Osteoarthritis and Cartilage (2000) **8,** 82–86

doi: 10.1053/joca.1999.0275, available online at http://www.idealibrary.com on IDEAL®

1063–4584/00/020082+05 $35.00/0

Osteoarthritis and Cartilage

Journal of the OsteoArthritis Research Society International

Role of nitric oxide in the inhibition of BMP-2-mediated stimulation of proteoglycan synthesis in articular cartilage

P. M. van der Kraan, E. L. Vitters, H. M. van Beuningen, F. A. J. van de Loo and W. B. van den Berg

Department of Rheumatology, University Hospital Nijmegen, Geert Grooteplein 8, 6525 GA Nijmegen, The Netherlands

Summary

Objective: Bone morphogenetic protein-2 (BMP-2)-mediated stimulation of articular cartilage proteoglycan (PG) synthesis is suppressed in arthritic murine knee joints and by interleukin-1 (IL-1). The goal of this study was to investigate whether the gaseous mediator nitric oxide (NO) plays a crucial role in the inhibition of BMP-2 effects by IL-1.

Methods: Bone morphogenetic protein-2 alone or in combination with IL-1 was injected into the right knee joint of wild-type and NOS2 deficient C57Bl/6×129/Sv mice. Proteoglycan synthesis was measured *ex vivo* by incorporation of ^{35}S-sulfate on day 1, 2 and 3 after injection. To study the role of NO in the inhibition BMP-2-mediated stimulation of PG synthesis in arthritic joints, BMP-2 was injected intra-articularly in the joints of wild-type and NOS2 deficient mice with zymosan-induced arthritis. To check for NOS2 deficiency, NO production was measured in conditioned medium after challenge of patellae with surrounding tissue with IL-1.

Results: BMP-2 potently stimulated proteoglycan synthesis in articular cartilage of normal knees (up to 4-fold) but not in arthritic knees. Co-injection of BMP-2 with tumor necrosis factor α had no effect on BMP-2-mediated stimulation of PG synthesis but co-injection with IL-1 α resulted in a nearly total inhibition of BMP-2-mediated stimulation. In contrast, in NOS2 deficient mice IL-1 had no effect on BMP-2-mediated stimulation of PG synthesis. However, injection of BMP-2 into arthritic knee joints of NOS2 knock out mice did not result in significant stimulation of PG synthesis.

Conclusions: In this study we show that NO plays a role in the inhibition of BMP-2-mediated stimulation of PG synthesis by IL-1. However, NO, or at least NOS2, plays no dominant role in the inhibition of BMP-2 effects in arthritic knee joints. © 2000 OsteoArthritis Research Society International

Key words: Bone Morphogenetic Protein-2, Nitric Oxide, Interleukin-1, Articular cartilage.

Introduction

A major consequence of rheumatoid arthritis and osteoarthritis is degradation of articular cartilage of which proteoglycan (PG) depletion is a crucial aspect. This process results in severe impairment of the function of the affected joints. Factors that are able to increase the synthesis of PGs and stimulate the replenishment of PGs in the diseased cartilage could be of therapeutic value. Possible candidates to fulfil this role are the bone morphogenetic proteins (BMPs) of which BMP-2 is a representative known to stimulate cartilage PG synthesis *in vitro*[1] and, as shown by our group earlier, *in vivo*.[2,3]

The BMPs belong to the TGF β superfamily. This superfamily of dimeric molecules consist of at least 30 members of which the BMPs are the largest subgroup. All members signal by serine/threonine kinases.[4] BMP-2 appears to participate both in chondrogenesis as well as in the maintenance of the chondrocyte phenotype. BMP-2 was shown to be involved in the differentiation of embryonic mesenchymal skeletal progenitor cells to chondrocytes[5,6] and was also able to induce the transdifferentiation of adult fibroblast to chondrocytes.[7] In the articular cartilage of young calves BMP-2 stimulated proteoglycan synthesis as measured by ^{35}S-sulfate incorporation and by expression of aggrecan mRNA.[1] Moreover, in young adult C57Bl/6 mice a single intra-articular injection of BMP-2 resulted in potent stimulation of proteoglycan synthesis in articular cartilage of the knee joint.[3,4]

In contrast to the anabolic factor BMP-2, IL-1 appears to be an important catabolic factor which has been shown to be a crucial mediator of cartilage destruction in experimental models of arthritis.[8,9] Furthermore it has been recently shown in patients with rheumatoid arthritis that blocking of interleukin-1 with IL-1 receptor antagonist has a beneficial effect on the progress of joint destruction in these patients.[10] At least part of the effect of interleukin-1 on articular cartilage is mediated by the gaseous mediator nitric oxide (NO).

