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Expression of Heparanase in Normal, Dysplastic, and Neoplastic Human Colonic Mucosa and Stroma

Evidence for Its Role in Colonic Tumorigenesis

Yael Friedmann,* Israel Vlodavsky,* Helena Aingorn,* Ayelet Aviv,* Tuvia Peretz,† Iris Pecker,† and Orit Pappo‡

From the Departments of Oncology and Pathology,‡ Hadassah-Hebrew University Hospital, Jerusalem, and InSight Ltd.,† Rabin Science Park, Rehovot, Israel*

The human heparanase gene, an endo-β-glucuronidase that cleaves heparan sulfate at specific intrachain sites, has recently been cloned and shown to function in tumor progression and metastatic spread. Antisense digoxigenin-labeled heparanase RNA probe and monoclonal anti-human heparanase antibodies were used to examine the expression of the heparanase gene and protein in normal, dysplastic, and neoplastic human colonic mucosa. To our knowledge, this is the first systematic study of heparanase expression in human colon cancer. Both the heparanase gene and protein were expressed at early stages of neoplasia, already at the stage of adenoma, but were practically not detected in the adjacent normal-looking colon epithelium. Gradually increasing expression of heparanase was evident as the cells progressed from severe dysplasia through well-differentiated to poorly differentiated colon carcinoma. Deeply invading colon carcinoma cells showed the highest levels of the heparanase mRNA and protein associated with expression of both the gene and enzyme by adjacent desmoplastic stromal fibroblasts. A high expression was also found in colon carcinoma metastases to lung, liver, and lymph nodes, as well as in the accompanying stromal fibroblasts. Moreover, extracts derived from tumor tissue expressed much higher levels of the heparanase protein and activity as compared to the normal colon tissue. In all specimens, the heparanase gene and protein exhibited the same pattern of expression. These results suggest a role of heparanase in colon cancer progression and may have both prognostic and therapeutic applications. ***(Am J Pathol 2000, 157:1167–1175)***

For a malignant tumor cell to metastasize, it must break away from its neighbors, force its way through the surrounding stroma, and penetrate basement membranes to enter the circulation. When it arrives at its destination, these steps must be repeated in reverse order.[1] A critical event in the process of cancer invasion and metastasis is therefore degradation of various constituents of the extracellular matrix (ECM) including collagen, laminin, fibronectin, and heparan sulfate proteoglycans (HSPGs). The cell is able to accomplish this task through the concerted action of enzymes such as metalloproteinases, serine proteases, and endoglycosidases.[2,3]

Among these enzymes is an endo-β-glucuronidase (heparanase) that cleaves heparan sulfate (HS) at specific intrachain sites.[4–6] HSPGs are ubiquitous macromolecules associated with the cell surface and ECMs of a wide range of cells and tissues.[7,8] The basic HSPG structure consists of a protein core to which several linear HS chains are covalently linked.[7,8] The ability of HS to interact with ECM macromolecules such as collagen, laminin, and fibronectin and with different attachment sites on the cell membrane suggests a key role for this proteoglycan in the self-assembly and insolubility of ECM components, as well as in cell adhesion and locomotion.[9–11] HSPGs are prominent components of blood vessels.[12] In capillaries they are found mainly in the subendothelial basement membrane where they support the vascular endothelium and stabilize the structure of the capillary wall. Cleavage of HS, therefore plays an important role in extravasation of blood-borne cells.[4,5]

Previous studies performed by us and by other groups have demonstrated a correlation between the expression of heparanase and the metastatic potential of various tumor cell lines.[4,5] Moreover, heparanase activity was detected in the urine of patients with aggressive metastatic cancer but not in the urine of healthy donors.[13] Treatment of experimental animals with heparanase inhibitors (eg, nonanticoagulant species of low molecular weight heparin, polysulfated saccharides) markedly reduced the incidence of experimental metastases.[4,5,14,15]

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Address reprint requests to Dr. Israel Vlodavsky, Department of Oncology Hadassah Hospital, POB 12000, Jerusalem 91120, Israel. E-mail: vlodavsk@cc.huji.ac.il.

Apart from its involvement in the egress of cells from the vasculature, heparanase may play an accessory role in angiogenesis and tissue repair by releasing HS-bound growth factors[16–18] and promoting endothelial cell migration and basement membrane degradation. Recently, partial sequencing of heparanase purified from human placenta, platelets, and hepatoma cells, followed by screening of expressed sequence tag (EST) databases led to the cloning of a cDNA and gene encoding the heparanase protein.[19–22] Only one sequence was identified, consistent with the notion that this is the dominant endoglucuronidase in mammalian tissues.[19–22] The genomic locus which encodes heparanase spans ~40 kb. It is composed of 12 exons separated by 11 introns and is localized on human chromosome 4q21.3.[19,20,23] Expression of the cloned cDNA in insect and mammalian cells yielded 65-kd and 50-kd recombinant proteins. The 50-kd enzyme represents a N-terminal-processed enzyme which is at least 200-fold more active than the full-length 65-kd form. Processing was readily demonstrated during incubation of the full-length recombinant enzyme with intact or lysed tumor cells.[19] Nonmetastatic murine T-lymphoma cells transfected with the heparanase gene acquired a highly metastatic phenotype *in vivo*, reflected by a high rate of mortality because of massive liver infiltration of subcutaneously inoculated lymphoma cells.[19] Preferential expression of the heparanase gene was found in metastatic cell lines and specimens of human breast, colon, and liver carcinomas.[19]

