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## Altered Expression of Chondroitin Sulfate Proteoglycan in the Stroma of Human Colon Carcinoma

HYPOMETHYLATION OF PG-40 GENE CORRELATES WITH INCREASED PG-40 CONTENT AND mRNA LEVELS\*

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The connective tissue stroma of malignant tumors is a newly formed tissue that supports the growth and progression of neoplastic cells. Proteoglycans are intrinsic components of this complex structure and molecular changes in this class of macromolecules can significantly affect behavioral properties of transformed cells. We report that human colon carcinoma contained increased levels of a chondroitin sulfate proteoglycan that exhibited an altered glycosaminoglycan structure in which 0- and 6-sulfated units, as detected by specific monoclonal antibodies, predominated. Proteoglycans with such epitopes were localized primarily to the connective tissue stroma surrounding the tumor cells but not to the tumor cells themselves or the native, non-cancerous connective tissue. Analysis of mRNA encoding PG-40, the main chondroitin sulfate proteoglycan of colon tissue, revealed a 7-fold increase in the two transcripts encoding this gene product. This increase was evident whether the data were normalized to total RNA content or  $\beta$ -actin mRNA levels. The altered steady state levels of PG-40 mRNA did not correlate with any significant gene amplification or rearrangement of PG-40 in human colon cancer. However, when genomic DNA was tested for degree of methylation, the colon carcinoma tissue showed a marked hypomethylation of PG-40 gene locus, a finding that has been associated with increased gene activation. Interestingly, PG-40 gene was also hypomethylated in cultured colon fibroblasts, which express PG-40, but not in colon carcinoma cells which do not express this gene. These results indicate that specific proteoglycan changes occur in colon carcinoma and that these alterations are the product of stromal cells that are topologically associated with and functionally respondent to the growing malignant cells. This is the

first evidence that enhanced PG-40 expression in a human malignant tissue is associated with a hypomethylated gene and suggests that the control of PG-40 gene expression may represent an important factor in the progression of colon carcinoma.

The degree of local tissue invasion by colorectal cancer, the second most common cancer in the western industrialized societies, is directly correlated with the clinical outcome (1). In the vast majority of cases, the invasion of the colonic wall is preceded by an exophytic growth of neoplastic cells and stromal elements within the intestinal lumen (2). It is noteworthy that about half of the neoplastic mass is composed of stromal elements, primarily fibroblasts, smooth muscle, and endothelial cells, which together provide the necessary requirements for tumor growth and progression (3). Proteoglycans are intrinsic constituents of this newly formed connective tissue; and, particularly because of their complex polyanionic nature, they have been implicated in a number of biological processes. Proteoglycans form a molecular sieve, are responsible for retaining water, act as growth promoting agents, operate as receptors for matrix molecules, and influence the attachment and migration of cells along defined routes (4-7). It is conceivable, therefore, that the newly synthesized proteoglycan-enriched milieu that accompanies colon carcinoma can enhance the proliferation of this specific tumor by providing a favorable growth environment.

The main hypothesis of our research is that the structure and formation of the tumor matrix is under the direct control of the neoplastic cells and, through a feedback loop, altered proteoglycan composition can influence the behavior of tumor cells. This hypothesis is based in part on our observation that human colon carcinomas have elevated amounts of a structurally altered chondroitin sulfate that results from the abnormal production of these molecules by the connective tissue surrounding the neoplastic cells (8, 9). These changes could be partly reproduced *in vitro* using either tumor metabolites (10) or co-cultures of colon carcinoma and normal mesenchymal cells derived from human colon (11).

We have now extended these studies using immunological and molecular biological probes specific for chondroitin sulfate proteoglycan. The results indicate that previously undetected qualitative and quantitative changes in this gene product exist and further suggest that the aberrant expression of this proteoglycan occurs solely in connective tissue closely associated with neoplastic cells. We further show for the first time that enhanced expression of PG-40, determined by West-

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ern and Northern blotting, is associated with hypomethylation of PG-40 gene locus, a DNA change known to be directly linked to gene activation. Our results raise the novel possibility that neoplastic-induced demethylating events may affect specific proteoglycan genes and offer a molecular explanation for the previously reported proteoglycan alterations in this human neoplasm.

#### EXPERIMENTAL PROCEDURES

**Tissue Preparation and Proteoglycan Extraction**—Colectomy specimens, obtained immediately after surgery, were opened longitudinally, the surface was gently washed with isotonic saline, and samples were taken from tumor and normal appearing mucosa. In all cases, the diagnosis of adenocarcinoma was made using endoscopic biopsy prior to resection. The samples were quickly frozen in liquid nitrogen and then transferred to a  $-75^{\circ}\text{C}$  freezer. A total number of 23 normal colon and 24 colon carcinoma samples were studied. In addition, a benign colonic polyp, human colon fibroblasts, and colon carcinoma (10) cells were studied. The number of normal and neoplastic tissue samples studied by various methodologies is indicated under "Results." For proteoglycan analysis, tissue aliquots (100–500 mg) were homogenized in 4 M guanidine HCl, 0.5 M sodium sulfate, pH 5.8, containing 0.1 M NaCl, 2% Triton X-100 and various protease inhibitors (12). Aliquots of [ $^{35}\text{S}$ ]sulfate-labeled heparan sulfate proteoglycan isolated from human colon carcinoma cells (12) were added to monitor recovery during the subsequent purification steps. Samples were cleared by centrifugation a  $10,000 \times g$  for 1 h at  $4^{\circ}\text{C}$  and purified by sequential Sephadex G-50 and DEAE-Sephacel (12). Recovery of the radiolabeled carrier proteoglycan exceeded 90%.