Nitric oxide is a short-lived free radical which is synthesized by three distinct NO synthases (NOS), neural NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3).[11] Nitric oxide produced by NOS2 appears to be involved in the inhibition of articular cartilage proteoglycan

Received 3 April 1999; accepted 1 September 1999.

Corresponding author and offprint requests: P. M. van der Kraan, Ph. D., Department of Rheumatology, University Hospital Nijmegen, Geert Grooteplein 8, 6525 GA Nijmegen, The Netherlands. E-mail: P.vanderkraan@reuma.azn.nl. Tel: 31-24-3616568, Fax: 31-24-540403.

P. M. van der Kraan is supported by 'Het national Reumafonds' (Dutch League against Rheumatism).

synthesis by IL-1.[12,13] However, the role of NO in proteoglycan breakdown is less clear. Nitric oxide has been shown to activate metalloproteinases and the NOS inhibitor L-NMA induced increased instead of reduced proteoglycan degradation in bovine explants.[14,15]

In earlier studies using C57Bl/6 mice, BMP-2 appeared to be a potent stimulator of PG synthesis in normal joints but not in arthritic joints or in combination with IL-1. The goal of this study was to investigate whether NO plays both a crucial role in the inhibition BMP-2-mediated stimulation of PG synthesis by IL-1 and in arthritic knee joints.

Material and methods

ANIMALS

The animals used in this study, the NOS2 −/− knock out mice with a C57Bl/6×129/Sv background and the wild-type C57Bl/6×129/Sv, were bred in our specific pathogen-free animal housing facilities. The animals were kept in cages with a wood chip bedding and had unlimited access to a standard diet and acidified tap water.

EFFECT OF IL-1 AND TNF-α ON NO SYNTHESIS AND CONTROL OF NOS2 DEFICIENCY

Patellae with a standard amount of surrounding tissue were dissected from the knee joints of NOS2 −/− and wild-type mice as described before.[16] Patellae from knock-out and wild-type mice were cultured for 24 hours in culture medium (one patella/200 μl medium) in the presence of 10 ng/ml IL-1α, 10 ng/ml TNF α or medium alone. The conditioned medium was checked for NO production by measuring nitrite (NO_2^-), a stable end product of NO, using the Griess reaction. In brief, 100 μl conditioned medium was mixed with 100 μl Griess reagent (0.1% naphtylethylenediamine dihydrochloride (Sigma), 1:1 diluted with 1.0% sulfanilamide (Sigma) in 5% H_3PO_4). The optical density was measured using flat bottomed microtiter plates (Costar, Cambridge, MA, U.S.A.) at 454 nm with an ELISA reader. Standards of $NaNO_2$ (Merck, Darmstadt, Germany) were used as controls.

INJECTION WITH RECOMBINANT CYTOKINES

Recombinant human BMP-2 (a gift of E. Morris, Genetics Institute Inc. (Cambridge, MA, U.S.A.), recombinant murine IL-1α (donated by Pfizer Central Research, Croton, CT, U.S.A.), and TNF α (R & D Systems Ltd, Abingdon, U.K.) were dissolved in saline +0.1% ultrapure bovine serum albumine (Sigma, St Louis, MO, U.S.A.). Six microliter volumes of BMP-2 or BMP-2 plus IL-1α were injected into the joint cavity of the right knee joint. The left, control knee joint received an equal volume of vehicle simultaneously.

