Inhibition of p38 pathway leads to OA-like changes in a rat animal model

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Abstract

Objectives. The p38 mitogen-activated protein kinase (MAPK) signal transduction pathway is involved in a variety of inflammatory responses, including cytokine generation, cell differentiation proliferation and apoptosis. Here, we examined the effects of systemic p38 MAPK inhibition on cartilage cells and OA disease progression by both in vitro and in vivo approaches.

Methods. p38 kinase activity was evaluated in normal and OA cartilage cells by measuring the amount of phosphorylated protein. To examine the function of p38 signalling pathway in vitro, normal chondrocytes were isolated and differentiated in the presence or absence of p38 inhibitor SB203580 and analysed for chondrogenic phenotype. Effect of systemic p38 MAPK inhibition in normal and OA (induced by meniscectomy) rats were analysed by treating animals with vehicle alone [dimethylsulphoxide (DMSO)] or p38 inhibitor (SB203580). Damage to the femur and tibial plateau was evaluated by modified Mankin score, histology and immunohistochemistry.

Results. Our in vitro studies have revealed that a down-regulation of chondrogenic and an increase of hypertrophic gene expression occurs in the normal chondrocytes when p38 is neutralized by a pharmacological inhibitor. We further observed that the basal levels of p38 phosphorylation were decreased in OA chondrocytes compared with normal chondrocytes. These findings together indicate the importance of this pathway in the regulation of cartilage physiology and its relevance to OA pathogenesis. At the in vivo level, systematic administration of a specific p38 MAPK inhibitor, SB203580, continuously for more than a month led to a significant loss of proteoglycan, aggrecan and cartilage thickness. On the other hand, SB203580-treated normal rats showed a significant increase in Terminal dUTP nick end-labelling (TUNEL)-positive cells, cartilage hypertrophy markers such as Type 10 collagen, Runt-related transcription factor and MMP-13 and substantially induced OA-like phenotypic changes in the normal rats. In addition, meniscectomy-induced OA rat models that were treated with p38 inhibitor showed aggravation of cartilage damage.

Conclusions. In summary, this study has provided evidence that the component of the p38 MAPK pathway is important to maintain cartilage health, and its inhibition can lead to severe cartilage degenerative changes. The observations in this study highlight the possibility of using activators of the p38 pathway as an alternative approach in the treatment of OA.

Key words: osteoarthritis, cartilage, subchondral bone, hypertrophy, MAPK-p38 signalling pathway.

Introduction

OA, the most common multifactorial degenerative joint disease in the elderly, is characterized by the degeneration of articular cartilage, changes in subchondral bone, osteophyte formation and synovial inflammation. The cause of OA is still unknown and many mechanical, biochemical and molecular factors are known to be involved. In the progression of OA, the balance of synthesis and
degradation of articular cartilage shifts towards catabolism [1]. These synthetic changes involve alterations in specific signalling molecules and their respective downstream pathways [2]. Among the most conserved signal transduction systems in the cartilage is the mitogen-activated protein kinase (MAPK) cascade, which consists of sequentially acting protein kinases resulting in the activation of three terminal MAPKs: p38 kinase, extracellular signal-regulated protein kinase (ERK) and c-jun N-terminal protein kinase (JNK) [3].

It has been reported that p38 signalling controls terminal differentiation [4], proliferation and differentiation of chondrocytes with a delicate balance, interacting with the TGF-β1/Smads signaling pathway [5, 6]. Our observations indicate that the p38 pathway plays a major positive role in regulating the articular chondrogenic phenotype and its expression negatively regulates the cartilage hypertrophic phenotype [7]. Although p38 kinases were originally cloned as mediators of growth and development, recent results demonstrate important roles of these proteins in stress and inflammatory signals [8]. Studies have documented a key role for pro-inflammatory cytokine overproduction as a potential driving force for cartilage catabolism in OA [9]. One of the key signal transduction pathways involved in the production of pro-inflammatory cytokines is the p38 MAPK pathway [10]. p38 MAPK has been reported as a primary signal transduction pathway activated by degradative cytokines such as IL-1β and TNF-α [2, 11]. This suggests that strategies to target signalling pathways that lead to cytokine overproduction should be explored in an attempt to develop new OA therapeutics with the potential for disease modification. Given the dual anabolic and catabolic role of the p38 pathway in cartilage, it remains unknown whether the inhibition of p38 pathway has some impact in preventing cartilage from undergoing degeneration.

