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Hypomethylation of the decorin proteoglycan gene in human colon cancer

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We have previously reported that the connective tissue stroma of human colon carcinoma contains elevated amounts of decorin, a small proteoglycan involved in the regulation of matrix formation and cell proliferation. These biochemical changes were correlated with increased mRNA levels and general hypomethylation of the decorin gene in human colon cancer DNA. In this report we use a quantitative polymerase chain reaction method coupled with digestion of the DNA template by methylation-sensitive restriction endonucleases to investigate in detail the location of hypomethylated sites in decorin gene. We demonstrate that a specific site in the 3′ region of the gene, encompassing codons 360–361, is specifically hypomethylated in both colon carcinoma and benign polyp. In contrast, three *Hpa*II sites, clustered in the 5′ untranslated region, show full methylation in normal and neoplastic DNA. The lack of such changes in ulcerative colitis DNA suggests that chronic inflammation alone is not sufficient to alter cytosine methylation in the decorin gene. These results suggest the possibility that the 3′ region of the decorin-coding sequence may be involved in the control of decorin gene expression.

INTRODUCTION

The newly formed connective tissue that is associated with a clonal population of cells is a vital component of most epithelial neoplasms. This complex structure provides a vascular supply, comprises a suitable extracellular matrix conductive for growth, and can harbour a number of growth factors that in turn modulate the growth of tumour and mesenchymal cells [1–3]. For many years, the formation of neoplastic stroma has been regarded as a mere response to the tissue 'injury' caused by the disruption of the basal lamina and infiltration of the surrounding tissues of the host by the tumour cells. Only in recent years has there been a better appreciation of this important constituent of human tumours. This realization derives primarily from our increased knowledge regarding the pivotal role that extracellular matrix proteins play in regulating cell behaviour and gene expression [1–3]. The central hypothesis of our research is that the structural, compositional and molecular changes of the tumour stroma are not random events; rather, they are the result of specific exchanges of information between neoplastic and host mesenchymal cells. Understanding the mechanisms that control tumour stroma formation may lead to a better understanding of how tumour cells grow and invade surrounding tissues. Our laboratory has used human colon carcinoma tissue and cells derived therefrom to investigate changes in proteoglycan structure, amount and gene expression. We have shown that there is a specific increase in a small chondroitin sulphate proteoglycan in the tissue of human colon carcinoma and that the connective tissue cells are responsible for this enhanced proteoglycan biosynthesis [4,5]. Increased levels of proteoglycans with an altered glycosaminoglycan composition can be reproduced *in vitro* by exposing normal colon fibroblasts or smooth muscle cells to tumour cell metabolites [6] or by co-culturing experiments [7]. More recently, we have shown [8] that the small chondroitin sulphate that accumulates in the colon carcinoma stroma is decorin, a ubiquitous proteoglycan [9] involved in the regulation of collagen fibrillogenesis [10] and cell proliferation [11]. Analysis of decorin mRNA levels revealed a 7-fold increase in colon carcinoma as compared with normal colon [8]. These mRNA changes were correlated with hypomethylation of the decorin gene [8], a finding that has been associated with increased gene transcription [12–15].

The goal of the present study was to investigate in detail the specific location of hypomethylated sequences in the decorin gene and to establish whether these changes were specific for colon carcinoma. Using a quantitative PCR method, coupled with Southern blotting and digestion with methylation-sensitive endonucleases, we demonstrate a specific hypomethylation of the internal cytosine of the 5′-CCGG-3′ sequence encompassing codons 360–361 of the decorin gene in human colon cancer. In contrast, three other 5′-CCGG-3′ sequences in the 5′ untranslated region of the decorin gene were fully methylated, as in normal tissue. The results also showed that these changes were present in colonic polyps but not in ulcerative colitis, thus suggesting that inflammation alone is not sufficient to induce alterations in 5-methylcytosine.

EXPERIMENTAL

Preparation of tissue and cells

Tissue specimens were obtained immediately after surgery from patients undergoing colon resection because of colon cancer or ulcerative colitis. Samples were immediately frozen in liquid N_2 and then transferred to a −75 °C freezer until analysed. A total number of four normal colon and four colon carcinoma samples (paired samples), two benign adenomatous polyps and two ulcerative colitis samples were studied. As a negative control we used colon carcinoma WiDr cells, which do not express decorin proteoglycan [16,17] and exhibit full methylation of the decorin gene on Southern blotting [8].

