

Heparanase upregulation by colonic epithelium in inflammatory bowel disease

Matti Waterman¹, Ofer Ben-Izhak², Rami Eliakim¹, Gabriel Groisman³, Israel Vlodavsky⁴ and Neta Ilan⁴

¹Department of Gastroenterology, Rambam Health Care Campus, Haifa, Israel; ²Department of Pathology, Rambam Health Care Campus, Haifa, Israel; ³Department of Pathology, Hillel-Yaffe Medical Center, Hadera, Israel and ⁴Bruce Rappaport Faculty of Medicine, Cancer and Vascular Biology Research Center, Technion-Israel Institute of Technology, Haifa, Israel

Heparanase is an endo- β -D-glucuronidase capable of cleaving heparan sulfate (HS) side chains at a limited number of sites, yielding HS fragments of still appreciable size (~5–7 kDa). Heparanase activity has long been detected in a number of cell types and tissues. Importantly, heparanase activity correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the extracellular matrix barrier. Similarly, heparanase activity was implicated in neovascularization, inflammation and autoimmunity, involving migration of vascular endothelial cells and activated cells of the immune system. The involvement of heparanase in inflammatory processes of the gastrointestinal tract has not been examined. Here, we utilized immunohistochemical analysis to investigate heparanase expression in acute and chronic inflammatory conditions. Heparanase expression was not detected in specimens derived from normal colon tissue. In contrast, strong heparanase staining was observed in Crohn's disease and ulcerative colitis, but not in infectious colitis. Interestingly, heparanase staining was primarily observed in epithelial rather than immune cells. Importantly, un-fractionated as well as low molecular weight heparin (enoxaparin), which exhibit a strong inhibitory activity towards heparanase, have proven efficacious in ulcerative colitis and Crohn's disease patients, suggesting that heparanase is actively involved in these pathologies and thus may be considered as a target for the development of anti-inflammatory therapies. *Modern Pathology* (2007) 20, 8–14. doi:10.1038/modpathol.3800710; published online 13 October 2006

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The mammalian endoglycosidase heparanase is the predominant enzyme that degrades heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPG), the dominant proteoglycan in the extracellular matrix (ECM) and cell surfaces.^{1–3} HSPG consist of a protein core to which HS side chains are covalently attached. These complex macromolecules are highly abundant in the ECM and are thought to play an important structural role, contributing to ECM integrity and insolubility.^{4,5} Traditionally, heparanase activity was well correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the

ECM barrier.^{6–9} A proof-of-concept of this notion has recently been established by using a specific anti-heparanase ribosome and siRNA methodology,¹⁰ clearly implicating heparanase activity as a critical requisite for metastatic spread. Similarly, heparanase was implicated in cell dissemination associated with inflammation and autoimmunity.^{11–13} Furthermore, heparanase activity can liberate a variety of HSPG-bound biological mediators, including cytokines and chemokines such as interferon (IFN)- γ , MIP-1 β , RANTES and interleukins, thus contributing to the regulation of inflammation and other immune responses.¹⁴

Heparanase expression by the gastrointestinal tract has been documented by employing immunostaining, RT-PCR and heparanase activity analyses.^{15–17} In these studies, heparanase was not detected in normal looking mucosa, whereas high levels of expression were detected in primary and metastatic colon carcinoma.^{15–17} The involvement of heparanase in inflammatory processes typical for the gastrointestinal tract has not been so far

Correspondence: Professor I Vlodavsky, PhD, Bruce Rappaport Faculty of Medicine, Cancer and Vascular Biology Research Center, Technion-Israel Institute of Technology, Technion, PO Box 9649, Haifa 31096, Israel.
 E-mail: Vlodavsk@cc.huji.ac.il
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examined. Crohn's disease and ulcerative colitis are chronic inflammatory conditions of unknown origin. Current data suggest that the combination of genetic, immunologic and environmental factors lead to chronic inflammatory conditions.¹⁸ Here, we utilized immunohistochemistry to investigate heparanase expression in acute and chronic inflammatory conditions. Heparanase expression was not detected in specimens derived from normal colon tissue, whereas strong heparanase staining was observed in Crohn's disease and ulcerative colitis, but not infectious colitis. Heparanase staining was primarily observed in epithelial rather than immune cells. These results indicate that heparanase levels are elevated under chronic inflammatory conditions, and that the colonic epithelium is the major source for heparanase in chronic, but not acute inflammatory disorders.