**Multiple Western Blotting**—Agarose-acrylamide gel electrophoresis, trans-blotting to nitrocellulose, multiple immunoblotting, and subsequent visualization with [ $^{125}\text{I}$ ]labeled secondary antibodies was done as described (13). This technique has the advantage of analyzing small samples of tissue with quantitative recovery and permanent trans-blot that can be probed with various antibodies before and after enzymatic removal of glycosaminoglycans (13). The following monoclonal antibodies were used: (i) CS-56, directed against native chondroitin sulfate (14); (ii) 2B6, directed against chondroitin 4-/dermatan sulfate (15); (iii) 3B3, directed against chondroitin 6-sulfate (15); (iv) 1B5, directed against chondroitin 0-sulfate (15), and (v) 7D4 and 6C3, directed against native/oversulfated chondroitin sulfate (16, 17). In addition, we used a monospecific antibody raised against the amino terminal peptide of PG-40 (18). Multiple immunoblotting was made possible through removal of antibodies with 3 M sodium thiocyanate, 0.5% 2- $\beta$ -mercaptoethanol, 0.5% Tween 20, pH 9.5. The exposure of the trans-blot to this chaotropic solution appears to have no effect on the immobilized proteoglycans (13). To visualize unsaturated  $\Delta$  4,5 glcAgalNAc 0-SO<sub>4</sub>, 4-SO<sub>4</sub>, or 6-SO<sub>4</sub> residues attached to the protein core, the membranes were immersed in 0.05 units of protease-free chondroitinase ABC in 10 ml of 0.2 M Tris buffer, pH 8.0, for 18 h at  $37^{\circ}\text{C}$ . As shown before (13), the deglycosylated proteoglycans are totally retained in the membrane and can react with specific antibodies. Autoradiographs were quantified in a laser beam scanning densitometer (LKB Instruments) by comparing the area under a scan to that of a 250-ng bovine nasal cartilage standard, an A1D1 preparation containing 70 ng of uronic acid. This amount of standard was well within the range of a linear relationship between concentration and absorbance. The calculated amounts of proteoglycan are therefore estimates based on the content of epitopes, reactive with the various antibodies, as found in bovine nasal cartilage.

**Immunohistochemical Studies**—To investigate the tissue expression and localization of chondroitin sulfate proteoglycan in human colon and colon carcinoma, the monoclonal antibodies described above were tested on both deparaffinized formaldehyde-fixed or frozen sections. Immunoperoxidase staining before and after chondroitinase ABC digestion was performed as described (15–17).

**RNA Isolation, Northern, and Slot Blotting**—Total RNA was prepared from frozen tissue pulverized under liquid nitrogen according to the acid guanidinium thiocyanate method (19) with the addition of 10.5 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories). RNA was also isolated from human colon fibroblasts, colon smooth muscle, and carcinoma cells using 6–8 T-150 Falcon flasks ( $2\text{--}5 \times 10^7$  cells/flask) each. Poly(A<sup>+</sup>) RNA was purified on oligo(dT)-cellulose columns (20), separated by electrophoresis in 1% formaldehyde-agarose gels (21), and transferred to nylon membranes (Hybond, Amersham Corp.). Denatured RNA samples were analyzed

by slot blot using a Minifold II apparatus (Schleicher and Schull) and BA85 nitrocellulose paper. Both nylon and nitrocellulose membranes were treated at  $80^{\circ}\text{C}$  for 2 h under vacuum before prehybridization and hybridization with a full-length 1.8-kb<sup>1</sup> PG-40 cDNA. The insert was excised by *EcoRI* digestion, separated on 1.2% agarose gel from the plasmid, purified by GeneClean (Bio 101, San Diego, CA), and nick-translated using [ $^{32}\text{P}$ ]dCTP to reach a specific activity  $>10^8$  cpm/ $\mu\text{g}$  DNA (22). To determine more exactly the amount of poly(A<sup>+</sup>) RNA, the slot blots were hybridized with a full-length 2.1-kb cDNA encoding human  $\beta$ -actin (23). All the membranes were prehybridized at  $65^{\circ}\text{C}$  for at least 5 h in  $5 \times \text{SSC}$  ( $1 \times \text{SSC} = 0.15 \text{ M NaCl}$ , 15 mM sodium citrate, pH 7.0), containing  $1 \times$  Denhardt's solution, 0.1% sodium dodecyl sulfate, and 100  $\mu\text{g}/\text{ml}$  salmon sperm DNA, and then hybridized with  $^{32}\text{P}$ -labeled probe for 18 h at  $65^{\circ}\text{C}$ . The membranes were washed in  $2 \times$ ,  $1 \times$ , and  $0.5 \times \text{SSC}$  for 30 min each and subjected to autoradiography using intensifying screens at  $-75^{\circ}\text{C}$ . After various exposures, autoradiograms were quantified by densitometry as described above.

