

Published in final edited form as:

Arthritis Rheum. 2009 November ; 60(11): 3303–3313. doi:10.1002/art.24882.

DNA De-methylation at Specific CpG Sites in the *IL1B* Promoter in Response to Inflammatory Cytokines in Human Articular Chondrocytes

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Abstract

Objective—To determine whether changes in the DNA methylation status in the promoter region of the gene encoding interleukin-1 β (IL-1 β) account for expression of *IL1B* mRNA after long-term treatment of human articular chondrocytes with inflammatory cytokines.

Methods—IL-1 β or TNF α combined with oncostatin M (OSM), or 5-aza-deoxycytidine (5-aza-dC) were added twice weekly for 4–5 weeks to primary cultures of normal human articular chondrocytes, obtained from patients with a femoral neck fracture. Expression of *MMP13*, *IL1B*, *TNFA* and *DNMT1* were determined by SybrGreen-based qRT-PCR on genomic DNA and total RNA extracted from the same sample before and after culture. Bisulfite modification was used to identify which CpG sites in the *IL1B* promoter showed differential methylation between expressing and non-expressing cells. The percentages of cells that were methylated at that critical CpG site (–299bp) were quantified by a method that depended on methylation-sensitive restriction enzymes and real-time PCR. Secretion of IL-1 β was assessed by enzyme-linked immunosorbent assay (ELISA) of the culture media.

Results—Healthy chondrocytes did not express *IL1B* mRNA, but the levels were increased 5-fold by 5-aza-dC and 100- to 1000-fold by or TNF α /OSM. The % CpG methylation was decreased by 5-aza-dC, but reduced considerably more by IL-1 β and almost abolished by TNF α /OSM. The mRNA was translated into protein in cytokine-treated chondrocytes.

Conclusion—These novel findings indicate that inflammatory cytokines can change DNA methylation status at key CpG sites, resulting in long-term induction of *IL1B* in human articular chondrocytes.

INTRODUCTION

Idiopathic osteoarthritis (OA) is a late-onset, complex disease of the joint. It is characterized by progressive failure of the extracellular cartilage matrix and changes in the synovium and subchondral bone (1,2). Susceptibility genes for OA have been indentified (3), which represents potential risks, whereas getting the disease is due to interaction with the environment. This interaction may be mediated by epigenetically induced changes in gene expression that are then transmitted to generations of daughter cells (4). As in all adult

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somatic cells, the phenotype of normal adult chondrocytes is stabilized by epigenetic mechanisms, such as DNA methylation of CpG (Cytosine-P-Guanine) sites, modifications of histone tails and changes in chromatin structure. DNA methylation is generally stable in somatic cells throughout adult life (5,6), whereas histone modifications are readily reversible by specific enzymes (7). During DNA replication, the methylation pattern is rapidly reproduced on the nascent strand by DNMT1, the maintenance DNA methyl transferase. (8,9). The histone code can be re-established after cell division by interactions of methyl binding domains and DNMTs with histone methyltransferases and histone deacetylases (10,11). As a result, both the DNA methylation pattern and the histone code are reproduced during mitosis.

In normal adult articular cartilage, chondrocytes synthesize cartilage matrix proteins, such as collagens II, IX, and XI as well as aggrecan. These molecules impart tensile strength and compressive resistance to the joint. Normally, chondrocytes maintain a low turnover of cartilage matrix proteins, but in OA many chondrocytes undergo a phenotypic change and acquire a gene expression repertoire that is characterized by the aberrant expression of numerous catabolic genes, including matrix metalloproteases (MMPs), aggrecanases (ADAMTS-4 and -5), inducible nitric oxide synthetase (iNOS) and prostaglandins (12), IL1B and other cytokines (13,14), and many more genes (15,16). This change in gene expression does not take place in all OA chondrocytes, but predominantly in chondrocytes of the surface zone and near weight-bearing regions (17). To distinguish these chondrocytes from OA chondrocytes with a normal gene expression repertoire, they will be referred to as 'degradative' chondrocytes. Disease progression correlates with an increase in the number of 'degradative' chondrocytes.

Gene expression is regulated by both epigenetic and non-epigenetic mechanisms. Genes that are part of the repertoire of a particular cell type show DNA hypo-methylation and chromatin has an open structure. This permits binding of specific transcription factors in combination with cofactors, e.g. histone acetylases and methylases, that mediate rapid responses (minutes to hours) to inductive or repressive factors. Genes that are not part of a given repertoire tend to be permanently silenced by DNA hypermethylation; this prevents access of the relevant transcription factors to their promoter. Epigenetic silencing is essential to insure genomic stability throughout life. Epigenetic disruption may activate normally silent genes (18) or silence normally expressed genes. This is precisely the situation in degradative OA chondrocytes, where many non-chondrocytic genes are permanently activated. So far there only a few studies have dealt with the question whether the aberrant gene expression in OA is linked to loss of DNA methylation. Our group (19)(20) has demonstrated hypo-methylation at specific CpG sites in the promoters of MMP-3, MMP-9, MMP-13 and ADAMTS-4 in 'degradative' OA chondrocytes. Iliopoulos et al. (21) have showed that the aberrant induction of leptin in OA is associated with loss of DNA methylation. On the other hand, loss of OP-1 expression in aged chondrocytes is correlated with hypermethylation of the OP-1 promoter (22).

