Role of dentin matrix protein 1 in cartilage redifferentiation and osteoarthritis

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Abstract

Objective. The aim of this study was to test the possible involvement, relevance and significance of dentin matrix protein 1 (DMP1) in chondrocyte redifferentiation and OA.

Methods. To examine the function of DMP1 in vitro, bone marrow stromal cells (BMSCs) and articular chondrocytes (ACs) were isolated and differentiated in micromasses in the presence or absence of DMP1 small interfering RNA and analysed for chondrogenic phenotype. The association of DMP1 expression with OA progression was analysed time dependently in the OA menisectomy rat model and in grade-specific OA human samples.

Results. It was found that DMP1 was strongly related to chondrogenesis, which was evidenced by the strong expression of DMP1 in the 14.5-day mouse embryonic cartilage development stage and in femoral heads of post-natal days 0 and 4. In vitro chondrogenesis in BMSCs and ACs was accompanied by a gradual increase in DMP1 expression at both the gene and protein levels. In addition, knockdown of DMP1 expression led to decreased chondrocyte marker genes, such as COL2A1, ACAN and SOX9, and an increase in the expression of COL10A and MMP13 in ACs. Moreover, treatment with IL-1β, a well-known catabolic culprit of proteoglycan matrix loss, significantly reduced the expression of DMP1. Furthermore, we also observed the suppression of DMP1 protein in a grade-specific manner in knee joint samples from patients with OA. In the menisectomy-induced OA model, an increase in the Mankin score was accompanied by the gradual loss of DMP1 expression.

Conclusion. Observations from this study suggest that DMP1 may play an important role in maintaining the chondrogenic phenotype and its possible involvement in altered cartilage matrix remodelling and degradation in disease conditions like OA.

Key words: dentin matrix protein 1, chondrocytes, redifferentiation, osteoarthritis, cartilage.

Introduction

OA is characterized by progressive destruction of articular cartilage, subchondral bone sclerosis and osteophyte formation. A number of molecular factors have been implicated in the development of OA. Dentin matrix protein 1 (DMP1) is an acidic phosphorylated extracellular matrix (ECM) protein originally cloned from a rat incisor cDNA library [1]. Currently the precise function of DMP1 is unknown, although this protein can bind tightly to hydroxyapatite and can mediate cell attachment through the RGD (Arg-Gly-Asp) domain [2]. Although early data suggest that this gene was odontoblast specific [3–5], more recent observations have revealed the expression of DMP1 in soft and hard tissues such as brain and bone [6]. Toyosawa et al. [7] suggest that DMP1 is a specific marker gene for osteocytes and pre-osteocytes. Somewhat unexpectedly, our immunohistological examination of knee samples revealed strong localization of DMP1 in mature articular cartilage tissue of both human and rodent origin apart from its intense expression in bone ECM and osteocytes. Such positive staining of DMP1 in mature articular cartilage tissue is unexpected, because DMP1 is an acidic phosphoprotein and is known to be predominantly expressed only in mineralized tissues such as bone, dentin and cementum. In those hard tissues the importance of DMP1 for dentin and
Role of DMP1 in chondrogenesis

Bone mineralization has been demonstrated in many studies [8, 9].

Similar to our observations, recently Sun et al. [10] reported that DMP1 is abundant in extracts of the femoral head cartilage and further demonstrated elevated levels of DMP1 with age during the development of cartilage. Furthermore, it has been shown that mice devoid of the DMP1 gene have severe phenotypic changes in the growth plate of their long bones [11]. Such a high level of chondrocyte-specific expression of DMP1 in articular cartilage suggests its possible role in cartilage and bone development and/or maintenance of cartilage homeostasis. However, no systemic evaluation of function of DMP1 proteins in mature articular cartilage and its expression in articular cartilage diseases such as OA has been performed. Thus the goal of this study was to evaluate the direct functional role of DMP1 during chondrocyte redifferentiation. Since, it is known that chondrogenic events are severely hampered in diseases like OA, we further investigated the DMP1 expression pattern during OA development. The results obtained provide strong support for its role in driving chondrogenesis.