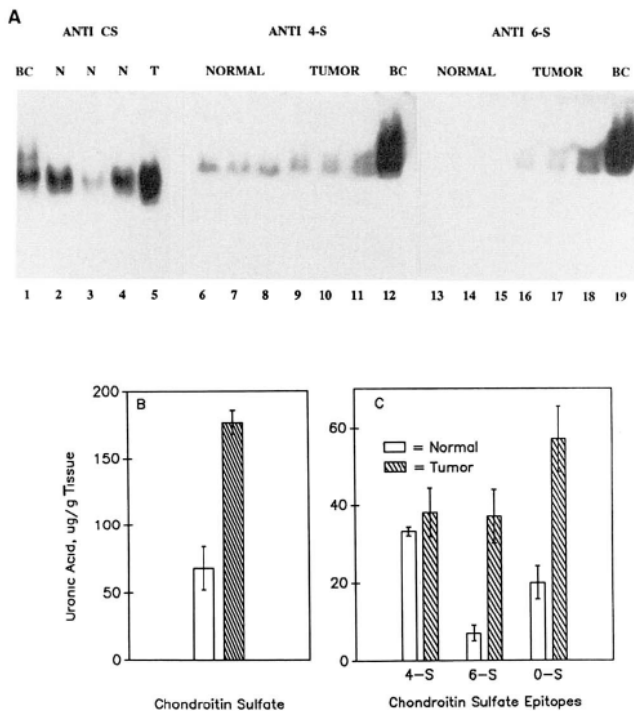
**DNA Isolation and Southern Blotting**—High molecular weight genomic DNA was prepared from 200 to 300 mg of tissue or from two to four T-150 flasks of confluent cells as described (24). Because of the intrinsic error in the solubilization of concentrated DNA solutions, equal amounts of DNA were initially digested with restriction enzymes as indicated below and run on 0.8% agarose gels in the presence of ethidium bromide (21). The gels containing digested and undigested samples were then photographed and each negative scanned. The results showed that the ratio of arbitrary absorbance units, determined by densitometry of the negatives, to that initially determined by spectrophotometry of DNA in solution was relatively constant with S.E. of  $\pm 7\%$  ( $n = 8$ ). In a typical experiment, 20  $\mu\text{g}$  of DNA were digested with *PstI*, *BglII*, and *HindIII* restriction endonucleases (5 units/ $\mu\text{g}$  DNA each) for 12 h at  $37^{\circ}\text{C}$ , separated in 0.8% agarose gels, and blotted onto nitrocellulose (25). The blots were hybridized under high stringency to the PG-40 cDNA or to a full-length cDNA encoding the human *c-src* proto-oncogene (26). To determine the degree of methylation of PG-40, DNA was digested with *HpaII* or *MspI*. *HpaII* cleaves 5'-CCGG-3' sequences only if the internal C is unmethylated (27), whereas *MspI* cleaves 5'-CCGG-3' sequences regardless of C methylation (28). In addition, the Southern blots of *HpaII*- and *MspI*-digested DNA were hybridized with  $\beta$ -actin cDNA.

#### RESULTS

**Proteoglycan Analysis and Western Blotting**—Western blot analyses of DEAE-Sephacel-purified proteoglycans using well characterized monoclonal antibodies against intact chondroitin sulfate (14) or distinct isomers (15–17) revealed a marked increase in chondroitin sulfate in the malignant tissue (Fig. 1, A and B). In both normal (Fig. 1A, lanes 2–4) and tumor tissue (Fig. 1A, lane 5), one broad band of chondroitin sulfate proteoglycan was detected by monoclonal antibody CS-56, a probe directed against intact chondroitin 4- or 6-sulfate chains (14). When the CS-56-reacted trans-blot was quantified by laserbeam densitometry (Fig. 1B), a 3-fold increase in chondroitin sulfate was detected in the tumor.

Following chondroitinase ABC digestion, an enzymatic degradation that unmasks the three major isomeric epitopes of chondroitin sulfate attached to the protein core (8), significant qualitative and quantitative changes were further detected. By inspecting the autoradiographs (Fig. 1A, lanes 16–18) it is evident that colon carcinoma tissue had a higher concentration of epitopes reactive with monoclonal antibody 3B3 directed against the chondroitin 6-sulfate isomer than the normal colon tissue (Fig. 1A, lanes 13–15). A similar increase in epitope reactivity of tumor tissue proteoglycan was also observed with monoclonal antibody 1B5 that reacts with chondroitin 0-sulfate epitopes (not shown). In contrast, the relative amount of chondroitin 4-/dermatan sulfate, an epitope detected by monoclonal antibody 2B6, was not appreciably changed (Fig. 1A, lanes 6–11). This major product was im-

<sup>1</sup> The abbreviation used is: kb, kilobase pair(s).



**FIG. 1. Immunohistochemical analysis of proteoglycans in tissue extracts of normal human colon and colon carcinoma.** Western blotting (A) of bovine nasal cartilage proteoglycan (BC), normal human colon (N), and colon carcinoma tissue (T) using monoclonal antibodies against native chondroitin sulfate chains (lanes 1–5), chondroitin 4-/dermatan sulfate epitopes (lanes 6–12), or against chondroitin 6-sulfate epitopes (lanes 13–19). In order to visualize the chondroitin sulfate epitopes, the trans-blots were subjected to chondroitinase ABC (protease-free) digestion before antibody reaction (A, lanes 6–19). Thus, lanes 6–19 represent chondroitin sulfate epitopes covalently attached to the protein core which is immobilized into the nitrocellulose membrane. B and C represent quantification of the autoradiograms by laserbeam densitometry as related to the concentration of uronic acid (28% of weight) in the standard cartilage proteoglycan run in parallel.

munologically related to PG-40, since it was visualized by the antibody raised against the PG-40 protein core (not shown). A minor faster moving band was evidenced following a longer exposure. Both bands, however, were reactive with anti-PG-40 antibody (not shown), indicating that the two species varied in their degree of glycosylation. When the chondroitinase ABC-treated trans-blots were quantified by laserbeam densitometry, the relative amount of chondroitin 6-sulfate epitope and 0-sulfate epitope was increased 6.3- and 3-fold (Fig. 1C), respectively. Taken together, these results indicate that colon carcinoma tissue contains elevated amounts of a proteoglycan which is structurally and immunologically related to PG-40, but with increased relative content of chondroitin 6- and 0-sulfate epitopes.