SB203580, a pyridinyl imidazole that selectively inhibits p38 MAPK phosphorylation, has been widely used for in vitro and in vitro studies to explore the function of p38 pathway. SB203580 has been demonstrated to attenuate the synthesis of inflammatory cytokines and MMPs in cartilage cells in several previous studies [12, 13]. One study showed a decrease in the chondrogenic phenotype in cartilage cells upon the addition of SB203580 [14]. Preclinical in vivo studies have shown that SB203580 inhibitors reduce inflammatory lung injury [15], RA [16] and cardiac dysfunction [17, 18]. Analysis of these data, collectively, suggests that the level of p38 phosphorylation plays a direct role in cartilage homeostasis. Therefore, the aim of this study was to investigate the p38 pathway in OA tissue samples and assess the effects of SB203580 in normal rats and rats challenged with OA.

Materials and methods

The study was conducted in compliance with the ethical principles derived from the Declaration of Helsinki and was approved by the local institutional review boards (Queensland University of Technology and Prince Charles Hospital Ethics Committees, number 0700000157).

All patients gave their informed consent to participate in this in vitro study. Animal ethics approval for this project was granted from the Queensland University of Technology and the Prince Charles Hospital Ethics Committees (Ethics number 0900001134). Male Wistar Kyoto rats (11–12 weeks old) were purchased from the Medical Engineering Research Facility (MERF) (Brisbane, Australia). Animals weighing about 300–350 g were used for this experiment.

Isolation of cartilage cells

Normal articular cartilage chondrocytes (ACCs) were obtained from knee medial compartment tibial joint cartilage from tissue donors (n = 4) who were undergoing above-the-knee amputations due to traumatic injury. Normal patients were healthy adults with no clinical signs or symptoms of joint, metabolic or hormonal diseases (osteoporosis). None of the patients were taking medications that might affect cartilage or bone metabolism. All patients selected for this study had ceased taking anti-inflammatory medication at least 2 weeks before surgery. Early-stage OA patients were excluded if the samples showed any evidence of cartilage changes such as softening of the hyaline articular cartilage, thinning and fibrous dislocation, ulcerations of the cartilage or light sclerosis of the subchondral bone. OA ACCs (n = 4) were sourced from the main defective area of medial compartment knee tibial joint cartilage from patients undergoing total knee replacement surgery. All radiographs were reviewed, and the patient samples were classified accordingly into three categories on the basis of a modified Mankin score [19, 20]. This score assesses structure (0–6 points), cellularity (0–3 points), matrix staining (0–4 points) and tidemark integrity (0–1 point), and has a maximum of 14 points. The final score for each cartilage was based on the most severe histological changes observed in multiple sections from each specimen. The Mankin score was again divided into three stages depending on the score: Grade 0–1 (normal cartilage), Grade 2 (mild to moderate degenerative change, 2–9 points), and Grade 3 (severe degenerative change, ≥10 points). In this study, normal cartilage showed Grade 0 and OA cartilage showed Grade 3 cartilage. Chondrocytes were isolated from both normal and OA patients using enzymatic digestion, as described previously [7, 21]. Passage 2–3 ACCs were used for the studies.

Detection of MAPK-p38 activation in ACC pellet cultures

The 2 × 105 cells of normal ACCs and OA ACCs were re-suspended in a serum-free chondrogenic media [high glucose DMEM supplemented with 10 ng/ml TGF-β3, 10 nM dexamethasone, 50 mg/ml l-ascorbic acid, 10 mg/ml sodium pyruvate, 10 mg/ml proline and ITS+ (final concentration 6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 mg/ml selenious acid, 5.33 mg/ml linoleic acid and 1.25 mg/ml BSA)] and centrifuged at 600g for 20 min to form a pellet. After 2 weeks of differentiation, protein from pellets was isolated and western blot technique was applied to see...
the expression difference of phosphorylated p38 (P-p38) in normal and OA ACCs.

