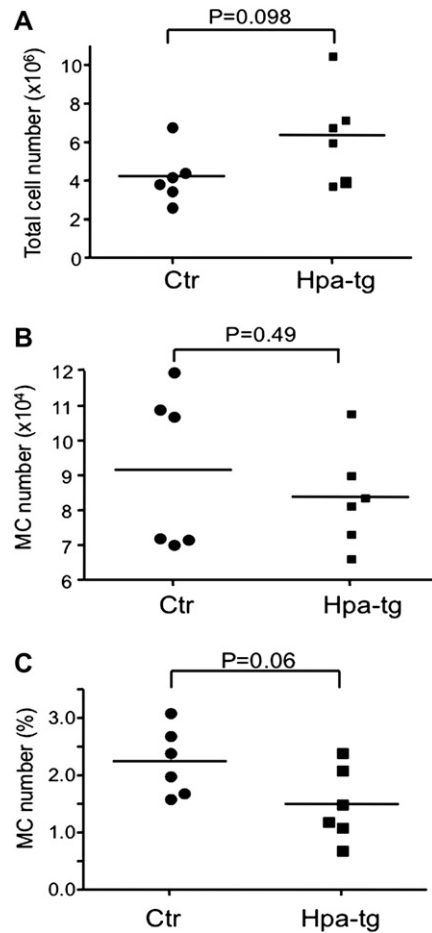


FIG E1. Assessment of total cell counts and MC numbers in peritoneal lavage fluid. **A**, Total peritoneal cell numbers were obtained by counting during phase contrast microscopy. **B**, MCs were quantified by means of FACS analysis of total peritoneal cells stained with FITC-conjugated anti-c-kit antibody. A minimum of 10,000 cell events was analyzed for each mouse ($n = 6$ per each group). **C**, Percentage of MCs in the total peritoneal cell population. *Ctrl*, Control.

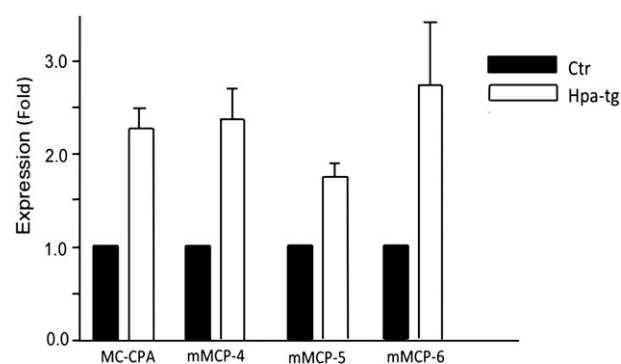


FIG E2. Expression of proteases in peritoneal MCs. Total RNA was extracted from control (*Ctr*) and *hpa-tg* cells collected from peritoneal lavage fluid ($n = 3$), as described in the [Methods](#) section. Quantitative RT-PCR analysis was performed with the primers presented in [Table E1](#). Signals for each protease gene were standardized against that of GAPDH (defined as 1). The *error bars* represent the average of duplicate samples of 2 analyses.