In the present study we applied antisense digoxigenin-labeled heparanase RNA probes and monoclonal anti-human heparanase antibodies to investigate the heparanase gene and protein distribution at various stages of human colon carcinoma progression. Expression of the heparanase gene and protein was detected at early stages of neoplasia, already at the stage of adenoma, whereas expression in the adjacent normal-looking colon was confined to nerves, ganglion cells, and smooth muscle around blood vessels. Gradually increasing expression of heparanase was evident as the cells progressed from severe dysplasia through well-differentiated to poorly differentiated colon carcinoma. Deeply invading colon carcinoma cells and adjacent desmoplastic stromal fibroblasts showed the highest levels of the heparanase mRNA and protein. Both the heparanase gene and protein were also highly expressed in colon carcinoma metastases to lung, liver, and lymph nodes, as well as in the accompanying stromal fibroblasts.

Materials and Methods

Tissue for Immunohistochemistry and in Situ Hybridization

Routinely processed formalin-fixed and paraffin-embedded specimens from 17 patients with colonic neoplasia operated on during 1996 to 1999 were retrieved from the files of the Departments of Pathology at the Hadassah University Hospital (Jerusalem) and the Tel-Aviv Sourasky Medical Center. The specimens included 16 cases of adenocarcinoma, five of which also had metastases to regional lymph nodes and three with distant metastases (two to lungs and one to liver). Two of the patients also had neoplastic polyps with severe dysplasia in their resected specimen. Another patient who underwent colectomy for tubulovillous adenoma was included. The specimens were evaluated according to standard criteria as detailed in Table 1.

Table 1. Patient Demographics and Histologic Grading and Staging

Patient no.	Age	Sex	Dukes	Grade
1	75	M	(TVA)	
2	64	F	A	PD
3	67	F	B2	MPD
4	78	F	B2	MD
5	85	M	B2	MD
6	81	F	C	MD
7	84	F	C	MD
8	70	M	C	MD
9	84	F	C	PD
10	82	M	C	MD
11	81	F	C	MD
12	76	M	D	MD
13	61	F	D	MD
14	47	M	D	MD
15	47	F	D	MPD
16	55	F	D	MD
17	55	F	D	MD

TVA, tubulovillous adenoma; PD, poorly differentiated; MPD, moderately to poorly differentiated; MD, moderately differentiated.

We studied the neoplastic, preneoplastic, and apparently uninvolved normal tissue from these cases.

Riboprobe Preparation and in Situ Hybridization

A 618-bp fragment of human heparanase was subcloned into the vector pBluescript KSII multiple cloning site (+/−) (Stratagene, La Jolla, CA). The vector was linearized and used as a template for *in vitro* transcription of antisense or sense (control) riboprobes using T7 and T3 RNA polymerase (Promega, Madison, WI), respectively. Riboprobes were labeled with digoxigenin RNA-labeling mixture (Boehringer-Mannheim, Mannheim, Germany). *In situ* hybridization was performed as described previously[19,24] with minor modifications. Briefly, 5-μm sections were mounted on SuperFrost Plus slides (Manzel-Glaser, Braumschweig, Germany), dewaxed, and rehydrated. Sections were first denatured with 0.2 N HCl for 10 minutes and then digested with proteinase K (20 μg/ml) at 37°C for 30 minutes. Digestion was stopped with two changes of H_2O. Slides were prehybridized and hybridized, as described.[24] Probe concentration was 2 μg/ml. Washes after hybridization, incubation with anti-digoxigenin antibodies, and colorimetric detection were performed as described before.[24] Some of the slides were counterstained with Mayer's hematoxylin.

Immunohistochemistry

Immunohistochemistry was performed as described before with minor modifications.[19] Briefly, 5-μm sections

were deparaffinized and rehydrated. Tissue was then denatured for 3 minutes in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0). Blocking steps included successive incubations in 0.2% glycine, 3% H_2O_2 in methanol, and 5% goat serum. The first two steps were followed by two washes in phosphate-buffered saline (PBS). Sections were incubated with a monoclonal mAb (92.4) anti-human heparanase antibody diluted 1:3 in PBS, or with Dulbecco's modified Eagle's medium supplemented with 10% horse serum as control, diluted as above, followed by incubation with horseradish peroxidase-conjugated goat-anti-mouse IgG + IgM antibody (Jackson, Bar-Harbor, ME). mAb 92.4 is directed against the N-terminus region of the 45-kd enzyme. The preparation and specificity of this mAb were previously described and demonstrated.[19] Color was developed using either Sigma Fast 3,3–diaminobenzidine tablet sets (Sigma Chemical Co., St. Louis, MO) or Zymed aminoethyl carbozole (AEC) substrate kit (Zymed, South San-Francisco, CA) for 10 minutes followed by counterstain with Mayer's hematoxylin.