Quantitative PCR and restriction endoculease digestion

High-molecular-mass genomic DNA was purified twice from 250–500 mg of tissue or from two to four 150 cm^2 flasks of

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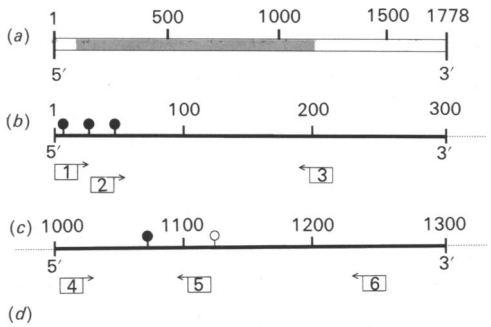

(d)

Primer number	Localization	Sequence
1	1–18	5′-GAATTCCGGTTACGTCTG-3′
2	28–45	5′-GGCAAATTCCCGGATTAA-3′
3	193–210c	5′-GTCATCAGGAACTTCTGG-3′
4	1006–1023	5′-TCAAGTGACTTCTGCCCA-3′
5	1105–1122c	5′-GACACATCTGAAGGTGGA-3′
6	1243–1260c	5′-CATTGCAGTTAGGTTTCC-3′

Fig. 1. Schematic representation of decorin cDNA, *Hpa*II and *Hha*I-sensitive sites and primers utilized for PCR amplification

(*a*) Schematic representation of the untranslated (empty bar) and translated (filled bar) regions of the decorin coding sequence [9]. Panels (*b*) and (*c*) show enlarged portions of the 5′ and 3′ regions of decorin gene and corresponding *Hpa*II (♦) and *Hha*I (♀) restriction sites, respectively. The 'boxed' numbers in (*b*) and (*c*) represent the locations and relative numbers of the various oligonucleotide primers used. The nucleotide sequence of each primer is listed in (*d*). The invert complementary sequence of primers is indicated by 'c'.

confluent cells as described before [18,19]. Primers were synthesized using a DNA synthesizer from Applied Biosystems Inc. The locations and sequences of the six 18 bp primers used in this study are shown in Fig. 1. A typical PCR amplification solution of 100 μl contained 50–450 ng of genomic DNA or 40 ng of decorin cDNA insert, 2.5 units of Taq polymerase (Perkin–Elmer Cetus) and 50–100 pmol of each primer [20]. The temperatures used for denaturation, annealing and primer extension were 95 °C (1 min), 52 °C (2 min) and 75 °C (3 min) respectively. Minor variations according to each primer set are described in the text and the Figure legends. The samples were subjected to 22 amplification cycles [21] using reagents and protocols from Perkin–Elmer Cetus in a TempCycler apparatus from Coy. About 10% of the original samples was analysed on 1.2–1.5% agarose gels with appropriate DNA size markers (BRL, Gaithersburg, MD, U.S.A.) as described [8]. The methylation status of the decorin gene was investigated by digesting 400–450 ng of DNA with the isoschizomeric restriction endonucleases *Hpa*II and *Msp*I, before PCR and trans-blotting. *Hpa*II cleaves 5′-CCGG-′3 sequences only if the internal C is unmethylated [22], whereas *Msp*I cleaves 5′-CCGG-3′ sequences regardless of internal C methylation [23]. Samples were also digested with *Hha*I, which cleaves 5′-GCGC-3′ sequences, but does not cleave if the internal C is methylated [24]. Following an 18 h digestion at 37 °C with an at least 3-fold excess of enzyme (60 units/μg of DNA), each sample was inactivated at 95 °C for 10 min before PCR [21].