**In vitro inhibition of P-p38 pathway using SB203580 in normal ACC pellets**

P-p38-specific inhibitor, SB203580, was used to study the pathway-mediated cellular phenotypic changes in the normal ACC pellets. Briefly, normal ACCs were incubated with or without SB203580 after dissolving the concentrated stock solutions of each inhibitor in dimethylsulphoxide (DMSO). The final concentration of DMSO never exceeded 0.1% (v/v) and the same amount of DMSO vehicle was added to the control medium. The medium was replenished every 3 days. Our previous experiments demonstrated an optimum concentration of 5 μM of SB203580 for p38 inhibition in ACC pellets [7]. At these concentrations there was no observable change in the proliferation rates between control cells and inhibitor-treated cells, nor was there any evidence of cytotoxicity, as assessed by lactose dehydrogenase (LDH) assays (data not shown). All experiments were performed in triplicate. After 2 weeks of differentiation in the presence or absence of inhibitor, some pellets were stained with Alcian blue or immunostained with Type 10 collagen (COL10) and Aggrecan (AGG) antibodies as described previously [7]. Some pellets were used to extract the total RNA to see the gene expression of hypertrophy and chondrogenic markers using protocols as described previously [7, 21].

**Rat OA models**

Rats were anaesthetized via intraperitoneal (i.p.) injection with Zoletil (tiletamine 15 mg/kg and 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 towards 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. A sham group on the left knee was subjected to the same surgical procedure, without excision of the ligament or any meniscus manipulation. After the surgery, all rats received pain killer (buprenorphine 0.05 mg/kg) and antibiotics (cephalothin sodium 20 mg/kg and gentamicin 5 mg/kg).

**Study design and drug administration**

The rats were divided randomly into the following four groups (n = 12 (six animals were used for histology + six animals for western blotting)): Group 1, normal rats treated with DMSO + saline (vehicle alone); Group 2, normal rats treated with SB203580; Group 3, OA rats treated with DMSO + saline; and Group 4, OA rats treated with SB203580. Rats received either i.p. SB203580 (50 mg/kg in 0.25 ml) or diluents only (DMSO + saline). This concentration was chosen based on the published reports known to sufficiently raise plasma drug levels [22–24]. On the 30th day of the experimental period, the rats were euthanized with lethabarb (200 mg/kg i.p.), and the knee joints were collected for downstream observations.

**Morphological characterization**

Whole knee joints were removed by dissection, fixed in 4% paraformaldehyde and decalcified in 10% EDTA. After dehydration and paraffin embedding, serial 5 μm sagittal sections from the lateral and medial compartment of the joint were cut. Two sections obtained at 100 μm intervals from the non-weight-bearing region and weight-bearing region of each knee joint were stained with Safranin-O/Fast Green. OA severity in the tibial plateau was evaluated according to modified Mankin histological grading system (Mankin score 0–14) [19, 20], and a cartilage destruction score was assigned for each knee sample by three independent assessors. For Safranin-O/Fast Green staining, 5 μm paraffin-embedded sections of tibia from mice were counterstained with haematoxylin before being stained with 0.02% aqueous Fast Green for 4 min (followed by three dips in 1% acetic acid) and then 0.1% Safranin-O for 6 min. The slides were then dehydrated and mounted with crystal mount medium.

**Cartilage thickness**

The depth of articular cartilage in the medial compartment tibial knee was measured using semi-automatic Image J software (National Institutes of Health) using sections stained with Safranin-O, which provided excellent colour discrimination between bone and cartilage. The regions of interest on the femoral condyles were drawn using software and divided on the basis of the load-bearing areas of the knee during locomotion. The total thickness of the cartilage was determined by measuring the distance from the medial compartment of the superficial border of non-calcified cartilage to the boundary with the zone of calcified cartilage according to the length proportion. For each condylar section, the average of three measurements was used for statistical analysis.