Heparanase Activity

Tissue specimens from three patients were freshly taken and homogenized in buffer containing 20 mmol/L phosphate/citrate, pH 6.0, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, and 0.5% Nonidet P-40. The tissue specimens were derived from the colon carcinoma and from normal-looking colon tissue away from the tumor region. The supernatant fractions were applied onto 0.3 ml of carboxymethyl (CM)-Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and the bound material was eluted with 10 mmol/L phosphate-citrate buffer, pH 6.5, supplemented with 1 mmol/L dithiothreitol, 1 mmol/L Ca^{++}, and 0.7 mol/L NaCl. Aliquots of the eluates containing 15 μg of protein were then tested for heparanase activity using as a substrate metabolically sulfate-labeled ECM, prepared as described.[5,15,19,25] Low-molecular weight sulfate-labeled HS degradation fragments released into the incubation medium were analyzed by gel filtration on a Sepharose CL-6B column.[5,15,19,25] We have previously demonstrated that these fragments elute from the column at $0.5 < K_{av} < 0.8$ (peak II, fractions 15 to 35). Nearly intact HSPG elutes just after the void volume of the column (peak I, $K_{av} < 0.2$).[26] Each experiment was done at least twice and the variation of elution positions (K_{av} values) did not exceed $\pm$15%.

Western Blot Analysis

For immunoblot analysis aliquots of the partially-purified material eluted from CM-Sepharose were loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins transferred to Immobilon-P membrane (Millipore, Bedford, MA), followed by successive incubations with block solution, anti-heparanase monoclonal antibodies in 1% bovine serum albumin, 10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 0.05% Tween-20, and horseradish peroxidase-conjugated anti-mouse antibodies (Jackson Laboratories, Bar-Harbor, ME), as described.[19] Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) reagent using luminol and p-cumaric acid (Sigma, St. Louis, MO). The light emitted by the chemical reaction was detected by exposure to Hyperfilm ECL (Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 to 120 seconds.[19]

Results

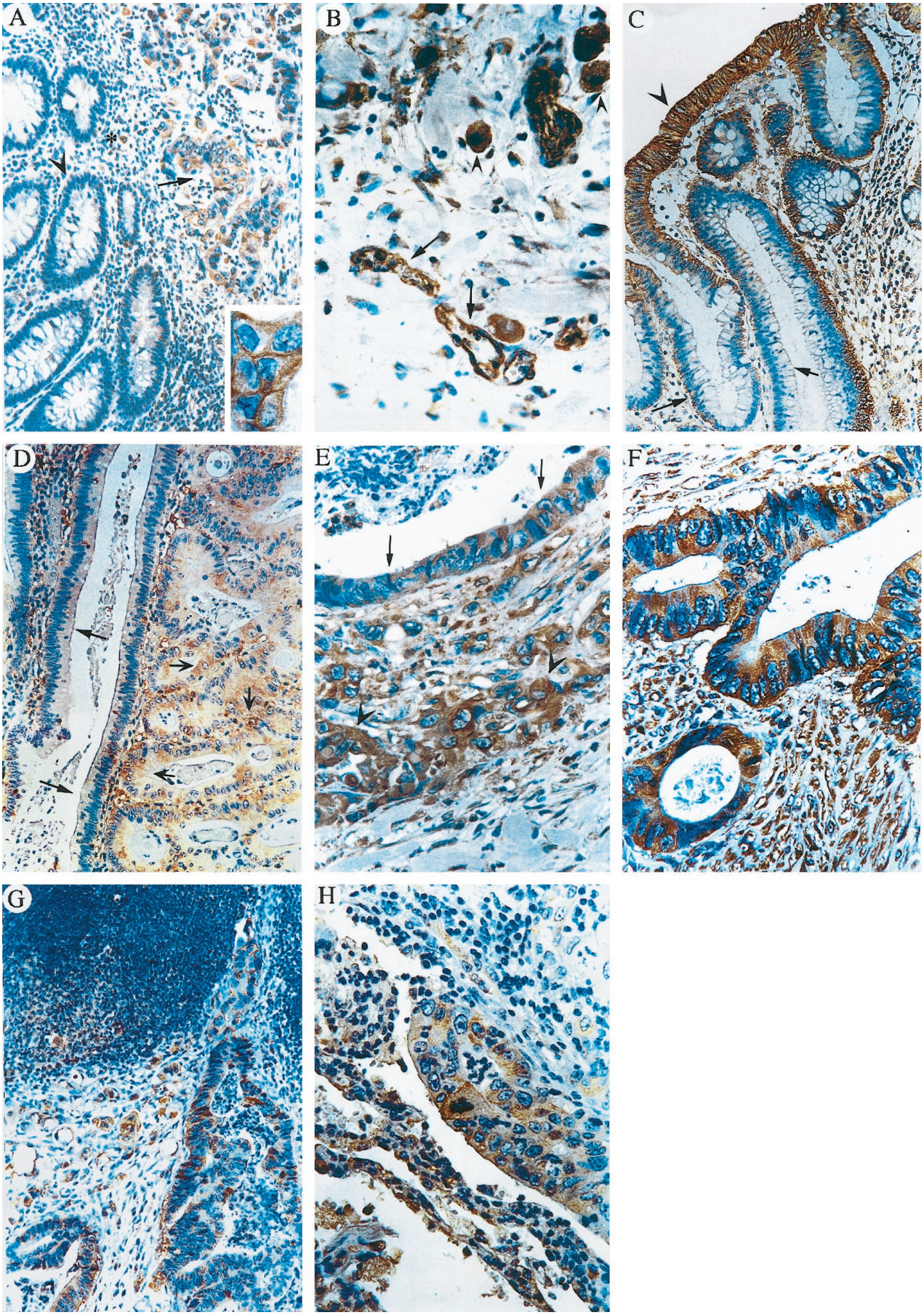
Expression Pattern of the Heparanase Protein