Materials and methods

Sample collection and cell culture

The study was conducted in compliance with ethical principles derived from the local institutional review board and was approved by the Queensland University of Technology Research Ethics Committee. All patients gave their informed consent to participate in this in vitro study. Isolation and characterization of bone marrow stromal cells (BMSCs) were done following procedures described previously [12, 13]. Articular chondrocytes (ACs) were obtained from the knees of OA patients. All radiographs were reviewed and the patient samples were classified into two categories on the basis of Mankin score [14, 15]. A Mankin score of 1–2 indicated mild OA cartilage and a score >5 indicated moderate to severe degenerative OA cartilage. ACs from mild OA cartilage were isolated using enzymic digestion, as described previously [16, 17]. Only passage 0–1 ACs that were characterized for chondrogenic phenotype were used for the studies. Mouse embryos were collected and prepared at the indicated embryonic day, as described previously [18].

Detection of DMP1 expression in in vitro chondrogenic pellet cultures

BMSCs or ACs (2 × 10^5 cells) were resuspended in a serum-free chondrogenic medium [Invitrogen, Mount Waverley, VIC, Australia] supplemented with 10 ng/ml TGF-β3 (R&D Systems, Gymea, NSW, Australia), 10 nM dexamethasone (Sigma Aldrich, Castle Hill, NSW, Australia), 50 μg/ml L-ascorbic acid (Sigma Aldrich), 10 μg/ml sodium pyruvate (Sigma Aldrich), 10 mg/ml proline and ITS+ (final concentration 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 5.33 μg/ml Linoleic acid and 1.25 μg/ml BSA) (Sigma Aldrich]) and centrifuged at 600 g for 20 min to form a pellet. After 2 weeks of differentiation, RNA was extracted from pellets as described previously to observe the expression pattern of DMP1 [19]. In addition, immunostaining of the pellets was performed as described previously [19] to see the abundance of DMP1 expression (the antibody for DMP1 was a kind gift from Professor Jian Feng, University of Texas, Austin, TX, USA). For histochemical staining of GAGs, the pellets were dehydrated, embedded in paraffin and 5 μm sections were cut and stained for 6 min in 0.1% Safranin O (Sigma Aldrich) containing 1% acetic acid and counterstained for 2 min in 1% Fast Green containing 7% acetic acid.

In vitro inhibition of DMP1 using small interfering RNA in AC micromass cultures

DMP1-specific small interfering RNA (siRNA) (Mission predesigned siRNA; Sigma Aldrich) was used to study DMP1-mediated cellular phenotypic changes in AC micromass cultures. Micromass culture was performed as described previously [17]. Briefly, trypsinized ACs were resuspended in DMEM with 10% fetal bovine serum (FBS) at a concentration of 10^5 cells/ml and drops of 10 μl were placed in a 24-well tissue culture dish. To study the role of DMP1 in ACs, siRNA transfection was performed 24 h after seeding the cells, followed by a medium change 6 h post-transfection, and cells were cultured in differentiation medium. Cells were transfected once more with siRNA at the beginning of day 5 to maintain knockdown efficiency. ACs were transfected with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions. Controls were incubated with the scrambled siRNA. These concentrations were carefully optimized by performing a preliminary dose-dependent study. All experiments were performed in triplicate. After redifferentiation in the presence or absence of siRNAs, some micromasses were stained with Alcian blue on day 7 to assess matrix deposition using protocols described previously [16, 17].