Because of the association of aberrant expression of proteases with DNA demethylation, it is of interest to identify factors causing loss of DNA methylation. The aberrant expression of non-chondrocytic genes that occurs in OA can arguably be reproduced *in vitro* by treating healthy chondrocytes with either IL-1 β (23) or TNF α , especially in combination with OSM (24). However, it is not known whether this aberrant induction depends solely on non-epigenetic regulation by transcription factors or whether epigenetic changes are also involved. In the first case, one would expect expression to be induced within hours and be readily reversible by cytokine withdrawal. In the second case, induction might take days or weeks, but expression would probably persist after cytokine withdrawal. We hypothesized that the latter situation is linked to de-methylation of specific CpG sites in the relevant

promoter regions, whereas this is not the case for short-term induction. To test our hypothesis, mRNA expression of *IL1B*, an aberrantly induced gene, was compared with DNA methylation status in primary human chondrocytes cultured in the absence and presence of inflammatory cytokines.

MATERIALS AND METHODS

Chondrocyte isolation

Human articular cartilage was dissected from femoral heads obtained within six hours of operation with ethical permission and patients' consent. Cartilage was obtained after hemiarthroplasty following a fracture (#) of the Neck of Femur (#NOF) or after total hip arthroplasty for OA. A previous study (25) had shown that the deep zones of #NOF patients contain healthy, albeit aged chondrocytes, which did not aberrantly express proteases and cytokines. However, the superficial zone did contain a few cells with aberrant expression. This zone typically displayed a pinkish colouring so that it was relatively easy to separate this zone with a scalpel from the deep zone. In OA patients, a significant amount of cartilage had already been degraded, especially at weight-bearing regions. To obtain enough cartilage, we collected the "surface zone" irrespective of whether this was superficial, intermediate or deep zone. To obtain proper deep-zone OA cartilage was only possible if thick cartilage had remained at non-weight bearing regions, which was not always the case. To liberate the cells, cartilage pieces were cut into small fragments and digested with 10% trypsin (LONZA, Wokingham, UK) in PBS for 30 minutes, 1mg/ml hyaluronidase (Sigma-Aldrich, Gillingham, UK) in PBS for 15 minutes and 10 mg/ml collagenase B (Roche, Lewes, UK) in α -Modified Eagle Medium (α -MEM) (Sigma-Aldrich, Gillingham, UK) for 12 to 15 hours at 37°C. In total, 21 #NOF patients and 12 OA patients were used in this study.

Chondrocyte culture

For culture only non-OA chondrocytes from the deep zone of #NOF patients (25) were used. Before treatment, chondrocytes were cultured for 48 hours at a density of 2 to 4 $\times 10^5$ cells/25 cm² flask in 5ml α -MEM supplemented with 10% fetal calf serum (FCS; Invitrogen, Paisley, UK), 1% ITS (Sigma-Aldrich, Gillingham, UK), 100 U/ml penicillin and 100 μ g/ml streptomycin (LONZA, Wokingham, UK) and 100 μ g/ml ascorbic acid (Sigma-Aldrich, Gillingham, UK) in an atmosphere of 5% CO₂ at 37°C. In the first experiment, chondrocytes were cultured until confluence, passaged once, incubated with a single addition of 10 ng/ml IL-1 β /10ng/ml OSM, and harvested after 24 or 72 hours. Other P1 cultures were treated with cytokines at each medium change for three weeks. Half of the cultures were then harvested by trypsinization, whereas the other half was passaged again and cultured without cytokine treatment until confluence at around 2 weeks.

In another experiment, chondrocytes were divided into five groups immediately after isolation: i) non-cultured; ii) cultured without treatment (control culture); iii) cultured with 2 μ M 5-aza-deoxycytidine (5-aza-dC); iv) cultured with 10 ng/ml IL-1 β and v) cultured with a mixture of 10 ng/ml TNF α /10ng/ml OSM. For group iii), the histone deacetylase inhibitor Trichostatin A (TSA, 300nM) was added just once at the first treatment to facilitate access of 5-aza-dC, a cytidine analogue that inhibits the activity of DNMT1 (26). This results in non-specific loss of DNA methylation during cell division. Media were changed twice per week, when reagents were also added. These primary cultures were maintained for 4 to 5 weeks until confluence.

DNA and RNA extraction

Genomic DNA and total RNA were extracted simultaneously from the harvested chondrocytes by AllPrep DNA/RNA Mini Kit (Qiagen, West Sussex, UK) according to the

manufacturer's instructions. RNA was immediately reverse-transcribed with AMV reverse transcriptase and both oligo(dT)₁₅ and random primers (27).