INDUCTION OF ARTHRITIS

Zymosan-induced arthritis (ZIA) was evoked by injection of 180 μg zymosan A (Saccharomyces Cerevisiae, 6 μl) in the right knee joint. Before injection zymosan was emulsified by boiling and sonification. The contralateral knee joint was injected with an equal volume of saline. Cytokines were injected on day three after induction of arthritis. In the ZIA model, arthritis is fully established at this time point.[13]

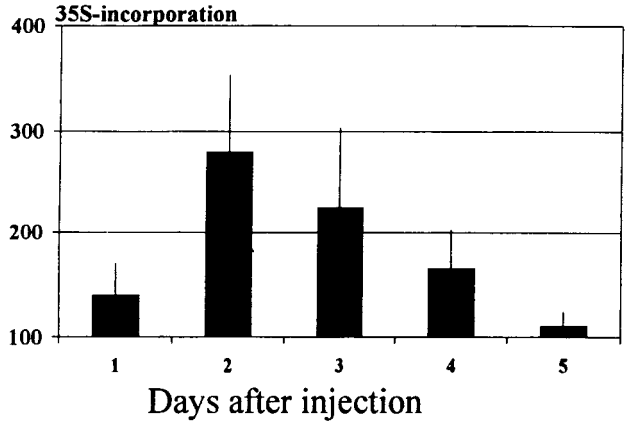

Fig. 1. Stimulation of PG synthesis in patellar cartilage, as measured by ^{35}S-sulfate incorporation, in male C57Bl/6×129/Sv mice effect by a single injection with 200 ng BMP-2. The ^{35}S-sulfate incorporation is expressed as a percentage of the vehicle-injected left knee joint. Each value represents the mean±SD of six animals.

TREATMENT OF ARTHRITIC MICE WITH L-NAME

Starting at one day after the induction ZIA wild-type mice were treated with the general NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME, Sigma). Mice were injected intraperitoneally every day until the end of the experiment with 50 mg/kg L-NAME. On day three after induction of arthritis both L-NAME treated and control mice were injected with BMP-2 in the right knee joint and proteoglycan synthesis was measured 24 hours later.

DETERMINATION OF PROTEOGLYCAN SYNTHESIS

Proteoglycan synthesis was measured *ex vivo* according to the method of van den Berg *et al*.[16] Patellae with a standard amount of surrounding tissue were dissected from the knee joints whereafter the patellae were labeled for 2 hours with ^{35}S-sulfate ($Na_2{}^{35}SO_4$, Dupont, NEN products, Boston, MA, U.S.A.). Subsequently, patellae were washed, fixated and decalcified. After decalcification the patellar cartilage was stripped entirely from the underlying bone and dissolved in lumasolve (Lumac, Groningen, The Netherlands). The ^{35}S content of the cartilage of each patellae separate was measured by liquid scintillation counting.

STATISTICS

Data are expressed as the mean±SD. Statistical significance was tested using the student T-test in combination with the Bonferroni procedure. A $P<0.05$ was considered statistical significant.

Results

EFFECT OF BMP-2 ON PG SYNTHESIS IN NORMAL AND ARTHRITIC KNEE JOINTS

In accordance with earlier studies using C57Bl/6 mice BMP-2 strongly stimulated PG synthesis in the knee joints of normal C57Bl/6×129/Sv mice (Fig. 1). Injection of 200 ng BMP-2 significantly increased PG synthesis on

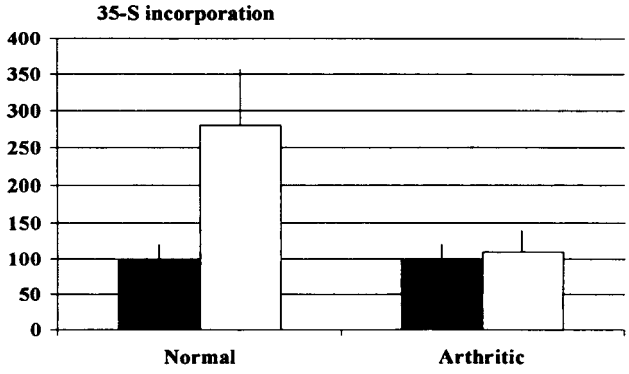

Fig. 2. Absence of BMP-2 effects on PG synthesis in arthritic C57Bl/6×129/Sv mice. BMP-2 was injected in the right knee joints of control mice and in joints of mice with zymosan arthritis. BMP-2 was injected 3 days after the induction of zymosan-induced arthritis. The values are expressed as percentage of the left non-injected controls and each value represents the mean (SD) (N=6). Shown is one representative experiment out of three. The ^{35}S-sulfate incorporation in the arthritic, non-BMP-2-injected knees was 40% of the incorporation in the left knees of the normal mice. (Control closed bars, BMP-2 open bars.)

