THE INHIBITORY EFFECT OF IL-1β AND TNF-α ON OSTEOPHYTE FORMATION IN VITRO

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ABSTRACT:

OBJECTIVE: Osteophytes are present in osteoarthritis, but not in rheumatoid arthritis (RA). It has been suggested that osteophytes are absent in RA due to the presence of the inflammatory cytokine tumor necrosis factor-α (TNF-α). We want to investigate whether TNF-α blocks osteophyte formation via the potent osteophyte inhibitor Dickkopf-1 (DKK-1) or via a different signaling pathway. We aimed to determine if interleukin-1β (IL-1β) and TNF-α were able to inhibit formation of growth factor induced osteophytes in vitro.
METHODS: We cultured bovine periosteum as explants. We stimulated them with transforming growth factor-β (TGF-β) and/or bone morphogenetic protein 2 (BMP2) to induce the formation of osteophytes. Then we combined growth factor stimulation with IL-1β or TNF-α to investigate whether these cytokines were capable of blocking osteophyte formation. We performed quantitative polymerase chain reaction for chondrogenesis- and osteogenesis-related genes and DKK-1. Furthermore, we performed microscopic analysis on the histology of the explants.
RESULTS: We observed chondrogenesis in the explants stimulated with TGF-β alone or combined with BMP2. No chondrogenesis was observed histologically in BMP2 stimulated explants. In the explants, exposure to TGF-β alone or combined with BMP2 up-regulated gene expression of aggrecan, but down-regulated osteogenesis related genes and DKK-1. IL-1β down-regulated collagen 2 (Col2) and aggrecan expression in all conditions, while TNF-α was only able to inhibit Col2 and aggrecan in the TGF-β stimulated explants.
CONCLUSION: There was no obvious effect of TNF-α on DKK-1 expression. IL-1β blocked osteophyte formation in growth factor stimulated explants. TNF-α inhibited osteophyte formation in any TGF-β stimulated explant completely, but partly inhibited TGF-β plus BMP2 induced osteophyte formation. With these results, absence of osteophytes in RA cannot be explained only by inhibitory effects of TNF-α through DKK-1.

WHAT'S KNOWN: Osteophytes are present in osteoarthritis, but not in rheumatoid arthritis (RA). It has been suggested that osteophytes are absent in RA due to the presence of the inflammatory cytokine tumor necrosis factor-α (TNF-α) via the potent osteophyte inhibitor Dickkopf-1 (DKK-1).

WHAT'S NEW: Although TNF- α was able to inhibit osteophyte formation in TGF- β stimulated chondrocytes, no effect on DKK-1 expression was found. The absence of osteophytes in RA can therefore not be explained only by inhibitory effect of TNF-α through DKK-1.

KEYWORDS: Osteoarthritis, periosteum, bovine, cytokine, growth factors

Introduction

Osteoarthritis (OA) is a degenerative joint disease involving cartilage, synovium and bone. It is characterized by cartilage breakdown and eventually osteophyte formation [1]. Rheumatoid arthritis (RA) is characterized by inflammation of the synovium and erosion of cartilage and bone [2]. OA and RA are both inflammatory diseases in which the inflammatory cytokines necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) have an important role [3, 4]. However, osteophytes are found in OA, but they are not found in human rheumatoid arthritis (RA). Primarily, we want to determine whether the absence of osteophytes in RA is due to the presence of the inflammatory cytokine TNF-α via the potent osteophyte inhibitor Dickkopf-1 (DKK-1) [5] or via a different signalling pathway. Secondly, we aimed to determine whether IL-1β and TNF-α were able to inhibit formation of growth factor induced osteophytes in vitro, which would explain the absence of osteophytes in RA.

