Blockade of Dickkopf (DKK)-1 induces fusion of sacroiliac joints

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ABSTRACT

Objective To study whether Dickkopf (DKK)-1, an inhibitor of wingless (Wnt) signalling, is involved in the fusion of sacroiliac joints.

Methods Mice transgenic for tumour necrosis factor (TNFtg mice), which develop bilateral sacroiliacitis, were treated with vehicle, anti-TNF antibody or anti-DKK1 antibody. Sacroiliac joints were analysed for histological signs of inflammation, bone erosion, osteoclast formation and ankylosis. Moreover, expression of collagen type X, β-catenin and DKK-1 was assessed by immunohistochemistry.

Results There were no signs of spontaneous ankylosis of the sacroiliac joints in TNFtg mice. TNF blockade effectively reduced inflammation, bone erosion and osteoclast numbers in the sacroiliac joints, but did not lead to ankylosis. Blockade of DKK1 had no effect on inflammatory signs of sacroiliacitis, but significantly reduced bone erosions and osteoclast counts. Moreover, DKK1 blockade promoted expression of collagen type X, the formation of hypertrophic chondrocytes and ankylosis of sacroiliac joints.

Conclusion DKK1 influences inflammatory remodelling of sacroiliac joints by prevention of joint ankylosis. This may indicate an important role of the Wnt signalling pathway in the structural bone changes of axial joint disease. Although this model does not reflect the entire spectrum of ankylosing spondylitis in humans, it helps to explain the pathophysiological processes of sacroiliac joint ankylosis, which is a hallmark of the spondyloarthritides.

Sacroiliitis is the hallmark of ankylosing spondylitis (AS) and other forms of spondyloarthritides (SpA) and can either emerge as unilateral or bilateral joint involvement. The affection of the sacroiliac joint is usually associated with characteristic symptoms, commonly grouped together as inflammatory back pain. Clinical symptoms emerging from sacroiliitis have thus become important diagnostic tools for SpA and their association with changes in MRI scans has shown to be predictive for AS.

Chronic inflammation of the sacroiliac joints typically leads to structural changes and remodelling of the joint architecture. This becomes evident from radiographic examinations, which initially present as erosive changes in the sacroiliac joints. During the course of disease, however, the joint spaces of the affected sacroiliac joints typically tend to be obliterated, leading to joint ankylosis. The radiographic scoring system of sacroiliitis reflects this transition from erosive changes into progressive ankylosis of sacroiliac joints. Moreover, higher levels of radiographic sacroiliitis score, which reflect proliferative changes, are still a central part of the diagnosis of ankylosing spondylitis. MRI has meanwhile improved the imaging of SpA and has revealed that inflammatory changes in the sacroiliac joints, as well as in the neighbouring iliac and sacral bones, are associated with profound structural damage in this joint region.

The mechanisms leading to remodelling of the sacroiliac joints are unknown. MRI studies dating back to the early 1990s demonstrated that bone marrow changes are an early sign of sacroiliitis and precede joint ankylosis by some time. MRI bone marrow changes have been directly identified as inflammatory lesions within the bone marrow on subsequent histology, and several histopathological studies have show inflammatory lesions in the bone marrow of patients with RA and patients with AS. Moreover, these investigations have shown that the small facet joint of patients with AS has an accumulation of osteoclasts in the juxta-articular bone marrow, suggesting that increased bone resorption takes place in the axial joints of patients with AS, but the investigations have also shown sites with increased bone formation, which are likely to be the basis for the progressive fusion of the spine of these patients. Inflammation and bone formation appear to occur at different time points during the pathogenesis of AS, with the former preceding the latter. Moreover, detailed histopathological analysis of inflammation and bone formation at enthesial sites suggests that these lesions occur at slightly different anatomical sites, suggesting some areas are prone to bone formation. As one of the most typical sites of ankylosis, the sacroiliac joint is of particular interest.

