FK506 protects against articular cartilage collagenous extra-cellular matrix degradation


Objective: Osteoarthritis (OA) is a non-rheumatologic joint disease characterized by progressive degeneration of the cartilage extra-cellular matrix (ECM), enhanced subchondral bone remodeling, activation of synovial macrophages and osteophyte growth. Inhibition of calcineurin (Cn) activity through tacrolimus (FK506) in vitro monolayer chondrocytes exerts positive effects on ECM marker expression. This study therefore investigated the effects of FK506 on anabolic and catabolic markers of osteoarthritic chondrocytes in 2D and 3D in vitro cultures, and its therapeutic effects in an in vivo rat model of OA.

Methods: Effects of high and low doses of FK506 on anabolic (QPCR/histochemistry) and catabolic (QPCR) markers were evaluated in vitro on isolated (2D) and ECM-embedded chondrocytes (explants, 3D pellets). Severe cartilage damage was induced unilaterally in rat knees using papain injections in combination with a moderate running protocol. Twenty rats were treated with FK506 orally and compared to twenty untreated controls. Subchondral cortical and trabecular bone changes (longitudinal microCT) and macrophage activation (SPECT/CT) were measured. Articular cartilage was analyzed ex vivo using contrast enhanced microCT and histology.

Results: FK506 treatment of osteoarthritic chondrocytes in vitro induced anabolic (mainly collagens) and reduced catabolic ECM marker expression. In line with this, FK506 treatment clearly protected ECM integrity in vivo by markedly decreasing subchondral sclerosis, less development of subchondral pores, depletion of synovial macrophage activation and lower osteophyte growth.

Conclusion: FK506 protected cartilage matrix integrity in vitro and in vivo. Additionally, FK506 treatment in vivo reduced OA-like responses in different articular joint tissues and thereby makes Cn an interesting target for therapeutic intervention of OA.

Introduction

FK506 (Tacrolimus, Prograf) is an immunosuppressive drug discovered by Kino et al. in the 1980s. Since then, it has been used clinically for an increasing number of immunological disorders. FK506 exerts its therapeutic effects by suppression of T-cell activation, without markedly affecting bone marrow cell differentiation and proliferation. Through binding to FK506-binding proteins (FKBPs), FK506 inhibits the activity of ubiquitously expressed calcium/calmodulin dependent calcineurin (Cn). As a consequence, the Cn mediated dephosphorylation of transcription factors of the nuclear factor of activated T-cells (NFATs) family (NFAT1-4) is inhibited.

Besides their role in T-cell activation, Cn and NFATs are now also known to play a role in physiological processes in many other cells.

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and tissue types and pathological conditions like cancer, degenerative brain diseases and cardiac hypertrophy. FK506 has proven to be useful in reducing inflammation and alleviating symptoms in patients with inflammatory (rheumatoid) arthritis. Interestingly, the Cn/NFAT signaling cascade is also reported to play a role in bone remodeling and chondrogenesis. FK506 has been shown to induce chondrogenic differentiation of murine chondroprogenitor cells. This suggests that patients with non-inflammatory joint diseases, like osteoarthritis (OA), also might benefit from a treatment with CN inhibitors.

OA is a complex progressive disease and a disturbed balance between anabolic and catabolic activity of chondrocytes is an early pathophysiological event leading to matrix degradation. Progression of OA finally results in severe deterioration of articular cartilage and involves pathological changes throughout the joint, like extensive subchondral bone remodeling and activation of synovial macrophages. We reported earlier that FK506 treatment of monolayer cultured osteoarthritic cells enhanced expression of anabolic markers like collagen type II (COL2), but suppressed relevant catabolic, hypertrophy and mineralization markers. Another Cn inhibitor, cyclosporine A (CsA) showed similar effects on anabolic and catabolic activity of OA chondrocytes and reduced cartilage damage in a condensation induced OA mouse model. However, this study only measured macroscopical and microscopic cartilage damage ex vivo and did not investigate possible effects of CN inhibition on other tissues of the joint, like bone and synovium.