Quantitative polymerase chain reaction (qRT-PCR)

Relative quantification of gene expression was performed with Applied Biosystems (Warrington, UK) ABI Prism 7500 detection system. Reactions were performed in triplicate with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Primer Express 3.0 software was used to design primers across exon-exon boundaries. The primers for *IL1B* were commercially designed by PrimerDesign Ltd (Southampton, UK) and the primers for *COL2A1* were obtained from (28). mRNA expression was quantified by the $2^{-\Delta\Delta C_t}$ method. The 25 μ l reaction mixture contained 1 μ l cDNA, 12.5 μ l 2x Power SYBR® Green PCR Master Mix and 500 μ M of each primer. Thermocycler conditions comprised an initial activation step at 95°C (10 min), followed by a 2-step PCR program of 95°C (15s) and 60°C (60s) for 40 cycles. A dissociation curve was obtained for each qPCR run.

Bisulfite Modification

Genomic DNA was modified with MethylDetector™ (Active Motif, Rixensart, Belgium), according to the manufacturer's instructions. The MethPrimer website (<http://www.urogene.org/methprimer>) was used to design primers which contained no CpG sites. 21 CpG sites located between -1300 and +15bp were investigated by three pairs of nested PCR primers (Figure 1). The sequences of all primers can be found at http://www.som.soton.ac.uk/research/dohad/groups/bone_joint/Who/Roach/default.htm

Thermocycler conditions comprised an activation step at 94°C (2 min), and followed by a 3-step PCR program consisting of 94°C (30s), 55°C (60s), 72°C (60s) for 35 cycles, and final extension at 72°C (3 min) as. The PCR products were diluted 50x and inner reactions were run. The PCR products were cloned with the TOPO® TA Cloning® Kit with One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, Paisley, UK), followed by purification of the plasmids by PureLink™ Quick Plasmid MiniPrep Kit (Invitrogen, Paisley, UK). Six plasmids were sequenced for each sample by Eurofins MWG (Ebersberg, Germany).

Determining the % methylation at -299 bp in the *IL1B* promoter

This CpG site was selected for quantification because it was differentially methylated in control versus cytokine-treated cultured cells (see results, Figure 1) and because it is recognized by a methylation-sensitive restriction enzyme, namely *HpyCH4IV* (New England BioLabs), Fully methylated DNA and fully non-methylated DNA were used i) to estimate non-specific digestion and incomplete digestion respectively, and ii) to generate a standard curve. CpGenome-Universal Methylated DNA was obtained from Millipore (Eastleigh, UK), while fully non-methylated DNA was generated from the fully methylated DNA by whole genome amplification with the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences). DNA concentrations were measured using Nanodrop (Thermo Scientific, UK) and were adjusted to 4ng/ μ l (20ng DNA in 5 μ l). *HpyCH4IV* was diluted to contain 2 Units/2.5 μ l enzyme solution. These concentrations had previously been determined as optimal (27). Each digestion sample consisted of a final volume of 8.3 μ l, containing 5 μ l of diluted DNA, 2.5 μ l diluted *HpyCH4IV*, and 0.8 μ l of 10x NE Buffer I. All prepared samples, together with controls, were incubated at 37°C for 12 hours, followed by heat inactivation at 65°C for 20 minutes.

PCR for % methylation analysis was performed as for RT-PCR. Each run included no-enzyme controls, fully methylated and non-methylated DNA controls, the standard curve and the enzyme-treated DNA samples. The DNA content of each enzyme-digested sample

was normalized to its corresponding no-enzyme control and the % methylation was calculated from the standard curve (27).

When #NOF samples were compared with OA samples for mRNA expression or % DNA methylation, all samples were analyzed in the same assay.

ELISA for IL-1 β in culture media

At the end of culture, the media containing the cytokines were removed and, after thorough washings to remove any remaining exogenous IL-1 β , 5ml fresh medium with 1% FCS was added. After 48 hours, the media were harvested and stored at -80°C until analysis with the Human IL-1 beta/IL-1F2 Quantikine® ELISA Kit (R&D Systems, Abingdon, UK).

Statistical analysis

The data for IL-1 β expression and % methylation were analyzed with Microsoft Excel® by the Wilcoxon's assigned-rank test and a significance level of 0.05. Other data were calculated as the mean \pm standard deviation.

RESULTS

Identifying differentially methylated CpG sites

Of the 21 CpG sites in the 1300bp sequence upstream of exon 1 (Figure 1), 16 were methylated in all cultures. This suggests that epigenetic regulation does not involve the region between -1300 to -590 bp. The *Ava*I site at -511 bp and the two CpG sites (-20 and $+13$) that encompass the transcription start site were essentially non-methylated in all groups. On the other hand, the two CpG sites at -299 and -256 bp were methylated in control samples, but had become demethylated in cytokine-treated chondrocytes. Because only the site at -299 bp was cleavable by a methylation-sensitive restriction enzyme, this site was selected for quantitative analysis in subsequent experiments.