Osteophytes

Osteophytes are newly formed bone spurs at the edge of the joint. They can cause serious medical problems, including restriction of joint movement and pain [6]. However, there is no clear correlation between presence and severity of osteophytes and pain in patients with OA [7]. Osteophytes might also have a positive effect by increasing the joint surface and thereby decreasing the pressure per surface unit, but this is still under debate [6]. Osteophyte formation originates from the periosteum, which is a tissue layer that covers the bone [6]. The process starts with chondrogenesis, followed by formation of cartilage-like tissue called a chondrophyte. Consecutively, the cells undergo hypertrophy and are replaced by bone (osteophyte) [8].

The formation of bone is called osteogenesis and takes place through two different mechanisms: endochondral bone formation (EBF) and intramembranous bone formation (IBF). In OA, osteophytes are mainly formed by EBF, thereby using cartilage as transition phase. In RA models, osteophyte formation through IBS is observed, in which mesenchymal stem cells directly differentiate into osteoblasts [2].

Transforming growth factor β superfamily signaling

In this study we stimulated periosteal cells derived from bovine periost with transforming growth factor β (TGF-β) and/or bone morphogenetic protein 2 (BMP2), both ligands of the transforming growth factor β superfamily, as an in vitro model for osteophyte formation. TGF-β superfamily signalling is involved in many cellular processes, including cell growth, migration, differentiation and apoptosis. TGF-β superfamily signalling is activated when a TGF-β superfamily ligand binds cognate transmembrane receptor kinases.

TGF-β and BMP2 are involved in a variety of development processes including chondrogenesis [8, 9]. In patients with osteoarthritis TGF-β is suggested to have an important role in the pathogenesis, since elevated values of active TGF-β are found in the synovial fluid. Moreover, knee joints which are exposed to TGF-β show changes in OA cartilage and after TGF-β overexpression in the knee joint, osteophyte formation occurred[10]. TGF-β is suggested to initiate the process of the formation of osteophytes by enchondral ossification in OA, whereas BMP2 is important in later phases of this process[8].

Interleukin-1β and tumor necrosis factor-α

The cytokines IL-1β and TNF-α are major mediators of local inflammatory processes in the joint which inhibit cartilage matrix synthesis by chondrocytes. It is also known that IL-1β and TNF-α induce the breakdown of extracellular matrix molecules of articular cartilage [11]. IL-1β not only breaks down extracellular matrix molecules, it also inhibits the synthesis of collagen type II and proteoglycans which are essential components of osteophyte formation [12]. IL-1β is therefore suggested to have an inhibitory effect on osteophyte formation [4].

TNF-α is secreted during inflammation and promotes bone destruction. In RA, TNF-α is suggested to inhibit osteophyte formation via DKK-1, a potent Wingless and Int homolog (Wnt) signalling pathway inhibitor. In human RA joint sections increased DKK-1 levels are found compared to OA sections [5].

Wnt signalling is a key trigger for bone formation. By inhibiting Wnt via DKK-1 in TNF-α transgenic mice, osteophyte formation was absent [5]. In earlier mouse models, we did not find inhibition of osteophyte formation with TNF-α via DKK-1. We found no differences in osteophyte formation in TNF-α knockout mice, compared to wild type mice. We also found that TNF-α does not decrease the incidence of osteophyte formation in RA models. This indicates that the inhibition of Wnt signalling by TNF-α suggested by Diarra et al did not occur in our model [13].

In this project, we will determine whether IL-1β and TNF-α are able to inhibit osteophyte formation. Moreover, we question whether TNF-α has a negative effect on osteophyte formation via DKK-1 as suggested by Diarra et al, since we found inconsistent findings in earlier mice experiments. In these previous experiments we found that TNF-α has a possible role in osteophyte inhibition in RA via another pathway: Smad2/3 phosphorylation. We found low expression of Smad2/3 phosphorylation during streptococcal cell wall induced arthritis [13]. Therefore we want to investigate whether we can reproduce the TNF-α induced osteophyte inhibition via DKK-1.