**Immunohistochemical Studies**—We next examined the immunohistochemical distribution of native protein-bound chondroitin sulfate chains (14), the three major chondroitin sulfate isomers (15–17), or the PG-40 protein core (18). Both normal and neoplastic epithelia were unreactive with all the anti-chondroitin sulfate proteoglycan antibodies (Fig. 2), indicating that these epithelial cells express little or no chondroitin sulfate. In contrast, the connective tissue of normal and malignant colon expressed various degrees of immunoreactivity with all the antibodies tested. Strikingly, the tissue distribution of the chondroitin 6-sulfate epitope (Fig. 2, B and C) was markedly different from that of the chondroitin 4-/dermatan sulfate epitope (Fig. 2, E–G). Whereas the latter

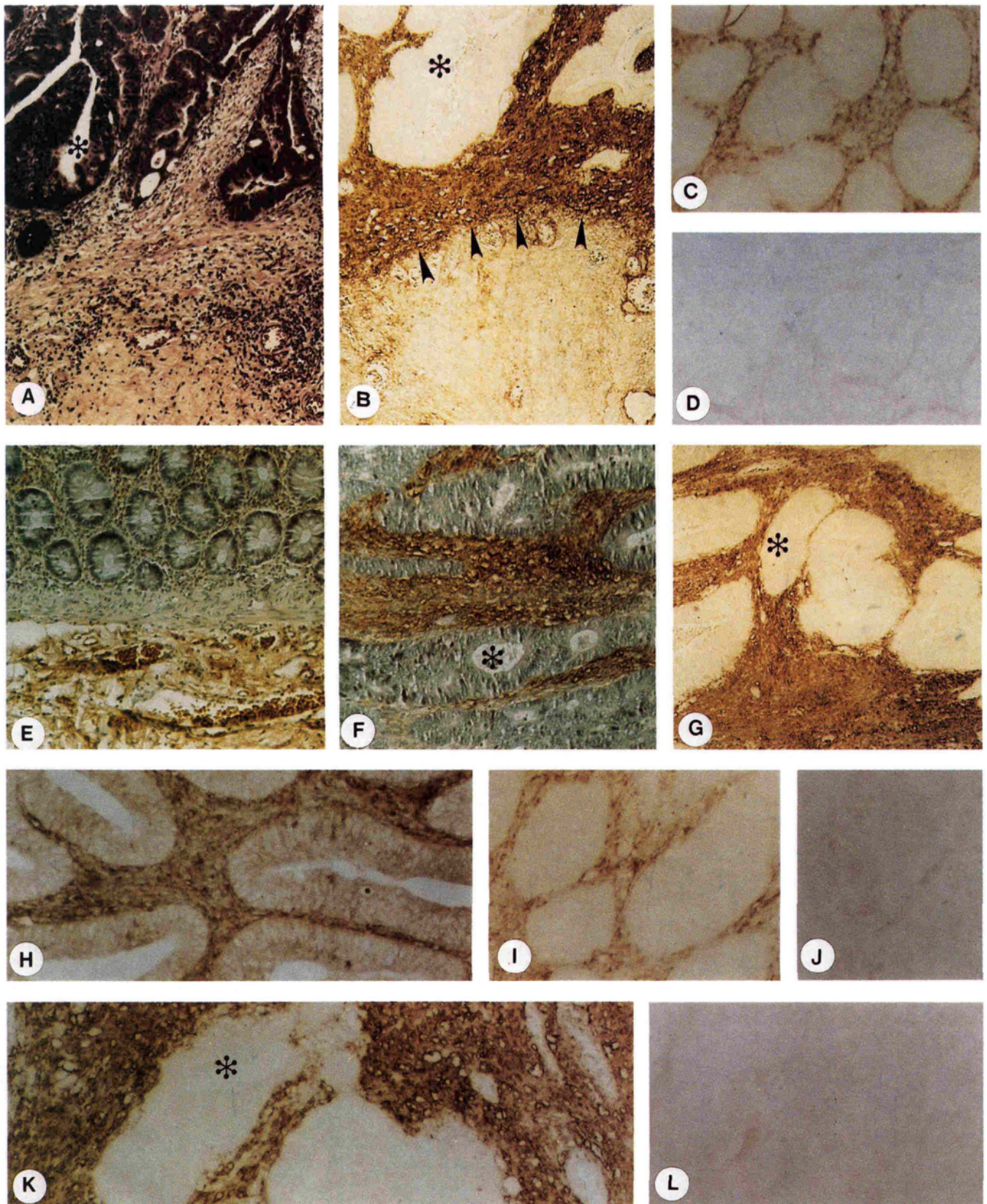
epitope was present in both normal and malignant stroma at approximately equal intensities (Fig. 2, E–G), the chondroitin 6-sulfate epitope was present almost exclusively in the connective tissue intimately associated with the malignant cells (Fig. 2B). The tumor stroma, although morphologically indistinguishable from native connective tissue (Fig. 2A), could be clearly separated from the pre-existing stroma by the high concentration of chondroitin 6-sulfate epitopes (Fig. 2B, arrowheads). A similar distribution was also observed for the chondroitin 0-sulfate epitope (not shown) and the PG-40 protein core epitope (Fig. 2K). Two other monoclonal antibodies, 7D4 and 6C3, which are comparable with the CS-56 in that they recognize native chondroitin sulfate chains (16, 17), exhibited a distribution similar to that of chondroitin 4-/dermatan sulfate (Fig. 2, H–I). A summary of the immunohistochemical data is presented in Table I. These results indicate that the changes detected biochemically can be directly correlated with an increased deposition of chondroitin 6-sulfate and 0-sulfate in the tumor-associated stroma.

**Analysis of RNA, Northern, and Slot Blotting**—We next examined whether the steady state levels of mRNA encoding PG-40 protein core were altered in human colon carcinoma. First, quantification of total RNA extracted from normal and tumor tissue showed a reproducible 2.5-fold increase in total RNA in colon carcinoma (Fig. 3A). When equal amounts of poly(A<sup>+</sup>) RNA were analyzed by Northern blotting (Fig. 3B), the two major transcripts of 1.9 and 1.6 kb typical of PG-40 (18) were observed in both normal and tumor samples. It was evident, however, that the amount of PG-40 transcripts was markedly increased in the tumor (Fig. 3B, lane 2) as compared with normal colon (Fig. 3B, lane 3). Similar transcripts were also observed in several mesenchymal cells including skin fibroblasts (Fig. 3B, lane 1), colon fibroblasts, and smooth muscle cells (not shown), but were not detected in WiDr colon carcinoma cells, a cell line that does not express this gene product (12). These findings are consistent with the notion that PG-40 is a product expressed primarily by connective tissue cells (7) and are in close agreement with the immunohistochemical data presented above.