For IL-1β treatment, cells were deprived of serum 24 h before the start of IL-1β experiments. Concentrations of IL-1β ranging from 1 to 5 ng/ml (R&D Systems) were used to study the expression of DMP1. Quantitative real-time RT-PCR and western blotting were performed as described previously [16, 17]. At mRNA level we detected the expression levels of DMP1, chondrogenic markers (COL2A1, ACAN, SOX9), degradative markers (MMP13) and hypertrophic markers (COL10A1). ΔΔCT was determined by subtracting ΔCT of the control sample from ΔCT of the target sample. The relative mRNA quantification of the target gene was calculated by two −ΔΔCTs. Day 0 undifferentiated ACs were used as a calibrator. Primer sequences used for this study are as follows:

DMP1: forward, GCATCCTGTCAATGTTCTTTTG; reverse, GAGCACAATGACCCTTCCATT
COL10A: forward, CAAGGCAACTTCCAGGAA; reverse, AAAGGATTGGTGGCAGCATTT
MMP13: forward, ACTTCAGATGGCATTGCTG; reverse, CATAAATTGGCCCAGGAG
GAPDH: forward, TCGAATGGCTCTCCTGAC; reverse, TCTGGTAGCCAGTGATGC

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18S rRNA: forward, CGAACCTCCGACTTCGTTC; reverse, TTCGGAACTGAGGCCATGAT
SOX9: forward, TTTCAGAGACAAAACATGA; reverse, AAGTCCAGTTTCTCGTTGA
ACAN: forward, CACTGTTACCGCCACTTCCC; reverse, ACCAGGGGAAGTCCCCTTCG
COL2A: forward, CTGGCAAAGATGGTGAGACAGGTG; reverse, TCCCGGCCAGCCAGGTCC

Induction of OA
Animal ethics approval for this project was granted by the Queensland University of Technology and the Prince Charles Hospital Ethics Committees. Male Wistar Kyoto rats (11–12 weeks old) were purchased from the Medical Engineering Research Facility (MERF) (Brisbane, Qld, Australia). Animals weighing ~300–350 g were used for this experiment. Rats were anaesthetized via intraperitoneal injection with Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg) and Xylazil (xylazine 10 mg/kg). OA was induced by transecting the medial collateral ligament just below its attachment to the meniscus, so that when the joint space opens, the meniscus is reflected toward the femur. The meniscus was then cut at its narrowest point without damaging the tibial surface, resulting in complete medial meniscus transection. The surgical wound was then closed by suturing in two layers. The other group was subjected to the same surgical procedure on the left knee, without excision of the ligament or any meniscus manipulation. After the surgery, all rats received pain killers (buprenorphine 0.05 mg/kg) and antibiotics (gentamicin 5 mg/kg). At specific time points (weeks 0, 4 and 8) knee samples were harvested to detect time-dependent OA changes in DMP1 expression.

Statistical analysis
The mean values and s.d.s were computed from the individual experiments utilizing Excel (Microsoft, Bellevue, WA, USA). A test for analysis of variance was performed using InStat software (version 3; GraphPad Software, La Jolla, CA, USA) and a post-hoc test was performed for statistical evaluation. Results were considered significant for P-values ≤ 0.05.

Results
DMP1 expression was detected in cartilage development sites
To verify the physiological relevance of DMP1 in cartilage differentiation, we examined its in vivo expression pattern during limb chondrogenesis of mouse embryos. Immunohistochemistry data demonstrated that the expression of DMP1 protein was higher and almost overlapping with Safranin O staining at the embryonic day 14.5 chondrogenic phase. For 14.5 days post-coitum embryos, DMP1 was not expressed in undifferentiated mesenchymal cells surrounding precartilage condensations, but was clearly detected in the condensation cores of both hind limbs and vertebrae (Fig. 1A and B). Furthermore, tibiae and femoral heads from post-natal (P) day 0 and post-natal day 4 littermates were stained with Safranin O for proteoglycans and anti-DMP1 antibody. Safranin O staining consistently indicated enhanced density of proteoglycans in both P0 and P4 littermates. Also, immunohistological analysis of DMP1 showed a positive reaction in resting chondrocytes and in the growth plate (Fig. 1C).