Recently, we established a novel rat OA model using a combination of papain injections with a running protocol to induce severe knee joint articular cartilage damage together with prominent involvement of subchondral bone and synovial macrophages. The current study aimed to elucidate the effects of systemic FK506 treatment in this OA animal model. We first characterized whether both low and high concentrations of FK506 modulate anabolic markers in OA chondrocytes in monolayer cultures. Since chondrocytes reside in an extra-cellular matrix (ECM) in vivo, we additionally investigated whether low dose FK506 treatment remains beneficial for ECM-embedded chondrocytes in ex vivo explants and pellet cultures. Finally, we tested modulating effects of FK506 in a synthetic knee model for severe cartilage degradation and analyzed articular cartilage (ex vivo µCT and histology), subchondral bone (in vivo µCT) and synovial macrophages activation (in vivo SPECT/CT) 6 and 12 weeks after induction of cartilage damage.

**Methods**

**FK506 effects on osteoarthritic chondrocytes in vitro**

Human articular cartilage was explanted from macroscopically normal areas of femoral condyles and tibial plateaus of patients (N = 9, between 55 and 82 years old) undergoing total knee replacement surgery for OA (MEC2004-322). Isolation of primary osteoarthritic human articular chondrocytes from cartilage under standard conditions (cytokine-free Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/L glucose, 10% fetal calf serum (FCS), 50 µg/mL gentamycin and 1.5 µg/mL fungizone; all Invitrogen, Paisley, Scotland, UK) adjusted to 380 mosm by adding sterile sodium chloride) and monolayer experiments were performed as described earlier. In short, passage 1 cells were seeded in 2D monolayer, stimulated with 0, 62 or 620 nM FK506 after 24 h and harvested for RNA analysis (quantitative RT-PCR) 6 days later. Experiments were performed at least in technical duplicates from four OA donors. In addition to 2D cultures, passage 2 cells from four OA donors were cultured as 3D pellets (2 × 10^5 cells/pellet) for 21 days in medium (380 mosm) with or without addition of 62 nM FK506.

To investigate the effects of FK506 on OA chondrocytes embedded in their ECM, 6 mm diameter full-thickness explants from femoral condyles and tibial plateaus of five OA donors were cultured as described before and cultured in medium (380 mosm) with or without 62 nM FK506 for 7 days.

**RNA and protein analysis by RT-QPCR and histology**

RNA from monolayer and explant cultures was extracted, purified and quantified, and cDNA was synthesized and quantified by RT-QPCR reactions as described earlier. RNA abundance was normalized to an index of the three most stable reference genes (GAPDH, HPR1, 18sRNA or UBC) replicate values were averaged per condition per patient and gene expression was calculated as fold change of control condition (0 nM FK506). Primer sequences for COL2, ACAN, MMP1, MMP13, ADAMTS4 and ADAMTS5 were adopted from Utterlinden et al., for COL1 from Das et al., and for VCAN/CSPG2 from Martin et al.. To quantify expression of COL9 and COL11, the following primers were tested for similar amplification efficiency and specificity, and were used as respectively 20 µl TaqMan and SYBR Green 1 reactions: HsCOL9A1_Fw AGGAGAGTTGAGAATTGGGAATC, COL9A1_Rv TGG CATGGCAAGTTTCTCT, COL9A1_R GCTTTGCTGTGCTGGGAAAA and COL11A1_Fw AGGAGGTGAGAATTGGGAATC, COL11A1_Rv TGG TGATCGAGATGACATTCG.

The expression data are presented as 2^-ΔΔCt values based on publication by Livak & Schmittgen. The CDA abundances of each gene of interest were normalized to an index of three stable expressed reference genes to generate a normalized, so-called 2^-ΔΔCt value. Replicate values were averaged per condition per patient, and finally expressed as fold change difference relative to the control condition (i.e., without FK506 treatment) and representing a 2^-ΔΔCt value. The COL2/Col1 ratio, like the VCAN/CSPG2 ratio, relates the expression levels of both genes to one another. Relative higher Col2 expression, as compared to Col1, is indicative of a relatively better preserved chondrocyte-specific gene expression. This also holds for VCAN/CSPG2 ratios, and was used earlier as a measure of de-differentiation of chondrocytes and for (Immuno)histochemical staining for COL2 and GAG on 3D pellet cultures was performed as described before. Staining intensities of pellets were quantified using ImageJ 1.42 software (http://rsb.info.nih.gov/ij/download.html).

