We report a detailed analysis of heparan sulfate (HS) structure using a model of human colon carcinogenesis. Metabolically radiolabeled HS was isolated from adenoma and carcinoma cells. The chain length of HS was the same in both cell populations (M, 20,000; 45–50 disaccharides), and the chains contained on average of two sulfated domains (S domains), identified by heparinase I scission. This enzyme produced fragments of approximate size 7 kDa, suggesting that the S domains were evenly spaced in the intact HS chain. The degree of polymer sulfation and the patterns of sulfation were strikingly different between the two HS species. When compared with adenoma HS, the iduronic acid 2-O-sulfate content of the carcinoma-derived material was reduced by 33%, and the overall level of N-sulfation was reduced by 20%. However, the level of 6-O-sulfation was increased by 24%, and this was almost entirely attributable to an enhanced level of N-sulfated glucosamine 6-O-sulfate, a species whose data implied was mainly located in the mixed sequences of alternating N-sulfated and N-acetylated disaccharides. The results indicate that in the transition to malignancy in human colon adenoma cells, the overall molecular organization of HS is preserved, but there are distinct modifications in both the S domains and their flanking mixed domains that may contribute to the aberrant behavior of the cancer cell.

Heparan sulfate is a widespread complex linear polysaccharide that consists of alternate hexuronic acid and N-substituted glucosamine residues. The ability to bind protein effectors such as growth factors or protease inhibitors (e.g. antithrombin III; Refs. 1 and 2) is strongly influenced by the position and density of sulfate residues that occur most frequently as N-sulfates but are also present as sulfate esters at the 6-O- (or less commonly the 3-O-) position of glucosamine or the 2-O-position of iduronic acid (3). The complexity of HS is achieved through the location of sulfate groups in domains of high and low sulfation (4), the conformational flexibility of idurionate residues (5, 6), and the degree of polymorphism manifest in different tissues (7–9). The strongly anionic zones of HS, the S domains, consist of contiguous glucosamine N-sulfate-containing disaccharides that bear a variable number of O-sulfate moieties. Domains of less sulfated sequences called mixed sequences are believed to flank the S domains, separating them from the unsulfated domains, and these are liberated as tetrascarbohydrates by low pH nitrous acid, a reagent that cleaves HS at N-sulfated glucosamine-containing disaccharides (i.e. GlcNSO\textsubscript{2}−α1−4-hexuronic acid).

The ability of HS to act as a growth factor activator (10–13) and as a component of focal adhesions (14) has focused attention on this molecule as a potential therapeutic target in diseases of aberrant cellular growth or migration such as cancer, diabetic retinopathy, or coronary arterial restenosis. A number of studies have investigated the structural changes in HS in animal models of malignancy with the earliest detailed analyses being performed on SV-40-transformed murine embryo cell lines (15–17). These studies showed that transformation was accompanied by a reduction in charge density that was largely due to a decrease in 6-O-sulfation in nitrous acid-resistant tetrascarbohydrates. Further analyses of normal and transformed mouse mammary basement membrane proteoglycans confirmed that carcinogenesis was associated with reductions in 6-O-sulfation and also of 3-O-sulfation and a reduction in the size of intact HS (18), changes that were associated with a reduced affinity for antithrombin III.

There have been no detailed studies of HS structure in human malignant tissues, but a crude comparison of normal liver and hepatoma tissue showed that there was a reduction in the charge density of HS in the neoplastic tissue (19).

The first in vitro model of the progression of human colon cancer from adenoma to carcinoma was developed recently by Paraskeva and co-workers (20). A cell line with the phenotype of an adenoma was derived from a polyp taken from a patient with familial adenomatous polyposis. Through chemical transformation, a second cell line was derived that was tumorigenic in nude mice and anchorage-independent in soft agar, in contrast to the adenoma cell line. We have used these cell lines to identify the structural changes in HS during the progression from human colon adenoma to carcinoma.

**EXPERIMENTAL PROCEDURES**

*Materials—d-[6-3H]Glucosamine hydrochloride (20–45 Ci/mmol) and Na\textsubscript{35}SO\textsubscript{4} (carrier free; 1200–1400 Ci/mmol) were obtained from NEN Life Science Products. Heparinase I (Flavobacterium heparinum; heparin lyase, EC 4.2.2.7), heparinase II (F. heparinum; no EC number assigned), and heparinase III/ heparinase (F. heparinum; heparanase). Mannitol; GAG, glycosaminoglycans; GlcUA, glucuronic acid; GlcNAc; N-acetylated glucosamine; GlcNS: N-sulfated glucosamine; GlcNS(6S); N-sulfated glucosamine 6-O-sulfate; HPLC, high performance liquid chromatography; IdceA, iduronic acid; IdceA(2S), IdceA 2-O-sulfate; PBS, phosphate-buffered saline; SAX, strong anion exchange; UA, uronic acid.*
sulfate lyase, EC 4.2.2.8) were obtained from Grampian Enzymes (Aberdeen, UK). Materials for cell culture were obtained from Life Technologies, Inc. All other chemicals were from Sigma.

