Heparanase expression by Barrett's epithelium and during esophageal carcinoma progression

Rita Brun¹, Inna Naroditsky², Matti Waterman¹, Ofer Ben-Izhak², Gabriel Groisman³, Neta Ilan⁴, and Israel Vlodavsky^{4*}

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 ⁴Cancer and Vascular Biology Research Center, The Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel

Enzymatic activity responsible for the cleavage of heparan sulfate, commonly known as heparanase, is abundant in tumor-derived cells. Heparanase cleaves heparan sulfate side chains, presumably at sites of low sulfation, thus facilitating structural alterations of the extracellular matrix and basement membrane underlying epithelial and endothelial cells. Traditionally, heparanase activity was correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of heparan sulfate cleavage and remodeling of the extracellular matrix barrier. More recently, heparanase upregulation was documented in an increasing number of human carcinomas and hematological malignancies, correlating with increased tumor metastasis, vascular density, and shorter post-operative survival of cancer patients. Although heparanase upregulation and its pro-malignant features are well documented, the instance of its induction in the course of tumor development was less investigated. Here, we used immunohistochemical analysis to investigate heparanase expression in normal esophagus, Barrett's esophagus without dysplasia, Barrett's esophagus with low-grade dysplasia, Barrett's esophagus with high-grade dysplasia, and adenocarcinoma of the esophagus. We report that heparanase expression is already induced in Barrett's epithelium without dysplasia, and is further increased during progression through distinct pathological stages, namely, low-grade dysplasia, high-grade dysplasia, and adenocarcinoma. Notably, heparanase induction correlated with increased cell proliferation index revealed by Ki-67 staining. These findings suggest that heparanase function is not limited to the process of tumor metastasis, but rather is engaged at the early stages of esophagus carcinoma initiation and progression.

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Heparanase is an endo- β -D-glucuronidase, the predominant enzyme that degrades heparan sulfate side chains of heparan sulfate proteoglycans.^{1,2} These complex macromolecules are highly abundant in the extracellular matrix and are thought to have an important structural role, contributing to extracellular matrix integrity and insolubility.^{3,4} Traditionally, heparanase activity was correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence

of heparan sulfate cleavage and remodeling of the extracellular matrix barrier.^{1,2} A proof-of-concept to this notion has been established by using specific anti-heparanase ribozyme and siRNA methodologies, clearly implicating heparanase-mediated heparan sulfate cleavage as a critical requisite for metastatic spread.⁵ Similarly, heparanase activity was implicated in cell dissemination associated with inflammation and angiogenesis.^{5,6} More recently, heparanase upregulation was documented in an increasing number of human carcinomas and hematological malignancies.^{7,8} In many cases, heparanase induction correlated with increased tumor metastasis, vascular density, and shorter post-operative survival of cancer patients, thus providing a strong clinical support for the pro-metastatic and pro-angiogenic functions of the

*Correspondence: Professor I Vlodavsky, Cancer and Vascular Research Center, Bruce Rappaport Faculty of Medicine, Technion, PO Box 9649, 7 Efron street, Haifa 31096, Israel.

E-mail: vlodavsk@cc.huji.ac.il

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enzyme and encouraging the development of heparanase inhibitors such as anti-cancer drugs.^{9–12}

Heparanase expression by the gastrointestinal tract has been studied employing immunostaining, RT-PCR, and enzymatic activity analyses. In these studies, heparanase was not detected in normal-looking epithelia, whereas high levels of expression were observed in colon and gastric carcinomas.^{13–17}