**FK506 therapeutic effects in a rat model for severe OA**

Forty-six-week-old male Wistar rats (Charles River Netherlands BV, Maastricht, the Netherlands) were housed in the animal facility of the Erasmus Medical Centre, with a 12-h light–dark regimen, at 21°C during the experimental period, and received standard food pellets and water ad libitum. OA-like articular destruction was induced in all animals, which were then divided over two groups: 20 rats served as untreated controls and 20 rats were treated during the experiment with FK506. FK506 treated animals received FK506 suspension (1 mg/kg) through oral probing, 5 days a week, not on weekends.

Severe cartilage damage was induced using intra-articular papain injections in the left knee joints combined with exposure to a moderate exercise protocol as described before. In short, all animals received three intra-articular injection that consisted of 15 µl 4% (w/v) papain solution (type IV, double crystallized, 15 units/mg, Sigma–Aldrich, St. Louis, MO, USA) with 15 µl 0.03 M L-cystein (Sigma–Aldrich) through an internal knee joint served as an internal healthy control. All rats were forced to run on a motorized rodent treadmill (LE-8700; Panlab Harvard Apparatus, Barcelona, Spain) for 6 weeks covering a total distance of 15 km.
During the study all animals were longitudinally monitored at baseline, 6 weeks and 12 weeks with μCT to measure subchondral bone changes\(^{24}\). At 6 and 12 weeks, 10 rats in both groups were selected for a full analysis sequence. This sequence consisted of SPECT/CT to quantify in vivo macrophage activation\(^{25}\), and ex vivo Equilibrium Partitioning of an Ionic Contrast agent using μCT (EPIC-μCT) and histology to measure cartilage quality\(^{29}\). The details of these procedures were described earlier\(^{18}\). The animal ethics committee of the Erasmus Medical Center, Rotterdam, the Netherlands, approved all conducted tests. A detailed planning scheme of all groups and conducted tests is given in Fig. 1.

**Subchondral bone measurements on μCT scans**

Both knees of all animals were μCT scanned under isoflurane anesthesia, using a Skyscan 1176 in vivo μCT scanner (Skyscan, Kontich, Belgium). Ten minutes of scan time was required per knee at an isotropic voxel size of 18 μm, at a voltage of 65 kV, a current of 385 mA, field of view of 35 mm, using a 1.0 mm aluminum filter, over 198° with a 0.5 degree rotation step, and a 270 msec exposure time. All datasets were segmented with a local threshold algorithm\(^{27}\). Cortical and trabecular bone were automatically separated using in-house software\(^{28}\). Using Skyscan software, both subchondral plate thickness (Sp. Pl. Th. in μm) and trabecular plate porosity (Sp. Pl. Por. in mm\(^3\)) of the medial and lateral compartment of the tibial plateau were measured\(^{24}\). In the tibial epiphysis, the trabecular thickness (Th. in μm) and trabecular bone volume fraction (BV/TV), representing the ratio of trabecular bone volume (BV, in mm\(^3\)) to endocortical tissue volume (TV, in mm\(^3\)) were measured. Ectopic bone formation (mm\(^3\)) was also quantified as a measure for osteophyte growth in these longitudinal μCT scans.

**Determination of activated macrophages by SPECT/CT using \(^{111}\)In-EC0800**

Activated macrophages express the folate receptor-β allowing monitoring macrophages in vivo using folate-based radio-tracers\(^{25,30}\). Phosphate buffered saline (PBS, pH 6.5) DOTA-Bz-folate (EC0800, kindly provided by Endocyte Inc., West Lafayette, USA)\(^{31}\) was labeled with \(^{111}\)InCl\(_3\) (Covedien, Petten, The Netherlands) as described previously\(^{14}\). Quality control was performed with ITLC-SG and revealed a radiochemical yield of ~91% at a specific activity of 50 MBq/μg. \(^{111}\)In-EC0800 (55 MBq) was administrated via the tail vein 20 h prior to scanning. SPECT/CT scans were performed with a four-head multiplex multi-pinhole small animal SPECT/CT camera (NanoSPECT/CT\(^{TM}\), Bioscan Inc., Washington DC, USA). All knee joints were scanned with both helical μCT (acquisition time 5 min) and SPECT (acquisition time 30 min). All scans were analyzed using InVivoScope processing software (Bioscan Inc.). To reduce inter-individual variation, the absolute difference in measured radioactivity (kBq/mm\(^3\)) of the OA knee joint compared to their internal control joint was calculated. This absolute difference was used when comparing means of untreated animals with FK506 treated animals.