We hypothesize that IL-1β and TNF-α can block growth factor induced osteophyte formation in vitro. We suggest that TNF-α has no negative effect on osteophyte formation via DKK-1. To investigate our hypothesis, the effect of these cytokines on growth factor induced osteophytes in vitro was determined.

Materials and Methods

Osteophyte formation by culturing periosteal explants

The materials and methods used to obtain periosteal explants, to culture them and to induce osteophyte formation are shown in appendix I. In our first experiment, we determined whether growth factors were able to induce osteophyte formation by histology and by gene expression after 4 and 6 weeks. We stimulated the explants with TGF-β, BMP2 or both. An endpoint of the experiment of 6 weeks was chosen, the same endpoint of culturing performed by O'Driscoll et al [14]. The explant sections used for histology were stained with Safranin O and Fast Green (appendix II). Safranin O is specifically stains products of cartilage including proteoglycans red and Fast Green stains subchondral bone and fibrous tissue blue [15, 16]. We measured the mRNA expression of Glyceraldehyde-3-phospate dehydrogenase (GAPDH), DKK-1, aggrecan, type I collagen (Col1), Col2 and Col10, runt-related transcription factor-2 (Runx2) and osteocalcin to determine the effect of growth factors on the explants. The housekeeping gene GAPDH was used as internal control, since it is expressed continuously and is involved in cell catabolic processes [17]. Cartilage contains the extracellular matrix components Col2 and the proteoglycan aggrecan, whereas bone contains Col1 [18]. Col2 and aggrecan are increased in the early process of chondrogenesis during EBF. Col10 is a marker for late-stage chondrocyte hypertrophy associated with EBF [19, 20]. Runx2 is a master regulator of osteoblast and terminal chondrocyte differentiation and is an accepted early marker for osteogenesis [20, 21]. Osteocalcin is a bone specific protein and therefore a measure for calcification [22]. The relative changes in mRNA expression of these genes in response to growth factors was measured by quantitative polymerase chain reaction (Q-PCR) analysis.

Table 1 *Q-PCR primer list.*

Gene	Forward primer[1]	Reverse primer[1]
Aggrecan	TGAAACCACCTCCACCTTCCATGA	TCAAAGGCAGTGGTTGACTCTCCA
Col1	AGTCAAGAACTGGTACAGAAATTCCAA	CTGGGTACCACCGTTGATAGTTT
Col2	TGATCGAGTACCGGTCACAGAA	CCATGGGTGCAATGTCAATG
Col10	CCATCCAACACCAAGACACAGT	TGCTCTCCTCTCAGTGATACACCTT
DKK-1	GCAGCAAGTACCAGACCATTGAC	CGACAGGCGAGGCAGATT
GAPDH	CACCCACGGCAAGTTCAAC	TCTCGCTCCTGGAAGATGGT
OC	CCCAAGAGGGAGGTGTGTGA	GCCGATAGGCTTCCTGGAA
Runx2	TGCACCACCACCTCGAATG	CTTCCGTCGGCGTCAAC

[1] Forward and reverse primers are shown from 3' to 5', OC, osteocalcin

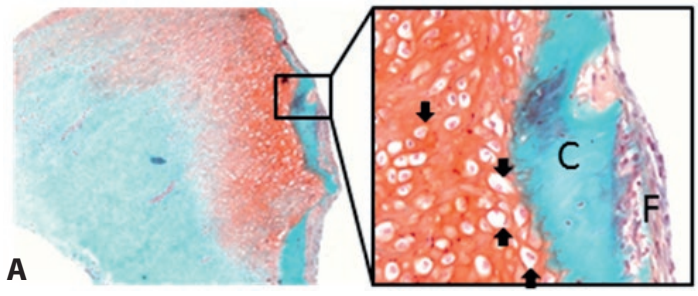
The effect of TNF-α and IL-1β on osteophyte formation