Heparanase induction has similarly been observed in carcinoma of the esophagus, and its expression correlated with tumor malignancy and poor survival.^{18–21} Although heparanase upregulation and its pro-malignant features are well documented, the timing of its induction in the course of tumor development is less investigated and understood. Barrett's esophagus is defined as the replacement (metaplasia) of the normal esophageal squamous mucosa with columnar epithelium.^{22,23} This condition most commonly arises in the setting of chronic gastroesophageal reflux disease, in which repeated mucosal injury is believed to stimulate metaplasia. The abnormal Barrett's epithelium predisposes patients to the development of epithelial dysplasia and, ultimately, to adenocarcinoma.^{22,23} Adenocarcinomas seem to progress in a step-wise manner, from low-grade to high-grade dysplasia, and to intramucosal and finally to invasive carcinoma. Here, we used immunohistochemistry analysis to investigate heparanase expression in normal esophagus, Barrett's esophagus without dysplasia, Barrett's esophagus with low-grade dysplasia, Barrett's esophagus with high-grade dysplasia, and adenocarcinoma of the esophagus.

Materials and methods

Experimental Design

The study included archived paraffin-embedded esophageal sections obtained from 66 patients who underwent esophagogastroduodenoscopy with biopsy or surgical esophageal resection. The cohort included 11 samples of normal esophagus, 21 Barrett's esophagus without dysplasia, 5 Barrett's esophagus with low-grade dysplasia, 12 Barrett's esophagus with high-grade dysplasia, and 17 adenocarcinomas, diagnosed at the Rambam Health Care Campus, Haifa, and the Hillel-Yaffe Medical Center, Hadera, Israel. The grade of dysplasia was defined according to the degree of nuclear atypia and glandular architectural changes.²⁴ Paraffin sections were stained with anti-heparanase antibodies and heparanase staining intensity was correlated to pathological staging and proliferation index revealed by anti-Ki-67 staining. The study protocol was approved by the Institutional Review Board.

Immunostaining

Staining of formalin-fixed, paraffin-embedded 5- μ m sections for heparanase was performed essentially

as described.^{25,26} Briefly, slides were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched (30 min) by 0.3% 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 non-specific binding, followed by incubation (20 h, 4°C) with polyclonal anti-heparanase 733 antibodies,²⁷ diluted 1:100 in blocking solution. 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. After additional washes, color was developed using the AEC reagent (Dako), sections were counterstained with hematoxylin and mounted, as described.^{25,26} Immunostained specimens were examined by a senior pathologist and were scored according to the intensity of staining (0 = none; 1 = weak to moderate; and 2 = strong). In addition, staining localization was classified as nuclear (n), cytoplasmic (c), or both (n + c). Slides were similarly stained with anti-Ki-67 monoclonal antibody (MIB-1; Dako). Biotinylated secondary antibody was applied for 10 min, followed by incubation with streptavidin peroxidase (Dako) for 10 min. Slides were then washed extensively and color was developed using AEC reagent (Dako) and counterstained with hematoxylin as above. Cell proliferation index was assessed by a senior pathologist and categorized as low (<5%), moderate (6–50%), and high (>50%) according to the percentage of Ki-67-positive cells.

Statistical Analysis

The data were evaluated by SPSS software, version 15 (SPSS, Chicago, IL, USA). Fisher's Exact Test and Pearson's χ^2 -test were used for detection of differences in the prevalence of the staining intensity between different diagnoses. Differences between heparanase intensity and the percentage of Ki-67 staining were assessed by non-parametric Mann–Whitney *U*-test. *P* < 0.05 was considered significant.

Table 1 Heparanase staining intensity in normal and pathological esophageal specimens

Staging	Heparanase staining intensity			Total
	0 (%)	1 (%)	2 (%)	
Normal	11 (100)			11
Barrett's esophagus	10 (48)	7 (33)	4 (19)	21
Low grade	2 (40)	3 (60)		5
High grade	0	8 (67)	4 (33)	12
Adenocarcinoma	0	2 (12)	15 (88)	17
	23	20	23	66

Results

Heparanase staining was not detected in normal esophageal epithelium (Table 1, Figure 1a). In contrast, positive staining of heparanase was observed in 52% (11/21) of the patients diagnosed

with Barrett's esophagus without dysplasia (Figure 1b; Table 1), an increase that is statistically highly significant ($P=0.0045$). In Barrett's esophagus, heparanase was mainly confined to intestinal-type epithelium and was localized at the basal aspect of the epithelium in a spotted manner (Figure 1b).

