CONCISE REPORT

DNA methylation is not likely to be responsible for aggrecan down regulation in aged or osteoarthritic cartilage

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Background: Expression of aggrecan is reduced during aging and osteoarthritic cartilage degeneration. CpG methylation may have a role in the down regulation of aggrecan transcriptions.

Objective: To investigate whether a correlation between gene methylation and expression of aggrecan in chondrocytes exists.

Methods: The human aggrecan promoter region was analysed computationally for CpG-rich regions. These were investigated for the methylation of C residues in normal (aged) and osteoarthritic chondrocytes by the bisulphite method for modifying DNA as well as sequence analysis using DNA directly extracted from normal and osteoarthritic cartilage tissue. Additionally, chondrocytic cell lines were investigated for methylation within the aggrecan promoter region.

Results: The CpG-rich promoter region of the human aggrecan gene contains a 0.6 kb region that meets the criteria of a CpG island as defined by prediction programmes. A significant correlation of aggrecan mRNA expression levels and methylation status in normal (aged) and osteoarthritic chondrocytes as well as in different chondrocytic cell lines was not found.

Conclusions: Expression of aggrecan in normal cartilage and diseased states is not modulated by gross changes of CpG methylation of its promoter region. CpG methylation does not have a central role in the switch off of aggrecan promoter activity in human adult articular chondrocytes.

Site-specific methylation within promoters is associated in many cases with the transcriptional silencing of specifically regulated genes. Aberrant methylation patterns are often found in neoplastic and aging cells, causing the modulation of transcription of linked loci.[1–3]

Aggrecan, a large chondroitin sulphate proteoglycan, is one essential structural component of the cartilage matrix. The down regulation of aggrecan mRNA expression during aging[4] and also associated with osteoarthritic joint disease[5] represents a characteristic and functionally important process paralleling cartilage degeneration. The promoter region of the human aggrecan gene is highly enriched in GC sequences. As methylation of genes increases with age in some genes[6] and osteoarthritis is an age related disease, we suggested that increased methylation of the aggrecan promoter might be associated with the reduction in expression levels both in aging and osteoarthritic chondrocytes. Therefore, we examined the human aggrecan promoter region for methylation of C residues in normal, aged, and osteoarthritic chondrocytes.

MATERIAL AND METHODS

Cell lines, tissue samples, and DNA isolation

Eleven normal (age 60–90 years) and six osteoarthritic (62–79 years) samples were ground under liquid nitrogen as described previously,[7] and genomic DNA was isolated using a DNeasy kit (Qiagen, Hilden, Germany). Human genomic DNA from lymphocytes (Promega, Madison) was used as control DNA. Positive control DNA with methylated CpG sequences was generated from human genomic DNA by treatment with *Sss*I methylase in vitro as described previously.[8] Different chondrocytic cell lines were used, either isolated from human chondrosarcomas (SM, AD, SG, 105Kc, Fscp-1)[9] or obtained after transfection of human chondrocytes with SV40 large Tantigen (C28I2, C28a4, C28a2, C20A4).[10] Treatment with the methylation inhibitor 5-aza-2′-deoxycytidine was performed according to Suzuki *et al*.[11]

RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated and levels of aggrecan mRNA were determined by real time PCR as described previously.[7]

Methylation analysis

The genomic DNA was treated with bisulphite,[6 12] and specific regions of the chemically modified genomic sequences were amplified by PCR as described (http://www3.mdanderson.org/leukemia/methylation/bisulfite.html (accessed 16 December 2004)). Primers for PCR amplification were selected and the fragments AP1 (position 57 to 271), AP2 (position 248 to 407), AP3 (position 735 to 951), and AP4 (position 384 to 725) were amplified by the primers AP1F-AP1R (GGGAATTTGAAGATTTAGAGTT, AAATTCTACAACTAAAAC TAACCA), AP2F-AP2R (TGGTTAGTTTTAGTTGTAGAATTT, CTAAAAAACCCGCATCTACATTAC), AP3F-AP3R (GGGGTT CGTAGAGTTGAGGA, CTACGCACCCCTCCTCCTA), AP4F-AP4R (GTAATGTAGATGCGGGTTTTTTAG, CTCAACTCTACG AACCCCCTC).