Materials and methods

Experimental Design

The study included archived formalin-fixed, paraffin-embedded colon sections obtained from ulcerative colitis ($n=17$), Crohn's disease ($n=17$) and infectious colitis patients ($n=8$) who underwent surgical colonic resection or colonoscopy at the Rambam Health Care Campus, Haifa, Israel. Socio-demographic and clinical profile (age at diagnosis, gender, disease duration before surgery, type of resection, disease extent and behavior) were collected from medical records. Crohn's disease and ulcerative colitis patients were diagnosed by expert gastroenterologists reviewing endoscopic, pathology and radiology reports. The disease extent and behavior were determined according to Gasche *et al.*¹⁹ Briefly, Crohn's disease patients were considered to have penetrating disease if intra-abdominal, peri-anal fistulas, inflammatory masses and/or abscesses were evident at any time in the course of disease, excluding postoperative intra-abdominal complications. Stricture disease was defined as the occurrence of constant luminal narrowing with pre-stenotic dilatation or obstructive signs/symptoms without presence of penetrating disease. By exclusion, all other Crohn's disease patients were considered to have an inflammatory disease. Crohn's disease location was classified as terminal ileal, colonic, ileocolonic and upper-gastrointestinal (disease limited proximal to the ileum). Ulcerative colitis location was classified as proctitis, left-sided colitis or pancolitis.

Microbiological laboratory data of stool cultures from infectious colitis patients were reviewed. Positive cultures for *Salmonella*, *Shigella*, *Campylobacter*, parasites or *Clostridium difficile* toxin, were retrieved.

Normal colonic samples were obtained from 17 patients who underwent surgical colon resection for colorectal carcinoma. Formalin-fixed, paraffin-

embedded sections of normal-looking mucosa distant from the tumor margins were analyzed.

The study protocol was approved by the Rambam Health Care Institutional Review Board.

Evaluation of Inflammation Activity

Tissue specimens were stained with hematoxyllin and eosin and classified by an expert pathologist as normal mucosa, mild, moderate or severe colitis, according to the proportion of sample crypt cross-sections containing intraluminal or intraepithelial neutrophils, that is, crypt abscesses or cryptitis. For each sample, an activity score was determined by an expert pathologist ranking 0–3 (0, no activity; 1, mild activity (<50% of crypts); 2, moderate activity (at least 50% of crypts, but no erosion/ulceration); 3, severe activity (erosion/ulceration present)).

Heparanase Immunostaining

Staining of formalin-fixed, paraffin-embedded 5 μ m sections for heparanase was performed essentially as described.^{17,20} Briefly, slides 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 in phosphate-buffered saline (PBS) for 60 min to block nonspecific binding and incubated (20 h, 4°C) with anti-heparanase 733 or 810 antibodies, diluted 1:400 and 1:100, respectively in blocking solution. Antibody 733 was raised in rabbits against a 15 amino-acid peptide (KKFKNSTYSRSSVDC) that maps at the N-terminus of the 50 kDa heparanase subunit, and preferentially recognizes the 50 kDa heparanase subunit vs the 65 kDa latent pro-enzyme (Figure 1b).²⁰ Antibody 810 was raised in rabbits against a 13 amino-acid peptide (GTKTDFLIFDPKK) located at the C-terminus of the 8 kDa heparanase subunit and preferentially recognizes the 8 kDa heparanase subunit vs the 65 kDa latent enzyme (Figure 1b).²¹ 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. Immunostained specimens were examined by senior pathologists, blind to clinical data of the patients and were scored according to the intensity of staining (0, no staining; 1, weak; 2, moderate; 3, strong staining). Specimens that were similarly stained with pre-immune serum, or applying the above procedure but lacking the primary antibody, yielded no detectable staining.