The mechanisms of osteophyte formation leading to ankylosis of the joints are only partly understood, but likely involve molecules that regulate bone formation, such as wingless (Wnt) proteins and bone morphogenic proteins (BMPs). Recently, we have shown that activation of Wnt signalling by blocking their natural inhibitor Dickkopf (DKK)-1 leads to formation of osteophytes in peripheral joints. We thus hypothesised that Wnt proteins may participate in the ankylosis of sacroiliac joints. Therefore, we blocked DKK-1 by neutralising antibodies in human tumour necrosis factor transgenic (hTNFtg) mice. This animal model of arthritis is not a standard model of sacroiliitis as it does not spontaneously lead to anabolic bone changes, but is characterised by inflammation of synovial tissue, which affects the peripheral joints and both sacroiliac joints leading to bilateral sacroiliitis.
METHODS

Animals and treatment
The heterozygous Tg197 transgenic mice have been described previously.15 These mice develop a chronic inflammatory and destructive arthritis within 5 weeks after birth. All mice (N = 24) were bred and maintained in a CBAxC57BL/6 genetic background. Mice were fed a standard diet with water ad libitum. Mice were treated with vehicle (phosphate-buffered saline (PBS); control group; N = 6), infliximab, a chimaeric antibody against human tumour necrosis factor (αTNF; N = 6) intraperitoneally at a dose of dose 10 mg/kg three times a week, a rat antibody against mouse DKK-1 (αDKK; N = 6) intraperitoneally at a dose of 10 mg/kg three times a week and a combination of αTNF and αDKK (N = 6) as described above. Treatment was initiated at the stage of early arthritis (week 6) and was continued for 4 weeks. Animals were killed by cervical dislocation 10 weeks after birth. The local ethical committee approved all animal procedures. Dosing of antibody was according to previous experience in treatment of inflammatory arthritis in hTNFtg mice.13 14

Histological assessment
Pelvises were fixed in 4.0% formalin overnight and then decalcified in 14% ethylenediaminetetra-acetic acid (EDTA; Sigma, St Louis, Missouri, USA) at 4°C (pH adjusted to 7.2 by addition of ammonium hydroxide (Sigma)) until the bones were pliable. Serial paraffin sections (2 μm) were stained with toluidin blue and for tartrate-resistant acid phosphatase (TRAP) activity. TRAP staining was performed as previously described.13 14 For quantification of the areas of inflammation, bone erosion and for osteoclast numbers, TRAP sections were evaluated. TRAP stained sections were counterstained with haematoxylin. Cartilage area was determined from toluidin blue stained serial sections. Osteoblasts were assessed by their typical cuboid-shaped form and localisation along bone. Synovial inflammation, bone erosions, osteoclast numbers and cartilage destruction were quantified with the use of a Zeiss Axioskop 2 microscope (Zeiss, Marburg, Germany) equipped with a digital camera and image analysis system (Osteomeasure, OsteoMetrics, Decatur, Georgia), as described previously.13 14

Immunohistochemistry
Paraffin embedded sections (N = 5 per group) were used for immunohistochemistry staining. Sections were deparaffinised, dehydrated by isopropanol and incubated with citrate buffer, pH 6, for antigen retrieval. Non-specific binding was blocked by addition of 10% rabbit serum in 5% bovine serum albumin (BSA)/PBS for 60 min at room temperature and then incubated with antibodies specific for collagen type X,16 β-Catenin and DKK-1 (both from Santa Cruz Biotechnology) overnight at 4°C. The sections were then incubated with alkaline phosphatase streptavidin for 30 min at room temperature before detection with fast red TR/Naphtol solution (Sigma), resulting in red staining. Slides were counterstained with Meyer haematoxylin. Samples were photographed and analysed by microscope (Nikon, Japan) on 400× for percentage assessment of positive cells.

Serum analyses
The serum level of osteoprotegerin (OPG) was assessed by a sandwich enzyme immunosorbent assay (Quantikine mouse OPG Immunoassay; R&D, Minneapolis, Minnesota, USA).

Statistical analysis
Data are shown as mean (standard error of the mean (SEM)). Group mean values were compared by Wilcoxon signed rank test.
RESULTS

TNF, but not DKK1, blockade inhibits sacroiliac joint inflammation

Based on our previous observations of bilateral synovitis of the sacroiliac joint in TNFtg mice and our investigations on the role of DKK1 in the remodelling of peripheral joints, we were interested in whether and how sacroiliitis is affected by DKK1 blockade. In contrast to TNF blockade, which effectively inhibited sacroiliac joint inflammation, inhibition of DKK1 by a neutralising antibody did not significantly reduce synovial inflammation in the sacroiliac joint (figs 1A and 2). Combination of TNF and DKK1 blockade was equally effective as TNF blockade alone in reducing synovial inflammation within the sacroiliac joints.