**Western blotting**

Total protein lysates were harvested by lysing the cartilage tissue samples with a lysis buffer containing 1 M Tris–HCl (pH 8), 5 M NaCl, 20% Triton X140, 0.5 M EDTA and a protease inhibitor cocktail (Roche, Castle Hill, Australia). The cell lysate was clarified by centrifugation and the protein concentration was determined by a bicinchoninic acid protein assay (Sigma, Castle Hill, Australia). Five micrograms of protein were separated by 12% SDS–PAGE, transferred to a nitrocellulose membrane and blocked with a Tris–TWEEN buffer containing 5% non-fat milk. The membranes were incubated with phosphorylated p38 or tubulin primary antibodies (1:1000) overnight at 40 °C. After washing the membranes three times in Tris buffered saline (TBS)–TWEEN buffer, they were incubated with anti-rabbit secondary antibody at 1:2000 dilutions for 1 h. The protein bands were visualized using the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Castle Hill, Australia) and exposed on X-ray film (Fujifilm, Stafford, Australia).
Immunohistochemistry

Immunohistochemistry was carried out using an indirect immunoperoxidase method. Tissue slices were dewaxed in xylene and dehydrated in ethanol. Endogenous peroxidases were blocked by incubation in 0.3% peroxide in methanol for 30 min following repeated washing in PBS. The sections were then incubated with proteinase K (DAKO Multilink, CA, USA) for 20 min for antigen retrieval. Next, all sections were treated with 0.1% BSA with 10% swine serum in PBS. Sections were then incubated with optimal dilution of primary antibody overnight at 4°C [P-p38, 1:100 (Genesearch Pty. Ltd, Queensland, Australia); COL10, 1:50 (Santa Cruz biotechnology, USA); AGG, 1:200 (Millipore, New South Wales, Australia); and MMP-13, 1:200 (Labvision, Fremont, CA, USA)]. The optimum concentration of antibodies was determined by using a series of dilutions. Next day, sections were incubated with a biotinylated swine-anti-mouse, rabbit, goat antibody (DAKO Multilink, CA, USA) for 15 min, and then incubated with horseradish peroxidase-conjugated avidin-biotin complex for 15 min. Antibody complexes were visualized by the addition of a buffered diaminobenzidine substrate for 4 min and the reaction was stopped by immersion and rinsing of the sections in PBS. Sections were lightly counterstained with Mayer's haematoxylin and Scott's blue for 40 s each, in between 3 min rinses with running tap water. Following this, they were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a coverslip using DePeX mounting medium.

Antibody complexes were visualized by the addition of a buffered diaminobenzidine substrate for 4 min and the reaction was stopped by immersion and rinsing of the sections in PBS. Sections were lightly counterstained with Mayer’s haematoxylin and Scott’s blue for 40 s each, in between 3 min rinses with running tap water. Following this, they were dehydrated with ascending concentrations of ethanol solutions, cleared with xylene and mounted with a coverslip using DePeX mounting medium. Controls for the immunostaining procedures included conditions where the primary antibody or the secondary (anti-mouse IgG) antibodies were omitted. In addition, an irrelevant antibody (anti-CD-15), which was not present in the test sections, was used as a control. For semi-quantitative data, at 400× magnification, the positive cells were counted from each field of observation from medial compartment femur knee. At least three observation fields were selected from each section and three sections were selected from each sample. To eliminate the difference in total cell number in each observation field, the positive cell numbers were normalized to the cell number per 100 total cells in each specific group.

Terminal dUTP nick end-labelling analysis

Terminal dUTP nick end-labelling (TUNEL) is a specific immunohistochemical technique that enables sensitive and specific staining of the high concentrations of DNA 3'-OH ends that are localized in apoptotic bodies. TUNEL analysis on tissue sections was performed by first permeabilizing the tissue with proteinase K solution for 30 min at 37°C. Following permeabilization, the slides were washed with PBS and the TUNEL reaction was performed using the fluorescein in situ cell death detection kit (Roche, Germany) following the manufacturer’s protocol. As a positive control, sections were treated with DNase 1 for 10 min at room temperature prior to the labelling procedure to induce DNA strand breaks. The TUNEL reaction mixture/terminal transfection was omitted for the negative control. Slides were viewed and analysed using the microscope and the percentage of positive cells was calculated as described above.

Statistical analysis

Statistical analysis was performed using the statistical package from GraphPad Prism (version 4.0). The unpaired Student’s t-test was used for comparisons of P-p38 expression in Grade 0 and 3 cartilages, the differential expression of COL2, AGG, COL10, CBFa1 and MMP-13 in the ACC culture, and the difference of positive cell numbers for AGG, COL10 and MMP-13 in normal, sham and SB203580 application rats. The Mannik score and cartilage thickness in different treatment groups (normal + vehicle, normal + SB203580, OA + vehicle and OA + SB203580) were subjected to one-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) tests. Data were expressed as the mean (± S.D.). A P-value <0.05 was considered to be statistically significant.