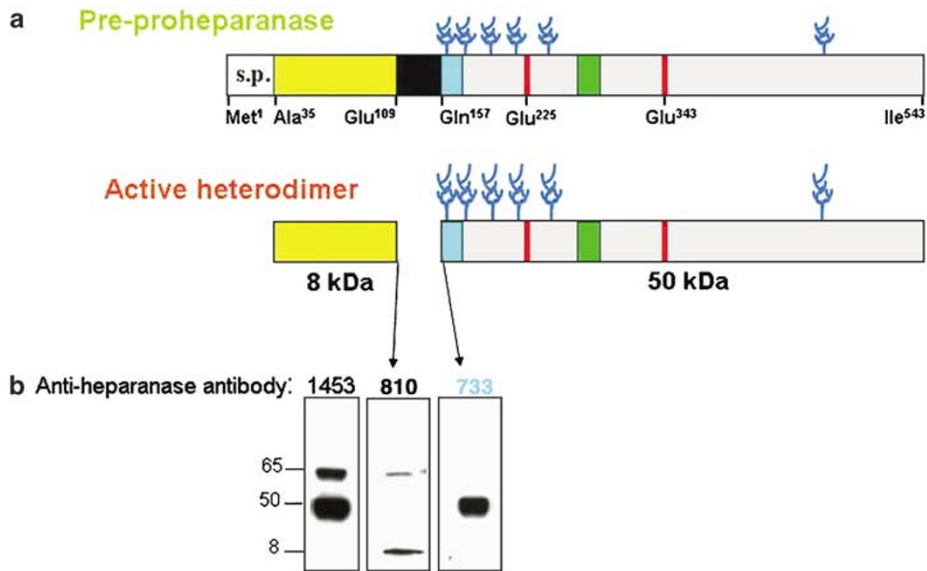


Figure 1 Heparanase primary structure, critical amino acids and antibody specificity. **(a)** Primary structure. Pre-proheparanase harbors a signal peptide (SP, Met¹-Ala³⁵) which is removed upon entering the ER. The protein is then subjected to glycosylation at six N-glycosylation sites (⚡) and secreted as a latent ~65 kDa protein (upper panel). Proteolytic processing removes the linker domain (Ser¹¹⁰-Gln¹⁵⁷), resulting in 8 kDa (Gln³⁶-Glu¹⁰⁹) and 50 kDa (Lys¹⁵⁸-Ile⁵⁴³) subunits (middle panel) that heterodimerize to yield an active enzyme. **(b)** Antibody specificity. Total cell lysate of heparanase-transfected 293 cells was subjected to immunoblotting applying antibody 1453 (left panel), antibody 733 (right panel) and antibody 810 (middle panel). Note that antibodies 733 and 810 preferentially recognize the 50 and 8 kDa heparanase subunits, respectively, vs the latent 65 kDa protein.

Statistical Analysis

Statistical analysis was performed using SPSS 11.5 software. Kruskal–Wallis test was applied to define differences of inflammation severity and heparanase staining intensity between the four groups included in the study. Mann–Whitney test was performed to check the above-mentioned differences between pair of groups, and Spearman’s correlation was employed to define correlation between inflammation severity and the intensity of heparanase staining.

Results

Patient Clinical Characteristics

We utilized immunohistochemistry analysis to study heparanase expression in specimens collected from patients with acute or chronic inflammatory conditions. Patients clinical and sociodemographic data are summarized in Table 1. Infectious colitis pathogens included *Salmonella* (three patients), *Campylobacter* (three patients), *Hymenolepis nana* (one patient), and *Clostridium difficile* (one patient). Inflammatory activity score was compared between 17 surgical specimens of normal colonic tissue, 17 specimens of ulcerative colitis (13 surgical, four endoscopic), 17 specimens of Crohn’s disease, and eight specimens of acute infectious colitis. Inflammation activity score was significantly higher in ulcerative colitis and infectious colitis biopsies compared with biopsies of Crohn’s disease (Table 1; $P=0.008$).

