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Paricalcitol inhibits the Wnt/beta-catenin signaling pathway and ameliorates experimentally induced arthritis

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Background/aim: The Wnt/β-catenin pathway has important biological activities, including the differentiation of cells and joint formations. The aim of our study was to determine the effect of paricalcitol on experimentally induced arthritis.

Materials and methods: Type II collagen combined with Freund's adjuvant was applied to induce arthritis in Wistar albino female rats. Paricalcitol (0.3 μg/kg daily) was subcutaneously injected starting 1 day after collagen applications (prophylactic group) or 1 day after the onset of arthritis (therapeutic group), until day 29.

Results: The 29th day arthritis scores were lower compared to the 13th day scores in the paricalcitol groups ($P < 0.05$), while they were higher in the arthritis group ($P < 0.05$). Marked cartilage-bone destruction and extensive perisynovial inflammation were detected in the arthritis group. Decreased cartilage-bone destruction and perisynovial inflammation in the paws were observed in the paricalcitol groups. The tissue mRNA levels of DKK1, Wnt5a, and axin-2 were higher in the arthritis group than in the control group. In the paricalcitol groups, mRNA expressions were lower than in the arthritis group.

Conclusion: The present study shows that the Wnt/β-catenin signaling pathway is active in arthritis. Moreover, paricalcitol ameliorates arthritis via inhibiting the Wnt/β-catenin pathway. Paricalcitol and the Wnt/β-catenin pathway are candidates for research in human rheumatoid arthritis.

Key words: Rheumatoid arthritis, Wnt/beta-catenin signaling pathway, paricalcitol

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease. Synovial inflammation leads to cartilage destruction and bone erosions in RA; however, the etiopathogenesis of RA is not yet fully known (1). Synovial fibroblasts (SFs) and increased secretion of various cytokines, chemokine, and growth factors play prominent roles in its pathogenesis. The proliferated SF activates the inflammatory pathways and leads to the secretion of matrix metalloproteinases (MMPs) (2–5). Thus, bone remodeling in RA is defective. It is now clear that inflammation also impacts osteoblast and osteoclast differentiation and function. Bone resorption and erosion are increased owing to increased activity of osteoclasts, whereas bone formation by osteoblasts is suppressed (6).

The Wnt/β-catenin signaling pathway plays a role in embryogenesis, limb development, and cell adhesion (7). Previous studies (8–14) showed that the Wnt/β-catenin signaling pathway affects the proliferation and functions of several inflammatory cells and the productions of several cytokines and chemokines, those having a prominent effect on the pathogenesis of RA. Moreover, increased synovial tissue expressions of Wnt5a, Wnt7a, and Wnt10b have also been documented in patients with RA (8–10). In vitro Wnt5a and Wnt7a applications enhance the productions of cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, and IL-15, forming SFs (8,9). Wnt1 administration induces the synthesis of pro-MMP3 from SFs (11). On the other hand, Wnt proteins are expressed by T cells (13). The Wnt/β-catenin

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signalingpathway affects T-cell proliferation (12,13) and extends the survival of regulatory T cells (14). The levels of Dickkopf-related protein 1 (DKK1), which is an inhibitor of the Wnt/β-catenin signaling pathway, are increased in the sera and synovial fluids of patients with RA (15).

Paricalcitol is an active synthetic analog of vitamin D that has biological activity similar to that of vitamin D (16,17). Several recent studies have indicated that paricalcitol has immunomodulatory and antiinflammatory effects in various experimental models, except the arthritis model (18,19). Paricalcitol significantly reduced inflammatory cytokine levels, such as those of IL-18, IL-6, and TNF-α. Conversely, it increased serum IL-10 levels (20). Several recent studies showed that paricalcitol inhibits Wnt/β-catenin signaling and blocks β-catenin-mediated gene transcription (17).

The aim of the present study was to evaluate the efficacy of the inhibition of the Wnt/β-catenin signaling pathway by paricalcitol for prophylactic and therapeutic effects on an experimental arthritis model of collagen-induced arthritis (CIA).