Elevated expression of DMP1 in the course of chondrogenesis in BMSCs and redifferentiation of ACs
To examine the expression profile of DMP1, we used a pellet culture system to differentiate BMSCs and ACs, which is a well-established in vitro cell model. Pellets of both BMSCs and ACs were strongly stained by Safranin O after chondrogenic induction for 14 days in a time-dependent manner. Similar to Safranin O, the
immunostaining showed a gradual increase in the expression level of DMP1 in both BMSCs and ACs compared with day 3 (Fig. 2A). We further determined the dynamic expression pattern of DMP1 during in vitro redifferentiation of ACs and chondrogenesis of BMSCs using a quantitative RT-PCR assay. In this model, DMP1 expression increased gradually during chondrogenesis in parallel with gene expression of COL2A1, in both BMSCs and ACs (Fig. 2B). These data indicate that the expression of DMP1 increases during differentiation of BMSCs and ACs, and this is consistent with its high expression of chondrogenic marker COL2A1. Furthermore, a positive correlation was observed when COL2A1 and DMP1 expression was compared in ACs. These data show that DMP1 may play an important role in maintaining chondrogenic phenotype (Fig. 2C).

Knockdown of DMP1 inhibits chondrogenic phenotype and promotes a hypertrophy-like phenotype

Knowing that DMP1 is time dependently enhanced during AC redifferentiation, we next determined whether endogenous DMP1 is required to maintain the chondrocyte phenotype by knocking down DMP1 using the siRNA approach in an AC micromass culture model. Real-time PCR and western blotting techniques were performed to verify RNA and protein levels of DMP1 in transfected and non-transfected cells. Transfection with siRNA resulted in a reduction in DMP1 mRNA in AC micromasses at all time points (Fig. 3A). At protein level, knockdown of DMP1 was

(A) Safranin O staining and immunostaining of DMP1 during the differentiation of BMSCs and ACs cultured in the three-dimensional pellets for 14 days. (B) Real-time PCR assay. Total RNA was prepared from pellet cultures of differentiated BMSCs and ACs at various time points, as indicated, and the mRNA expression of DMP1 and COL2A1, 18S rRNA and GAPDH (serving as an internal control) were examined by real-time PCR. mRNA levels of chondrogenic differentiated ACs were normalized to undifferentiated control cells from day 0. (C) Correlation between COL2A1 gene expression with DMP1 gene expression in ACs. The COL2A1 expression of all samples was plotted on the y-axis vs DMP1 expression. All results are representative of experiments performed on three different donors with three replicates each time and presented as mean (s.d.). *P < 0.05. ACs: articular chondrocytes; BMSCs: bone marrow stromal cells; DMP1: dentin matrix protein 1.
Expression of DMP1 is decreased in severe OA cartilage graded according to disease severity

To examine changes in DMP1 expression in human OA articular cartilage, immunostaining and quantitative RT-PCR were performed on samples prepared from human knee OA articular cartilage that were graded according to disease severity. Both immunostaining (Fig. 4A) and gene expression (Fig. 4B) studies revealed that DMP1 expression in articular cartilage from severe OA (Mankin score 8–10) was significantly lower than that in relatively normal cartilage (obtained from the same individuals; Mankin score 1–2). The specificity of the staining was confirmed using a non-immune control IgG. These data demonstrate abnormally reduced DMP1 expression in OA cartilage, which appears to decrease with increased disease severity and loss of matrix.

IL-1β treatment decreased DMP1 expression in ACs

IL-1β is one of the critical mediators of OA, and IL-1β stimulation of ACs causes gene expression patterns similar to those of OA cartilage. To analyse the effects of IL-1β on the expression of DMP1 in ACs, we performed quantitative RT-PCR. In response to IL-1β stimulation, the expression of DMP1 was markedly decreased in a dose-dependent manner. Taken together, these results show reduced DMP1 expression in the context of IL-1β-induced OA-like changes in AC gene expression (Fig. 4C).