Monoclonal anti-heparanase antibodies were applied to detect heparanase protein in specimens from 17 patients with colonic neoplasia. In 15 cases the samples included apparently uninvolved normal-looking colonic tissue, located either in close proximity or away from the tumor, in which no heparanase stain was detected even though in the nearby region, stained tumor cells were seen (Figure 1A). In all of the immunoreactive cells staining was characterized by being both cytoplasmic and membrane associated with many cells showing preferential cell surface localization of the enzyme (Figure 1A, inset), suggesting that heparanase may be membrane associated and/or secreted. Rarely, a weak positive staining was found on the free luminal surface of normal-looking epithelium adjacent to the cancer (not shown). In the majority of cases however, the surface epithelia were negative, as were the fibroblasts in the lamina propria (Figure 1A). Occasional endothelial cells in blood vessels stained weakly positive (not shown).

In all specimens, heparanase stain was observed in macrophages (Figure 1B), ganglion cells, and nerves (not shown), regardless of whether tumoral or nontumoral regions were examined.

Three tubulovillous adenoma specimens were available for investigation. Two adenomas from patients, who had also colonic carcinomas, displayed moderate to focal severe dysplasia with superficial invasive carcinoma. The third one showed moderate dysplasia. In all three cases very little or no heparanase was detected in mildly dysplastic cells (Figure 1, C and D), whereas moderately and severely dysplastic cells expressed high levels of the heparanase protein (Figure 1, C and D). The most intense staining was found in areas of carcinomatous transformation (Figure 1D). Stain was negative in fibroblasts and endothelial cells, as well as in adjacent normal-appearing mucosa (not shown). Occasional inflammatory cells including macrophages, plasma cells, and neutrophils were stained (Figure 1C).

Heparanase staining yielded a similar pattern in all cases of colon adenocarcinoma. The more differentiated regions showed a weakly to moderately positive heparanase expression in a proportion of the malignant epithelial cells, whereas in poorly differentiated areas stronger and more abundant staining was seen (Figure 1E). No differences were detected between the central regions of the tumor and areas of transition between malignant and normal-looking tissue. However, stronger and more dif-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fuse staining was found in deeply invading tumor areas and was particularly prominent in desmoplastic regions (Figure 1F). In these areas heparanase staining was noted also in the proliferating fibroblasts in the stroma surrounding the invasive cancer cells (Figure 1F). The stain intensity gradually increased in both tumor cells and fibroblasts with increasing depth of invasion, being most prominent at the leading edge of invasion and in tumor cells in the serosal fat (not shown). Occasional stain was noted in endothelial cells of small blood vessels in the tumor (Figure 1B).

In all metastases, whether to regional lymph nodes (Figure 1G), liver (not shown), or lung (Figure 1H), moderate to marked heparanase expression was found in the majority of metastasizing malignant epithelial cells. Endothelial cells and fibroblasts in the metastases were weakly positive.

Expression Pattern of the Heparanase mRNA

To investigate the correlation between the localization of the heparanase protein and mRNA, we performed *in situ* hybridization for heparanase on representative samples from four patients with metastatic colon carcinoma, two of whom also had synchronous tubulovillous adenomata. As shown in Figure 2, heparanase mRNA pattern of expression was similar to that of the heparanase protein (Figure 1).

No labeling of the heparanase mRNA was noted in normal-looking tissue adjacent to the neoplasm (Figure 2, A and D). Labeling in these areas was confined to ganglion cells and occasional endothelial cells of small blood vessels (not shown).

In the tubulovillous adenomas, intense heparanase-mRNA labeling was found in dysplastic cells (Figure 2A). Mildly dysplastic cells were faintly labeled (Figure 2A) whereas the more superficially situated, severely dysplastic cells, were heavily labeled (Figure 2A), similar to the immunostaining pattern of the heparanase protein (Figure 1C).