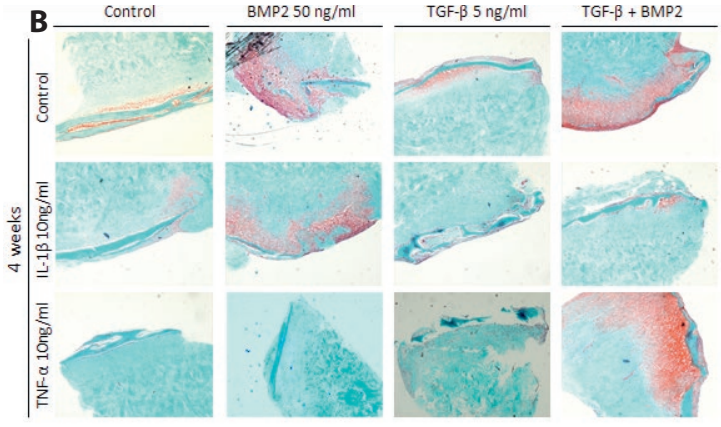
To identify the role of TNF-α and IL-1β on osteophyte formation we stimulated bovine periosteal explants with TGF-β, BMP2 or both, combined with TNF-α and IL-1β exposure. The effects of TNF-α and IL-1β on growth factor induced osteophyte formation were determined by histology and gene expression.

mRNA isolation and Q-PCR

mRNA was extracted from the periosteal explants through different steps (appendix III). Eventually, gene expression of the genes of interest (Aggrecan, Col1, Col2, Col10, DKK-1, GAPDH, osteocalcin and Runx2) (table 1) were determined by Q-PCR. The primer mix consisted of a forward and a reversed primer of each gene.

All primers were obtained from Biolegio BV (Nijmegen, the Netherlands). Q-PCR quantified the results in CT (threshold cycle). The threshold was put at 0.2, which is at the linear part of the curve in the amplification plot. The CT values of the interested genes of a sample were corrected for GAPDH, which leads to the ΔCT value. Then the ΔCT values of the interested genes in stimulated explants were corrected for the unstimulated control explants which eventually leads to a ΔΔCT value.





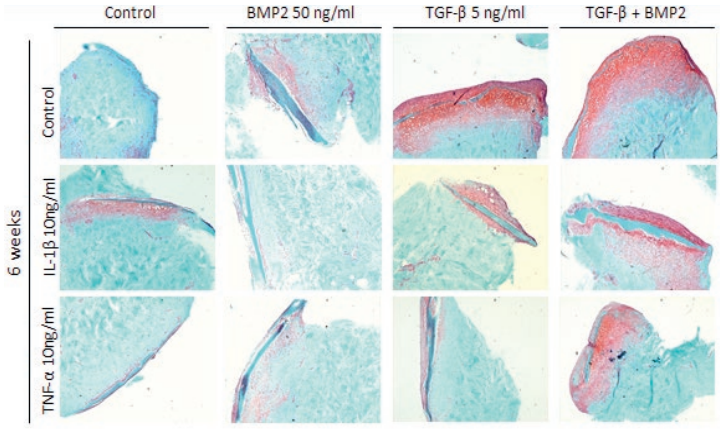


Figure 1 *Histology of periosteal explants.*

A: example to interpret histological sections. Chondrocytes (arrows), a cambium layer (C) and fibrous tissue (F) are shown (magnification 50x to 200x).
B: periosteal explants cultured for 4 and 6 weeks, stimulated with BMP2 and/or TGF-β, combined with exposure to IL-1β or TNF-α. Sections were stained with Safranin O and Fast Green (magnification 50x).

Results

The effect of growth factor induced osteophyte formation
BMP2 was not capable of inducing osteophyte formation. We observed proliferation of fibrous cells, but no cartilage-like cells were seen. On the other hand, TGF-β was capable of inducing osteophyte formation. Cartilage-like structures were observed after 4 weeks which were enlarged by 6 weeks. The combination of TGF-β and BMP2 was also capable of inducing osteophyte formation in periosteal explants.