Results

Patient demographics

The mean age and BMI of OA study participants were 63.7 (2.8) years and 26.8 (0.6) kg/m², respectively. The mean age and BMI of normal study participants were 59.3 (2.4) years and 25.1 (0.7) kg/m², respectively. Both normal and OA cartilage samples were obtained from males only. No significant statistical differences were observed with respect to all the above parameters when compared in-between the groups.

Increased p38 expression in cartilage tissue and ACCs collected from OA patients

Characterization of normal and OA ACCs were performed to ensure the phenotypic stability by analysing the gene expression of COL2 and AGG. It was found that both normal and OA ACCs expressed significantly higher levels of COL2 and AGG. However, reduced levels of COL2 and AGG were observed in OA compared with normal ACCs (Fig. 1A). Levels of phosphorylated p38 were determined in the protein lysate collected from normal ACC and OA ACC pellets after differentiating for 2 weeks under chondrogenic conditions. It was found that the levels of P-p38 were reduced significantly.
In OA ACCs compared with normal ACCs (Fig. 1B).

Knee tibia joint sections from OA patients were collected and graded according to the disease severity based on the Mankin scale. Immunohistochemistry further confirmed the in vitro findings showing a decrease of p38 phosphorylated forms in OA cartilage tissue, suggesting that the p38 MAPK pathway may be more active in normal compared with OA cartilage (Fig. 1C, D and E).

In vitro inhibition of P-p38 pathway using SB203580 led to a decrease of chondrogenic and an increase of hypertrophic phenotype in normal ACCs.

Normal ACC pellets were differentiated for 2 weeks in the presence or absence of SB203580. After 2 weeks, ACC pellets were stained with Alcian blue to analyse the GAG deposition and chondrogenic phenotype. When normal ACC pellets were differentiated in the presence of SB203580, a decrease in GAG deposition and AGG expression and an increase in hypertrophic marker COL10 were found in ACC pellet cultures (Fig. 2A). These observations were further confirmed by the gene expression showing a decrease in the expression of COL2 and AGG in the presence of SB203580 (Fig. 2B). On the other hand, COL10, Runt-related transcription factor 2 (RUNX2) and MMP-13 were increased in the presence of SB203580, emphasizing the importance of this p38 signalling pathway in maintaining cartilage function (Fig. 2C).

Tissue p38 MAPK measures in rats

High basal activity of p38 was observed in the normal untreated animal groups compared with OA groups. Both normal and OA rats were treated with SB203580 (50 mg/kg in 0.25 ml i.p.) or diluents only (DMSO + saline). On the 30th day of the experimental period, western blot with the cartilage homogenate was performed to verify whether that SB203580 inhibits the activity of p38 MAPK in the in vivo cartilage. The results showed that the induction of p38 MAPK activity was suppressed significantly to a level far below baseline when animals were administered SB203580, demonstrating the efficacy of the drug to block p38 activity (Fig. 3A). Down-regulated P-p38 expression was associated with an increased Mankin score (Fig. 3B) and a decrease in cartilage thickness (Fig. 3C). As expected, the Mankin score was 0 in untreated groups, and of interest, the degenerative changes were substantially increased in sham and OA animals upon administration of SB203580.

Effect of SB203580 in normal and OA-induced rats

After determining the efficacy of SB203580, both a normal and an OA experimental animal model (induced by meniscectomy) were used to evaluate the effects of SB203580 treatment on the development of the clinical and pathological manifestations of the disease.
In the joints of normal (treated with vehicle alone) rats, no macroscopic changes were detected on the articular surfaces of femoral condyles and tibial plateau. Normal untreated animals showed the healthy appearing cartilage with intact superficial, mild and deep zones that stained deeply with Safranin-O (red) for GAG. The chondrocytes were arranged in columns in untreated animals. However, when the animals were treated with SB203580, a significant loss of matrix staining was observed, which is consistent with decrease of P-p38 immunostaining in the cartilage (Fig. 4A, B and C).