Granular diffuse cytoplasmic labeling was found in malignant epithelial cells, ranging from well-differentiated (Figure 2B) to poorly differentiated (Figure 2C) grade, and from superficially (Figure 2D) to deeply invasive carcinoma (Figure 2E). Labeling of stromal cells was only focal in the deeply invasive and less differentiated tumor areas (Figure 2, C and 2). In these areas, there was labeling in occasional endothelial cells lining small blood vessels.

As with the heparanase protein, colon carcinoma cells metastasizing into a regional lymph node, as well as their surrounding stromal cells, expressed the heparanase mRNA (Figure 2F).

Expression of Heparanase Activity and Protein

Evaluation of heparanase activity and protein levels was performed to support the proposed involvement of the enzyme in colon tumorigenesis. For this purpose, freshly taken specimens derived from colon carcinoma and from normal-looking colon tissue located away from the tumor region were homogenized and partially purified on CM-Sepharose. Supernatant fractions were subjected to 1) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis, and 2) evaluation of heparanase activity. As shown in Figure 3 (inset), a prominent 50-kd protein in the tumor tissue reacted with the anti-human heparanase antibodies (lanes 2 and 4), as compared to a very weak (lane 1) or no (lane 3) immunoreactivity in samples derived from normal-looking colon tissue specimens. The prominent immunoreactive protein preferentially detected in extracts from the colon tumor further demonstrates the strict specificity of the anti-heparanase antibodies applied in the immunostaining studies.

Incubation of equal protein amounts, taken from the above supernatant fractions, with sulfate-labeled ECM resulted in release of high levels of HS degradation fragments (Peak II, fractions 15 to 35) in samples derived from the colonic tumor as compared to much lower levels of HS degradation products released during incubation of the ECM with extracts derived from a normal-looking colon tissue (Figure 3). Degradation fragments eluted in peak II were shown to be degradation products of HS, as they were fivefold to sixfold smaller than intact HS side chains (K_{av} ~0.33) released from the ECM by treatment with either alkaline borohydride or papain; and were resistant to further digestion with papain or chondroitinase ABC and susceptible to deamination by nitrous acid.[26] Thus, the preferential expression of the heparanase transcripts and protein seen by *in situ* hybridization and immunostaining of the colon carcinoma is also reflected by an increased heparanase activity found in fresh tissue samples derived from the tumor *versus* the normal colon tissue.

Discussion

Our results obtained by immunohistochemistry and *in situ* hybridization strongly suggest that heparanase expres-

Figure 1. Immunohistochemical analysis of the heparanase protein in neoplastic colon. Staining was performed as described in Materials and Methods. Positive staining is reddish-brown. Counterstain of nuclei is blue-purple. **A:** Heparanase immunostaining of tumor tissue (**arrow**) and lack of staining in the adjacent normal-looking tissue (**arrowhead**). A few macrophages in the normal-looking tissue (*) are positive. Cell surface expression of heparanase is demonstrated in the **inset** of the figure. **B:** Staining of blood vessels (**arrows**) and macrophages (**arrowheads**) in the proximity of the tumor. **C** and **D:** Sections of tubulovillous adenoma representing several presumed stages in tumor progression ranging from very mild dysplasia (**arrows**) in which almost no heparanase protein is detected, through moderate and severe dysplasia (**arrowheads**) showing a stronger stain, to a carcinomatous area (**concaved arrows**) in which high levels of heparanase are detected. **E:** Heparanase staining of well-differentiated (**arrows**) and more intense staining of poorly differentiated (**arrowheads**) adenocarcinoma. **F:** Highly positive heparanase staining of deeply invading tumor cells as well as of the desmoplastic stromal cells surrounding the tumor cells. **G** and **H:** Metastases of colon carcinoma derived from two different patients, in a regional lymph node (**G**) and in lung (**H**), showing high cytoplasmic expression of heparanase in the metastatic cells. Original magnifications: ×200 (**A**, **C**, and **D**); ×400 (**B**, **E**, **F**, and **H**); ×100 (**G**); ×1,000 (**inset** in **A**).