**Cartilage evaluation with contrast enhanced μCT and histology**

Equilibrium partitioning of a contrast agent using μCT (EPIC-μCT) has a strong correlation with cartilage sulfated-glycosaminoglycan (sGAG) content\(^{26}\). Animals were euthanized directly after the last SPECT/CT scan and both knee joints were harvested for EPIC-μCT analysis. All specimens were incubated in 40% solution of ioxaglate (Hexabrix320, Mallinckrodt, Hazelwood, MO, USA) for 24 h at room temperature\(^{33}\). EPIC-μCT was performed on the 1176 in vivo μCT scanner (Skyscan), using the following scan settings: isotropic voxel size of 18 μm, a voltage of 65 kV, a current of 385 mA, field of view 35 mm, a 0.5 mm aluminum filter, 198° with a 0.5 degree rotation step, and a 235 msec exposure time. In all EPIC-μCT datasets, X-ray attenuation (arbitrary gray values inversely related to sGAG content) and cartilage thickness (μm) was calculated for cartilage of the medial and lateral plateau of the tibia\(^{15}\).

After EPIC-μCT, the separated parts of the knee joints were fixed in 3.7% phosphate buffered formaldehyde, decalcified with formic acid and embedded in paraffin. Sagittal sections were made at 300 μm intervals and stained with safranin-O with a fast green counterstain to image the distribution of the GAGs. Sections were stained all at once, to minimize artifacts between different samples.

**Statistical analysis**

Statistical analysis of vitro studies was performed as described before\(^{11}\). Briefly, replicate raw expression data of multiple donors was tested for the effect of FK506 using Linear Mixed Model regression and ‘donor’ was incorporated as a random effect to correct for basal differences in expression between donors (SPSS Inc., Chicago, USA).

For the in vivo study, differences between means of OA induced and healthy knee joints within the same animal were tested using paired t-tests at each time point for all outcome parameters (GraphPad Software, San Diego, California, USA). When comparing differences between means of untreated animals and FK506 treated animals, an unpaired t-test was used at each time point for all outcome parameters (GraphPad Software). Longitudinal data from in vivo μCT were additionally analyzed using generalized estimating equations (SPSS). For all tests, P values ≤0.05 were considered significant.

**Results**

**In vitro effects of FK506 on human osteoarthritic chondrocytes**

Inhibition of Cn activity by FK506 in monolayer cultured passage 1 osteoarthritic chondrocytes increased expressions of cartilage specific collagens. Both low and high concentrations of FK506
positively stimulated COL2/COL1 ratio (62 nM FK506 by ±two-fold, \( P = 0.067 \); 620 nM by ±three-fold, \( P = 0.001 \)) and COL9 expression (62 nM FK506 by ±1.8-fold, \( P = 0.037 \)), while no effects were found on the ACAN/VCAN ratio or COL11 expression [Fig. 2(A)]. In cartilage explants, the osteoarthritic chondrocytes are embedded in a matrix, which might limit chondrocyte exposure to FK506. Despite the large standard deviations in the explants, we found clear matrix-protective trends after FK506 treatment. A low dose of FK506 was enough to induce a similar trend of increased anabolic marker expression in explants as seen in monolayer cultured chondrocytes [Fig. 2(B)]. Moreover, the FK506 induced changes in chondrogenic marker expression seem to be even higher in the explants. In line with our previous work on monolayer chondrocytes, FK506 also reduced the expression of the catabolic MMPs and important aggrecanases in the explant cultures [Fig. 2(B)].

The FK506 induced increase in collagen expression was confirmed by immunohistology on 3D pellets cultures of osteoarthritic chondrocytes. FK506 clearly increased COL2 protein expression (to 121.4% of control, \( P = 0.009 \)) in chondrocyte pellet cultures, while no clear effect was seen on GAG staining [Fig. 2(C)].

In vivo effects of FK506 treatment

Bodyweight of all untreated rats at baseline was 416.4 g (411.3–421.5 g), during 6 weeks of treadmill running this decreased non-significantly to a mean weight of 408.3 g (398.2–418.3 g). During subsequent 6 weeks of rest, all rats increased in their mean bodyweight to 485.5 g (473.0–498.0 g). FK506 treated animals (mean weight at baseline was 413.6 g; 409.4–417.8 g) also did not increase in bodyweight during induction of OA-like articular destruction (mean weight after 6 weeks was 418.5 g; 412.9–424.1 g). After 12 weeks their mean bodyweight was 507.1 g (498.8–515.4 g), which was significantly higher compared to untreated controls (\( P = 0.004 \)) (Supplementary Fig. 1 online). During the course of the experiment, none of the animals showed signs of FK506-induced cytotoxicity, like weight or hair loss.