Relative PG-40 transcript levels were measured by slot blot hybridization of RNA run in triplicate and further quantified by laserbeam densitometry and computer integration. The amount of specific PG-40 mRNA was increased nearly 3-fold in the tumor tissue *vis à vis* the normal colon tissue when normalized on a milligram of total RNA (Fig. 3C). However, when the values were corrected for changes in total RNA content (Fig. 3A) and expressed as relative hybridization/g of tissue (Fig. 3D), there was a 7.4-fold increase in this transcript in the tumor. Normalization of the PG-40 mRNA levels to human  $\beta$ -actin mRNA levels showed similar PG-40 mRNA increases in colon carcinoma tissue, since  $\beta$ -actin mRNA levels were not significantly different from those of normal colon (not shown).

These results indicate that the increase in chondroitin sulfate proteoglycan detected biochemically and immunohistochemically is associated with elevated steady state levels of PG-40 mRNA.

**Analysis of DNA and Southern Blotting**—To investigate further the molecular basis for the changes in PG-40 mRNA levels described above, we purified genomic DNA from samples of normal colon ( $n = 6$ ) and colon carcinoma ( $n = 8$ ) tissue and determined the relative amounts by scanning ethidium bromide-stained agarose gels. In contrast to the RNA content, the total amount of DNA did not significantly vary between normal and tumor tissue (2.6–2.8 mg/g tissue). Therefore, the changes reported for mRNA are equally sig-



**FIG. 2. Gallery of light micrographs of human colon and colon carcinoma immunostained with antibodies to various epitopes of chondroitin sulfate proteoglycan.** A, colon carcinoma tissue stained with hematoxylin and eosin. B, a sequential section from the same block stained with 3B3 monoclonal antibody directed against chondroitin 6-sulfate epitope; note the marked labeling of the tumor stroma by this antibody and the clear demarcation (*arrowheads*) from the native connective tissue; the malignant cells (*asterisk*) are totally unreactive. C, cross-section of normal colonic mucosa reacted with 3B3 monoclonal antibodies. D, control section in which the specific antibody was omitted. E, vertical section of normal colon reacted with 2B6 monoclonal directed against chondroitin 4-/dermatan sulfate epitopes; note the delicate distribution of this epitope in the interstitium of the

TABLE I

Distribution of immunoreactive chondroitin sulfate epitopes in the stroma of normal and malignant human colon tissue

The values represent a semiquantitative distribution of chondroitin sulfate proteoglycan epitopes in the stroma of normal human colon and colon carcinoma specimens. The relative intensity of immunostaining ranges from very strong (++++), to weak ( $\pm$ ), to undetectable (-). A total of 23 normal and 24 tumor samples were analyzed immunohistochemically. The first five monoclonal antibodies listed below recognize either specific disaccharide stubs remaining on the protein core following digestion with chondroitinase ABC (2B6, 3B3, and 1B5) or the native chondroitin sulfate chains covalently linked to the protein core (7D4 and 6C3). The anti-PG-40 antibody is a monospecific rabbit anti-peptide antibody generated against eighteen amino acid comprising the amino terminus of PG-40/decorin protein core, a region that also contains the glycosaminoglycan binding domain (18). For additional details, see Fig. 2.

Antibody	Epitope	Normal		Malignant peritumorous stroma
		Mucosa	Submucosa	
2B6	Chondroitin 4-S/Dermatan sulfate	+	++	+++
3B3	Chondroitin 6-S	$\pm$	-	++++
1B5	Chondroitin 0-S	+	$\pm$	++++
7D4	Native chondroitin sulfate	++	+	+++
6C3	Native chondroitin sulfate	++	++	+++
PG-40	PG-40 protein core, "decorin"	+	++	++++

nificant when normalized to total DNA content.

Genomic DNA was examined by Southern blot hybridization following digestion with *Pst*I, *Bgl*II, and *Hind*III restriction endonucleases. Both the pattern and relative intensity of PG-40 hybridization was similar in normal and tumor tissue (Fig. 4A), indicating that there was no gross genomic alteration for the PG-40 locus in this human tumor, *i.e.* no significant chromosomal rearrangement or gene amplification. A relatively limited number of fragments (two to five) were detected by PG-40 cDNA probe (Fig. 4A). These results are similar to those obtained using the same restriction endonucleases on human placental DNA (18) and suggest that not more than one or two genes encode PG-40.

As a further control for the integrity of the purified DNA and for the hybridization data described above, we examined Southern blots of parallel DNA digests using a full-length cDNA encoding *c-src* proto-oncogene, a gene expressed by variety of tissues (26). The *c-src* probe reacted intensely with two major DNA fragments only in the *Pst*I-digested DNA (Fig. 4B). The pattern and relative intensity of the hybridization were similar in normal and tumor tissue (Fig. 4B), indicating no significant genomic alteration in the *c-src* and further validating the hybridization with PG-40 probe described above.