The amplification conditions were 0.8 pmol/µl primers, 2.5 U AmpliTaq Gold (Perkin Elmer), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and buffer according to the supplier. The reactions were carried out at 94℃ for 30 seconds, 35 cycles 94℃ for 30 seconds, 60–64℃ for 1 minute, 72℃ for 30 seconds, and one cycle 72℃ for 7 minutes.

The PCR products AP1, AP2, and AP3 were tested with diagnostic digestions using *Hinc*II, *Taq*I, and *Pvu*I (MBI Fermentas), and fragments were analysed on agarose gels. The AP4 amplification product was cloned into the TA cloning vector pGemTeasy (Promega), transformed into *E coli* DH5α, and individual clones were selected, analysed by restriction enzyme digestion and sequencing.

RESULTS

Analysis of the methylation pattern of the aggrecan promoter

The promoter region of the human aggrecan gene (−784, +485) contains a 0.6 kb region that meets the criterion of a CpG island as defined by prediction programmes (fig 1A).[13] To define the specific methylation of CpG sites (fig 1B), the bisulphite method for modifying DNA was used, converting

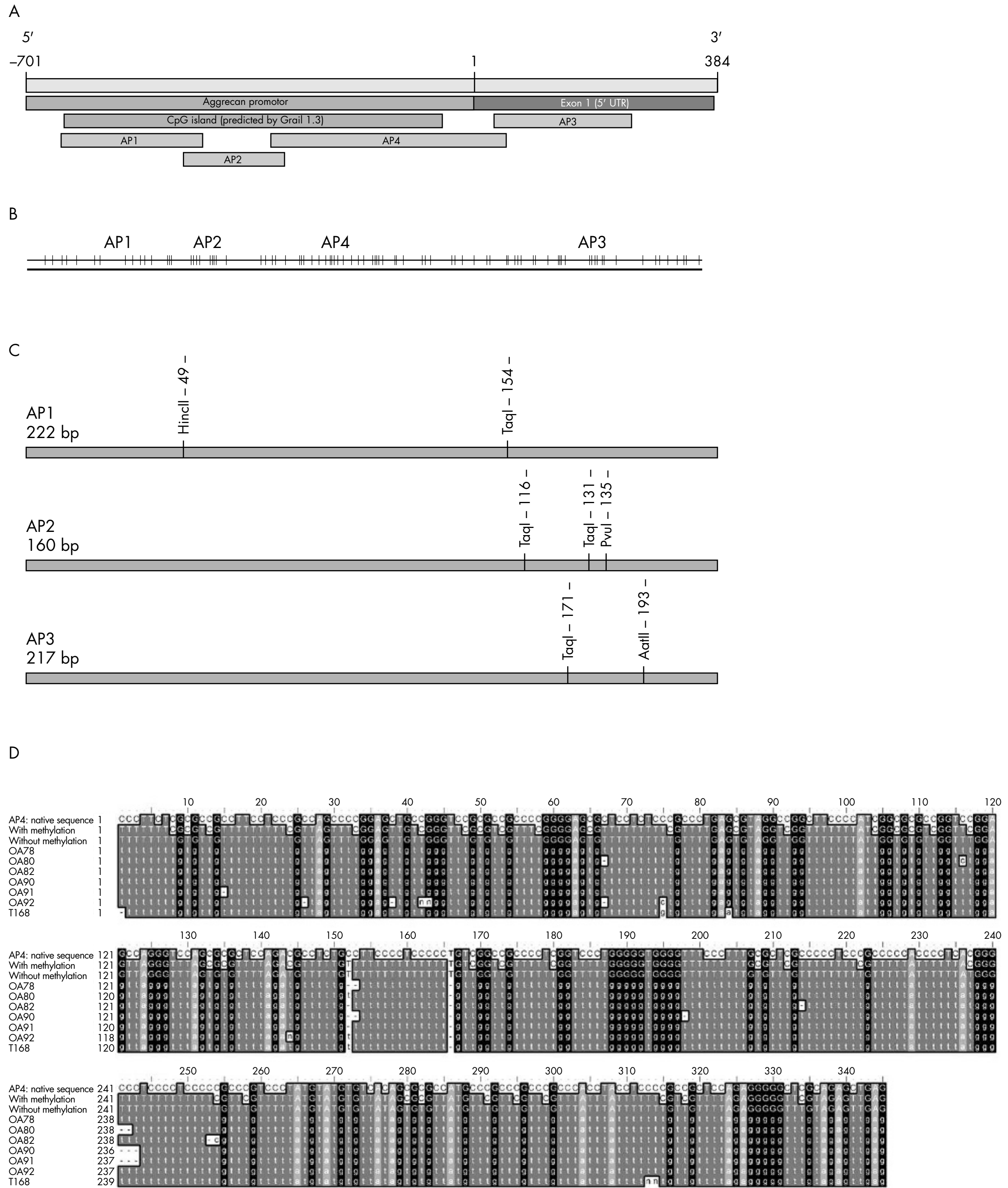

Figure 1 Genomic organisation of the human aggrecan promoter region. (A) Promoter region and exon 1 (GB: AF031586) include a predicted CpG island (Grail 1.3, CpG Island Searcher). The regions AP1-AP4 were analysed for their methylation status by PCR amplification after bisulphite treatment of genomic DNA. (B) Distribution of CpG sites (vertical lines) in the promoter region. (C) Map of new restriction sites within the fragments AP1-AP3, generated by bisulphite