2. Material and methods

2.1. Animals

The study protocol was approved by the local ethics committee. This study was performed on 40 female Wistar albino rats. They were 8 to 10 weeks old and their weights were between 200 and 250 g. These rats were purchased from the Experimental Animal Research Center of Fırat University. During the experiment, animals were kept at a temperature of 21 ± 4 °C and humidity of 52 ± 8% with a 12-h light dark cycle.

2.2. Experimental applications

The experimental animals were randomized into four groups (n = 10): control, arthritis (sham), prophylactic paricalcitol, and therapeutic paricalcitol groups. Collagen injections were applied to induce arthritis in the arthritis and paricalcitol groups. Type II collagen (Sigma-Aldrich, St. Louis, MO, USA), which was diluted with 0.1 M acetic acid (1 mg in 1 mL), was emulsified with an equal amount of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). The prepared solution (totally 200 µg to each rat) was intradermally injected into the dorsal tail (100 µg) and back paws (50 µg to each paw) on the first day. After seven days, a volume of solution of 100 µg was applied to the dorsal tail (booster). Each rat was individually evaluated for development of arthritis after the first collagen injection. Arthritis scorings were done according to the previously described method (21).

The rats in group III (prophylactic paricalcitol group) received paricalcitol subcutaneously starting 1 day after collagen applications. Paricalcitol applications were started 1 day after the onset of arthritis in group IV (therapeutic paricalcitol group). Since prominent arthritis was observed in the collagen-injected groups on the 12th and 13th days (Figure 1), paricalcitol application was started on the 13th day in the therapeutic paricalcitol group. Paricalcitol at 0.3 µg/kg was given subcutaneously and daily until the 29th day. Physiologic serum (equal volume to paricalcitol) was injected intraperitoneally in the control and arthritis (sham) groups (22).

2.3. Samples

The animals were euthanized on the 29th day. Back paws were amputated for further examination. Joint samples were divided into two sections for real-time PCR analysis and histopathological examinations. The section for the real-time PCR analysis was kept at –80 °C. The other part was put into 10% formaldehyde for histopathological examinations.

2.4. Histopathological examinations

Joint samples, which were fixed in a formalin solution, were decalcified in 10% nitric acid (30 days) and were embedded in paraffin blocks. Then the sectioned paraffin blocks were stained with hematoxylin and eosin (H&E). They were examined under 40×, 100×, 200×, and 400× magnifications with a light microscope. Pannus formation, inflammatory cell infiltration, and bone destruction in the joints were assessed. The inflammatory status and cartilage-bone destruction were scored for histopathological scoring on a scale between 0 and 4 points (23).

2.5. Real-Time PCR analysis

TRIzol reagents (Invitrogen, Carlsbad, CA, USA) were used to isolate total RNA from joint tissue. cDNA was generated by reverse transcription of total RNA with a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). PCR reactions were performed in triplicate and heated to 50 °C

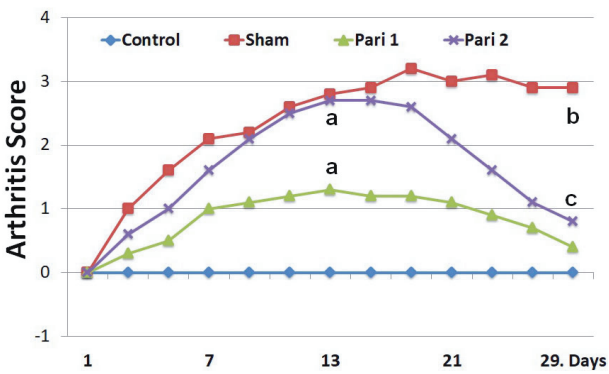

Figure 1. Daily arthritis scores in the all study groups. [a] P < 0.05 when compared to the control group. [b] P < 0.05 when compared to both paricalcitol groups. [c] P < 0.05 when 13th and 29th day arthritis scores were compared in the Pari 2 group. Pari 1: Prophylactic paricalcitol group, Pari 2: therapeutic paricalcitol group.

for 2 min followed by 40 cycles of denaturation at 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. For each sample, standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference. Real-time PCR analysis, using Tag Man Master Mix (Applied Biosystems, Foster City, CA, USA), was performed with the ABI Prism 7500 Fast Real Time PCR Instrument (Applied Biosystems). All results were standardized to the levels of GAPDH (24). The comparative *Ct* (ΔΔ*Ct*) method was used to quantify axin-2, Wnt5a, and DKK1 genes as described in the Assays-on-Demand User's Manual.