IL1B, but not TNFA, is differentially expressed in control and OA cartilage

To investigate the zonal distribution of *IL1B*-expressing chondrocytes *in vivo*, mRNA expression was assessed separately for the superficial and deep zones of OA and control cartilage. Expression was 10- to 1000-fold higher in the surface zone of OA cartilage compared with #NOF cartilage (Figure 2A). The variability among patients bore some relation to age in that, with one exception, expression increased markedly with age. In contrast to the superficial zone, no expression of *IL1B* was apparent in the deep zone of OA cartilage. In the #NOF samples, very low expression was found in the surface, but not in the deep zone. These findings confirm that the deep zone of #NOF cartilage contains chondrocytes that do not express *IL1B* and are therefore suitable for culture experiments. A previous study of ADAMTS-4 expression showed similar differential expression (20). In contrast to *IL1B*, *TNFA* expression was not significantly different in the surface and deep zone (Figure 2B); this supports the notion that IL-1 β , but not TNF α , is the major cytokine involved in OA pathology.

Only long-term cytokine treatment results in persistent aberrant expression

A single treatment with IL-1 β /OSM induced the aberrant expression of *MMP3* (not shown), *MMP13* (Fig. 3A) and *IL1B* (Fig. 3B) within 24 hours (Figure 3A). By 72 hours enzyme expression had declined or disappeared. By contrast, when expression was induced by repeated cytokine addition during 3 weeks of incubation, the expression persisted after 2 more weeks without further cytokines treatment (Figure 3D,E). *COL2A1* expression was reduced after a single cytokine addition and remained low throughout (Fig. 2C,F)

Experimental de-methylation increases expression of *IL1B*

If DNA de-methylation underpins aberrant *IL1B* expression in chondrocytes, then experimentally-induced de-methylation should result in increased expression. When normal chondrocytes were cultured with 5-aza-dC, the expression of *IL1B* increased 3.6- to 8.6-fold (mean = 5.5 ± 2.2) compared with control cultures (Figure 4A). To check that 5-aza-dC treatment had actually resulted in loss of DNA methylation in the *IL1B* promoter, the % of methylation was quantified. As shown in Figure 4B, 61.0 ± 6.1 % of chondrocytes were methylated in uncultured chondrocytes. In cultured samples, this was reduced to 44.8 ± 9.9 %, indicating culture *per se* could cause limited loss of DNA methylation. However, 5-azadC further reduced the % DNA to 33.6 ± 5.7 %. The results show that experimentally induced loss of DNA methylation results in increased gene transcription, thus confirming a cause-effect relationship between DNA de-methylation and transcription.

Long-term exposure to cytokines increases expression of *IL1B* 100 to 1000-fold

Healthy chondrocytes were cultured for 4 to 5 weeks with twice-weekly additions of either IL-1 β or TNF- α /OSM (Figure 4C). Absence of expression in non-cultured chondrocytes was confirmed, and the low expression induced by culture alone was set to 1. IL-1 β increased its own expression 15.8- to 197-fold, while the combination of TNF- α /OSM increased expression of *IL1B* by 306- to 1750-fold compared with control cultures ($P < 0.01$). This showed that the capacity of the cytokines to increase expression was several orders of magnitude greater than that of 5-aza-dC.

Long-term exposure to cytokines causes loss of DNA methylation

Quantitative analysis showed that culture alone reduced DNA methylation in two cases (patients Nr 406 and 421), but this decrease was not significant in the other patients. By contrast, the effects of the cytokines were very prominent. Only 4 to 25% (mean = 17.9 ± 6.9 %) of IL-1 β -treated chondrocytes were still methylated at -299 bp and with the combined TNF α /OSM treatment only 0–8 % (average 4.5 ± 3.7 %) of cells remaining methylated. Although there is not a direct linear correlation between the fold increases in gene expression and the loss of DNA methylation, the trends are very clear: those samples with the greatest loss of DNA methylation showed, on the whole, the greatest induction of gene transcription.

Increased *IL1B* is associated with protein release into the medium

Significant amounts of IL-1 β protein were produced by chondrocytes that had been cultured with either IL-1 β or TNF α /OSM, but no IL-1 β was detected in the control cultures or those treated with 5-aza-dC. This indicates that the small increases in transcription by 5-aza-dC did not produce measurable amounts of IL-1 β protein. Treatment with exogenous IL-1 β resulted in the release of 0.011 to 0.225 pg IL-1 β / μ g DNA/hr, while TNF α /OSM treatment caused release of 0.066 to 0.169 pg IL-1 β / μ g DNA/hr. These findings indicate that the increased expression of *IL1B*, a likely result of transcriptional promoter activation, resulted in increased synthesis of IL-1 β protein.