Consistent decrease of DMP1 expression during OA development in a rat model

Time-dependent DMP1 changes in OA were observed in a rat meniscectomy model. We observed that meniscectomy in rats resulted in morphological surface changes that resemble those occurring in some stages of human OA. Cartilage showed rough surface and fibrous structure by week 8 compared with week 4 and week 0 in meniscectomy-induced OA rats. A reduction in cartilage proteoglycan, extensive alterations characterized by increased cellularity resulting from clonal expansion and numerous osteophytes on the margins were often evident in week 8 OA rats. By Safranin O staining, typical signs of progressive proteoglycan loss in the operated knees of week 4 and 8 rats were observed, whereas strong staining in the knee cartilage of week 0 rats was noted (Fig. 5A).

The severity of histological changes in articular cartilage was evaluated using a Mankin scale. Mankin score analysis confirms time-dependent OA changes in the meniscectomy OA models. Time course analysis of week 0 cartilage revealed that DMP1 expression was positive in most of the chondrocytes in non-calcified cartilage. In contrast, there was a clear, significant, gradual decrease in the expression of DMP1 in the diseased tissue of weeks 4 and 8. Statistical evaluation for the cell score revealed a clear decrease in the number of chondrocytes staining positive for DMP1 in week 4 and week 8 articular cartilage (Fig. 5B). Furthermore, a negative correlation was found when the Mankin score and DMP1 expression were plotted (Fig. 5C). These results indicate that DMP1 expression was reduced in a time-dependent manner as...
OA progressed in menisectomy-induced OA rat models, even before the observation of severe lesions (i.e. at 8 weeks OA), suggesting that these changes in expression may be symptomatic of early changes in OA.

Discussion

Identification of the regulatory genes and signalling molecules that control chondrogenesis in mature articular cartilage is crucial in understanding normal and abnormal cartilage physiology. In addition to COL2A1, the ECM of the cartilage contains many types of non-collagenous matrix protein, including DMP1. Many studies have been carried out regarding the role of DMP1 in mineralized tissue, however, there are only a few reports referring to the presence of DMP1 in non-mineralized cartilaginous tissues [10]. This study is the first to demonstrate an important role of DMP1 in maintaining the chondrogenic phenotype of mature ACs and its progressive decrease during OA development.

In this study we first detected strong localization of DMP1 in the mature articular cartilage of both humans and rodents, suggesting its important role in cartilage function. Our data show that DMP1 mRNA expression is markedly up-regulated during chondrocyte differentiation in an in vitro three-dimensional pellet culture system in both BMSCs and ACs, indicating that DMP1 is an important gene required for the chondrogenic phenotype. The expression of DMP1 mRNA correlated with the up-regulation of the early chondrogenic marker COL2A1. Supporting the in vitro results, DMP1 protein levels displayed similar higher up-regulation during the chondrocyte differentiation phase of embryonic day 14.5 mice. These findings indicate that the up-regulation of DMP1 does not appear to be an artefact of the micromass culture system, but reflects the physiological processes of its important role in cartilage differentiation.

In addition, our in vitro loss-of-function studies demonstrated that DMP1 silencing could significantly affect the chondrogenic phenotype in ACs, particularly on day 7. Furthermore, we were able to demonstrate that DMP1 silencing could significantly affect chondrogenic gene expression. This belief is consistent with the findings from the DMP1 knockout studies, which showed that mice devoid of the DMP1 gene have severe phenotypic changes in the growth plate of their long bones [22]. Recently it has been demonstrated that the different fragments of DMP1 have distinct roles in different tissues. For example, in the ECM of bone and dentin, DMP1 occurs mainly as proteolytically processed fragments, including a 37-kDa fragment (DMP1-N) and a 57-kDa fragment (DMP1-C), originating from the NH2- and COOH-terminal regions, respectively, of the DMP1 amino acid sequence [23]. In addition to the core protein form (i.e., DMP1-N), the NH2-terminal fragment of DMP1 also occurs as a proteoglycan, which shows that this particular fragment plays an important role in cartilage compared with DMP1-C [10, 24, 25]. Interestingly, a recent in vitro study showed that DMP1-PG dose-dependently inhibited mineral accumulation [26]. An in vivo study showed that the level of DMP1-PG was remarkably elevated in the poorly mineralized long bones of hypophosphataemic mice [27]. Taken together, these findings indicate that DMP1-PG may function as an inhibitor of mineralization for cartilage and bone.