day 1 and resulted in a maximal stimulation of nearly threefold on day 2. Thereafter PG synthesis decreased and was comparable to controls on day 5. However, in arthritic knee joints, a similar BMP-2 dose (200 ng) was unable to stimulate PG synthesis in articular cartilage (Fig. 2). On day 2 after BMP-2 injection PG synthesis in normal knee joints was approximately 270% while in arthritic knee joints BMP-2 injection did not result in a significant stimulation of PG synthesis.

EFFECT OF IL-1 ON PG SYNTHESIS IN KNEE JOINTS OF NORMAL AND NOS2−/− MICE

To investigate whether the absence of stimulation of PG synthesis by BMP-2 in the inflamed joints could be caused by the presence of pro-inflammatory cytokines in these joints, BMP-2 (200 ng) was co-injected with IL-1 (10 ng) and TNF α (10, 100 ng) in normal murine knee joints (NOS2 controls). On day 1, 2 and 3 after the intra-articular injections PG synthesis was measuered *ex vivo* in patellar cartilage by ^{35}S-sulfate incorporation. Injection of BMP-2 together with IL-1 resulted in almost complete inhibition of stimulation of PG synthesis by BMP-2 (Fig. 3A). While on day 1, 2 and 3 BMP-2 alone significantly stimulated PG synthesis, co-injections of BMP-2 with IL-1 did not lead to increased PG synthesis. Co-injection of BMP-2 with TNF α had no significant effect on the BMP-2-mediated stimulation of PG synthesis (Fig. 3A). Co-injection of 10 or 100 ng TNF α with BMP-2 did not result in a diminished incorporation of ^{35}S-sulfate compared to injection of BMP-2 alone. TNF α had no stimulatory effect on NO production during incubation of patellae *in vitro* (Table 1).

As it has been shown that at least part of the IL-1 effects on articular cartilage are mediated by NO, the experiment described above was repeated with NOS2 knock out mice. The NOS2−/− mice had no elevated production of NO after in-vitro incubation of patella with IL-1 in contrast to control mice (Table 1). As can been seen in Fig. 3B, in NOS2−/− mice the combination of BMP-2 and IL-1 stimulated the PG synthesis in articular cartilage as potent as BMP-2 alone. This indicates that NO plays an important role in the