Cytokine treatment does not increase *TNFA* expression in chondrocytes

Because IL-1 β induced its own expression *in vitro*, we explored whether the same was true for TNF α . Non-cultured chondrocytes expressed *TNFA* (Figure 2B) and culture had no further effect. Expression of *TNFA* in chondrocyte cultures treated with IL-1 β or TNF α /OSM was not significantly different from that of cultured controls, with mean increases in expression of 0.59 ± 0.44 and 0.91 ± 0.49 fold, respectively. Thus, in contrast to *IL1B*, inflammatory cytokines did not increase *TNFA* expression *in vitro*.

Culture and cytokine treatment decreases *DNMT1* expression

To determine whether decreased expression of *DNMT1* contributed to the observed loss of DNA methylation, we investigated expression by qRT-PCR. Indeed, *DNMT1* expression in surface-zone OA chondrocytes was half that in the #NOF control chondrocytes (Figure 5A). Culture alone also reduced *DNMT1* expression to around half of that in non-cultured chondrocytes, which may, in part, explain the loss of DNA methylation in control cultures. IL-1 β or TNF α /OSM addition caused only a small further decrease (Figure 5B).

DISCUSSION

The novel findings of the present study are that inflammatory cytokines have the capacity not only to induce changes in gene expression, but also de-methylation of specific CpG sites in the proximal *IL1B* promoter. IL-1 β is at the apex of several inflammatory cascades and is arguably the most important cytokine in OA, although the debate is ongoing. Because IL-1 β has a very short half-life and mRNA transcript levels correspond to protein levels, it is important to understand transcriptional regulation of this protein. Non-epigenetic regulation of the *IL1B* promoter has been studied extensively in monocytes/macrophages. In these cells, *IL1B* is readily induced by a variety of stimuli, including IL-1 β itself (29,30). The CpG region between -131 and +12 bp is sufficient to direct expression of a reporter gene (31), but an inducible enhancer, located between -3134 and -2729 bp (31,32), is also involved in signal-dependent expression. In monocytes, this promoter is packaged into a non-transcribed, but “poised” promoter structure that is characterized by a histone-free transcription start site and constitutive association with the transcription factors PU.1 and C/EBP β (30). Monocytes are thus primed for rapid activation in response to pathological or other stimuli.

Articular chondrocytes do not normally express *IL1B*, as confirmed here by qPCR. The low level of expression observed in the superficial zone of #NOF patients probably indicates an age-related change to ‘degradative’ chondrocytes in a few cells. By contrast, the chondrocytes located near the surface of OA cartilage showed very high levels of *IL1B* expression, whereas this was undetectable in deep-zone OA cartilage. We hypothesized that *IL1B* was silenced by DNA methylation in normal chondrocytes and that demethylation had occurred in OA, as previously reported for MMPs and ADAMTS-4 (19). To investigate the epigenetic regulation of *IL1B*, we studied the proximal promoter because its activity regulates cell-type specific expression (33). When the CpG locations are compared with the transcription factor binding sites (Figure 6), the latter are concentrated in two CpG-free regions, each bracketed by CpG sites. Interestingly, the site at -511 bp and the two CpG sites around the transcription start site (-20 and +10 bp) were un-methylated, whether or not *IL1B* was expressed. The two CpG sites that are differentially methylated and where methylation status inversely correlated with expression (-256 and -299 bp) were located between the two regions that contain multiple transcription factor binding sites and bracket a NF- κ B binding site. Both IL-1 β and TNF α activate NF- κ B (34–36), which, in turn, activates many genes (37), including *IL1B* and other catabolic genes induced in OA. Perhaps more relevant in the present context is the fact that NF- κ B may be involved in demethylation (38), although details are not clear. The observation (39) that, in intestinal inflammation, the TNF α -induced NF- κ B expression was associated with demethylation of CpG islands supports the inference that NF- κ B may play a role in demethylation in the experiments reported here.

Having identified the differentially methylated CpG sites, we determined the percentage of cells that were methylated at -299 bp (27). Even in control chondrocytes, which do not express *IL1B*, the % methylation was only ~60% and not the expected 100%. This raises the question why 40% of non-methylated cells did not express *IL1B*. Hypomethylation is a

necessary, but not sufficient, condition for gene activation, inasmuch as histone modifications are also required. Deacetylated histones or methylated H3K9 and H3K27 can in theory silence a gene even in the absence of DNA methylation. An alternative possibility is that normal articular chondrocytes do not contain the transcription factors required for *IL1B* expression. Thus the rapid induction after 24 hours could depend on cytokine-induced histone modifications and/or activation of transcription factors in those cells with pre-existing de-methylation. In support of this notion, IL-1 α has been shown to translocate to the nucleus and to interact with histone acetyltransferase in yeast strains (40). Whether this also applies to IL-1 β is not known. Upregulation of *IL1B* after short-term treatment did not persist, yet after long-term treatment the expression of *IL1B* persisted for at least two weeks after the inducer had been withdrawn. This is consistent with permanent epigenetic changes. Because the pathology of OA involves long-term aberrant induction of IL-1 β and proteases, short-term *in vitro* experiments are unlikely to model the *in vivo* situation.