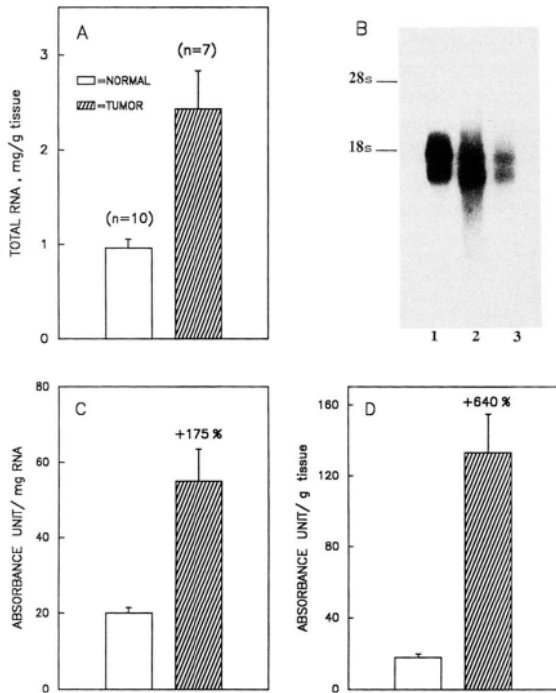
**Hypomethylation of PG-40 Gene in Colon Cancer**—It has been shown that gene activation is associated with a decrease in DNA methylation, a covalent modification of the mammalian genome that occurs almost exclusively at the dinucleotide CG (29–31). Here we wanted to test whether PG-40 was indeed activated via a decrease in methylation and determine whether DNA from actively growing colon fibroblasts and colon carcinoma cells showed similar changes. To test these possibilities we used two methylation-sensitive restriction endonucleases, *Hpa*II and *Msp*I. *Hpa*II cleaves 5'-CCGG-3' sequences only if the internal C is unmethylated (27), whereas *Msp*I cleaves 5'-CCGG-3' regardless of the methylation state of the internal C (28). Southern blotting revealed that normal

colon tissue gave relatively larger *Hpa*II fragments than either malignant or benign tumor tissue (Fig. 5A, compare lanes 1 and 2 with lanes 3–6 or 9, respectively), in spite of the fact that the ethidium bromide-stained gels showed no detectable differences in *Hpa*II sensitivity (not shown). Interestingly, DNA isolated from cultured colon carcinoma cells (Fig. 5A, lane 7) was resistant to *Hpa*II in contrast to colon fibroblasts (Fig. 5A, lane 8) in which a major band of about 7 kb appeared.

Restriction fragments generated by *Msp*I (Fig. 5B) were essentially invariant among the tissues and cells examined. This indicates that the changes in *Hpa*II restriction pattern shown in Fig. 5A were due to the presence of the modified base 5-methylcytosine in the PG-40 gene. Specifically, six different DNA fragments of about 7, 5.5, 3.8, 2, 1.2, and 0.85 kb were generated by *Msp*I digestion. The first five fragments were detected in all the tissues examined, whereas the 0.85-kb fragment was present in about half of the specimens. These results are consistent with genetic polymorphism, rather than with gene rearrangements, since no differences were noted in the restriction fragments generated by other three enzymes (*cf.* Fig. 4).

The degree of hypomethylation of PG-40 gene (Fig. 6A) was determined by calculating the absorbance ratio of the smaller *Hpa*II fragments (*group* II, Fig. 5A) to the that of the total *Hpa*II fragments (*group* I plus *group* II, Fig. 5A). The Southern blots were also hybridized with human  $\beta$ -actin cDNA and the degree of hypomethylation calculated as above (Fig. 6B). The PG-40 gene from colon carcinoma ( $n = 8$ ) was about three times more hypomethylated than normal colon ( $n = 5$ ) (Fig. 6A), in contrast to that of  $\beta$ -actin that showed no significant difference (Fig. 6B). Colon carcinoma cells exhibited the lowest degree of hypomethylation (Fig. 6A), consistent with the fact that colon carcinoma cells do not express this gene product (12) and do not show any immunoreactivity with anti-PG-40 antibody both *in vitro* and *in vivo* (11). In contrast, human colon fibroblasts, which express this product (10), showed a significant degree of hypometh-

lamina propria and the submucosal connective tissue. *F* and *G*, sections of tumor tissue reacted with 2B6 monoclonal antibodies; note the marked reaction of the tumor stroma and the lack of reaction of the malignant epithelial cells (*asterisk*). *H*, colon carcinoma; *I*, normal colon reacted with 7D4 monoclonal antibody directed against native chondroitin sulfate; notice again the stromal labeling and the intensity in the tumor stroma. *J*, control section for 7D4 antibody. *K*, cross-section of tumor tissue reacted with monospecific anti-peptide antiserum which recognizes the amino terminal peptide of PG-40; notice the stromal distribution similar to the chondroitin 4- and 6-sulfate epitopes. *L*, control section for anti-PG-40 antibodies ( $A-L \times 200$ ). A summary of the immunostaining is provided in Table I.



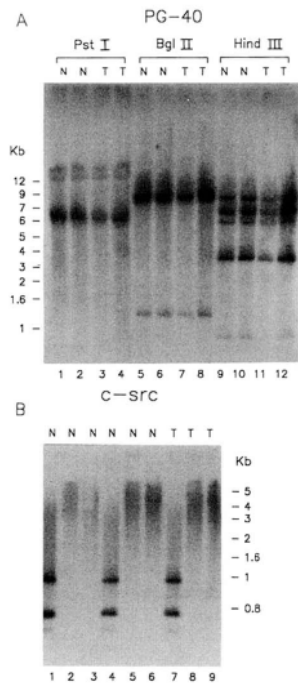
**FIG. 3. Analysis of RNA in normal human colon and colon carcinoma tissue.** A, total amount of RNA. B, Northern blottings of 10  $\mu$ g of total RNA isolated from skin fibroblast (lane 1), and 5  $\mu$ g each of poly (A<sup>+</sup>) RNA from colon carcinoma (lane 2) and normal colon (lane 3). The blots were reacted with a full-length cDNA encoding PG-40 under high stringency as described in detail under "Experimental Procedures." Notice the two transcripts of 1.9 and 1.6 kb typical of PG-40 in both colon fibroblasts and colon tissues. Similar transcripts were observed in human colon smooth muscle cells but not in colon carcinoma cell RNA (not shown). Relative hybridization for PG-40 transcripts was determined by laserbeam scanning densitometry of slot blots run in triplicate and expressed as absorbance units/mg of RNA (C) or corrected for changes in total RNA content and expressed as absorbance units per gram of tissue (D). When the data were expressed on  $\beta$ -actin hybridization, similar increases in PG-40 mRNA levels were obtained (not shown).

ylation (Fig. 6A). Finally, DNA isolated from an adenomatous polyp exhibited a pattern of methylation similar to the malignant tissue (Fig. 6A), suggesting that hypomethylation is associated with connective tissue activation and remodelling occurring in neoplasia. Analysis of the data presented in Fig. 6B clearly show that the PG-40 values would not significantly change if normalized to  $\beta$ -actin hybridization values.