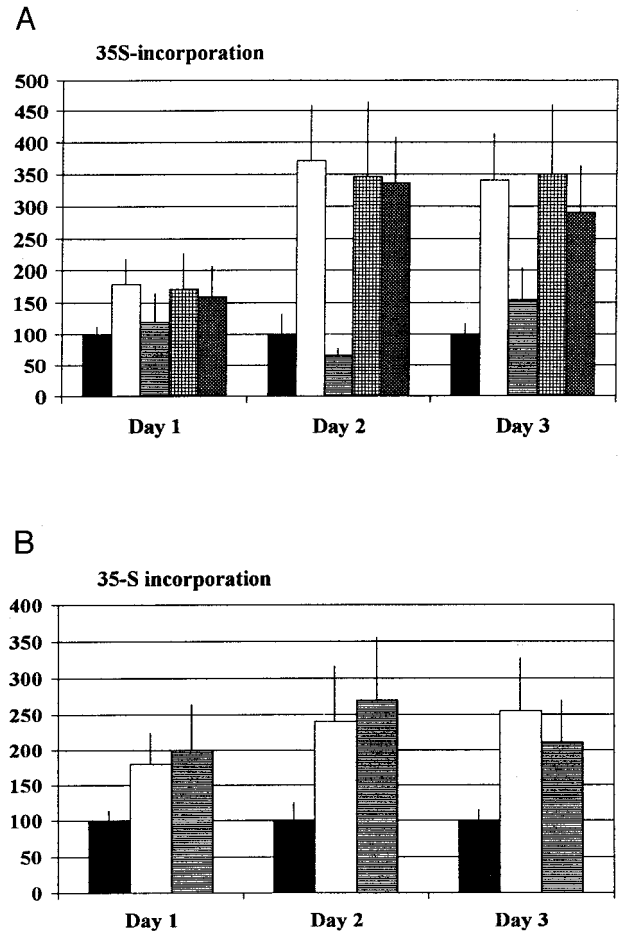

Fig. 3. Inhibition of BMP-2-mediated stimulation of PG synthesis by IL-1α and TNF α in wild type and NOS2−/− mice. BMP-2 (200 ng) was injected alone or in the presence of cytokine in the right knee joint of wild-type or NOS2−/− mice. In wild-type mice co-injection of BMP-2 with IL-1α resulted in a nearly complete inhibition of the stimulation of PG synthesis by BMP-2 on day 1, 2 and 3 after injection of the cytokines (3A). However, in mice deficient in NOS2, IL-1α had no significant effect on the stimulation of PG synthesis by BMP-2 (3B). On all time points BMP-2 significantly stimulated PG synthesis. Expressed are the means (SD) of at least six mice per group of a representative experiment out of three. The ^{35}S-sulfate incorporation values are a percentage of the values in the vehicle-injected left knee joints. (Control closed bars, BMP-2 open bars, BMP-2/IL-1 striped bars, TNF α 10 ng/BMP-2 bars with squares, TNF α 100 ng/BMP-2 hatched bars.)

inhibition of BMP-2 stimulated PG synthesis by IL-1 in articular cartilage.

EFFECT OF BMP-2 IN ARTHRITIC KNEE JOINTS OF NOS2−/− AND L-NAME TREATED MICE

BMP-2 stimulated the PG synthesis in murine knee joints of NOS2−/− mice in the presence of IL-1α. Therefore we investigated whether BMP-2 also retained its stimulating activity on PG synthesis in arthritic knee joints of NOS2−/− mice. Although NOS2 deficiency protects from the inhibition of BMP-2 stimulation of PG synthesis by IL-1, in arthritic joints of NOS2−/− mice BMP-2 still lacked significant stimulating effects on PG synthesis (Fig. 4). In addition,

Table I
Effect of IL-1α (10 ng/ml), TNF α (10 ng/ml) and medium alone on the production of NO (μM) by patellae from NOS2−/− and wild-type mice during a 24 hours culture period. The conditioned medium was checked for NO by measuring nitrite. Expressed are the mean (SD) of six patellae

Mouse strain	IL-1α (10 ng/ml)	TNF α (10 ng/ml)	Medium
C57Bl/6×129/Sv+/+	25.6 (4.9 μM)	7.8 (4.8 μM)	7.3 (3.4 μM)
NOS2−/−	4.2 (1.4 μM)	3.4 (1.2 μM)	3.8 (1.8 μM)

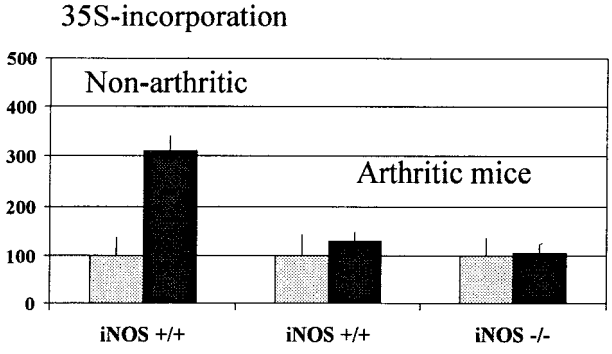

Fig. 4. Effect of BMP-2 in arthritic knee joints of wild-type and NOS2−/− mice. Although BMP-2 strongly stimulated PG synthesis in non-arthritic wild-type mice no stimulation was observed in arthritic wild-type or in arthritic NOS2−/− mice. Expressed are the means (SD) of at least six mice per group of an representative experiment out of three. The ^{35}S-sulfate incorporation values are a percentage of the values in the vehicle-injected left knee joints. (Control striped bars, BMP-2 dotted bars.)

mice treated daily with 50 mg/kg L-NAME, a general inhibitor of NO synthesis, did not show an increased synthesis of PG after intra-articular injection of BMP-2 in the inflamed joints. Injection of BMP-2 (200 ng) in the arthritic joints of L-NAME treated mice resulted in an increase in PG synthesis of 6±5% while in the control joints of L-NAME treated mice an 76±15% increase in PG synthesis was observed one day after injection of BMP-2.