Heparanase Immunostaining

We have developed a polyclonal antibody (733) that preferentially recognizes the 50 kDa heparanase subunit over the latent 65 kDa pro-enzyme (Figure 1b). Thus, immunostaining of archival material can be correlated with heparanase enzymatic activity. We utilized antibody 733 to examine heparanase expression in specimens collected from patients with acute (infectious colitis) and chronic (inflammatory bowel disease) inflammation. No staining of heparanase was detected in normal colon tissue (Table 2, Figure 2a and b), in agreement with previous findings.^{15,17} Similarly, no detectable heparanase staining was evident in infectious colitis specimens (Table 2, Figure 2d and e). In contrast, heparanase staining was markedly increased in Crohn’s disease (Table 2, Figure 2g and h) and ulcerative colitis (Table 2, Figure 2j and k) specimens. Surprisingly, only weak heparanase staining was evident in immune cells (Figure 2), whereas prominent staining was detected in epithelial cells of the inflamed colon (Figure 2g–l). In all positively stained cases, more than 50% of the epithelial cells reacted with the anti-heparanase antibody. The strongest glandular and surface epithelium (Figure 2j) staining was observed in ulcerative colitis and Crohn’s disease patients (scored as 1.7 ± 0.66 and 1.4 ± 0.75 , respectively, Table 2) compared to scoring of 0.3 ± 0.4 and 0.44 ± 0.42 in normal colonic and infectious colitis specimens, respectively, differences that are statistically highly significant ($P=0.008$). Moreover, the intensity of heparanase

Table 1 Patient characteristics and inflammation activity

	Ulcerative colitis (n = 17)	Crohn's disease (n = 17)	Infectious colitis (n = 8)
Age at diagnosis (years, mean ± s.d.)	32.9 ± 19.7	27.4 ± 15.9	NA
Males (%)	13 (76)	9 (53)	6 (75)
Disease duration before surgery/endoscopy (years, mean ± s.d.)	6.6 ± 8.5	6.1 ± 6.6	2.3 weeks
Indication for surgery	Intractable disease: 12/17 IBD surveillance: 2/17 IBD exacerbation: 2/17 Bowel obstruction: 1/7	Intractable disease: 4/17 Bowel obstruction: 5/17 Severe dysplasia: 1/17 Disease penetration: 6/17 Unknown: 1/17	NA
Type of surgery	Total proctocolectomy: 13/17 Colonoscopy: 4/17	Rt hemicolectomy: 12/17 Lt. hemicolectomy: 2/17 Total colcetomy: 2/17 Sigmoidectomy: 1/17	NA
Disease extent	Proctitis: 0/17 Left-sided colitis: 4/17 Pancolitis: 13/17	Terminal ileum: 2/17 Ileo-colic: 11/17 Colon-only: 4/17	NA
Disease behavior	NA	Inflammatory: 4/17 Penetrating: 7/17 Strictureing: 6/17	NA
Inflammation activity score (mean ± s.d.)	1.88 ± 0.82	0.74 ± 0.69	1.62 ± 0.52

Table 2 Heparanase staining intensity in normal colonic vs colitis biopsies

	Heparanase 733 staining intensity in colonic epithelial cells (mean ± s.d.)	Proportion of positively stained specimens
Normal colon	0.32 ± 0.43	4 ^c /17
Ulcerative colitis	1.74 ± 0.66 ^{a,b}	17/17
Crohn's disease	1.41 ± 0.75 ^{a,b}	15/17
Infectious colitis	0.44 ± 0.42	2 ^c /8

^aCompared to normal colon $P=0.001$.

^bCompared to infectious colitis $P=0.008$.

^cWeak (+1) staining was observed in these cases.

staining by the inflamed epithelium correlated with inflammation activity in ulcerative colitis and Crohn's disease patients (Spearman's correlation index $r_s=0.404$, $P=0.018$). In ulcerative colitis specimens, similar heparanase staining was noted for endoscopic (4/17) and full-thickness (13/17) biopsies. No heparanase staining was observed in the muscularis and serosa layers, suggesting for epithelial-specific reactivity. In the inflamed tissue, heparanase staining was mainly confined to a supra-nuclear aspect of the colon epithelia (Figure 2h and k) in a pattern that somewhat resembled vesicle-like structures. These vesicles have been previously characterized as endocytic endosomes and lysosomes in cells maintained *in vitro*,^{20,22,23} as well as in tumor xenografts.²⁰ In order to further substantiate this unexpected staining pattern, biopsies were similarly stained with anti-heparanase 810 antibody. This antibody is directed against a peptide mapped at the C-terminus of the 8 kDa subunit that com-