When the effect of SB303580 was tested on the OA model, it was found that the meniscectomy-induced OA knees revealed an extensive degeneration of cartilage, surface roughness, fibrillation, small osteophytes or areas of peripheral fibrous tissue proliferation in both tibia and femur where the meniscus was removed; however, the damage appears much higher when OA models were treated with the SB203580 (Fig. 4A, B and C). These results collectively indicate that the inhibition of the p38 pathway led to more severe damage to the structure of cartilage.

Expression of cartilage markers and hypertrophy markers in the SB203580-treated animals

We found a strong presence of AGG (78% of total cartilage area) protein expression in articular cartilage of normal rats (treated with vehicle alone), whereas cartilage of the normal knee cartilage treated with SB203580 showed minimal AGG (40% of total cartilage area) staining (Fig. 5A, B and C). These data indicated to us that the deprived expression of p38 expression led to phenotypic changes of cartilage even in the normal animals. In contrast to the above findings, we found that the expression of COL10 (Fig. 5D, E and F) and MMP-13 (Fig. 5G, H and I) were significantly up-regulated in normal animals treated with SB203580 compared with corresponding controls, which indicated a transition to hypertrophy phenotype in the absence of the P-p38 pathway.
Inhibition of p38 pathway leads to OA in rat model

**Fig. 3** Inactivation of p38 kinase after treating animals with SB203580 led to an increased cartilage Mankin score and decreased cartilage thickness. (A) P-p38 activity levels were reduced to a level far below baseline when normal animals were administered SB203580, demonstrating the efficacy of the drug in blocking p38 activity. Representative bands obtained from six different animals with similar results are shown. (B and C) Graphs showing the increased Mankin score and decreased cartilage thickness in both normal and OA rats that were treated with SB203580, indicating that the lack of P-p38 activity is pathological for the articular cartilage. Results are shown as mean (s.d.) \( (n=6) \). Asterisk represents a significant difference \( (P \leq 0.05) \).

**Fig. 4** Histological findings demonstrating the detrimental effects on the cartilage of rats treated with SB203580. (A) Gross morphological cartilage changes in the normal and meniscectomy-induced OA rats that were treated with or without SB203580, demonstrating the efficacy of the drug in blocking p38 activity. Arrows point to damaged surface area. (B) Safranin-O staining demonstrated a higher rate of proteoglycan depletion in both normal and OA animal models when treated with SB203580 compared with their respective controls. Scale bars: 100 \( \mu \)m. (C) Immunostaining showing that p38 phosphorylation was significantly decreased in the animal models treated with SB203580. Scale bar: 50 \( \mu \)m.
Enhanced expression of TUNEL-positive chondrocytes in the SB203580-treated animals

Cartilage degradation of the joint is characteristically accompanied by apoptosis-related cascades. To test this hypothesis, normal rats that were treated with SB203580 were examined for apoptosis using the TUNEL assay. Very few apoptotic (TUNEL-positive green fluorescent) cells were observed in cartilage from untreated rats. Treatment with SB203580 caused a modest increase in the number of apoptotic cells in the cartilage (Fig. 6A, B and C). These results clearly demonstrate that a decrease in p38 phosphorylation is capable of inducing apoptosis of cartilage cells even in normal rats.

Animal behavioural studies

No significant differences were found with respect to behaviour, assessment of physical appearance and measurement of body weight in SB203580-treated vs untreated animals (data not shown).

Discussion

Growing evidence shows that the reversal of structural remodelling represents a key therapeutic target in OA management and treatment. Given the central role of p38 kinase in the regulation of cellular stress response mechanisms, modulation of p38 kinase activity represents an attractive therapeutic approach in the treatment of several diseases. Indeed, small molecule p38 inhibitors have been suggested to have potentially beneficial effects in pulmonary disease [25], septic shock [24] and RA disease [26]. The concept of manipulation of the phosphorylation level of p38 as a potential treatment for OA is based on the assumption that p38 kinase is activated in these diseases. However, it is surprising to note that the detailed change in p38 kinase activity in OA cartilage has not been previously reported.