These findings have a direct biological relevance to the present observations of this study. In humans, once growth formation is complete and has ceased, soft tissues such as articular cartilage tend not to undergo hypertrophic differentiation. However, chondrocyte hypertrophy and mineralization have been reported to be reinitiated in the course of pathological conditions such as OA [28]. During this very last phase, dramatic phenotypic changes occur in the behaviour of ACs, characterized by expression of bone-specific markers and mineralized matrix deposition [29]. Since DMP1-PG is an inhibitor of mineralization, its expression in cartilage may have a unique role in protecting normal cartilage undergoing hypertrophic differentiation and maintaining the chondrogenic phase of mature...
cartilage throughout life. Therefore it is possible that decreased expression of DMP1 in OA cartilage may lead to a hypertrophy phenotype and subsequent mineralization. It was indeed shown that the deposition of these minerals could influence expression of several degenerative pathways involving inflammatory cytokines and MMPs, all of which have been implicated in OA pathogenesis leading to subsequent cartilage degeneration [30]. Moreover, our results showed that DMP1 is highly expressed in normal human articular cartilage compared with OA cartilage, providing strong evidence for a potential regulatory role of DMP1 in cartilage tissue maintenance and homeostasis at the in vivo level. In line with this evidence, DMP1 expression was significantly down-regulated in the OA rat model in a time-dependent manner, raising the possibility that those gradual alterations in DMP1 expression are associated with OA development. How DMP1 regulates cartilage phenotype and its respective targeting transcription factors should be explored in future studies. In OA pathogenesis, several chondrocyte-specific genes, including COL2A1, ACAN and SOX9, are down-regulated. In contrast, the cartilage-degrading enzyme MMP13 is up-regulated [31]. Interestingly, we found that DMP1 knockout cells showed a decrease in chondrogenic expression and an increase in some hypertrophy and degradative markers. These results suggest that DMP1 regulates the overall balance of cartilage matrix synthesis and degradation. Previous analysis of the rat DMP1 promoter identified several regulatory target transcription factors. Some of the known transcription factors that interact with DMP1 include AP-1 family members, c-Fos, Ras and SP1 [32, 33]. Transcription factors such as AP-1 are known to be important for skeletogenesis by affecting chondrocyte differentiation [34]. Furthermore, it is interesting to note that the expression of SOX9 is slightly down-regulated by lack of DMP1 expression in primary chondrocytes, indicating that DMP1 somehow plays a regulatory role for this gene. It is possible that DMP1 may exert its effects on SOX9 through direct or indirect transcriptional activation of as-yet-unidentified genes.

Some of the important regulators of chondrocyte redifferentiation are fibroblast growth factors (FGFs). FGF2 has been implicated in stage-dependent regulation of chondrocyte proliferation and differentiation and is known to regulate SOX9 expression [35, 36]. In this context, it has been shown that DMP1 expression in osteoblasts and osteocytes requires FGF and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signalling [37]. Also, it has been demonstrated that members of the AP-1 family (c-Jun and c-Fos) are involved in the transcriptional regulation of the DMP1 gene and are also known to regulate SOX9. Therefore it is possible that DMP1 can interact with SOX9 with the aid of some growth factors or cross-talk with transcription factors to maintain cartilage homeostasis. In addition, we found that DMP1 is highly associated with the severity of articular cartilage damage in human OA and in the OA rat model, providing further evidence of a potential regulatory role of DMP1 in cartilage tissue maintenance and homeostasis at the in vivo level.

**Rheumatology key messages**

- This study shows that DMP-1 plays an important role in chondrocyte redifferentiation.
- Reduction of DMP-1 levels may be associated with OA development.

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