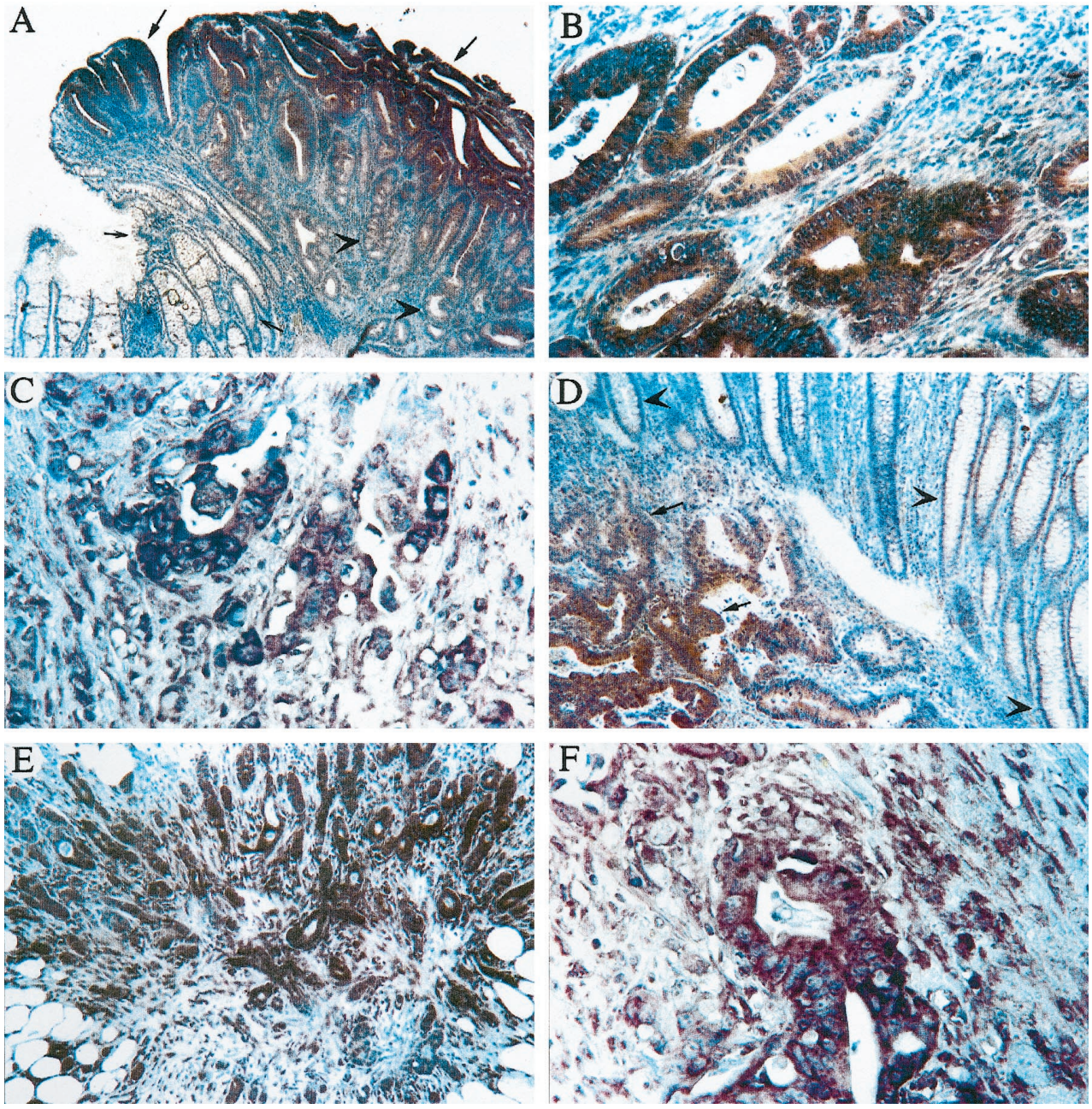

Figure 2. Heparanase mRNA (*in situ* hybridization) in neoplastic colon tissue. Hybridization was performed as described in Materials and Methods. Positive labeling is brown-black. Nuclei counterstain is blue. **A:** High levels of heparanase mRNA in the superficially situated, severely dysplastic cells (**arrows**) of a tubulovillous adenoma relative to a faint labeling of mildly dysplastic cells (**arrowheads**). No heparanase mRNA is detected in the normal-looking epithelium (**concaved arrows**). **B:** A well-differentiated adenocarcinoma showing intense heparanase mRNA expression in the cytoplasm of the tumor cells. No heparanase mRNA is detected in the majority of the stromal fibroblasts. **C:** A poorly differentiated adenocarcinoma expressing heparanase mRNA both in the tumor cells and stromal fibroblasts. **D:** Heparanase mRNA expression in tumor tissue (**arrows**) and no expression in the adjacent normal-looking tissue (**arrowheads**). **E:** Deeply invading tumor cells and their surrounding stroma express heparanase mRNA. **F:** Metastatic colon cells in a regional lymph node showing high levels of heparanase mRNA. The surrounding stroma also expresses heparanase mRNA. Original magnifications: ×50 (**A**), ×200 (**B**), ×400 (**C** and **F**), and ×100 (**D** and **E**).

sion is related to the presumed stages in colon carcinoma development. Expression was highly abundant in the primary carcinoma and in the metastases, both in the epithelium and desmoplastic stromal fibroblasts and less abundant in tubulovillous adenomas, particularly in mildly dysplastic cells. In most of the normal-looking colonic tissue, no heparanase was observed and in the few cases in which some staining was detected, it was weak and confined to the free surface of luminal epithelium. The results are summarized in Table 2, showing preferential expression of the heparanase gene and protein in correlation with tumor progression. Thus, overexpression of heparanase both in the tumor cells and surrounding stromal fibroblasts can be regarded a characteristic feature of colon cancer. These results are supported by the fact that both the heparanase protein, detected by Western blot analysis, and enzymatic activity, measured by release of HS degradation fragments from intact ECM,