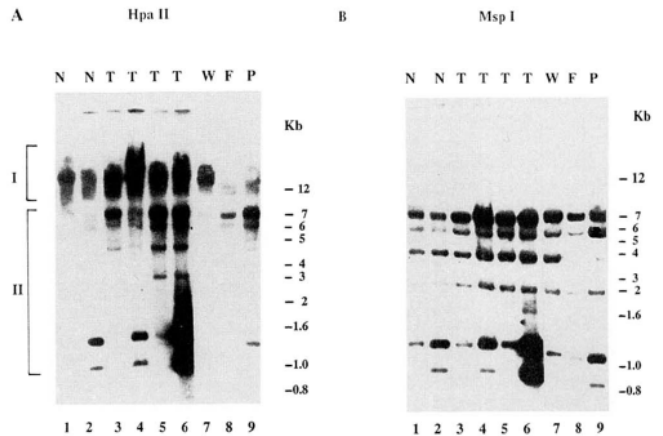
In summary, these results indicate that the expression of PG-40 gene is activated in colon carcinoma tissue and provide a plausible explanation for the increased PG-40 mRNA levels and proteoglycan content in this tumor.

**DISCUSSION**

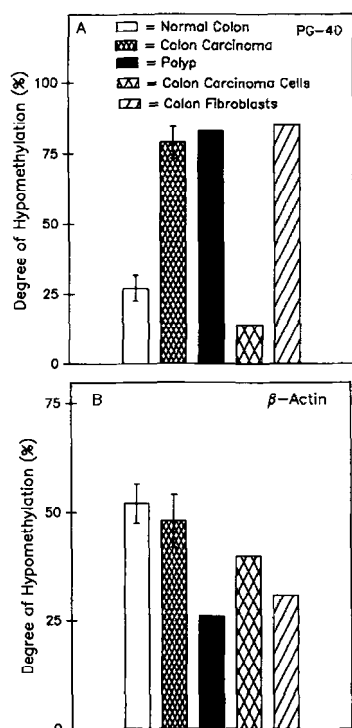
In this study we have used a variety of immunological and molecular biological approaches to investigate changes in the expression of chondroitin sulfate proteoglycan in human colon carcinoma. The results show a marked increase in a chondroitin sulfate proteoglycan with immunological and structural characteristics similar to the ubiquitous PG-40 proteoglycan (18), also named "decorin" for its ability to bind and decorate collagen fibrils (7). The glycosaminoglycan composition of tumor-associated proteoglycans appeared to be altered, displaying increased levels of 0- and 6-sulfate epitopes when compared with normal colon proteoglycans. The tumor distribution of this gene product was also quite striking since it localized predominantly to the newly formed connective



**FIG. 4. Southern blot hybridization analysis of genomic DNA from normal colon and colon carcinoma tissue with PG-40 (A) or c-src (B) cDNA.** Twenty  $\mu$ g of genomic DNA isolated from normal colon (N) and tumor tissue (T) was digested with various restriction endonucleases (*Pst*I, *Bgl*II, and *Hind*III) and reacted with <sup>32</sup>P-labeled PG-40 cDNA (A) or c-src proto-oncogene cDNA (B). In B, lanes 1, 4, and 7 are samples digested with *Pst*I; lanes 2, 5, and 8 are samples digested with *Bgl*II; and lanes 3, 6, and 9 are samples digested with *Hind*III. The size in kilobase pairs of DNA markers is indicated at the margins. Notice the presence of DNA fragments of comparable intensity and pattern in both normal and tumor tissue, indicating no significant amplification or rearrangements of either of these two genes.



**FIG. 5. Methylation patterns of PG-40 locus in normal human colon, colon carcinoma tissue, benign colonic polyp, and cultured cells.** Twenty  $\mu$ g of genomic DNA isolated from normal colon (N), colon carcinoma tissue (T), WiDr colon carcinoma cells (W), normal human colon fibroblasts (F), or benign adenomatous polyp of colon (P) were digested with *Hpa*II (A) or *Msp*I (B) restriction endonucleases and analyzed by Southern blotting using <sup>32</sup>P-labeled PG-40 cDNA insert. The size in kilobase pairs of DNA markers is indicated in the margins. Notice the presence of relatively smaller fragments in the *Hpa*II-digested DNA from the tumor tissue *vis á vis* the normal tissue. The ethidium bromide stained gel did not show any detectable change among any of the DNA samples (not shown). For additional details, see Fig. 6.



**FIG. 6. Quantitation of the degree of hypomethylation of PG-40 (A) and  $\beta$ -actin (B) genes in human colon tissue and cultured colon cells.** The degree of PG-40 hypomethylation was obtained by calculating the absorbance ratio of group II (the smaller molecular weight *Hpa*II fragments as indicated in Fig. 5A) to the total (group I plus group II) *Hpa*II fragments from several autoradiographs of Southern blots as described before (34). The proportion of II/I + II was determined for each digest and recorded as percent of hypomethylation. The Southern blots were heated at 100 °C in 0.1% sodium dodecyl sulfate, exposed overnight to check for complete removal of the probe, rehybridized with  $\beta$ -actin cDNA, and quantitated for methylation (B) as for the PG-40. The values for the normal colon ( $n = 5$ ) and colon carcinoma ( $n = 8$ ) represent the mean  $\pm$  S.E. The values for the remaining samples are the mean of duplicate determinations.