Further evidence for a cause-effect relationship between DNA methylation and gene expression was obtained in 5-aza-dC treated cultures, in which the experimentally induced loss of DNA methylation increased *IL1B* expression by 5-fold. Treatment with 5-aza-dC reduced, yet did not abolish, the % methylation. One reason may be that 5-aza-dC, whose half-life is around four hours (26), can act only on those cells that undergo cell division during that time. Because 5-aza-dC was added 3 times per week, it is likely that active 5-aza-dC was not present at all times when cell division took place. Treatment with cytokines rather than with 5-aza-dC was much more effective in inducing gene expression. This was especially true for the combination of the two cytokines (TNF α /OSM). TNF α with or without OSM has frequently been used to induce expression of degradative proteases in cultured chondrocytes (34,41). The findings presented here show that the TNF α /OSM combination is also highly effective in stimulating *IL1B* in chondrocytes, increasing its expression up to 1000-fold. OSM belongs to the IL-6 family, acts via JAK/STAT signalling, and can, on its own, induce cartilage degradation *in vitro* (42), but is usually used together with TNF α (43) or IL-1 β (24,41) to enhance the cytokine effect. The % DNA methylation correlated negatively with increases in *IL1B* expression, although this varied considerably among patients. Samples treated with TNF α /OSM had the highest expression level and the greatest loss of DNA methylation. Indeed DNA methylation was completely abolished in some samples. On the basis of these findings it is evident that long-term aberrant expression of *IL1B* was a consequence of DNA de-methylation, presumably caused by inflammatory cytokines. The question then arises of how cytokines can achieve greater DNA demethylation than 5-aza-dC. Because 5-aza-dC inhibits *DNMT1*, we asked whether cytokines also alter *DNMT1* expression. This was indeed reduced *in vitro* by the cytokines and in OA chondrocytes compared with #NOF chondrocytes *in vivo*. However, since culture *per se* significantly reduced expression, lower expression of *DNMT1* alone could not explain the results. We hypothesize that activation of NF- κ B plays a major role in the cytokine-induced DNA de-methylation (38), but further studies are required.

There are several caveats that should be borne in mind. When chondrocytes are grown in monolayer for several weeks, their phenotype is lost. However, explants of articular cartilage in organ culture, in which the chondrocytic phenotype is maintained, exhibit little or no loss of DNA methylation or gene induction. This suggests that the extracellular matrix protects the cells from loss of phenotype and possibly loss of DNA methylation. To maintain genomic stability *in vivo*, one would expect resistance to changes in DNA methylation status in healthy fully differentiated cells and the extracellular matrix might provide increased resistance in explant cultures. Monolayer cultures probably sensitize chondrocytes to epigenetic changes so that one can obtain within weeks the phenotypic changes that probably take years in OA. To what extent the protease-producing 'degradative' cells are still chondrocytes is a matter for debate.

One should also be careful about extending these *in vitro* findings to the *in vivo* situation. In *in vitro*, we observed decreased expression of *COL2A1* after a single cytokine addition, whereas *in vivo* we actually found increased *COL2A1* expression in surface-zone OA chondrocytes compared with #NOF chondrocytes (not shown), suggesting that the cultures do not model the *in vivo* situation as far as *COL2A1* is concerned. There is also some disagreement about the importance of IL-1 β in OA. We have observed significant expression of *IL1B* in chondrocytes of the superficial zone of OA cartilage, where cytokines have also been immunolocalized (44,45). By contrast, Fan et al. (46) found no significant upregulation of *IL1B* in OA patients, but had used early degenerate cartilage of the knee joint, with the deep zone included. In *IL1B* knock-out mice, experimentally-induced OA was either enhanced or reduced (47,48) depending on genetic background, whereas over-expression of human *TNFA* induced severe inflammatory arthritis (49). However the mouse models may not be representative of human OA.

Summary

Our novel findings support the conclusion that the long-term cytokine-stimulated induction of *IL1B* in human articular chondrocytes *in vitro* involves loss of DNA methylation. If applicable *in vivo*, then the mechanisms for OA progression may involve the following: An initial inflammatory episode in the synovium, perhaps as a consequence of mechanical stress, activates synovial macrophages to produce IL-1 β and TNF α . These diffuse into the articular cartilage. There the cytokines induce aberrant expression of proteases and *IL1B* in the chondrocytes (50). If this induction leads to the loss of DNA methylation, *IL1B* will now be included in the expression repertoire of the OA chondrocytes, even after synovial inflammation has abated. This scenario may explain why protease inhibitors, such as TIMPs, have little effect when injected into the joint, and why, once degradative processes have been operative, OA progression cannot be halted.