2.6. Statistics

Statistical analysis was performed using the SPSS 21.0 (IBM Corp., Armonk, NY, USA). Kruskal–Wallis variance analysis and the Mann–Whitney U test were selected for statistical analysis. The comparison of differences in the arthritis scoring between the 13th and 29th days was evaluated with the Wilcoxon rank-sum test. Bonferroni corrections were performed for multiple comparisons. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Clinical scoring of arthritis

Clinically prominent arthritis was observed at 12 to 13 days after collagen injections (Figure 1). The arthritis scores were higher in the arthritis (sham), prophylactic paricalcitol, and therapeutic paricalcitol groups compared to the control group ($P < 0.05$ for all). The 29th day arthritis scores were decreased in the paricalcitol groups compared to their own 13th day scores ($P < 0.05$ for both). The mean 29th day arthritis score was increased in the arthritis group compared to its own 13th day score ($P<0.05$).

3.2. Histopathological evaluations

Histopathological analysis showed marked cartilage-bone destruction and extensive perisynovial inflammation in the rats of the arthritis group, while these findings were not observed in the rats of the control group (Figure 2). Prophylactic and therapeutic paricalcitol applications decreased the cartilage-bone destruction and perisynovial inflammation in the joints (Figure 2).

3.3. Quantitative real-time PCR analysis

The tissue mRNA expressions of DKK1 (3-fold), Wnt5a (11-fold), and axin-2 (22-fold) were increased in the arthritis group compared to the control group. Conversely, their mRNA expressions were similar in the paricalcitol groups and the control group (Figure 3).

4. Discussion

The present study demonstrates that the Wnt/β-catenin signaling pathway is activated in the CIA model, an experimental model of RA. On the other hand, paricalcitol suppresses the Wnt signaling pathway and thus ameliorates arthritis.

Multiple signaling pathways are activated during RA development (25). The Wnt/β-catenin signaling pathway has considerable roles in the pathogenesis of RA (11,26). In our study, the mRNA levels of axin-2, Wnt5a, and DKK1 were increased in the arthritis group in comparison to the control group. These results suggest that the Wnt/β-catenin signaling pathway is activated in arthritis. Recent studies (9,27) showed that the β-catenin expression in synovial specimens harvested from RA patients is significantly higher than in the synovial specimens harvested from patients with osteoarthritis and healthy adults. β-Catenin and Wnt5a productions are increased in SFs harvested from patients with RA (8,11). Wnt/β-catenin pathway activation contributes to the activation of fibroblast-like synoviocytes (FLSs) in RA (11,26).

The Wnt/β-catenin pathway participates in many physiological activities, including cell differentiation, proliferation, and apoptosis (5). FLSs and inflammatory cells have prominent roles in the pathogenesis of RA (1,4,6). The activation changes the phenotype of FLSs. Active FLSs produce cytokines, chemokines, and MMP. The other pathogenic actors are inflammatory cells that invade synovial tissue, and activated/proliferated inflammatory cells also produce cytokines, chemokines, and MMP (1,4,6). The Wnt/β-catenin pathway is a candidate to affect activation, proliferation, and migration of inflammatory cells and FLSs. In in vitro settings it has been observed that when Wnt proteins are applied to the synovial cells harvested from control subjects, the productions of proinflammatory cytokines, which are IL-1, IL-6, and TNF-α, are enhanced (9). These cytokines are known to play an important role in RA pathogenesis. RA is an inflammatory disease, and cytokines are the prominent process of the RA pathogenesis.