**Cell Culture**—The adenoma and carcinoma cells were grown in conditions described by Williams et al. (20) and were used within 10 passages.

**Preparation Of Heparan Sulfate**—Glycosaminoglycans were prepared from cultured cells in four 175-cm² tissue culture flasks. The adenoma and carcinoma cells were labeled at 80% confluence by incubation in normal medium containing 5 mM NaCl (not shown). There was no difference in the GAG composition between the adenoma and carcinoma cells. The 3H/35S-labeled HS was isolated from the cell layer by treatment with Pronase in 1% (v/v) Triton X-100; the culture medium was also digested with Pronase, and the medium and cell layer extracts were then pooled and clarified by centrifugation (see “Experimental Procedures”). The GAGs were fractionated by anion-exchange chromatography, and the HS (identifiable by comparison with known reduced standards) were detected by their absorbance at 294 nm using the in-line UV detector in the HPLC apparatus.

**RESULTS**

Adenoma and carcinoma cells were taken at 80% confluence and metabolically radiolabeled for 48 h with [3H]glucosamine and 35SO₄. GAGs were extracted from the cell layer by treatment with Pronase in 1% (v/v) Triton X-100; the culture medium was also digested with Pronase, and the medium and cell layer extracts were pooled and clarified by centrifugation (see “Experimental Procedures”). The GAGs were fractionated by anion-exchange chromatography, and the HS (identifiable by its degradation by nitrous acid) eluted in a double-labeled peak at ~0.55 M NaCl (not shown). There was no difference in the NaCl concentration required to desorb the HS produced by adenoma and carcinoma cells. The 35S-labeled HS was cleared of any contaminating chondroitin sulfate and hyaluronic acid by treatment with chondroitinase ABC. The Sepharose CL-6B elution profiles of intact adenoma and carcinoma HS species were symmetrical, and the position of the peak of elution of each sample corresponded to a chain of average molecular mass 20 kDa, equivalent to 45–50 disaccharides (Fig. 1).

**Low pH Nitrous Acid Scission of HS**—The adenoma and carcinoma HS chains were cleaved with nitrous acid, which breaks HS at the glycosidic bond between N-sulfated glucosamine and uronic acids. The products of scission were eluted from the column by treatment with Pronase in 1% (v/v) Triton X-100; the culture medium was also digested with Pronase, and the medium and cell layer extracts were pooled and clarified by centrifugation (see “Experimental Procedures”). The GAGs were fractionated by anion-exchange chromatography, and the HS (identifiable by its degradation by nitrous acid) eluted in a double-labeled peak at ~0.55 M NaCl (not shown). There was no difference in the NaCl concentration required to desorb the HS produced by adenoma and carcinoma cells. The 35S-labeled HS was cleared of any contaminating chondroitin sulfate and hyaluronic acid by treatment with chondroitinase ABC. The Sepharose CL-6B elution profiles of intact adenoma and carcinoma HS species were symmetrical, and the position of the peak of elution of each sample corresponded to a chain of average molecular mass 20 kDa, equivalent to 45–50 disaccharides (Fig. 1).

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The heparinase III profiles demonstrate that in both HS species, the majority of the GlcUA-bearing disaccharides occur in contiguous sequences, as revealed by the large disaccharide peaks (Fig. 2, c and d). The fragments that are resistant to heparinase III digestion are the sulfated domains (S domains).

**Heparinase I Scission**—To gain further information on the structure and location of the S domains, the enzyme heparinase I was used. This enzyme cleaves HS essentially where GlcNS(\(\pm 6\)-S)-IdceA(2S) residues occur (23, 24), although the enzyme is active against glucuronate-2-O-sulfate (28), a rare constituent in HS (29, 30). Scission of the intact adenoma and carcinoma chains with heparinase I and elution of the products by Sepharose CL-6B gel filtration (Fig. 1) showed that the \(K_{av}\) of the main peak of eluted material was 0.7, suggesting that the average molecular weight of heparinase I-resistant fragments was 7,000 (21).