Osteoarthritic changes of articular cartilage

Intra-articular papain injections combined with moderate exercise in untreated controls induced severe sGAG depletion from both medial and lateral cartilage compartments of the tibia plateau. This sGAG depleted state persisted throughout the experiment [Fig. 3(A),(B)]. After the running protocol at 6 weeks, cartilage of the medial compartment was slightly reduced in thickness [Fig. 3(C)]. Lateral cartilage thickness was severely degraded [Fig. 3(D)] and resulted in almost completely denuded subchondral bone [Fig. 3(E)]. During subsequent 6 weeks of rest medial cartilage continued to degrade, in the lateral compartment an ongoing decline in cartilage thickness was absent [Fig. 3(C)]. Representative medial and lateral cartilage images from safranin-O stained histology from untreated controls at 6 and 12 weeks are shown in Fig. 4.

Compared to untreated controls, FK506 treated animals had similar sGAG depleted cartilage in medial and lateral compartments of the tibia plateau [Fig. 3(A),(B)]. Lateral cartilage was reduced in thickness to similar extent as untreated controls [Fig. 3(D)]. However, medial cartilage showed a trend towards thicker ECM compared to untreated control at 6 weeks, although this was not significant (\( P = 0.15 \)). In contrast to the progressive degradation of medial cartilage as seen in untreated controls at 12 weeks, medial cartilage thickness of FK506 treated animals remained constant and was significantly thicker at 12 weeks compared to untreated controls (\( P = 0.02 \)) [Fig. 3(C)].

Although lateral cartilage did not differ significantly in sGAG content or thickness between untreated and FK506 treated rats, we did observe a small difference between both groups. In four FK506...
treated rats, but in none of the untreated rats, there were small and focal regions of lateral tibia cartilage that showed intact but totally sGAG depleted ECM [Fig. 3(E)]. A difference that was also found on safranin-O stained histology sections as shown in Fig. 4.

**Subchondral bone changes**

Subchondral bone plate thickness of medial tibia compartment in untreated controls and FK506 treated rats was slightly reduced after 6 and 12 weeks of follow-up [Fig. 5(A)], but did not differ between both groups \( (P = 0.83) \). Medial plate porosity did not increase in both experimental groups throughout the experiment [Fig. 5(B)]. Lateral compartment subchondral bone thickness of untreated OA joints was clearly increased compared to their healthy control joint at 6 weeks \( (P < 0.0001) \), and there was also more subchondral plate porosity \( (P = 0.02) \) [Fig. 5(C),(G),(H)]. Subchondral plate thickness further increased during subsequent 6 weeks of rest \( (P < 0.0001) \). Plate porosity also seemed to increase further, but there was no significant difference compared to internal healthy control joints. FK506 treated animals also had a thicker subchondral bone plate at 6 \( (P < 0.0001) \) and 12 \( (P < 0.0001) \) weeks compared to their internal healthy control joints. When longitudinal subchondral bone changes in OA joints of both groups were analyzed with generalized estimating equations, FK506 treated rats had thinner lateral subchondral bone plates compared to untreated controls \( (P = 0.03) \) [Fig. 5(C),(G)]. FK506 rats did not develop subchondral plate porosity. This was significantly lower at 6 weeks \( (P = 0.02) \), but not at 12 weeks anymore [Fig. 5(D),(H)]. After 6 weeks of treadmill exercise-mediated trabecular bone thickness \( (P = 0.05) \) and BV/TV \( (P = 0.02) \) was lower in FK506 treated animals compared to untreated controls [Fig. 5(E),(F)]. Reduced trabecular thickness normalized during subsequent 6 weeks of rest, while the BV/TV ratio increased compared to untreated controls \( (P = 0.02) \).

During 6 weeks of moderate running, FK506 treated animals formed less ectopic bone formation compared to untreated animals \( (P = 0.007) \) [Fig. 6(B),(C)]. This difference in ectopic bone formation between FK506 treated animals and untreated controls was still measured after the subsequent 6 weeks of rest \( (P = 0.04) \) [Fig. 6(B),(C)].