Wnt proteins and Wnt/β-catenin signaling pathway-induced proteins such as DKK1 can be produced by inflammatory cell, and they affect the inflammatory cells. Previous findings showed that the Wnt/β-catenin signaling pathway is activated in RA synovium and that the activated Wnt/β-catenin signaling pathway enhances the production of proinflammatory cytokines such as IL-1, IL-6, and TNF-α (10,11). Similarly, increased Wnt1, Wnt5a, and Wnt7b in FLSs from RA patients induce the production of these cytokines and MMP3 (28). Moreover, when Wnt5a is silenced with siRNA the cytokine and chemokine productions significantly decrease (29).

In addition to inflammatory roles, the Wnt signaling pathway has an important regulative role in the balance of the osteoblast and osteoclast axis. The disturbed balance of osteoblasts and osteoclasts is one component of the pathogenesis of RA and leads to bone resorption and

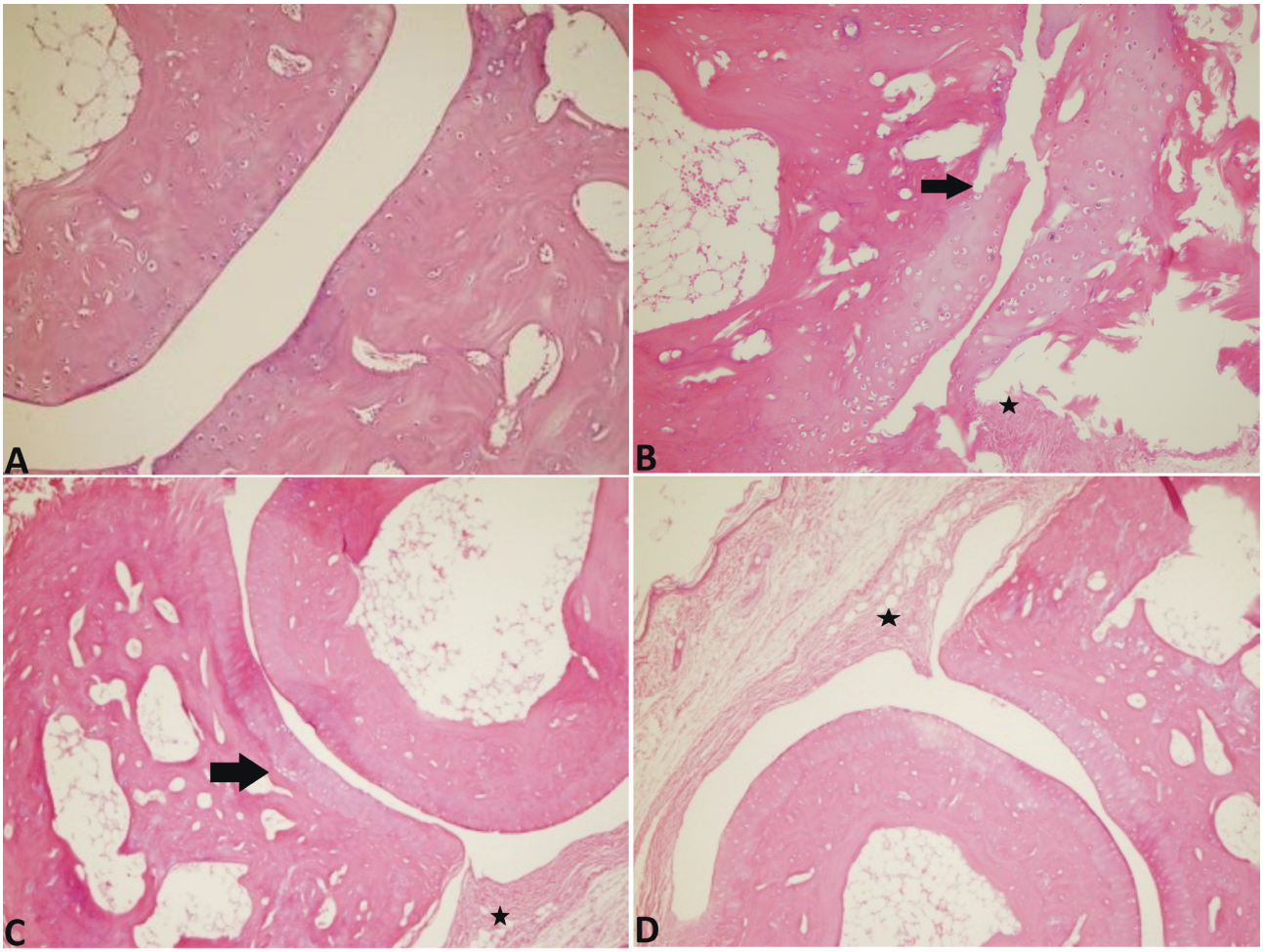

Figure 2. Histopathological sections of joints in the study groups (H&E, 400×). The appearances of perisynovial tissue and cartilage-bone were normal in the control group (A). The destruction of cartilage-bone was observed in the arthritis group (B). Minimal perisynovial inflammation and synovial hyperplasia were detected in the prophylactic (C) and therapeutic (D) paricalcitol groups. The destruction of cartilage-bone is marked by arrows and the inflammatory cell infiltrations and synovial hyperplasia are marked by asterisks.