Acknowledgments

We are grateful to the Orthopaedic Surgeons of Southampton General Hospital for supplying us with femoral heads following joint replacements. The critical reading of the manuscript by Dr. Felix Bronner (Connecticut) was much appreciated.

Supported by NIH grant R21-AR054887 to MBG and HIR, and Wessex Medical Research grant No M19 to HIR

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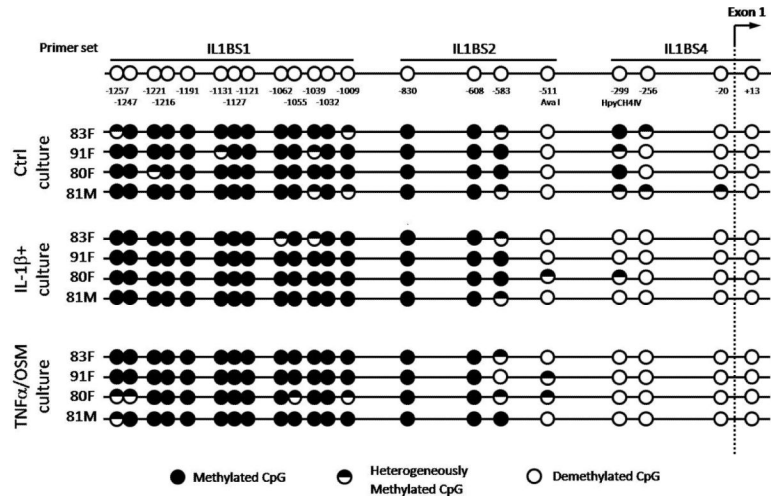


Figure 1. CpG methylation map for the proximal *IL1B* promoter

Normal chondrocytes were isolated from four #NOF patients and cultured for 4–5 weeks with or without the cytokines. Methylation status was determined by bisulfite modification. Each circle represents the average result of six sequenced clones. The CpG site at –299bp was selected for quantification.

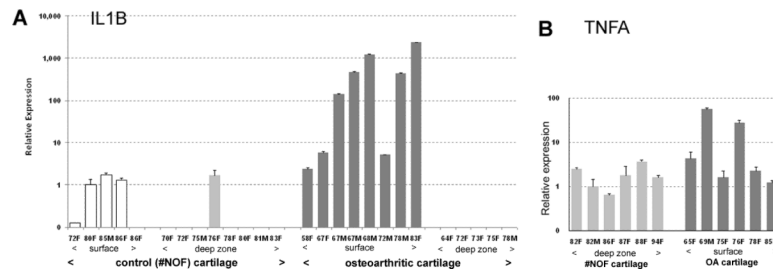


Figure 2.

Differential qPCR expression of *IL1B* (A) and *TNFA* (B). Articular cartilage was obtained from either #NOF patients or OA patients. The surface zones were removed and analyzed separately from the deep zones. Results are shown for individual patients, arranged in age order. For each gene, #NOF and OA samples were analyzed in the same assay. Low levels of *IL1B* in the superficial zone of #NOF patients contrasted the 100–1000 fold increased expression in the surface zone of OA cartilage. With one exception (72 year old male), expression increased with age. There was no expression of *IL1B* in the deep zones of #NOF (one exception) or OA cartilage. By contrast, *TNFA* was expressed in the control chondrocytes located in the deep zone of #NOF patients and expression did not increase significantly in surface-zone OA chondrocytes.

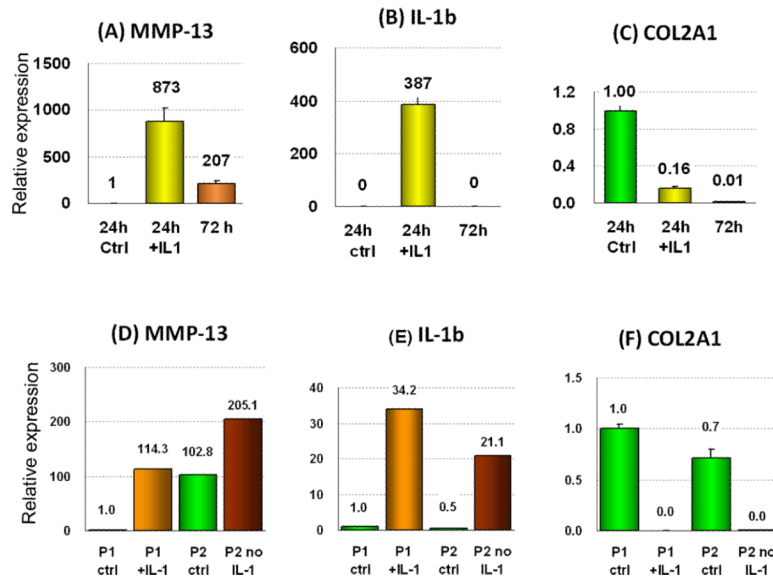


Figure 3.

