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

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CONCISE REPORT

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

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.

METHODS

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 SsoI methylase in vitro as described previously.9 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 T antigen (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,4,12 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 (GGGAATTTGAAGATTTAGGTT, AAATTCTACAATTAAAAAC TAAACCA), AP2F-AP2R (TGTTATGTTTATTTGATGTAATTT, CTAAAAAAACCTAGCTACATTAC), AP3F-AP3R (GGGATGT TAGATCCAGGTTA, CTACCGACCCCTCCCTCCA), AP4F AP4R (GTTATGAGTGGCGTTTTTTAG, CTGCACTCTACG AACCCCC). The amplification conditions were 0.8 pmol/μl primers, 2.5 U AmpliTaq Gold (Perkin Elmer), 2.5 mM MgCl₂, 0.2 mM dNTPs, and buffer according to the supplier. The reactions were carried out at 94°C for 30 seconds, 35 cycles 94°C for 30 seconds, 60–64°C for 1 minute, 72°C for 30 seconds, and one cycle 72°C for 7 minutes.

The PCR products AP1, AP2, and AP3 were tested with diagnostic digestions using HincII, TaqI, and PvuII (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

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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).
Methylation of the aggrecan gene

increase in transcript levels of aggrecan (data not shown). Together, these findings further support the conclusion from normal and osteoarthritic cartilage, 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 chondroeneogenesis. 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,” but not for the regulation of a gene in a given tissue or cellular context.

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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. Whether this is due to methylation or to the selection of mutations during the continuous culture remains unclear. Treatment of these cell lines with the methylation inhibitor 5-aza-2'-deoxycytidine 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 accumulation 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.1

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 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 remains unclear. Treatment of these cell lines with the methylation inhibitor 5-aza-2'-deoxycytidine also did not induce a significant increase in transcript levels of aggrecan. Together, these findings further support the conclusion from normal and osteoarthritic cartilage, that CpG methylation does not have a central role in the switch off of aggrecan promoter activity in human articular chondrocytes.

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