Smaller fragments were also produced by heparinase I, and these were examined by chromatography on Bio-Gel P-10. The results showed that the adenoma HS gave a significantly higher yield of low molecular weight products (disaccharides and tetrasaccharides) than the carcinoma HS (Fig. 2, e and f). Although 13% of the hexosaminidic linkages in the adenoma HS were susceptible to heparinase I, only 7% of such linkages were cleaved in the carcinoma HS. In addition, the chromatographs suggest that approximately 4% of the adenoma HS disaccharides were present as contiguous heparinase I-sensitive disaccharides, whereas the corresponding figure for the carcinoma HS was about 2% (Fig. 2, e and f). This was a consistently observed difference and was noted in three separate experiments.

**Total Disaccharide Composition**—HS from adenoma and carcinoma cells was completely depolymerized with heparinasin I, II, and III. The disaccharides were separated by strong anion-exchange HPLC, and the results are shown graphically (Fig. 3, a and b) and numerically (Table II). The major disaccharide in both HS species was \(\Delta\)UA-GlcNAc, which comprised just over half the total disaccharide units; the \(\Delta\)UA-GlcNSO\(_3\) was also a prominent constituent (Table II). However, a number of reproducible differences were present in the O-sulfated disaccharides. In particular, progression to carcinoma was associated with an increase in \(\Delta\)UA-GlcNAc(6S) but a reduction in \(\Delta\)UA(2S)-GlcNS. The latter disaccharide is most likely to contain IdceA(2S) in the HS chain and to be located in the S domains, where it will be cleaved by heparinase I. The findings on the content of IdceA(2S) in the two HS species are compatible with the reduced heparinase I sensitivity in the carcinoma-derived material noted earlier (Fig. 2, e and f).

The data also show that the average number of sulfate groups per 100 disaccharides in the adenoma and carcinoma HS was 73 and 61, respectively. The lower sulfation in the carcinoma HS was due to a 20% reduction in N-sulfation and a 33% reduction in 2-O-sulfation, the observed 24% increase in 6-O-sulfation only partially offsetting the fall in 2-O- and N-sulfation.

**Analysis of Disaccharides Liberated by Nitrous acid; Comparison with Heparinase-released Disaccharides**—The foregoing analyses indicated that transformation was associated with a reduction in 2-O-sulfation and an increase in 6-O-sulfation. To establish the principal locations of these changes, disaccharides and tetrasaccharides released by nitrous acid scission were examined further by SAX-HPLC. Disaccharides released by nitrous acid provide an indication of the composition of the S domains.

The relative proportions of each disaccharide were largely equivalent in the adenoma and carcinoma material except for a striking decrease in the IdceA(2S)-aMan\(_R\) in the carcinoma HS.
FIG. 2. Bio-Gel P-10 gel filtration chromatographic analysis of adenoma HS (a, c, and e) and carcinoma HS (b, d, and f) after scission with nitrous acid (a and b), heparinase III (c and d), and heparinase I (e and f). The insets in e and f represent enlargements of the $^3$H radiolabel profiles in each graph. Solid line, $^3$H label; dotted line, $^{35}$S label; dp, degree of polymerization; dp2, disaccharides; dp4, tetrasaccharides; dp6, hexasaccharides.
Heparan Sulfate, from Adenoma to Carcinoma

TABLE I

Summary of the comparative sensitivities of the adenoma and carcinoma HS species to specific reagents

|                        | Adenoma | Carcinoma |
|------------------------|---------|-----------|
| $M_r$ of intact chains | 20,000  | 20,000    |
| ($K_m$, on Sepharose CL-6B) | (0.5) | (0.5) |
| $M_r$ of heparinase I-resistant chains | 7,000 | 7,000 |
| ($K_m$, on Sepharose CL-6B) | (0.7) | (0.69) |
| % chain susceptible to nitrous acid scission | 37.0 | 32.0 |
| % chain-bearing contiguous disaccharides sensitive to nitrous acid | 16.0 | 13.0 |
| % chain susceptible to heparinase I scission | 13.3 | 7.3 |
| % chain-bearing contiguous disaccharides sensitive to heparinase I scission | 3.8 | 1.6 |
| % chain susceptible to heparinase III scission | 69.2 | 73.6 |
| % chain-bearing contiguous disaccharides sensitive to heparinase III scission | 59.7 | 61.3 |