TNF and DKK1 blockade inhibits bone erosions in the sacroiliac joints

As observed in human disease, inflammation of sacroiliac joints in TNF transgenic mice leads to osteoclast formation and bone erosions. We thus addressed whether blockade of DKK1 affects the formation of local bone erosions. At the age of 10 weeks numerous osteoclasts were found in the sacroiliac joints of untreated TNFtg mice and local bone erosions were formed. Blockade of TNF significantly reduced the number of osteoclasts by 75% and the size bone erosions by 67% (figs 1B,C and 2). Similarly, blockade of DKK1 was highly effective in reducing structural damage, as osteoclasts were reduced by 82% and bone erosions by 69%. Combination of DKK1 and TNF blockade resulted in a complete abrogation of osteoclasts and bone erosions in the sacroiliac joints. Simultaneously with decreased osteoclast formation serum levels of OPG, a Wnt-regulated protein and decoy receptor for receptor activator for nuclear factor κB ligand (RANKL), which blocks osteoclast activity, was significantly increased after DKK-1 blockade (see Supplementary material). This suggests that decreased bone resorption after DKK1 blockade is indirectly through induction of OPG. Osteoblast numbers were significantly increased upon blockade of DKK1 (Supplementary material).

DKK1, but not TNF, blockade enhances sacroiliac ankylosis

Based on our observations that DKK blockade enhances bone growth in peripheral joint we suspected that it might exert similar effects on the sacroiliac joints compartment, especially enhancing ankylosis. We thus searched for signs of new bone formation and quantified areas of bony proliferation in the sacroiliac joints. There were no signs of bone formation such as osteophytes or similar lesions containing hypertrophic cartilage in untreated TNF transgenic mice (figs 1D and 2). Moreover,
anti-TNF treatment did not lead to a significant proliferation of bone (figs 1D and 2). DKK-1 expression was found locally in the sacroiliac joint suggesting that it act at the local level in suppressing bone formation (Supplementary material). Blockade of DKK1 lead to a significant increase of bone proliferative responses in the sacroiliac joint compartment and this effect was further increased when DKK and TNF blockade were applied simultaneously. Bone formation was observed to be local within the sacroiliac joint gap but not in neighbouring structures, such as enthesial sites along the sacroiliac joint. When more pronounced, these changes lead to progressive ankylosis of the joint (figs 1D and 2). Expression of collagen type X indicating differentiation of hypertrophic chondrocytes was abundant after blockade of DKK-1 by neutralising antibody, but was only scarcely observed in untreated or anti-TNF treated TNF transgenic mice (fig 3). Moreover, also the expression of β-catenin in the sacroiliac joints was strongly increased after DKK-1 blockade indicating active Wnt signalling, which is a prerequisite for new bone formation (fig 4).

**DISCUSSION**

We have previously shown that DKK1 is a key factor for joint remodelling during inflammatory disease. DKK1 suppresses bone formation by interfering with the Wnt pathway and inhibits skeletal repair responses during inflammatory joint disease. The balance between proteins involved in bone formation such as Wnt proteins and those suppressing bone formation such as DKK is apparently one of the decisive steps in joint remodelling, particularly in determining whether an affected joints faces erosive damage or build up of bony spurs. This model has so far only been validated in peripheral joints, although its role in the axial skeleton might be important as well, given that the anatomical region primarily affected in SpA is the spine. Herein, we show that blockade of DKK1 by a neutralising antibody promotes ankylosis of the sacroiliac joint. These data suggest that the molecular mechanisms identified for the remodelling of peripheral joints play a role in the ankylosis of joints of the spine as well.

Involvement of the sacroiliac joint is the hallmark of spinal disease in all different forms of SpA. The two sacroiliac joints constitute tiny gaps between the sacrum and the iliac bones and allow only a minor range of motion. Nonetheless, when inflamed, they emerge as a major contributing factor for disease symptoms in SpA, particularly relevant for inflammatory back pain. Sacroiliitis is characterised by synovitis, concomitant erosion of juxta-articular bone as well as osteitis in the neighbouring bone marrow. Erosive bone changes are evident from radiographic examinations of the sacroiliac joint and indicate a destructive course of disease. Importantly, however, affection of the sacroiliac joints during SpA results in ankylosis that is often associated with an improvement of symptoms since the inflammatory tissue within the joint space is replaced by cartilage and bone. The mechanisms that allow a switch from erosive sacroiliac joint involvement to joint ankylosis are poorly understood. Importantly, they may share mechanisms that have been unravelled for peripheral joint disease.