erosion (1). There are different roles of the Wnt/β-catenin signaling pathway in the functions and differentiations of osteoblasts and osteoclasts. Thus, the Wnt/β-catenin signaling pathway has a pivotal role on bone turnover and the regulation of cartilage (30). In our study, it was observed that the Wnt/β-catenin signaling pathway is activated in experimentally induced arthritis.

Paricalcitol blocks β-catenin accumulation and activation mediated by its ability to inhibit the expression of multiple Wnt genes and to inhibit β-catenin-mediated gene transcriptions (16,17). It has also been shown that paricalcitol inhibits the production of proinflammatory cytokines and reduces the inflammatory cell infiltration of the kidneys. Treatment with paricalcitol attenuates inflammation and oxidative damage in animal models (17). Some reports showed that vitamin D deficiency has been linked to an increased incidence of RA and suggested that vitamin D supplementation might prevent the development of these diseases. In addition, a few scientists presented a hypothesis that paricalcitol can be used in combination with biological agents for treating RA, by improving the therapeutic efficacy of biologic agents and decreasing the incidence of infection (31). In the present study, paricalcitol ameliorated synovial hyperplasia, pannus formation, and cartilage-bone destruction. In addition, paricalcitol reduced the arthritis scores. Moreover, paricalcitol decreased Wnt5a expression in our study. These results may suggest that paricalcitol exerts antiarthritic action and depletes the Wnt signaling pathway.

Additionally, higher levels of several Wnt proteins such as Wnt1 and Wnt5a have been shown in FLSs and synovial tissue specimens from RA patients than in the osteoarthritis patients and healthy controls (8). Several