Southern blotting and hybridization conditions

The gels of PCR-amplified untreated or endonuclease-digested genomic DNA were trans-blotted overnight on to nitrocellulose membranes [25] and hybridized under high stringency using a full-length decorin cDNA insert [9]. The insert was originally excised by *Eco*RI digestion, separated on a 1.2% agarose gel from the plasmid and purified using GeneClean (Bio 101, San Diego, CA, U.S.A.) according to the manufacturer's instructions. About 100 ng of insert was labelled with [^{32}P]dCTP (specific radioactivity $\sim 10^9$ c.p.m./μg of DNA) using the random primed method [26] and the PROBE-EZE labelling kit (3 Prime-5 Prime, West Chester, PA, U.S.A.). The membranes were prehybridized at 65 °C for at least 5 h in 5 × SSC (1 × SSC = 0.15 M-NaCl/15 mM-sodium citrate, pH 7.0), containing 1 × Denhardt's solution, 0.1% SDS and 100 μg of salmon sperm DNA/ml, and then hybridized to ^{32}P-labelled decorin cDNA probes for 18 h at 65 °C. The membranes were sequentially washed at 65 °C in 2 ×, 1 ×, 0.5 × and 0.2 × SSC for 30 min each, and subjected to autoradiography using intensifying screens at −75 °C. After various exposures, ranging from a few minutes to several hours, autoradiograms in the linear range were quantified by densitometry [19].

RESULTS

Strategy and requirements

Because our previous study has shown a direct correlation between enhanced mRNA levels, proteoglycan content and hypomethylation of the decorin gene in human colon cancer [8], we wanted to determine the exact location of hypomethylated sites and investigate whether such sites may be involved in the regulation of decorin expression. To address such questions, we used a recently developed PCR assay performed before or after digestion with methylation-sensitive restriction endonucleases. This highly sensitive PCR method [21] uses primers that bracket *Hpa*II or *Hha*I sites. If the DNA is treated with these enzymes before the PCR, amplified products are seen only from methylated genomic templates. Before the study was possible, however, a number of requirements needed to be fulfilled. First, in order for the PCR to be successful, we had to determine that no intronic sequences were present in the regions that we wished to amplify. Second, we had to demonstrate lack of amplification following isoschizomeric enzyme digestion to establish that the cleavage of that CpG sequence and the subsequent lack of PCR product were indeed due to the presence of 5-methylcytosine. Third, we needed to establish optimal conditions for the PCR reaction, including a low number of amplification cycles and higher stringency, and, fourth, we had to generate standard curves for each primer combination.

Lack of intronic sequences in the 5′ and 3′ regions of the decorin gene

The decorin coding sequence contains only four *Hpa*II/*Msp*I sites and one *Hha*I-sensitive site (Fig. 1). Three of these 5′-CCGG-3′ tetranucleotides recognized by *Hpa*II/*Msp*I are clustered in the 5′ untranslated region (Fig. 1*b*), whereas the other one is located near the 3′ end of the coding sequence (Fig. 1*c*). The only *Hha*I-sensitive site, a 5′-GCGC-3′ tetranucleotide, is located very close to the 3′ end of the gene, just distal to the *Hpa*II site (Fig. 1*c*). The asymmetrical location of these restriction endonuclease cleavage sites allowed us to design a series of 18 bp primers (Fig. 1*d*) that could bracket at least four of the five available sites. Using either primers 1–3 or 2–3, amplification of decorin cDNA as an internal control and of genomic DNA isolated from normal colon, colon carcinoma and a human polyp resulted in the expected size fragments of 210 and 183 bp respectively (Fig. 2). These amplified products were recognized by the labelled decorin cDNA under high stringency. Also, when using a combination of primers 4–5 and 4–6 (Fig. 3), the expected fragments of 117 and 255 bp respectively were obtained in all of

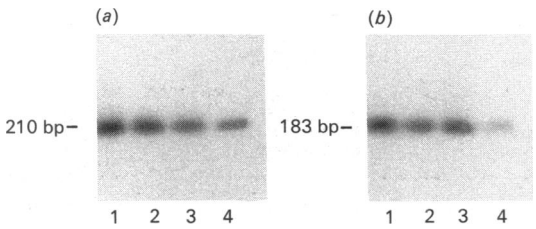

Fig. 2. Southern blotting of PCR-amplified products of the 5′ untranslated region of the decorin gene

Decorin cDNA insert (40 ng, lanes 1) or 400 ng of genomic DNA from human colon (lanes 2), colon carcinomas (lanes 3) or adenomatous polyp (lanes 4) was amplified using primers 1–3 (*a*) or 2–3 (*b*). Following a 10 min incubation at 95 °C, samples were amplified for 22 cycles. One cycle comprised a 1 min melting at 95 °C, a 2 min annealing at 52 °C and a 3 min extension at 75 °C. At the end of each amplification reaction, about one-fifth of the product was analysed on a 1.2% agarose gel, transblotted to nitrocellulose filters and hybridized to a ^{32}P-labelled decorin cDNA insert. Routinely, 10^6 c.p.m. of ^{32}P-labelled decorin cDNA insert/ml, labelled by random priming, was used to hybridize with the transblotting for between a few hours and overnight. Hybridization and washings were performed under high stringency as described in the experimental section. The numbers on the left indicate the sizes in bp of the amplified DNA fragments, as determined by running in parallel an appropriate DNA ladder (not shown). Exposure of the autoradiograms was for 20 min.

the samples analysed. These results indicate that there are no intronic sequences in either the 5′ or the 3′ regions of the decorin gene.