tissue surrounding the tumor cells. The presence of this proteoglycan within the tumor stroma was clearly demarcated from the native connective tissue by using monoclonal antibodies directed against the intact glycosaminoglycan chains, against the major chondroitinase ABC-generated disaccharide epitopes, or against the amino terminal peptide of PG-40 protein core. In contrast, in normal colon tissue, PG-40 proteoglycan was expressed at relatively lower levels, was primarily associated with blood vessels, and was only focally present in the interstitium. These findings corroborate our previous histochemical and autoradiographic studies which show a similar tissue distribution of chondroitin sulfate proteoglycans (8) and with our organ culture studies of human colon carcinoma which show enhanced synthesis of a small chondroitin sulfate proteoglycan by the tumor tissue (9). It is noteworthy that similar tumor-associated changes in connective tissue composition are also observed in distant metastases of colon carcinoma (2, 32) and in a number of carcinomas (33) with morphogenetic features similar to colon cancer, but are absent in malignant neoplasms that do not induce a stromal response (3). This argues that the neoplastic-associated stromal changes are tumor-specific and not just an epiphenomenon, *i.e.* a nonspecific response of the host (see below).

In the present studies we provide direct evidence that proteoglycans with immunological and structural character-

istics related to PG-40 are indeed the major source of these changes. We further show that the steady state levels of PG-40 mRNA were significantly elevated and that the total content of this specific transcript was over seven times greater than that of normal tissue, whether the values were normalized to total RNA content or  $\beta$ -actin mRNA levels. Because transformed colon epithelial cells do not express detectable levels of PG-40 mRNA nor express the proteoglycan *in vitro* (11, 12), the present results imply that connective tissue cells are stimulated to produce this gene product. Since our data are based on RNA extracted from whole tissue, it cannot be excluded that the changes in PG-40 mRNA levels are related to a greater number of connective tissue cells in the tumor. However, previous morphometric studies (8) have shown that the various cellular compartments of colon carcinoma tissue are not significantly different from the normal state.

Using Southern blot hybridization, we demonstrate that PG-40 gene was neither significantly amplified nor rearranged. However, when we tested the degree of methylation of the PG-40 gene using methylation-sensitive and -insensitive restriction endonucleases, we found a 3-fold decrease in the methylation state of this gene in colon carcinoma tissue. The specificity of these findings was validated by analysis of  $\beta$ -actin methylation, a gene that was equally hypomethylated in normal and colon carcinoma tissue. The DNA of a variety of genes has been found to be undermethylated in cells and tissues where they are expressed and to be methylated when they are not expressed (29–31). Methylation of DNA is a covalent modification of the mammalian genome, with about 70% of cytosine in CG sequences being methylated in normal cells (29–31). Recent studies have shown a nonrandom decrease in DNA methylation of specific genes in various tumors including colon carcinoma (34–36). In the present study, the degree of hypomethylation was not a general phenomenon, since ethidium bromide-stained gels before Southern blotting revealed no significant differences in *Hpa*II sensitivity between normal and neoplastic colon. These data confirm previous reports (34, 36), and further stress that specific genes show undermethylation when tested by Southern blotting, even in the absence of a general hypomethylation detectable with less sensitive methods. Our results are the first to demonstrate a hypomethylation in a gene encoding a ubiquitous proteoglycan and provide a plausible explanation for the increased PG-40 mRNA levels and proteoglycan content detected in this tumor.

Because several genes are undermethylated to the same degree in both benign and malignant colonic neoplasms, it has been proposed that hypomethylation is a consistent DNA alteration of human colonic neoplasms and that this alteration precedes malignancy (36). Implicit in these studies, however, is the concept that selective gene hypomethylation derives from the clonal neoplastic cells, whether benign or malignant, and no effort has been put on determining the cellular source of these alterations. In the present study, we have attempted to determine the cellular source of PG-40 undermethylation by comparing DNA isolated from normal and transformed colon tissues with that of normal stromal or transformed epithelial cells derived from human colon. All the data point to a selective undermethylation of PG-40 in the neoplastic stromal cells, which correlates well with the immunohistochemical localization of PG-40, rather than in the neoplastic cells themselves.

An important question that needs to be addressed is about the specificity of these changes, that is, to what extent these DNA alterations are due to a nonspecific connective tissue response associated with neoplastic growth. In this context,



we have recently found<sup>2</sup> in four different patients with ulcerative colitis, a chronic bowel disease characterized by active inflammation and tissue remodelling (1), that PG-40 gene is methylated to the same extent as normal colon. These results, together with PG-40 hypomethylation in a benign adenomatous polyp of the colon described in this report, suggest a significant degree of specificity for these DNA alterations and that these changes are associated with or induced by a clonal cell population.

The finding of PG-40 hypomethylation in proliferating colon fibroblasts, but not in colon carcinoma cells, which do not express PG-40 (12), is further evidence for a connective tissue activation in response to tumor cytokines. This concept is based on the fact that analogous changes in chondroitin sulfate proteoglycan can be induced *in vitro* by either exposing colon fibroblasts to soluble cytokines released by the colon carcinoma cells (10) or by co-culturing colon carcinoma cells with colon fibroblasts or smooth muscle cells (11). An additional regulatory mechanism could be provided by heparan sulfate proteoglycan. This proteoglycan binds a variety of growth factors (37), concentrates them in the extracellular matrix, and protects them from proteolytic attack (38, 39). Because colon carcinoma cells synthesize large amounts of heparan sulfate proteoglycan (12, 40), and these levels can be further increased by exposing the cells to transforming growth factor  $\beta$ ,<sup>3</sup> it is conceivable that the enhanced expression of PG-40 may be in turn induced by growth factors bound to the heparan sulfate proteoglycan.

In conclusion, we believe that modulation of extracellular matrix by neoplastic cells is an important step in tumor progression because it provides a proteoglycan-enriched well hydrated microenvironment which, by analogy with developmental systems, can favor the growth of tumor cells. Our results provide a molecular explanation for the previously reported proteoglycan changes in human colon carcinoma and raise the novel possibility that demethylating events associated with or induced by a clonal cell population may affect specific connective tissue genes.

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