prises, together with the 50 kDa subunit, the active heparanase heterodimer enzyme (Figure 1a).^{21,24} Moreover, antibody 810 preferentially recognizes the 8 kDa subunit vs the 65 kDa latent enzyme (Figure 1b)²¹ and is thus expected to yield staining pattern similar to antibody 733. Indeed, staining with antibody 810 resembled the staining with antibody 733 in terms of specificity, intensity and localization. Thus, no detectable heparanase staining was observed in normal (Figure 2c) and infectious colitis (Figure 2f) specimens. Heparanase was readily detected by antibody 810 in the glandular epithelium of Crohn's disease (Figure 2i) and ulcerative colitis (Figure 2l) specimens, assuming typical supra-nuclear localization (Figure 2i and l). Staining pattern (intensity and localization) with antibody 810 correlated with antibody 733 staining in 14 out of 16 IBD specimens examined (eight Crohn's disease and eight ulcerative colitis), supporting the notion that the colonic epithelium is a major source of heparanase under chronic, but not acute, inflammatory conditions.

Discussion

The etiology of inflammatory bowel disease, including Crohn's disease and ulcerative colitis, remains unknown and is likely multifactorial.²⁵ Clearly, the immune system plays a key role in either initiating or maintaining the disease state, largely owing to imbalance between pro-, and anti-inflammatory cytokines.¹⁸ The intestinal epithelium plays an active role in the maintenance of mucosal homeostasis and is considered to be part of the innate