Specificity of PCR amplification

We next wanted to establish the specificity of the PCR amplification described above by predigesting the DNA samples with *Msp*I or *Hpa*II [27,28] before testing with the primers flanking the specific restriction sites. Predigested genomic DNA would serve as template only if the *Hpa*II sites are methylated and thus refractory to the enzyme [21,29]. Also, it should be pointed out that the PCR method has the required sensitivity, but that the amplified product does not retain the 5-methylcytosine pattern of the genomic template DNA [29]. The results showed the predicted fragment of 210 bp, not only in the untreated decorin cDNA and normal genomic DNA (Fig. 4, lanes 1 and 2, respectively), but also in the *Hpa*II-digested genomic DNA (Fig. 4, lanes 8–12). In contrast, no amplified signal could be detected after *Msp*I digestion (Fig. 4, lanes 3–7). These results provide strong evidence that the amplification of the 210 bp fragment with primers 1–3 is due to the presence of internal 5-methylcytosine in all three *Hpa*II-sensitive sites in the 5′ untranslated region of the decorin gene (see below). This is in fact the only possibility, given the specificity of these two enzymes [22,23].

Generation of standard curves for each amplified product

In initial experiments we found that differences in cytosine methylation could not be detected if the concentration of DNA templates was relatively high (> 1–2 μg) or if the number of amplification cycles was ~ 30, a number conventionally used for PCR amplification [20]. This *Hpa*II PCR assay is so sensitive that even a minute amount of undigested contaminating DNA would give a visible band by Southern blotting [21]. Furthermore, original differences in DNA concentrations would be obliterated by a few extra cycles of PCR, since the signal is proportional to the template DNA only if primers and nucleotides remain in great excess [29]. To minimize this problem we generated standard curves for each primer set using 22 cycles and increasing concentrations of DNA ranging from 50 to 450 ng, which are equivalent to approx 7×10^3 and 6×10^4 cells respectively [29]. The intensity of the amplified bands was linearly related to the DNA concentrations tested using either primers 4–5 (Figs. 5*a* and 5*b*) or primers 4–6 (Fig. 5*c* and 5*d*). Similar results were also

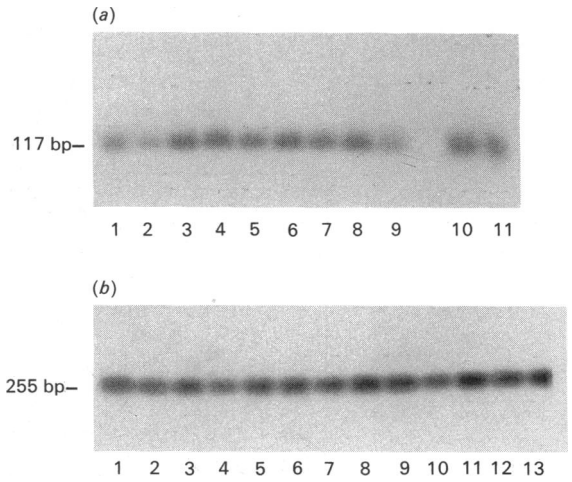

Fig. 3. Southern blotting of PCR-amplified products of the decorin 3′ end region

Decorin cDNA (40 ng) or 450 ng of genomic DNA from various sources was amplified by PCR using the conditions detailed in Fig. 2 and primers 4–5 (*a*) or 4–6 (*b*), transblotted to nitrocellulose filters and hybridized under high stringency to a ^{32}P-labelled decorin cDNA insert. (*a*) Lane 1, decorin cDNA; lanes 2–5, normal colon; lanes 6–9, colon carcinoma; lane 10, ulcerative colitis; lane 11, adenomatous polyp. (*b*) Lane 1, decorin cDNA; lanes 2–5, normal colon; lanes 6–9, colon carcinoma; lanes 10 and 11, ulcerative colitis; lanes 12 and 13, adenomatous polyp. The numbers on the left indicate the size in bp of the amplified DNA fragments, as determined by running appropriate DNA size markers (not shown). Exposure of the autoradiograms was for 30 min.