(Adnan, 2007; GlcNAc, 2009). This component corresponds to the Ahexuronic acid(2S)-GlcNS unit in the heparinase-released disaccharides (Table II). Disaccharides of structure GlcUA(2S)-aMan R(6S), that elute after IdceA(2S)-aMan R(6S) (peak 5) on SAX-HPLC were not detected. The data indicate that the reduction in 2-O-sulfation on transformation occurs chiefly in the S domains. The major 6-O-sulfated unit in the nitrous acid-released disaccharides was IdceA(2S)-aMan R(6S), and only very small amounts of the mono-6-O-sulfated units GlcUA/IdceA-aMan R(6S) were present (Fig. 3, c and d). This indicates that the majority of the $\Delta$UA-GlcNS (6S) detected in the heparinase digests (Table II) must be present in the tetrasaccharides released by nitrous acid, which correspond to the mixed sequences. Because of the close coupling of N- and O-sulfation in HS, the $\Delta$UA-GlcNAc (6S), which represents 12% of adenoma and 16% of carcinoma HS disaccharides, will also be present mainly in the mixed sequences, which thus contain significantly more 6-O-sulfates than the contiguous N-sulfated regions. Combining the data in Table II and Fig. 3, c and d, we can calculate that approximately 70 and 80% of the total 6-O-sulfates are present in the mixed sequences in the adenoma and carcinoma HS, respectively.

Analysis of Nitrous Acid-derived Tetrasaccharides—The structure of the tetrasaccharide products of nitrous acid scission were analyzed by SAX-HPLC and shown to contain non-, mono-, and disulfated species (Fig. 3, e and f). $^{35}$S radiolabel in these peaks is O-sulfate that is mainly present at C-6 of GlcNAc and C-6 of aMan R(6S) (see “Discussion”). The sulfation of each peak was based on the ratio $^{35}$S/3H. The results showed that the adenoma-derived nitrous acid-resistant tetrasaccharides consisted mainly of non- and mono-O-sulfated structures (Fig. 3e), whereas the carcinoma contained a significantly higher proportion of monosulfated structures, with some disulfated material also present (Fig. 3f). These differences were reproduced in three experiments. The results confirm that variation in O-sulfation of the mixed sequences is an important distinguishing feature between adenoma and carcinoma HS.

DISCUSSION

This paper describes a detailed analysis of the changes in structure of HS in a model of human colon carcinogenesis in vitro (20). The study shows that HS from cultured adenoma and carcinoma cells share a common molecular organization in the form of similar chain lengths and spacing of S domains (Fig. 1). When HS was degraded with either low pH nitrous acid or heparinase III (Fig. 2), the results showed that many of the linkages susceptible to these reagents were contiguous, especially in the case of heparinase III (Fig. 2). The data indicate that the HS from both samples conform to a domain structure that is characteristic of the HS family (4, 31). The adenoma and carcinoma HS were approximately 45–50 disaccharides in length (20 kDa), and the fragmentation by heparinase I to yield a major peak of resistant material with a molecular mass of 7 kDa (Fig. 1) implied that the HS chains contained two evenly

spaced S domains.

Progression to malignancy was associated with specific structural changes, including a reduction in 2-O-sulfation in the S domains and an increase in 6-O-sulfation in the mixed sequences, which consist of alternate N-sulfated and N-acetylated disaccharides. Previous studies have suggested that O-sulfates in these sequences are mainly present on C-6 of the amino sugars (32), and we assume that this is the case in the HS studied here. Structural changes in the mixed sequences have been reported before in murine models of transformation. These studies reported that tumor cells synthesized HS with a reduced content of 6-O-sulfate (15–17) and 3-O-sulfate (18) in the mixed sequences. Our results confirm that these regions are a major target of structural change in carcinogenesis. However, the data show that malignant transformation of human adenomas is accompanied by an increase in mixed sequence 6-O-sulfation mainly associated with GlcNAc residues (Table II). This increase is also revealed by the high content of mono-O-sulfated and di-O-sulfated tetrasaccharides in the nitrous acid scission products (Fig. 3, e and f).

The biosynthesis of HS is assumed to follow the heparin pathway of polymer modification in which the addition of 2-O-sulfates to iduronic acid residues occurs before 6-O-sulfation of GlcNS/GlcNAc (1, 33, 34). The composition of the S domains is compatible with this sequence of events, in that most of the 6-O-sulfate in these domains occur in disaccharides bearing 2-O-sulfate moieties (Fig. 3, c and d). However, in the adenoma HS and particularly in the carcinoma HS, there are more 6-O-sulfate residues in the flanking sequences than in the S domains. Taken in conjunction with the reduction in 2-O-sulfation in the carcinoma HS, the data suggest that the 6-O-sulfotransferases that act on the mixed sequences operate independently of the presence of 2-O-sulfate groups, in accord with findings on the structure of HS produced by a Chinese hamster ovary cell mutant defective in 2-O-sulfotransferase (35). It is interesting that in other species of HS the content of 6-O-sulfates is higher in the mixed sequences than in the S domains (28).