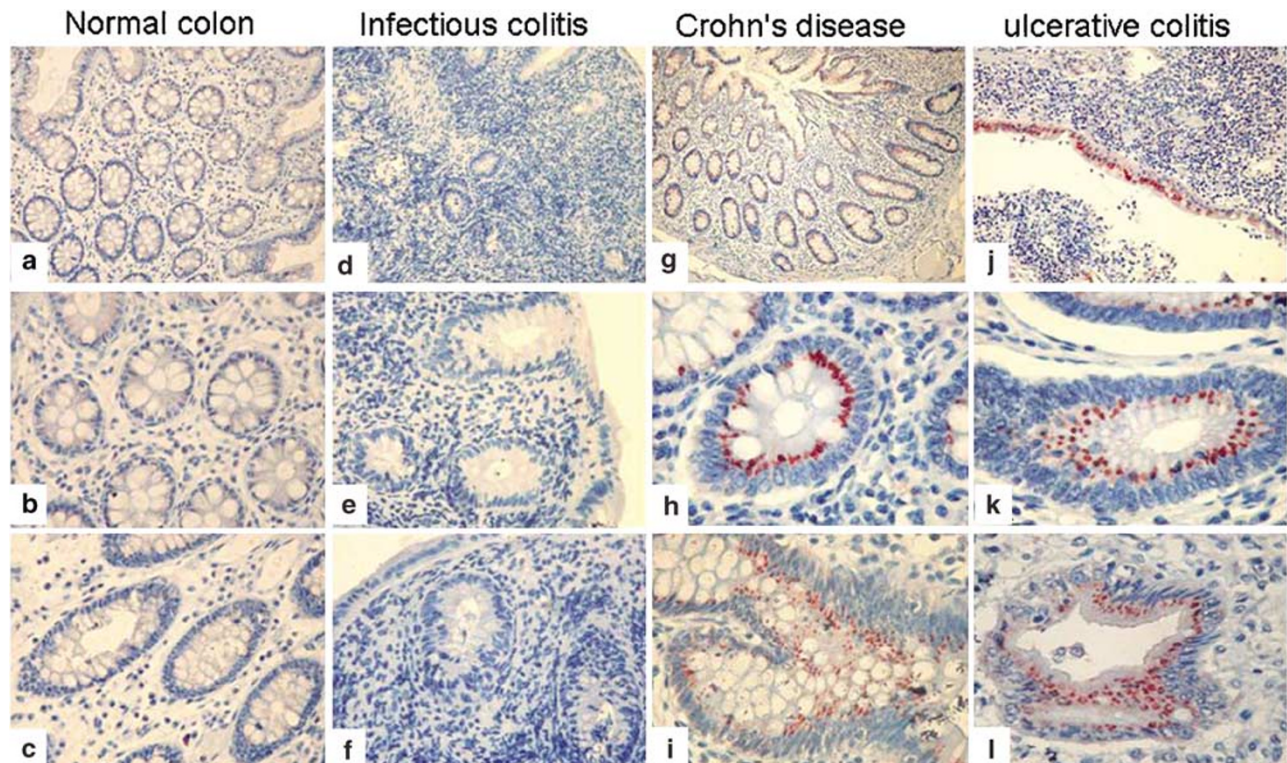


Figure 2 Immunohistochemical staining of heparanase in inflammatory bowel disease specimens. Formalin-fixed, paraffin-embedded 5 μ m sections of normal (a–c), infectious colitis (d–f), Crohn's disease (g–i) and ulcerative colitis (j–l) specimens were subjected to immunostaining of heparanase, applying anti-heparanase 733 (upper and middle panels) and 810 (lower panel) antibodies, as described under 'Materials and methods'. Shown are representative photomicrographs. Original magnifications: (a), (d), (g), (j), (k) \times 50; (b), (c), (e), (f), (h), (i), (l) \times 200. No detectable staining was observed with pre-immune serum, or when the primary antibody was omitted.

immunity system. Epithelial cells can act as antigen-presenting cells and, moreover, are avid producers of chemokines and cytokines considered central to the pathogenesis of inflammatory bowel disease such as tumor necrosis factor (TNF)- α , interleukin-1 and interleukin-6.²⁶ Aberrant secretion of these pro-inflammatory mediators by the epithelium is an integral part of the dysregulated immune response that initiates or perpetuates intestinal inflammation.²⁶ The results presented here clearly suggest that colonic glandular and surface epithelial cells are also avid producers of heparanase. Among the few cell types that express heparanase under normal physiological conditions are placental cytotrophoblasts, skin keratinocytes and blood borne cells such as lymphocytes, neutrophils, macrophages and platelets.^{2,3} Heparanase was localized to tertiary granules of neutrophils^{6,27} and mast cells,²⁸ and its release by degranulation has been implicated in diapedesis of a number of immune cells, including neutrophils, macrophages, mast cells, dendritic cells and lymphocytes,^{13,29,30} thus strongly implying heparanase as a pro-inflammatory mediator. Surprisingly, however, only a weak heparanase staining was observed in immune cells of IBD specimens (Figure 2). In contrast, the inflamed colonic epithelium exhibited strong reactivity with the anti-heparanase antibodies employed (Table 2, Figure 2), suggesting

that colonic epithelium rather than immune cells is the major heparanase producer under chronic inflammatory conditions. We have recently reported that heparanase expression is induced in a delayed-type hypersensitivity inflammatory model in mice.³¹ In the delayed-type hypersensitivity model, the vascular endothelium emerged as the major cellular source of heparanase, and pro-inflammatory mediators such as TNF- α and IFN- γ were able to induce heparanase expression by cultured endothelial cells.³¹ It is likely that elevated heparanase levels produced locally by endothelial cells enhance the remodeling of the vascular basement membrane, allowing extravasation of immune cells.³¹ It therefore appears that vascular-, and immune cell-derived heparanase, contributes to the early phases of an inflamed condition,³¹ whereas prolonged exposure to pro-inflammatory cytokines results in heparanase induction by the colonic epithelium, contributing to the sustained, chronic nature of the disease. Interestingly, un-fractionated as well as low molecular weight heparin (enoxaparin), which exhibit a strong inhibitory activity towards heparanase,^{32–35} have proven efficacious in ulcerative colitis and Crohn's disease patients,^{36–38} suggesting that heparanase is actively involved in these pathologies and thus may be considered as a target for the development of anti-inflammatory therapies.

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