**Macrophage activation and osteophytes**

Each animal received 54 ± 2 MBq of \(^{111}\)In-EC0800 under isoflurane anesthesia. There were no significant differences of injected activity between experimental groups. After completion of the running protocol, both untreated and FK506 treated rats revealed similarly increased radioactive uptake in their papain injected knee joints compared to their internal healthy control joints [Fig. 6(A),(C)]. After 6 subsequent weeks of rest, radioactive uptake in OA induced joints of FK506 treated animals dropped to control levels. The absolute difference in radioactive uptake between OA induced and healthy control joints in FK506 treated animals was lower compared to the absolute differences measured in untreated controls [Fig. 6(A),(C)].

**Discussion**

OA is characterized by a loss of cartilage matrix, because chondrocytes cannot maintain tissue homeostasis due to a disturbed balance between anabolic and catabolic activities. Inhibiting Cn activity with immunosuppressive agents like cyclosporin A\(^ {13} \) or FK506\(^ {11,12} \) increases the anabolic, while suppressing the catabolic, activity of osteoarthritic chondrocytes. In this study, we found that both high and low concentrations of FK506 improved the COL2/COL1 ratio and COL9 expression in monolayer cultured human osteoarthritic chondrocytes [Fig. 2(A)]. Then, in 3D chondrocyte
pellet cultures, FK506 clearly increased COL2 content, while no effect was seen on sGAG staining [Fig. 2(C)]. These data indicate that Cn inhibition through FK506 may protect the structural integrity of the ECM. Next, we studied the effects of low dose FK506 treatment in cartilage explants, in which the chondrocytes are still embedded in their native ECM. The explants were harvested from macroscopically 'healthy' cartilage areas of the degenerated side. However, on microscopic level there might be still big differences in grade of degeneration between explants of the same donor. To limit the effects of these differences, explants were first pooled before assigning them to a certain culture condition. Despite the large standard deviations, we found a clear trend towards stimulated anabolic but reduced catabolic activities after FK506 treatment [Fig. 2(B)].

Finally, we evaluated whether FK506 also exerts similar favorable effects in a severe OA in vivo model with a different response in medial and lateral compartments of tibia plateau cartilage. Six weeks of OA-like damage induction severely erodes lateral compartment cartilage and results in complete denudation of subchondral bone. Medial cartilage becomes sGAG-depleted with a slightly degraded ECM, a process that continued progressively during the course of the experiment [Figs. 3 and 4]. FK506 treatment in vivo did not increase sGAG levels nor did it protect against sGAG loss [Fig. 3(A);B]. However, it did protect against structural matrix degradation [Fig. 3(C)], which was in line with our in vitro results [Fig. 2]. Loss of lateral cartilage matrix could not be prevented with FK506 treatment [Fig. 3(D)]. Longitudinal μCT analysis showed reduced sclerotic bone formation in the lateral compartment of FK506 treated animals [Fig. 5(C)]. Previous experiments with this severe OA model suggest that subchondral sclerosis might develop when cartilage is completely lost and subchondral bone is denuded. In some FK506 treated animals we found focal regions of cartilage on the lateral tibia plateau that showed totally sGAG depleted but partially intact ECM. This suggests, that FK506 might have delayed lateral cartilage matrix degradation and thus reduced formation of subchondral sclerosis. However, Cn inhibition is also known to modulate bone turnover and therefore may have reduced sclerosis through direct modulation of osteoclast and osteoblast activity. CsA and FK506 have both been described to induce osteopenia through anti-anabolic effects on osteoblastic cells and to reduce bone formation through inhibition of osteoblast differentiation. This could be another explanation for the reduced development of subchondral sclerosis in the lateral compartment, but has to be further investigated.