Figure 1 Immunohistochemical staining of esophageal specimens. Heparanase staining. Formalin-fixed, paraffin-embedded 5- μ m sections of normal (a), Barrett's (b), low-grade (c), high-grade (d), and adenocarcinoma (e) esophageal biopsies were subjected to immunostaining of heparanase, applying anti-heparanase polyclonal Antibody no 733, as described under 'Materials and Methods'. Specimens that were similarly stained applying the above procedure, but lacking the primary antibody, yielded no detectable staining (f, adenocarcinoma). Original magnification: (a) $\times 200$; (b–f) $\times 400$.

Gastric–cardiac epithelium and gastric body-type epithelium often mingled with intestinal epithelium of Barrett's esophagus, and rarely stained for heparanase. Further enhancement in heparanase staining was observed in high-grade dysplasia ($P=0.005$; Figure 1d), wherein all cases stained positively (Table 1). Similarly, all cases diagnosed as esophagus adenocarcinoma stained positive for heparanase (Table 1; Figure 1e). Moreover, although in high-grade dysplasia 33% of the cases (4/12) exhibited strong (+2) staining of heparanase, 88% (15/17) of the adenocarcinoma biopsies exhibited such staining ($P=0.0045$) (Table 1). Unlike the spotted appearance noted in Barrett's esophagus (Figure 1b) and low-grade dysplasia (Figure 1c), heparanase staining appeared diffuse throughout the cell cytoplasm of high-grade dysplasia (Figure 1d) and adenocarcinoma (Figure 1e) specimens. Careful examination revealed further differences in heparanase cellular localization. Thus, although in high-grade specimens heparanase staining appeared mostly cytoplasmic (Figure 1d; Figure 2a), 71% (12/17) of the adenocarcinoma cases also exhibited nuclear localization of heparanase (Figure 2b) compared with only one case of high-grade dysplasia exhibiting such localization ($P=0.0018$) (Figure 2c).

To further elucidate the clinical significance of heparanase expression in esophagus cancer, cell proliferation was evaluated by staining for Ki-67. As expected, a low rate of cell proliferation was noted in normal and Barrett's epithelium (Figure 3a, b, respectively). In Barrett's esophagus, Ki-67 staining primarily decorated the intestinal epithelium. Cell proliferation was markedly increased in high-grade dysplasia (Figure 3d) and adenocarcinoma (Figure 3e). Significantly, heparanase staining correlated with cell proliferation index ($P=0.0006$) (Table 2). Thus, although 87% of the cases that were stained negative for heparanase exhibited a low (<5) proliferative index, most heparanase-positive cases (70%) showed medium to high proliferation rate (Table 2). These findings imply that heparanase may have a role in esophageal cancer initiation and progression.

Discussion

It is now generally accepted that Barrett's epithelium can progress through a metaplasia–dysplasia–carcinoma sequence.^{22,23} Barrett's epithelium is, therefore, considered a premalignant condition with

Figure 2 Heparanase cellular localization. Shown are representative photomicrographs of cytoplasmic (a) and nuclear (b) staining of heparanase in esophageal adenocarcinoma. Original magnification: $\times 400$. Distribution (percent) of cases exhibiting cytoplasmic vs nuclear localization of heparanase in high-grade dysplasia and adenocarcinoma is presented graphically in (c).

Figure 3 Immunohistochemical Ki-67 staining of esophageal specimens. Formalin-fixed, paraffin-embedded 5- μ m sections of normal (a), Barrett's (b), low-grade (c), high-grade (d), and adenocarcinoma (e) esophageal biopsies were subjected to immunostaining of Ki-67 as described under 'Materials and Methods'. Original magnification: $\times 200$.