Here, we found that P-p38 is highly expressed in normal human cartilage, which suggests that cartilage must generate significant levels of P-p38 for tissue maintenance and homoeostasis. However, P-p38 is significantly
reduced in diseased OA cartilage, confirming and expanding on our previous data for P-p38 levels at the cellular level of normal and OA chondrocytes. In line with this evidence, P-p38 expression is significantly down-regulated in the OA animal model \textit{in vivo}, raising the possibility that those alterations in P-p38 expression are associated with the earliest stages of OA pathogenesis.

To understand the precise role of the p38 pathway, normal chondrocytes pellets were incubated with SB203580 to inhibit phosphorylation of the p38 pathway. We found that the lack of p38 expression led to a decrease in chondrogenic phenotype and an increase in the hypertrophic phenotype. Consistent with our results, Li \textit{et al.} [27] showed regulation of chondrogenesis by the p38 pathway as a delicate balance, interacting with the TGF-β1/Smads signalling pathway. Similarly, Zhang \textit{et al.} [28], using genetic inhibition studies, showed a role of p38 in chondrocyte differentiation and suggested that Sox9 is a downstream target of the p38 MAPK pathway. Furthermore, it has been shown previously that complete disruption of p38 is lethal, suggesting that maintenance of normal levels of p38 activity is necessary for normal embryonic development [29]. The data obtained in this study show that apoptosis in normal rat cartilage (TUNEL-positive chondrocytes) is significantly increased when treated with p38 inhibitor. These observations suggest that a significant proportion of chondrocytes die by apoptosis in the absence of basal p-38 phosphorylation levels. Under normal conditions mature articular chondrocytes are kept in a state of maturational arrest. In contrast to this phenotype, it has been shown that OA chondrocytes express hypertrophic differentiated markers such as COL10, ALP and RUNX2 [30]. It has been reported that hypertrophy of chondrocytes can lead to degenerative changes in the cartilage by up-regulating the expression of MMPs [31]. These results together indicate that loss of P-p38 activity can lead to phenotypic changes of articular chondrocytes to a hypertrophic phenotype, which could be pathological to the adult mature cartilage structure. In line with our findings, Stanton \textit{et al.} [4] showed elevated RUNX2, Osterix and OC transcript levels in chondrocyte cultures upon inhibition of p38 activity with a pharmacological inhibitor, suggesting that loss of p38 signalling leads to chondrocyte hypertrophy during skeletal maturity.

At the \textit{in vivo} level we demonstrated for the first time that an inhibitor of the p38 pathway (SB203580) worsened cartilage degeneration in both normal and OA animal models, evidenced by an increase in the Mankin score and a decrease in cartilage thickness. The decrease in P-p38 levels was associated with a decrease in the chondrogenic and an increase in the hypertrophic phenotype.
Consistent with our findings it has been recently demonstrated that the genetic reduction of p38 MAPK activity in cartilage leads to phenotypic changes in bone and/or cartilage in adult mice [32]. Although this study suggests a protective role for p38 kinase both in vitro and in vivo, a number of studies have shown the destructive role of p38 when it is up-regulated. It has been reported that up-regulated p38 phosphorylation levels can lead to increased cytokine production by several mechanisms, such as direct phosphorylation of transcription factors [33]. One study has also found attenuation of cartilage degeneration and OA pain in animal models due to up-regulated p38 levels, suggesting that p38 inhibitors may be a useful approach in the treatment of OA [34]. These results indicate the phosphorylation levels of p38 need to be well-balanced at a physiological level in order to maintain cartilage health, and both inhibition and up-regulation of the p38 signalling are detrimental to cartilage. Several studies do support a considerable cross-talk between the different MAPKs subtypes, including interactions between inflammatory/stress-activated signal pathways and hormone/growth factor-activated signal pathways [35]. In our previous study we demonstrated the cross-talk between ERK and p38 pathways, and showed that an up-regulation of ERK phosphorylation levels can lead to a decrease in P-p38 levels and vice versa, which in turn can lead to a cartilage hypertrophy and degradation, indicating the dynamic and balanced interactions of MAPK pathways are required for cartilage homeostasis [7].

Rheumatology key messages

- The present study revealed reduced p38 kinase activity as a causative factor in OA.
- Normalization of p38 kinase activity may represent a new preventive approach against progression of OA.

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