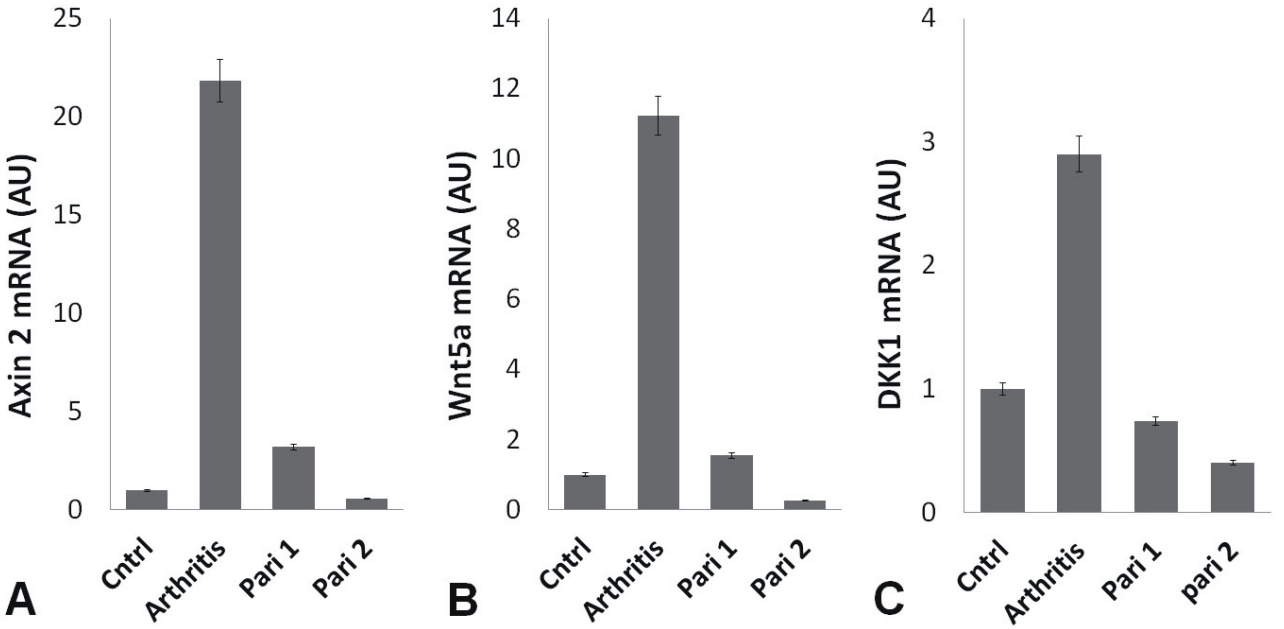

Figure 3. Tissue mRNA expressions of axin-2, Wnt5a, and DKK1 in the study groups. Cntrl: Control, Pari 1: prophylactic paricalcitol group, Pari 2: therapeutic paricalcitol group, DKK1: Dickkopf-1.

studies showed that Wnt5a-mediated intracellular signaling in RA aggravates the production of inflammatory cytokines, chemokines, MMPs, and angiogenic factors (7,8,11). While increased Wnt5a expression upregulates the expression of cytokines and chemokines, decreased Wnt5a expression reduces their synthesis and secretion. The Wnt5a-mediated production of the cytokines and chemokines was at least partly induced by NF-κB. Moreover, the Wnt5a-mediated signaling pathway can also promote angiogenesis of the synovium by upregulating angiogenic regulators (28). Recent studies have also shown that Wnt5a enhanced osteoclast formation (32).

Dickkopf-1, an endogenous inhibitor of Wnt/β-catenin signaling, is secreted by FLSs in response to inflammation in RA. DKK1 is important for the balance of osteoblasts and osteoclasts and it repairs bone damage in RA (31). The levels of DKK1 were significantly higher in RA patients and increased DKK1 inhibits the Wnt/β-catenin signaling pathway in osteoblasts. Thus, DKK1 inhibits the osteoblastic differentiation in RA. In an experimental model of RA, blockade of DKK1 attenuated bone erosion to induce new bone formation (15,28,33). In our study, the tissue mRNA expression of DKK1 was greatly increased in the arthritis group compared to the control group. DKK1 expressions were decreased with administration of paricalcitol and were similar in the paricalcitol and control groups. These observations show that DKK1 might be an interesting new structural biomarker in early RA and DKK1 may be a promising therapeutic target in RA.

Axin-2 is a negative regulator and a target of Wnt/β-catenin signaling. Axin-2 is expressed in cell populations responsive to Wnt/β-catenin signaling. T-cell factor proteins played a key role in axin-2 induction. The increase in axin-2 expression in the arthritis group and decrease in paricalcitol groups may be associated with this interaction in parallel with the Wnt/β-catenin pathway (34).

The present study has several limitations. First, it documents the early effects of paricalcitol applications on arthritis; however, its long-term effects should also be researched. Second, radiographic progressions need to be evaluated.

In conclusion, these results show that the Wnt signaling pathway is active in arthritis. Moreover, paricalcitol inhibits the Wnt/β-catenin signaling pathway and thus ameliorates arthritis. Paricalcitol and the Wnt/β-catenin signaling pathway are candidates for further research in human RA.

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