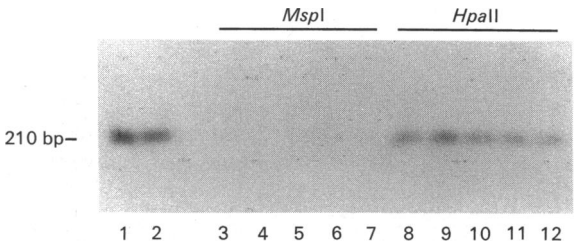

Fig. 4. Specificity of PCR amplification reaction

Aliquots (450 ng) of genomic DNA, which was digested overnight with either *Msp*I (lanes 3–7) or *Hpa*II (lanes 8–12), were amplified with the primers 1–3 as detailed in the legend to Fig. 2 and hybridized under high stringency to a ^{32}P-labelled decorin cDNA insert. Note that, following digestion with *Msp*I, no bands were detected in the DNA samples from normal and neoplastic colon tissues (lanes 3–5 and lanes 6 and 7, respectively); in contrast, bands of the predicted size are seen in the corresponding *Hpa*II-treated samples (lanes 8–12). Lanes 1 and 2 represent decorin cDNA and undigested normal colon DNA respectively. The number on the left represents the size in bp of the amplified DNA fragment, as determined by running in parallel an appropriate DNA ladder (not shown). Exposure of the autoradiogram was for 30 min.

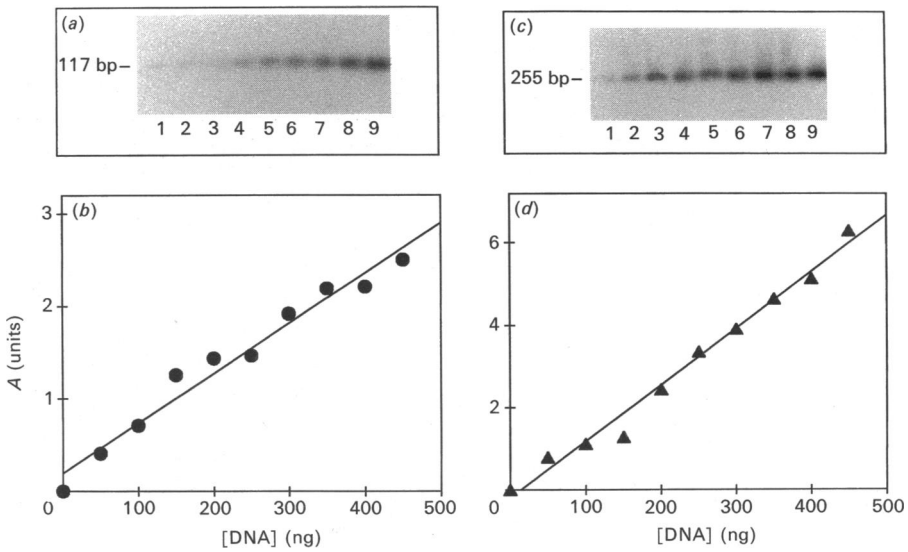

Fig. 5. Generation of standard curves for PCR-amplified products

Increasing amounts of normal genomic DNA (50–450 ng) were amplified using the protocol detailed in Fig. 2, transblotted to nitrocellulose and hybridized under high stringency to a ^{32}P-labelled decorin cDNA insert. Panels (*a*) and (*c*) represent autoradiographs of Southern blotting using primer sets 4–5 and 4–6 respectively. Exposure of the autoradiograms was for 20 min. Panels (*b*) and (*d*) represent the densitometric values from the corresponding Southern blotting shown in (*a*) and (*c*) respectively, which were obtained by computer integration of the scanned areas. The numbers on the left (*a* and *c*) represent the size in bp of the amplified DNA fragments, as determined by running in parallel appropriate DNA size markers (not shown).

obtained using primers 1–3 or 2–3 (results not shown). These standard curves allowed us to design precise amplification conditions for each set of primers and served as an additional control for the integrity of the DNAs tested.