a 50–100-fold increased risk of cancer.^{22,23} The etiology of malignant Barrett's esophagus remains largely unknown and is likely multifactorial. Previous studies showed that heparanase expression is induced in carcinoma of the esophagus.^{18,20} Heparanase induction correlated with enhanced tumor cell invasion into the muscular and adventitia

layers, increased tumor metastasis, and advanced tumor stage, altogether associated with poor survival.^{18,20} High heparanase staining in esophageal adenocarcinoma biopsies noted in this study (Table 1) further supports this notion. Interestingly, however, increased heparanase expression was already noted in Barrett's epithelium, representing

Table 2 Heparanase expression associates with cell proliferation revealed by Ki-67 staining

Heparanase status	Ki-67		
	<5 (%)	6–50 (%)	>50 (%)
Negative	87	13	0
Positive	30	45	25

$P=0.0006$.

the earliest stage of esophagus carcinogenesis (Table 1; Figure 1). Further increase in heparanase expression paralleled the progression through distinct pathological stages, namely, low-grade dysplasia, high-grade dysplasia, and adenocarcinoma (Table 1, Figure 1). These findings suggest that heparanase function is not limited to the process of tumor metastasis, but rather is engaged at the early stages of esophagus carcinoma initiation and progression to adenocarcinoma. Intimate involvement in esophageal cancer development is further supported by the close association found between heparanase expression and cell proliferation. Clearly, biopsies stained positively for heparanase exhibited a higher proliferative index than did heparanase-negative specimens (Table 2). Reduced proliferation rates noted after heparanase gene silencing in tumor-derived cells²⁸ supports this notion and suggest that its pro-proliferative function is a common feature. Thus, heparanase inhibitors, such as non-anticoagulant glycol-split heparin^{12,29} may be considered as a prophylactic treatment for Barrett's patients. This possibility is supported by recent studies showing no adverse side effects in mice treated with this drug for a relatively long period of time,³⁰ yet it awaits in-depth preclinical and clinical evaluation. Whether heparanase plasma levels could serve as a biomarker for distinguishing Barrett's cases that are more likely to progress into adenocarcinoma is yet to be revealed and requires long-term follow-up and a large cohort of patients. A recently developed heparanase ELISA assay^{31–33} may be applied to monitor heparanase levels in Barrett's patients. This approach would not only provide another indication for heparanase upregulation and secretion into the circulation but may also ideally substitute for the invasive endoscopic surveillance currently employed to monitor Barrett's patients.

Esophagus cancer progression was also associated with alterations in heparanase cellular localization. In Barrett's and low-grade dysplasia, heparanase appeared punctuated and was localized to the basal aspect of the epithelium (Figure 1b, c). Interestingly, a similar staining pattern was noted in adenoma of the colon and in inflammatory bowel disease specimens,^{14,26} both representing premalignant conditions. In contrast, heparanase appeared more diffusely distributed in the cell cytoplasm of high-grade dysplasia (Figure 1d) and adenocarcinoma

specimens (Figure 1e) representing, possibly, heparanase protein that is more readily secreted. This is in agreement with the elevation of heparanase levels in the plasma and urine of cancer patients.^{31–33} The significance of this observation critically emerges from recent studies showing that secreted heparanase augments signal transduction pathways, leading to activation of certain protein kinases (that is, Akt, Src, EGFR) and increased gene transcription (that is, VEGF, TF, COX2) associated with enhanced tumor progression.^{21,28,34–39}

Previous studies have shown that heparanase also assumes nuclear localization,^{40,41} and that nuclear heparanase is associated with a favorable prognosis of head and neck, gastric, and esophageal cancer patients.^{20,41–43} It was unclear, however, at what stage of tumor initiation/progression nuclear translocation takes place. Likewise, nuclear localization of heparanase was observed in the present study (Figure 2b, c), supporting its prevalence in carcinoma of the esophagus. Notably, nuclear localization was observed primarily in esophagus adenocarcinoma, and only occasionally in high-grade dysplasia (Figure 2c). These findings suggest that nuclear translocation of heparanase takes place mainly at the final stages of tumor progression into adenocarcinoma. Identifying the molecular mechanism underlying this process and the associated favorable outcome is a major challenge for future research focusing on heparanase.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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