After 4 weeks we observed more chondrogenesis in the culture stimulated with TGF-β combined with BMP2, compared to TGF-β alone. After 6 weeks the osteophytes were enlarged in the culture of combined stimulation with TGF-β and BMP2 (figure 1b).

Periosteal explants which were exposed to IL-1β alone, formed cartilage-like cells after 6 weeks, but hardly any cartilage matrix was seen. Furthermore, IL-1β inhibited cell proliferation in BMP2 stimulated explants. IL-1β completely blocked TGF-β induced osteophyte formation at 4 and 6 weeks. Strikingly, despite the block of osteophyte formation we did observe a fibrous layer that had formed on the explants after 6 weeks of TGF-β and IL-1β stimulation. When we combined TGF-β with BMP2, IL-1β inhibited osteophyte formation completely, but the thick fibrous layer was also present on the explants after 6 weeks similar to the layer observed when TGF-β alone was combined with IL-1β. This fibrous layer was not seen after 4 weeks on the explants (figure 1b).

We observed less cell proliferation after 4 weeks when we exposed BMP2 stimulated explants to TNF-α compared to BMP2 stimulation alone. After 6 weeks, TNF-α did not inhibit BMP2 induced proliferation. TNF-α was able to prevent osteophyte formation including the fibrous layer completely at both time points in TGF-β stimulated explants. However, TNF-α was not capable of inhibiting TGF-β plus BMP2 induced osteophyte formation after 4 weeks. After 6 weeks TNF-α was able to reduce TGF-β plus BMP2 induced osteophyte formation, but not completely (figure 1b).

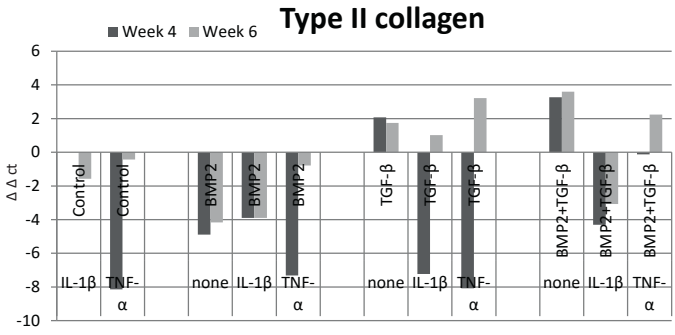


Figure 2 *Relative mRNA levels expression of type II collagen (Col2). CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

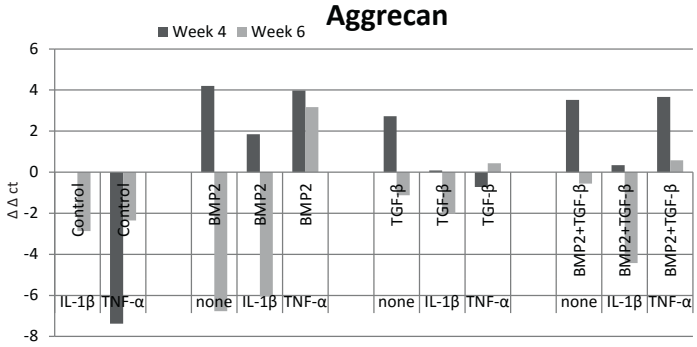


Figure 3 *Relative mRNA levels expression of aggrecan. CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

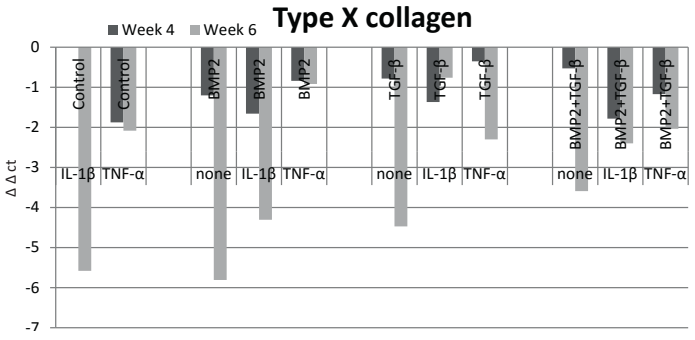


Figure 4 *Relative mRNA levels expression of type X collagen (Col10). CT values were first corrected for GAPDH, and then corrected for the unstimulated control*