Discussion

In this study we show that NO plays an important role in the inhibition of BMP-2-mediated stimulation by IL-1. Co-injection of BMP-2 with IL-1 blocked the effects of BMP-2 on PG synthesis in articular cartilage in wild-type mice but not in NOS2 knock out mice. Remarkably, NO plays no significant role in the inhibition of BMP-2 effects in arthritic joints.

The absence of BMP-2 effects on PG synthesis in arthritic joints appears to be comparable with the phenomenon of IGF non-responsiveness which has been observed in arthritic joints earlier. Patellar cartilage from murine arthritic knee joints is insensitive for stimulation of PG synthesis by IGF-I, this in contrast to cartilage from normal knee joints.[17] However, in contrast to the effects of IL-1 on BMP-2, IL-1 alone is not sufficient to induce IGF non-responsiveness in articular cartilage.[18] Moreover, cartilage from arthritic joints of NOS2−/− mice remained responsive to IGF-I *in vitro*[13] while arthritic knee joints from NOS2−/− mice were non-responsive to BMP-2 as shown in this study. These observations indicate that although BMP-2 and IGF-I non-responsiveness have some aspects in common the overall mechanism is most likely different.

The mechanism of inhibition of BMP-2 response by IL-1 and NO can be multiple. Down-regulation or inactivation of BMP receptors on the cell membrane by NO or NO products is a possibility. An alternative explanation is that IL-1/NO reduce the availability of the intracellular second mediators involved in BMP signaling (SMAD 1 and 5) or up-regulate the inhibitory SMADs and in this way block the effects of BMP-2 on PG synthesis. An overall non-responsiveness of the chondrocytes to anabolic factors appears to be unlikely since PG synthesis of chondrocytes from arthritic knee joints or after injection of IL-1 can still be stimulated by TGF β.[2,19] The latter observation also implies that down-regulation of SMAD 4, involved in both BMP and TGF β signaling, can not be involved in the inhibition of BMP-2-mediated stimulation of PG synthesis.

Our results show that although NO (NOS2) plays a crucial role in mediating the effects of IL-1 on the inhibition of BMP-2 stimulated PG synthesis, in arthritic joints NOS2 deficiency is not sufficient to restore the response of the chondrocytes to BMP-2. Moreover, treatment of arthritic mice with L-NAME also did not result in maintenance of the response to BMP-2 in the arthritic knee joints. These results indicate that in arthritic joints other factors than NO appear to be involved in the inhibition of BMP-2. Factors in arthritic knee joints, such as IL-17 and leukemia inhibitory factor (LIF), can play a role in inhibition of BMP-2 effects. However, a mediator suggested to be involved in articular cartilage pathology, TNF α,[20] had no significant effects on BMP-2-mediated stimulation of PG synthesis but TNF α had also no stimulatory effect on NO production *in vitro* in the dose tested.

The effects of the arthritis process in general and of IL-1 and NO in particular on BMP-2 signaling can have substantial consequences for articular cartilage homeostasis and repair. Under the conditions of accelerated or even normal catabolic processes in articular cartilage the inhibition of anabolic factors will eventually result in breakdown of the cartilage matrix. Repair of damaged articular cartilage will be seriously impaired by inflammatory mediators in the arthritic joint due to the inhibition of growth factors such as BMP-2 which can potentially play an important role in the repair process. Moreover, since it has been shown that NO is significantly produced in joints of patients with osteoarthritis, one could speculate that repair of articular cartilage is not only impaired in joints of arthritis patients but also in joints of patients with degenerative joint diseases.[21]

In conclusion, our data show that stimulation of PG synthesis by BMP-2 is strongly impaired in arthritic knee joints. Although this process can be mimicked with IL-1 and it appears that NO plays a role in IL-1-induced inhibition of BMP-2 in arthritic knee joints, deficiency of NOS2 does not lead to restoration of the BMP-2-mediated stimulation under arthritic conditions. The inhibition of anabolic factors by the arthritic process can have severe effects on homeostasis and repair of articular cartilage.

Acknowledgments

A breeder pair of the NOS2−/− mice was kindly provided by J. Mudgett, Rahway, U.S.A.

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