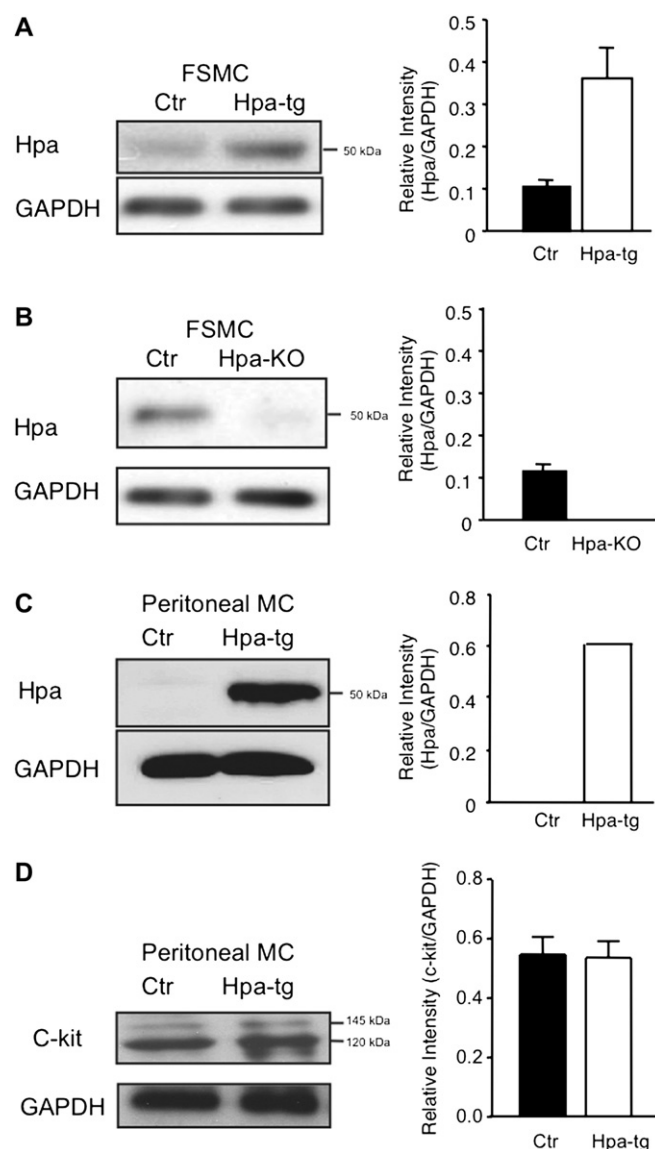


FIG E3. Western blot analysis of heparanase and c-kit. **A-C**, Heparanase expression in *hpa*-tg FSMCs (Fig E3, A), *hpa*-KO FSMCs (Fig E3, B), and *hpa*-tg peritoneal MCs (Fig E3, C). FSMCs were matured *in vitro*, as described in the Methods section. Adult MCs were isolated from the total peritoneal lavage fluid of 7 control (Ctrl) and 7 *hpa*-tg mice by means of FACS after immunostaining with anti-c-kit antibody (see Fig E2). Cells were lysed in SDS-PAGE sample buffer, and the supernatants were subjected to SDS-PAGE analysis. Total protein of 50 μ g (for FSMCs) or 20 μ g (for peritoneal MCs) was applied and blotted to polyvinylidene difluoride membranes that were probed with anti-heparanase antibody 733 diluted 1:1000 (FSMCs) and 1:500 (for peritoneal MCs). As loading control, the membranes were reprobed with anti-GAPDH antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, Calif). The relative intensity of heparanase to GAPDH is shown in the right panels. It should be noted that the level of heparanase in Ctrl peritoneal MCs is less than the detection limit under the conditions used. **D**, c-kit Expression in cells from peritoneal lavage. Total protein of 20 μ g was applied.