The three *Hpa*II sites in the 5′ untranslated region of the decorin gene are fully methylated in normal and neoplastic tissue

Quantification of the degree of methylation in various tissues was achieved by comparing the intensity of the various bands after *Hpa*II digestion with that of untreated internal controls which were run in parallel. The results showed that the three *Hpa*II-sensitive sites clustered in the 5′ untranslated region of the decorin gene were fully methylated in all of the DNA templates tested. In particular, all of the DNA samples tested by Southern blotting and scanning densitometry showed a degree of methylation very close to the values (100 %) of untreated controls (results not shown). Similar results were also obtained using DNA from human colon carcinoma cells, which do not express decorin when cultured *in vitro* [16,17] and show a fully methylated pattern for the decorin gene on Southern blotting of genomic DNA [8].

The 3′ coding region of the decorin gene contains a hypomethylated *Hpa*II sequence in human colon neoplasia

We next wanted to determine whether the 3′ end of decorin gene, which contains only one *Hpa*II site encompassing codons 360–361 (bp 1076–1080, cf. Fig. 1), was hypomethylated in human colon neoplasia. Using primers 4–5 and *Hpa*II digestion, we found that amplification of the 117 bp predicted fragment was markedly inhibited by the enzyme only in DNA from colon carcinoma tissue and colonic polyp (Fig. 6*a*). Specifically, the degree of methylation of the cytosine in position 1077 of the decorin-coding region was 34 ± 5 % ($n = 4$) in colon carcinoma, 48 % ($n = 2$) in colonic polyp and 91 ± 4 % ($n = 4$) in normal colon tissue (Fig. 6*b*). This locus was fully methylated in DNA from ulcerative colitis (96 %; $n = 2$) (Fig. 6*b*).

Finally, we wanted to test whether the only *Hha*I site, located at codons 376–377 (bp 1128–1131, cf. Fig. 1), was also differentially affected in its degree of cytosine methylation. *Hha*I is a restriction endonuclease that cleaves 5′-GCGC-3′sequences only if the internal C is unmethylated [24]. Using primers 4–6 and *Hha*I digestion, we found a general hypomethylation (about 40 % of untreated controls) in all of the samples tested, without significant differences amongst the tissues analysed (results not shown).

DISCUSSION

There is a vast body of evidence indicating that there is an inverse relationship between methylation and gene expression and, in particular, that either spontaneous or experimentally induced demethylation of certain genes can lead to their abnormal expression [12–15]. The results of the present study provide direct evidence for hypomethylation of the decorin gene in human colon cancer and support our previous observations of a correlation between enhanced expression of decorin and hypomethylation of this gene in human colonic neoplasia [8]. This correlation, however, should not be generalized to other connective tissue genes, since we have not detected a linkage between hypomethylation and increased transcription of versican proteoglycan gene in human colon cancer [19]. In the present study, using an *Hpa*II/PCR method, we show that enhanced decorin gene expression correlates with specific hypomethylation of an *Hpa*II site in the 3′ coding region of the gene. In contrast, neither the methylation state of the three *Hpa*II sites in the 5′ untranslated region nor that of the *Hha*I recognition sequence in the 3′ end correlated with enhanced expression of the decorin gene. These results, however, do not exclude the possible effects of methylation of the 5′ flanking region on the expression of the decorin gene. In this context, it should be pointed out that our study has investigated only a limited subset of potential CpG methylation sites and that additional sites which do not form part of a 5′-CCGG-3′ or 5′-GCGC-3′ sequence may be equally

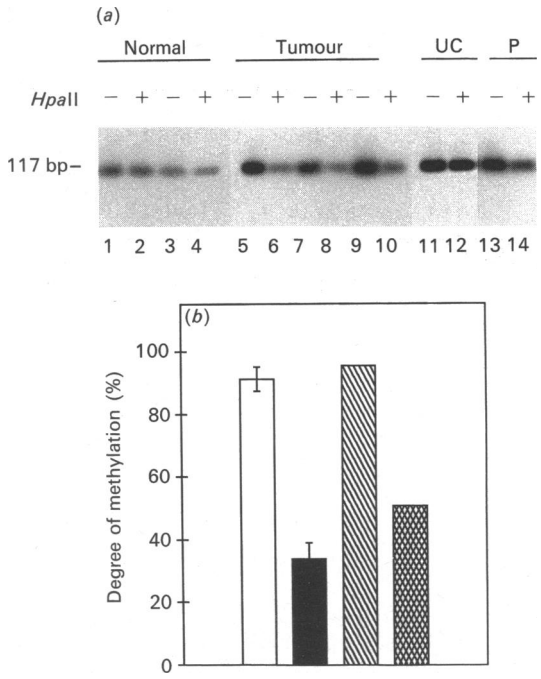

Fig. 6. Hypopmethylation of a *Hpa*II site in the 3′ end of the decorin gene in colon neoplasms