treatment of CpG methylated sites. (D) Sequence analyses of cloned amplification products of AP4 after bisulphite treatment of genomic DNA. The unconverted sequence of the region AP4 (lane 1), the converted sequences after bisulphite treatment assuming complete CpG methylation (lane 2) or without methylation (lane 3) are compared with the sequences of six OA samples (lanes 4–9) as well as one normal case (lane 10) after bisulphite treatment, amplification, and cloning. The OA samples showed no indication of CpG methylation, as all cytosines were converted to thymines as seen with the non-methylated sequences (lane 3).

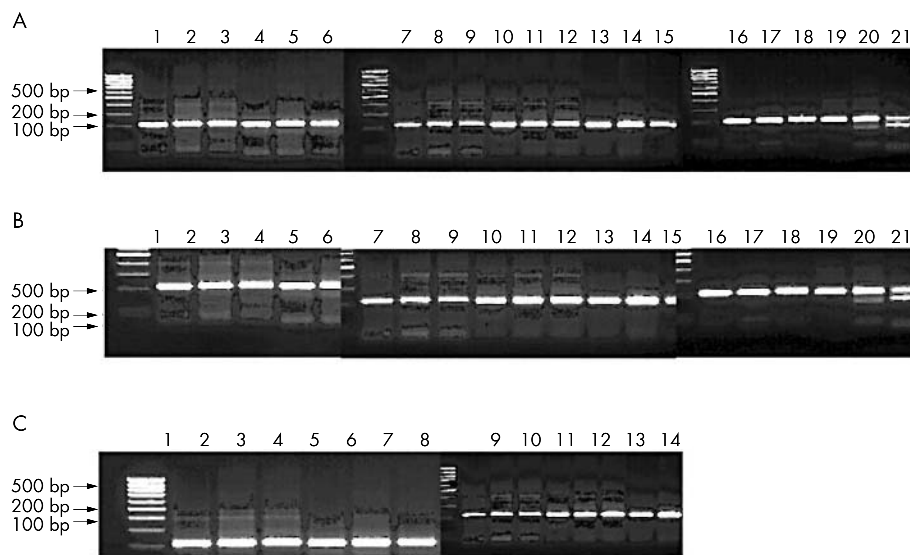

Figure 2 Detection of new restriction sites in the regions AP1-AP3 after bisulphite treatment. The fragments AP1-AP3 were amplified after bisulphite treatment and potential new restriction sites were detected by restriction digestions with the indicated enzymes (see fig 1B). (A) Region AP1 from three normal samples (1–3, 4–6, 7–9), three osteoarthritic (OA) samples (10–12, 13–15, 16–18), and control DNA treated with *Sss*I methylase (19–21). Fragments were either untreated (1, 4, 7, 10, 13, 16, 19), restricted with *Hinc*II (2, 5, 8, 11, 14, 17, 20), or restricted with *Taq*I (3, 6, 9, 12, 15, 18, 21). The appearance of new restriction sites is only apparent in *Sss*I treated control DNA. (B) Region AP2 was analysed as in (A) but restrictions were done with *Taq*I and *Pvu*I, respectively. (C) Region AP3 was analysed from three OA samples (1–2, 3–4, 5–6), three normal samples (7–8, 9–10, 11–12), and a positive control (13–14).