Arthritis in TNFtg mice is driven by mesenchymal over expression of TNF and inflammatory lesions are confined to anatomical sites with synovial tissue such as diarthrodial joints.

**Figure 3** Collagen type X expression in sacroiliac joints. Tumour necrosis factor (TNF)tg mice were either treated with (A) vehicle, (B) anti-TNF antibody (infliximab, aTNF), (C) anti-Dickkopf (DKK)-1 antibody (aDKK) or (D) a combination of both antibodies. Microphotographs show sections of sacroiliac joints stained for collagen type X (red colour). Original magnification 5× (left) and 20× (right).
including the sacroiliac joints. In contrast, regions without synovial tissue such as the intervertebral spaces are spared from inflammatory lesions. Thus, TNFtg mice can be regarded as an excellent model of bilateral erosive sacroiliitis, but not as a model of SpA per se, since pathological changes along the intervertebral spaces including syndesmophyte formation are absent. This is in contrast to the HLAB27 transgenic rat and the proteoglycan immunisation model of balb/c mice, which both develop spinal fusions as well as the model of male DBA mice, which shows anklylosis of peripheral joints without a preceding erosive phase of disease.28 Whereas anklylosis of intervertebral spaces and peripheral joints is well mimicked by these models, sacroiliac joint involvement is not covered by any of these models, although it constitutes a major part of the disease process of human SpA.

Sacroiliitis in hTNFtg mice is a bilateral erosive process based on synovial inflammation, which mimics human sacroiliitis. Although this model is an experimental model of synovitis rather than spondyloarthropathy, as spontaneous anabolic bone responses are absent and the disease process does not affect intervertebral spaces, it constitutes a model of bilateral sacroiliitis. Immunohistochemical analysis of sacroiliitis reveals macrophages, T cells, neutrophils and only very few B cells within the inflamed joint space. Joint anklylosis does not form spontaneously, suggesting that TNF is not the driving factor for joint fusion. By contrast, TNF blockade does not induce joint fusion, suggesting that TNF blockade does not lead to sufficient Wnt activation, which is necessary to induce bony proliferations. Only after direct blockade of DKK1 did we observe progressive bony proliferations emerging from both joint surfaces, which finally lead to progressive anklylosis of the joint. These data strongly support a role of Wnt signalling in the fusion of axial joints, which is a major component of the disease process in SpA. It is as yet unclear whether anklylosis of sacroiliac joints occurs by endochondral ossification or by membranous bone formation and chondroidal metaplasia. Studies in humans and mice have shown morphological features of hypertrophic chondrocytes as a sign of endochondral bone formation in axial and peripheral joints, and also direct evidence for membranous bone formation and chondroidal metaplasia has been obtained especially at enthesial sites.22 The observation of abundant collagen type X expression, however, suggests that endochondral ossification at least plays an important role in this process.

Inflammation per se was not affected by blockade of DKK1, and inflammatory cells were still found in the joint. Similar observations have also been obtained, when another key antagonistic player of bone formation, noggin, a negative regulator of the bone morphogenetic protein pathway was inhibited. Thus, haploinsufficiency of noggin did not affect inflammatory features of arthritis but did affect structural integrity of joint, particularly articular cartilage. Most strikingly, bone erosions and osteoclasts were dramatically reduced upon DKK1 blockade, which reinforces the concepts that the Wnt pathway is a potent regulatory mechanism for osteoclastogenesis. Very similar observations have been previously made in peripheral joints, showing that Wnt signalling activates OPG and in consequence blocks RANKL-induced osteoclast formation and bone erosion in the joint. Increased OPG levels have also been found in this experiment suggesting that inhibition of osteoclast formation is by interference with RANKL–RANK interaction and osteoclast differentiation.

Effective blockade of bone erosion is of central importance to allow bone growth, which requires an excess of bone formation with respect to bone resorption. This is exactly what Wnt proteins accomplish during joint anklylosis: They stimulate ossification by inducing the differentiation of osteoblasts and simultaneously block osteoclast activity. These data support this concept and suggest that similar mechanisms are apparently also relevant for sacroiliac joint anklylosis.

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