The data also indicate that there is a reduction in 2-O-sulfation after malignant transformation (Table II; Fig. 2, e and f). Since the 2-O-sulfotransferase has a similar amount of iduronate (Table I, heparinase III-resistant fraction) to act on in both HS species, the implication is that the enzyme is acting more efficiently in the adenoma than the carcinoma.

Approximately 70% of both the adenoma and carcinoma HS species were sensitive to heparinase III (Fig. 2, c and d), which acts mainly on GlcUA-containing disaccharides (23, 24). IdceA-containing disaccharides are relatively poor substrates by comparison. Although activity has been demonstrated against IdceA-containing disaccharides (25, 26), studies with desulfated heparins indicate that in polymeric structures, cleavage of such units is substantially incomplete (27). Our data show that only 13% of the adenoma HS and 7% of the carcinoma HS
FIG. 3. Strong anion-exchange HPLC analysis of disaccharides and tetrasaccharides derived from adenoma HS (a, c, and e) and carcinoma HS (b, d, and f). Panels a and b show the separation of disaccharides released by complete depolymerization of HS using heparinases I, II, and III (peak 1, ΔUA-GlcNAc; peak 2, ΔUA-GlcNS; peak 3, ΔUA-GlcNAc(6S); peak 4, ΔUA(2S)-GlcNAc; peak 5, ΔUA-GlcNS(6S); peak 6, ΔUA(2S)-GlcNS; peak 8, ΔUA(2S)-GlcNS(6S)). Panels c and d show the separation of nitrous acid-liberated disaccharides (peak 1, IdceA/GlcUA-aManR; peak 2, IdceA(2S)-aManR; peak 3, GlcUA-aManR(6S); peak 4, IdceA-aManR(6S); peak 5, IdceA(2S)-aManR(6S); panels e and f show the analysis of nitrous acid-resistant tetrasaccharides (peak 1, nonsulfated; peak 2, monosulfated; peak 3, disulfated). Solid line, 3H label; dotted line, 35S label.
Heparan Sulfate, from Adenoma to Carcinoma

**Comparative disaccharide compositions of the adenoma and carcinoma HS species**

HS samples were degraded by combined heparinase I, II, and III digestion, and the resulting disaccharides were analyzed by SAX-HPLC. The results represent the mean of values obtained from three determinations, with the S.E. values in all cases being \( \leq 1.5\% \).

|                        | Adenoma | Carcinoma |
|------------------------|---------|-----------|
| UA-GlcNAc              | 51      | 54        |
| UA-GlcNS               | 16      | 14        |
| UA-GlcNAc(6S)          | 12      | 16        |
| UA(2S)-GlcNAc          | 2       | 2         |
| UA-GlcNS(6S)           | 6       | 6         |
| UA(2S)-GlcNS           | 10      | 4         |
| UA(2S)-GlcNAc(6S)      | 0       | 0         |
| UA(2S)-GlcNS(6S)       | 3       | 4         |
| %N-sulfation            | 35      | 28        |
| % 2-O-sulfation         | 15      | 10        |
| % 6-O-sulfation         | 21      | 26        |
| No. sulfates/100 disaccharides | 73  | 61        |

**Fig. 4. Domain structures of adenoma and carcinoma HS species.** HS from both adenoma and carcinoma cells is depicted with two S domains (open rectangles) evenly spaced along the chain from the core protein (left) to the nonreducing terminus (right). These S domains will contain the sites of cleavage for heparinase I. Distinct O-sulfation patterns are present in the S domains and the adjacent mixed sequences. In the carcinoma HS, the mixed sequences are relatively enriched in 6-O-sulfate, but a lower concentration of 2-O-sulfate occurs in the S domain. As a consequence of this reduction in 2-O-sulfate, the carcinoma HS has fewer sites of cleavage for heparinase I than the adenoma HS. Circles, GlcUA-GlcNAc; squares, GlcUA/Idcea-GlcNSO3, respectively; thin arrows, linkages susceptible to nitrous acid scission; thick arrows, linkages susceptible to heparinase I.

**Table II**

|                      | Adenoma | Carcinoma |
|----------------------|---------|-----------|
| No. sulfates/100 disaccharides | 73  | 61        |

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