Effects of cytokine withdrawal following short-term (A–C) or long-term (D–F) treatment with IL-1 β /OSM on expression of the indicated genes. The catabolic genes MMP13 and IL1B are up-regulated several 100-fold after 24 hours following a single addition of IL-1 β /OSM, whereas COL2A1 is down-regulated. After 72 hours with no further cytokine treatment the expression of the catabolic genes reduced again to near-normal, but the expression of COL2A1 was not re-gained.

(D–F) When IL-1 β /OSM was added twice a week for 3 weeks to passage 1 (P1+ IL-1) cells, the catabolic genes MMP13 and IL1B were again up-regulated considerably. Cells were then passaged and cultured for a further 3 weeks during which no cytokines were added (P2 no IL-1). This time expression was maintained even in the absence of cytokines, consistent with a permanent induction of gene expression. COL2A1 expression was abolished by the cytokines and remained low throughout. Representative examples of four experiments.

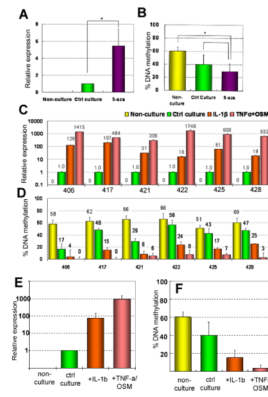


Figure 4.

Long-term treatment with cytokines causes DNA de-methylation together with aberrant gene expression. Chondrocytes were cultured with 5-aza-dC (**A,B**); or IL-1 β or TNF- α /OSM (**C,D,E,F**). Relative mRNA expression of *IL1B* was compared with the % DNA methylation at -299bp in the *IL1B* promoter.

(**A,B**) Culture *per se* induced some expression and a 20% loss in methylation, 5-aza-dC increased expression 5-fold compared to control culture (**A**) and caused a further loss of DNA methylation (**B**). Means and SDs of six samples; * = P<0.05. (**C**) Relative expression of *IL1B* in chondrocytes from six individual patients, labelled with the patients' numbers and treated as indicated in the key. No *IL1B* was detectable in non-cultured chondrocytes. Culture itself induced low expression (set to =1), but IL-1 β , and TNF- α /OSM increased expression considerably. (**D**) The % DNA methylation in the same samples as above. Before culture ~60% of cells were methylated. Culture alone could reduce DNA methylation, which was significant in patient 406 and 421. However, cytokine treatment caused greater loss of DNA methylation, particularly the combined treatment. (**E,F**) Means and S.D. of the samples shown in C and D. The differences between all groups are significant with P<0.05.

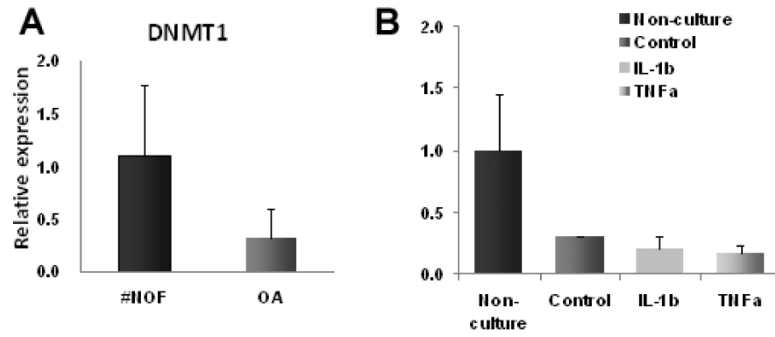


Figure 5. Expression of DNMT1 in articular chondrocytes in vivo (**A**) and in vitro (**B**). DNMT1 expression was less than half in OA chondrocytes compared with control (#NOF) chondrocytes ($P < 0.05$). *In vitro*, culture alone had the greatest effect ($P < 0.01$) and the decrease in expression was of a similar in magnitude to the decrease in OA. In addition, TNF- α /OSM decreased expression further ($P < 0.01$). Means and SDs of $n=5$ for all groups.

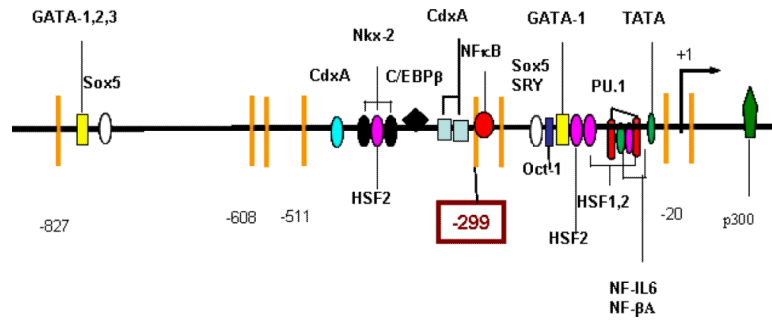


Figure 6. Proximal promoter of IL1B. Vertical bars indicate the position of CpG sites. The CpG sites at -299 and -256bp bracket a NF-κB binding site