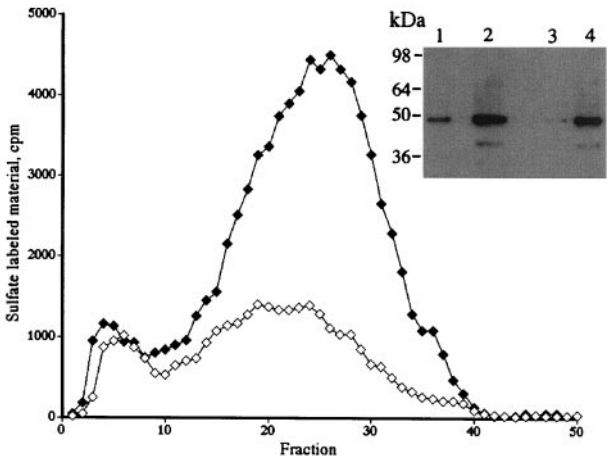

Figure 3. Expression of heparanase activity and protein in colon carcinoma *versus* normal-looking colon tissue. Partially purified samples derived from normal-looking colon tissue (**open diamonds**; **lanes 1** and **3**) and from the colon tumor (**filled diamonds**; **lanes 2** and **4**) of the same patient were 1) tested for heparanase activity and 2) subjected to Western blot analysis using monoclonal anti-heparanase antibodies (**inset**). For heparanase activity, samples containing 15 μg of protein were incubated (24 hours at 37°C, pH 6.2) with sulfate-labeled ECM. The incubation medium was then subjected to gel filtration on Sepharose 6B. Similar results were obtained using tumor and normal tissues from three different patients. The amount of protein applied in **lanes 1** and **2** was 0.9 μg/well *versus* 0.3 μg protein/well in **lanes 3** and **4**.

were found in much higher levels in the colon tumor tissue than in the adjacent normal-appearing tissue. The low heparanase activity expressed by the normal tissue is most probably because of the presence of blood inflammatory cells and platelets in the tissue homogenate. Platelets and activated cells of the immune system were previously shown to express a high heparanase activity.[27]

A similar pattern of expression was observed both at the mRNA (*in situ* hybridization) and protein (immunohistochemistry) levels, indicating that both the heparanase gene and protein begin to be expressed in the early stages of carcinogenesis and continuing through later stages. The Western blot analysis clearly reflects the specificity of the anti-heparanase antibody for the heparanase protein, demonstrating its preferential expression in the tumor *versus* the normal tissue.

Altogether our results, using descriptive and functional approaches, support the proposed involvement of heparanase in tumor cell invasion and metastasis.[4,5,14,19]

The nonradioactive *in situ* hybridization allowed accurate identification of the cells which actually synthesize the heparanase mRNA. The co-expression of heparanase mRNA and protein implies that heparanase is synthesized and stored in the same cell, whether neoplastic epithelial cells or stromal fibroblasts. Some of the protein was detected on the cell surface, which suggests that it is membrane-associated or secreted. In case of secretion to the ECM, the protein cannot be detected because it is lost during tissue processing.

The most significant prognostic feature in colon tumors is the degree of bowel wall penetration by the tumor and the presence or absence of distant metastases,[28,29] expressed by the Dukes grading system. This prognosis is also affected by the number of involved lymph nodes. Meticulous lymph node dissection, however, is often overlooked. It is believed that many patients with invasive tumor whose lymph nodes were not infiltrated and had no evidence of distant metastases but died of metastatic disease, have often had occult micrometastases that were likely missed at the time of diagnosis.[30] It seems that heparanase immunostaining in the cancer cells of Dukes A and B2 samples was weaker than in Dukes C and D. In addition, there was no expression of heparanase in the stromal fibroblasts of Dukes A and one of the Dukes B2 tumors. The two other Dukes B2 specimens did express heparanase in the fibroblasts, whereas the desmoplastic stromal fibroblasts in Dukes C and D were heavily labeled. Heparanase stain may thus serve as an additional prognostic marker, and detection of strong heparanase staining may possibly allow patients selection for systemic therapies at an earlier stage. However, to verify this assumption, more cases should be investigated and followed-up for the occurrence of metastases.

Interestingly, a relatively similar correlation between the tumor stage and the expression of type IV collagenase was reported.[31] The enzyme was expressed in significantly lower amounts by Dukes A and B carcinomas than by Dukes C carcinomas and was expressed even in lower levels by adenomas. In addition, type IV collagenase expression level was retained or slightly augmented in metastases compared with the matched primary tumor.[31]

The degree of tumor differentiation is next in prognostic importance to staging. Tumor grade correlates with the likelihood of venous and local spread, as well as with lymphatic and bowel wall penetration.[32] The incidence of nodal spread increases as the tumor becomes undifferentiated and invades deeper into the bowel wall.[33] Black and Waugh[34] reported that 30% of low-grade adenocarcinomas metastasize to regional lymph nodes compared with 81% metastasis of high-grade lesions. The number of involved lymph nodes also increases with tumor grade. We found that heparanase expression was inversely correlated to tumor grade, being the strongest in poorly