Another cell type that is modulated by FK506 are macrophages. We determined activated macrophages using 111In-EC0800 and quantitative SPECT/CT [Fig. 6(A)]. During OA progression, macrophages become activated and their TGF/BMP-2 production has previously been related to osteophyte development. In our in vivo experiment, animals developed clear osteophytes at the margins of the patella [Fig. 6(B)]. FK506 treatment reduced osteophyte development while the total amount of activated macrophages was equal after 6 weeks of follow-up. This may suggest that FK506 treatment limits cytokine production by activated synovial macrophages and previous reports on Cn inhibition in macrophages showed reduced cytokine production.
FK506 may initially activate Toll-like receptors (TLR) pathways in activated macrophages, which can enhance NFKb activity\(^4\) and stimulate expression of cytokines, like TNFa, IL1\(\alpha\), IL1\(\beta\), IL12 and iNOS\(^4\). However, prolonged exposure to Cn inhibitors has been shown to also secondarily inactivate this TLR induced pro-inflammatory cytokine expression by negative feedback loops\(^4\). As such, continuous FK506 exposure may eventually suppress NFKb pathways, but activate caspas 3 and 9 to enhance macrophage apoptosis\(^4\). Possibly, FK506 induced macrophage apoptosis may explain why radioactive folate uptake in our experiments was restored to levels comparable to healthy control joints [Fig. 6(A)].

Osteoclasts are large multinucleated cells of the monocyte–macrophage hematopoietic lineage and are also influenced by FK506. During osteoclastogenesis macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-\(\kappa\)B ligand (RANKL) stimulate precursor cells to acquire osteoclast characteristics\(^4\). NFATc1 is an essential terminal differentiation factor of osteoclastogenesis and can be blocked in a dose-dependent fashion using CsA or FK506\(^4\). CsA and FK506 treatment suppress RANKL stimulated osteoclastogenesis\(^4\), and especially inhibits late stages of the osteoclast life cycle\(^4\). By this mechanism Cn inhibition can diminish the activity of mature osteoclasts and reduce bone resorption\(^4\). FK506 mediated suppression of osteoclast maturation and subsequently hindered subchondral bone resorption may therefore explain why less subchondral pores were measured in FK506 animals.

Systemic FK506 treatment is known to induce toxic side effects\(^4\). Throughout the experiment our animals gained weight (see online Supplementary Fig. 1), and FK506 animals increased more in weight from 6 to 12 weeks of follow-up. However, this result could not clearly be related to side-effect of FK506, which usually results in a loss of weight. At the end of the 12 week experiment liver function (AST, ALT, alkaline phosphatase) and kidney function (creatinin and urea) were normal (see online Supplementary Fig. 2(A)). Liver and kidney histology gave no indication that FK506 induced liver or kidney fibrosis (see online Supplementary Fig. 2(B)–(C)). Despite these promising findings, systemic FK506 treatment cannot be translated towards clinical OA care directly. FK506 induced side effects are well described in patients after long term use and is unacceptable for a therapeutic strategy in human OA patients. To reduce toxicity, local intra-articular treatment may be used, but repetitive intra-articular injections also increase the risk of iatrogenical arthritis and should be avoided. FK506-coupled biodegradable delivery systems might be able to prolong intra-articular FK506 exposure and sustain long term therapeutic action\(^4\), hopefully without systemic adverse effects.

![Subchondral bone changes analyzed with longitudinal in vivo µCT in untreated OA (circles) and FK506 treated (squares) animals. Subchondral plate thickness (Sh. Pl. Th.; A, C) and porosity (Sh. Pl. Por.; B, D) were measured in the medial (A, B) and lateral (C, D) compartment of the tibial epiphysis. Changes in trabecular thickness (Th. Th.; E) and trabecular bone volume fraction (BV/TV; F) were measured in tibial epiphyseal bone marrow. Representative sagittal images from binary µCT scans (G) show pore development (indicated with \(\nabla\)) and development of subchondral sclerosis (indicated with dashed line and \(*\)). Three-dimensional top views of the tibial plateau at different time points (H) show subchondral pore (red color) development. \(\ast\): \(P < 0.05\), \(\ast\ast\): \(P < 0.01\), \(\ast\ast\ast\): \(P < 0.001\), error bars indicate 95% confidence intervals.](image)
Conclusion

Inhibition of Cn activity with FK506 stimulated anabolic activity, while reducing catabolic productivity of osteoarthritic human chondrocytes. Systemic treatment with FK506 in a rat model for severe OA also protected against cartilage ECM degradation. Additionally, there was also less development of subchondral sclerosis, macrophage activation and osteophyte formation. Altogether, our data suggests that Cn inhibition with FK506 proves to be a promising candidate for therapeutic management of OA.

Contributions

All authors have substantially contributed to the conception and design of the study, acquisition of data, or analysis and interpretation of data. All authors have participated in the writing process and approved the final version of the manuscript.

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Competing interests

All authors indicate that they have no conflicts of interest.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2014.02.003.

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