Purified samples from normal colon (normal), colon carcinoma (tumour), ulcerative colitis (UC) and polyp (P) were digested with *Hpa*II, and 400 ng portions were amplified by PCR using primer set 4–5. Panel (*a*) shows the autoradiogram of Southern blotting of PCR-amplified DNA following overnight incubation with (+) or without (−) the enzyme. The number on the left represents the size in bp of the amplified DNA fragments, as determined by running in parallel appropriate DNA size markers (not shown). Exposure of autoradiograms was for 30 min. Panel (*b*) shows quantification of the degree of methylation from four normal colon (□, mean ± S.D.), four colon carcinoma (■, mean ± S.D.), two ulcerative colitis (▨, mean), and two polyp (▩, mean) samples.

important. Ulcerative colitis, an inflammatory bowel disease with significant tissue remodelling, showed no changes in methylation of the *Hpa*II site at the 3′ end of decorin gene. This indicates that the changes reported here are not related to a non-specific process of repair, but that they are linked to the presence of a neoplastic clonal cell population.

The site-specific hypomethylation in the coding sequence of a connective tissue gene is relatively unusual. In fact, the vast majority of the changes in methylation described in other genes occur in CpG islands in the 5′ flanking regions which contain regulatory elements [12–15]. Indeed, a pattern of 5′ undermethylation and 3′ methylation is typical of housekeeping genes which are constitutively expressed in all tissues [12–15,30]. Interestingly, it has been reported that methylating various structural regions of the herpes thymidine kinase gene has a strong inhibitory effect on its expression [30]. In contrast, methylation of the 3′ end of the glucose-6-phosphate dehydrogenase gene correlates with augmented transcriptional activity [31]. More recently, prolactin gene expression has been shown to be enhanced when specific *Hpa*II sites in the fourth exon are hypomethylated [32,33], a finding that closely resembles the changes reported here for decorin methylation. As previously proposed [34], methylation of control regions could be the primary switching mechanism in gene control, whereas methylation of coding sequences could provide 'fine tuning'. These sequences could influence transcription without directly altering recognition sequences for RNA polymerase. This can be achieved, for instance, if methylated 3′ end sequences bind to a transacting factor which induces a conformational change in DNA that in turn hinders RNA polymerase interaction with the start site [33].

It is noteworthy that the sequences of both bovine decorin [35] and human biglycan [36] contain asymmetrically located *Hpa*II sites. Particularly similar is biglycan, which contains two *Hpa*II sites in the 5′ untranslated region and two in the 3′ end of the coding sequence [36]. Deduced amino acid sequences of decorin and biglycan strongly suggest that they are derived from a gene duplication, probably through a series of short gene tandem duplications that code for the leucine-rich repeats [36]. It would be interesting to determine whether increased biglycan mRNA levels also correlate with hypomethylation of these 3′ end *Hpa*II sites.

In conclusion, this study shows specific changes in the methylation status of the internal cytosine of a 5′-CCGG-3′ tetranucleotide located in the 3′ region of the coding sequence of decorin gene. Whereas DNA isolated from normal human colon or ulcerative colitis patients shows no evidence of hypomethylation, the DNA from human colon carcinoma and adenomatous polyp shows only 30–40% of the normal methylation levels for this specific sequence. That these changes are not due to a general hypomethylation of DNA in neoplastic tissue is clearly demonstrated by the presence of complete methylation in three analogous sequences located in the 5′ untranslated region. Lack of a change in decorin gene methylation in ulcerative colitis, an inflammatory bowel disease characterized by severe ulceration and tissue remodelling, points out the specificity of these tumour-associated DNA alterations and suggests that the inflammatory process alone is not sufficient to induce these changes in 5-methylcytosine.

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