cytosine to uracil, whereas 5-methylcytosine remains non-reactive.[6 12] Genomic DNA was extracted from eight normal (aged) samples and 12 samples of osteoarthritic, articular human cartilage. After treatment with bisulphite, the four regions AP1-AP4 corresponding to the aggrecan promoter (figs 1A and B) were amplified by PCR. The presence of methylated, therefore unaltered, cytosines, in AP1-AP3 could be detected after bisulphite conversion by diagnostic restriction digestions with *Hinc*II, *Taq*I *Pvu*I, and *Aat*II (fig 1C). In no case were these new restriction fragments detected (figs 2A–C), indicating that no significant levels of CpG methylation are found at these specific sites in OA samples.

To define the methylation status of all cytosine residues, the amplification products of the promoter proximal fragment AP4 were cloned and used for sequencing (fig 1D). From the comparison of the genomic sequence and the sequences derived from bisulphite treated AP4 fragments, no evidence was seen for significant changes of the methylation status of this major part of the CpG island of the aggrecan promoter in normal or osteoarthritic DNA chondrocytes (fig 1D).

Analysis of chondrocytic cell lines

Chondrocytic cell lines derived from chondrosarcomas or from non-neoplastic chondrocytes after SV40 transformation were analysed for the expression of aggrecan by quantitative RT-PCR and shown to express only minimal amounts of aggrecan-specific mRNA (data not shown). Therefore, the DNA from these chondrocyte cell lines was examined for evidence of modified methylation patterns of the aggrecan promoter. Indeed, no or only partial methylation was detected in some of the cell lines in the aggrecan promoter as seen by the generation of 0–3 new sites at the eight positions investigated (fig 1B) tested (data not shown). Treatment of these cell lines with the methylation inhibitor 5-aza-2′-deoxycytidine[11] also did not induce a significant increase in transcript levels of aggrecan (data not shown).

DISCUSSION

The CpG-rich promoter region of the human aggrecan gene (−784, +485) contains a CpG island. However, no evidence was seen for significant changes of the methylation status of the CpG island of the aggrecan promoter in normal aged or osteoarthritic DNA chondrocytes. Thus, the decrease in aggrecan expression of normal aging or osteoarthritic articular human cartilage does not correlate with increased methylation of the CpG island in the aggrecan promoter. Although a potential contribution of individual CpG sequences at distinct sites cannot be excluded, distinct mechanisms are most likely responsible for the down regulation of aggrecan expression, like the response to cytokines or modulation of transcription factor activity. This is further supported by the fact, that some chondrocytes in osteoarthritic cartilage even show increased aggrecan mRNA expression.[5]

Previous observations indicated that cell lines often have significant differences in their methylation patterns compared with corresponding tissues.[2] Chondrocytic cell lines, either derived from SV40 transfected chondrocytes or isolated from chondrosarcomas[9 10] showed no or only partial methylation in some of the cell lines in the aggrecan promoter. Whether this is due to methylation or to the selection of mutations during the continuous culture[14] remains unclear. Treatment of these cell lines with the methylation inhibitor 5-aza-2′-deoxycytidine[11] also did not induce a significant increase in transcript levels of aggrecan. Together, these findings further support the conclusion from normal and osteoarthritic chondrocytes, that CpG methylation does not have a central role in the switch off of aggrecan promoter activity in human articular chondrocytes.

Genomic methylation appears to be an important factor for tissue and cell-specific differentiation during development, including chondroneogenesis.[15] Thus, the methylation status of a gene appears to be important for its general activity in a defined tissue of cell type such as chondrocytes,[15 16] but not for the regulation of a gene in a given tissue or cellular context.

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