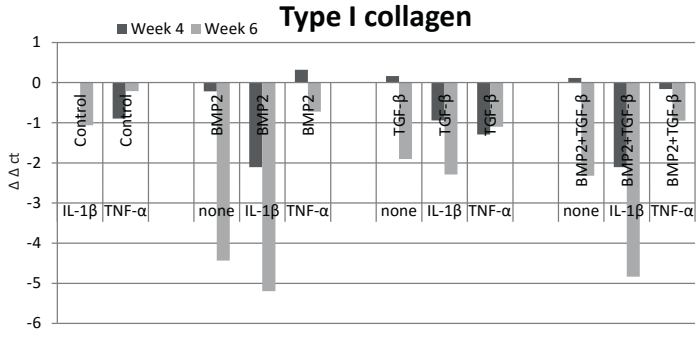


Figure 5 *Relative mRNA levels expression of type I collagen (Col1). CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

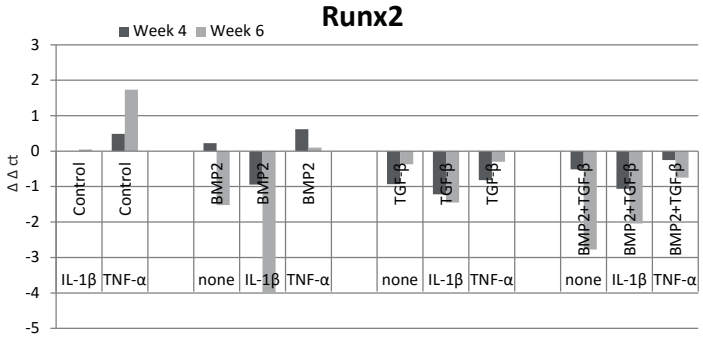


Figure 6 *Relative mRNA levels expression of runt-related transcription factor-2 (Runx2). CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

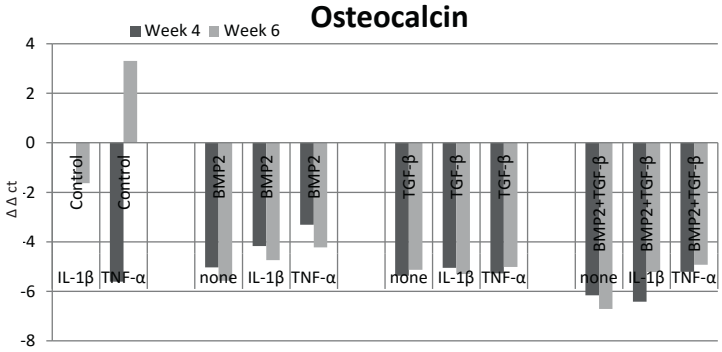


Figure 7 *Relative mRNA levels expression of osteocalcin. CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

Taken together, only conditions containing TGF-β resulted in osteophyte formation. The addition of BMP2 to TGF-β stimulation even induced chondrogenesis more than TGF-β alone. IL-1β inhibited chondrogenesis in all explants, but did not block the formation of a fibrous layer on the explant. TNF-α only blocked osteophyte formation in TGF-β stimulated explants. When we combined TGF-β with BMP2, TNF-α was no longer able to prevent osteophyte formation.