Table 2. Summary of the Immunohistochemistry (IH) and *in Situ* Hybridization (ISH) Experiments

	Normal		Dysplastic		Primary tumor		Metastatic	
	IH	ISH	IH	ISH	IH	ISH	IH	ISH
Epithelial	0/15*	2/4	3/3	2/2	12/12	4/4	8/8	2/2
Stromal	0/15	0/4	0/3	0/2	11/12	3/4	6/8	2/2
Endothelial	4/15	3/4	0/3	0/2	10/12	3/4	7/8	2/2

*In some cases (see Discussion) very weak luminal staining in normal epithelial cells neighboring the tumor was observed.
Presented is the ratio between the number of cases in which the cells expressed heparanase in the normal and the progressive stages of neoplasia (dysplasia, primary tumor, and metastasis) and the total number of cases tested for that stage.

differentiated tumor areas. This suggests that heparanase may reflect the differentiation status and metastatic nature of the cells.

High-grade dysplasia represents the extreme end of the spectrum of abnormal histological changes, short of invasive carcinoma in the adenoma-carcinoma continuum. The presence of severe dysplasia strongly correlates with a contiguous invasive carcinoma.[35] Although adenomas probably constitute the precursor lesions for most carcinomas, a vast gap exists in the prevalence rates of adenoma and carcinoma, indicating that 90 to 95% of adenomas will never become malignant during a person's lifetime.[36] In tubulovillous adenoma, heparanase expression gradually increased as transition of the tissue from normal through dysplastic to carcinomatous appearance took place. Heparanase expression thus correlates with the histological changes that take place during this transition. Moreover, it appears that expression of heparanase increased even as the tubulovillous adenoma progressed from mild through moderate to severe dysplasia. In addition, heparanase was not detected in normal-looking colonic epithelial cells, whereas the adjacent transformed cells were clearly positive and deeply invading tumors showed the highest level of heparanase expression. Heparanase expression pattern might thus predict the biological behavior of polyps, especially in polyps with carcinomatous transformation and invasion of the stalk. Again, the *in situ* hybridization and immunolocalization of the heparanase gene and protein showed the same pattern.

Overexpression of heparanase both in cancer cells and stromal fibroblasts was noted in advanced stages of tumor progression. The stromal component of colonic carcinoma varies from little fibrous stroma to frankly scirrhous tumor.[37] Overexpression of heparanase by cancer cells may be because of a mutation in the heparanase gene itself or in genes regulating its expression. By expressing heparanase, the tumor cells may facilitate their ability to invade the surrounding tissue and metastasize, as previously demonstrated.[19] On the other hand, fibroblasts in the tumor area are thought to be normal in the sense that they have neither mutated oncogenes nor mutated tumor-suppressor genes.[38] Still, it is known that stromal fibroblasts in the vicinity of the tumor actively proliferate. Activated fibroblasts produce collagen and other ECM proteins as well as growth-promoting factors that induce the desmoplastic reaction.[39,40] Overexpression of heparanase by both the tumor and connective tissue cells may contribute to this process by means of releasing heparin-binding growth factors that are stored in the ECM as a complex with HSPG.[17,18,41] Expression of genes for ECM components and their degradative enzymes has been shown to be coordinated with and necessary for tissue remodeling.[42] Matrix-degrading enzymes play a role in the turnover of ECM proteins of connective tissues in general[43] and the stroma of pancreatic,[44] gastrointestinal, and other tumors.[38] Heparanase expression by stromal fibroblasts may thus be induced by the tissue remodeling that takes place during tumor progression and may reflect a physiological response to alterations in the local tissue caused by the tumor growth.

Our immunohistochemical and *in situ* hybridization studies point to the cancer cells as the major site of synthesis of heparanase, particularly at early stages of tumorigenesis. At later stages, the enzyme was also expressed by surrounding stromal elements such as fibroblasts. Immunohistochemistry and *in situ* hybridization done by other groups revealed abundant expression of urokinase in cancer cells of colon adenocarcinomas with occasional staining in stromal fibroblasts.[45,46] Immunolocalization of MMP-9 and to a lesser extent MMP-2 in breast carcinoma specimens revealed staining of tumor cells compared with a weak staining of the tumor stroma.[47] MMP-3, on the other hand, is specifically expressed in stromal cells of breast carcinomas.[48]

To our knowledge this is the first systematic study describing the progressive expression pattern of heparanase in human colon cancer. Altogether, we have shown preferential expression of the heparanase mRNA and protein in colon tumors as compared to the normal-looking colon epithelium. A correlation between the degree of tumor invasiveness, differentiation level of the cells and expression of the heparanase protein and mRNA by the carcinoma and stromal cells was noted. Expression increased as the tumor cells were more invasive and less differentiated. These results suggest a role for heparanase in colon tumor progression, most probably through its effect on the tumor microenvironment, resulting in an enhanced tumor cell invasiveness and vascularization.

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