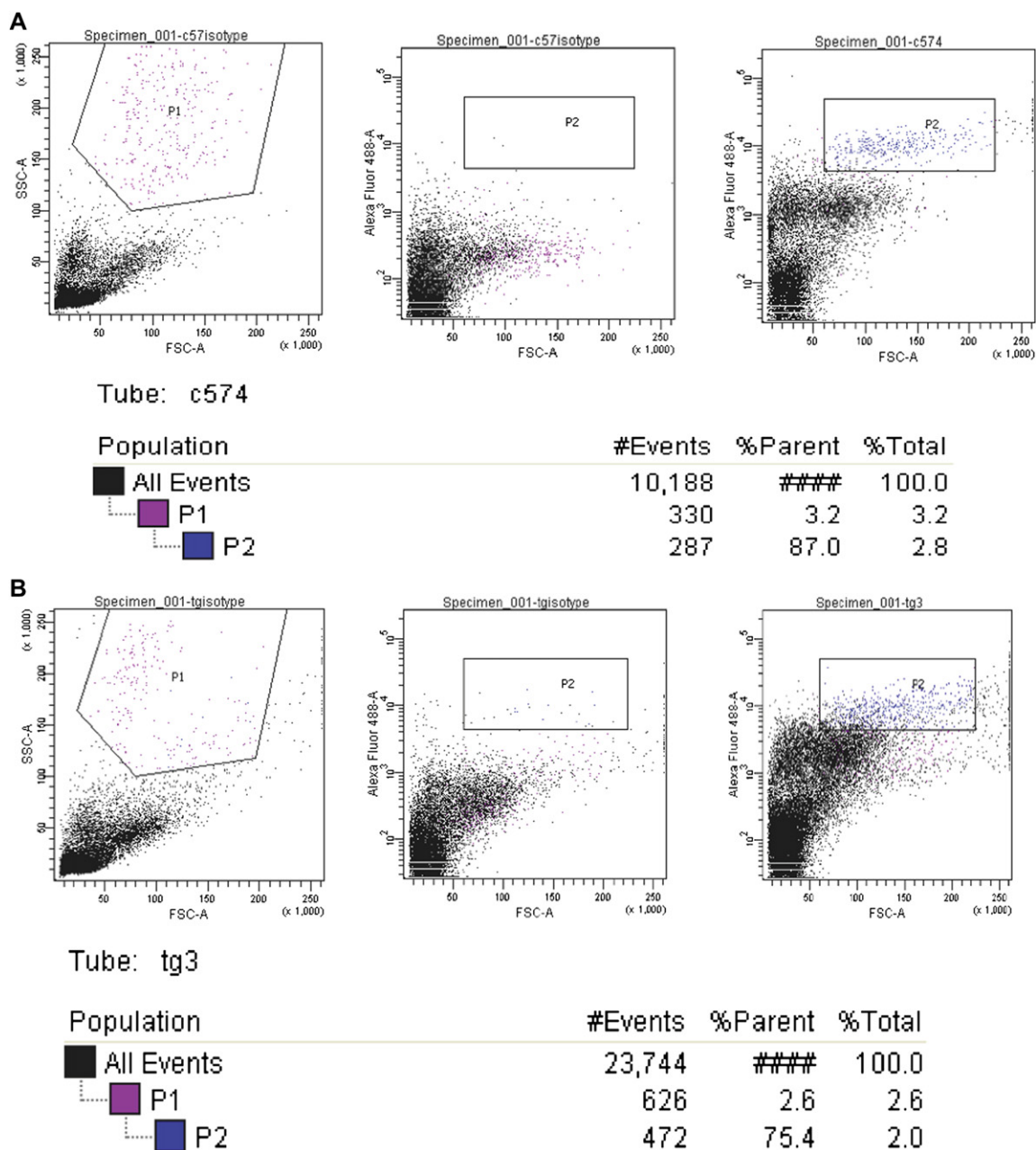


FIG E4. Representative graphs of FACS analysis. Data on control (*Ctrl*; **A**) and *hpa-tg* (**B**) cells with or without anti-c-kit immunostaining are shown. *Left panels* show cells sorted by cellular density (side scatter) and size (forward scatter), where *P1* represents MCs. The *middle panels* show cells stained with FITC-conjugated isotype IgG. The *right panels* show cells stained with FITC-conjugated anti-c-kit antibody. *P2* represents c-kit⁺ cells.

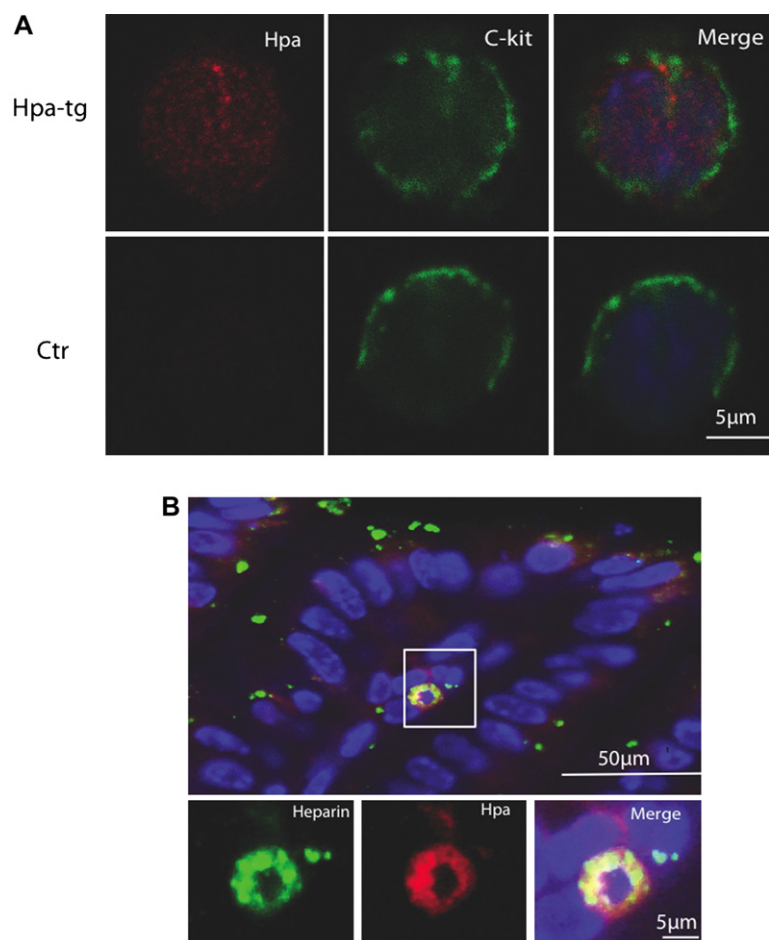


FIG E5. Immunohistologic analysis of heparanase expression in MCs and intestine. **A**, Peritoneal lavage cells were seeded on cover slips coated with fibronectin and cultured overnight. After extensive washing, the cells were stained with antibodies against c-kit (*green*) and heparanase (*red*). Nuclei are stained with DAPI. **B**, Sections of paraffin-embedded intestine from *hpa-tg* mice were double-stained with anti-heparin (*green*) and anti-heparanase (*red*) antibodies. The boxed MC is enlarged in the *lower panels*. *Ctrl*, Control.

TABLE E1. Primers used in quantitative PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
<i>mHpse</i>	TGTCCTGAACCTCCATAATGTC	TACGTATCCACTGGTTTCCTGA
<i>mGAPDH</i>	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA
<i>mMCP-4</i>	GCCAAAGAGACTCCCTCTGTGATT	GCATCTCCGCGTCCATAAGATACA
<i>mMCP-5</i>	TTGCCAGCCTGTGAGGAAA	TACAGACAGGCCAGATCGCAT
<i>mMCP-6</i>	CATTGATAATGACGAGCCTCTCC	CATCTCCCGTGTAGAGGCCAG
<i>MC-CPA</i>	TGACAGGGAGAAGGTATTCCG	CCAAGGTTGACTGGATGGTCT