Gene expression of periosteal explants

Col2 mRNA expression

The gene expression of extracellular matrix molecule Col2 was decreased by BMP2 stimulation. TGF-β alone and when combined with BMP2 increased Col2 mRNA expression. These findings were consistent with histology, since we observed chondrogenesis in TGF-β alone or combined with BMP2 stimulated explants and no chondrogenesis in BMP2 stimulated explants in histology (figure 2).

IL-1β had no effect on the expression of Col2 in BMP2 stimulated explants. When the explants were stimulated with TGF-β alone or combined with BMP2, IL-1β decreased Col2 expression after 4 weeks. The Col2 decrease of IL-1β in TGF-β stimulated explants was no longer present after 6 weeks, but Col2 was still decreased after 6 weeks when TGF-β was combined to BMP2. On histology, we also found that IL-1β inhibited chondrogenesis (figure 2).

TNF-α decreased Col2 expression after 4 weeks in BMP2 stimulated explants, while there was an increase after 6 weeks. TNF-α down-regulated Col2 expression in TGF-β stimulated explants after 4 weeks and up-regulated Col2 after 6 weeks. TNF-α decreased Col2 expression after 4 and 6 weeks both when the explants are combined stimulated with TGF-β and BMP2 (figure 2).

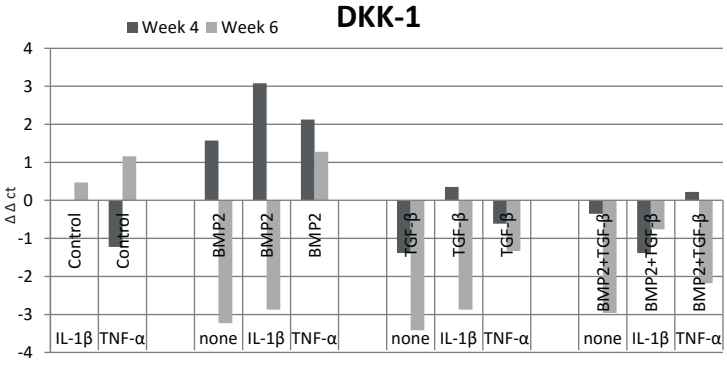


Figure 8 *Relative mRNA levels expression of DKK-1. CT values were first corrected for GAPDH, and then corrected for the unstimulated control.*

Aggrecan mRNA expression

The expression of aggrecan was up-regulated in all growth factor groups after 4 weeks and dropped after 6 weeks. This aggrecan drop is also seen in chondrogenesis of embryonic stem cells after 30 days [23] and 10 weeks [24].When we exposed the explants to IL-1β, aggrecan expression was down-regulated in all groups after 4 and 6 weeks. TNF-α did not inhibit aggrecan expression in BMP2 or TGF-β plus BMP2 stimulated explants. TNF-α had no obvious effect on aggrecan expression of TGF-β stimulated explants (figure 3).

Col10, Col1, Runx2 and osteocalcin mRNA expression

Gene expression of the hypertrophic chondrocyte stage marker (Col10) and osteogenesis markers (Col1, Runx2 and osteocalcin) was down

regulated in aproximately all periosteal explants. IL-1β and TNF-α administration blocked the inhibition of chondrocyte hypertrophy (Col10) when the explants were stimulated with BMP, TGF-β or combined after 6 weeks (fig. 4). The different conditions downregulated or barely had any effect on Col1 (fig. 5), Runx2 (fig. 6) and osteocalcin (fig. 7) expression. This downregulation indicates that bone formation was not induced. This is consistent with the histology findings, where no bone was seen.

DKK-1 mRNA expression

The proposed osteophyte inhibitor DKK-1 was downregulated by TGF-β and TGF-β plus BMP2. When these explants were exposed to IL-1β, DKK-1 expression was upregulated in all conditions, except for the explants stimulated with BMP2 plus TGF-β after 4 weeks. TNF-α, also partially blocked the down-regulation of DKK-1 in all groups (figure 8).

Discussion

In this study, we induced bone formation in bovine derived periosteal cells by adding different mediators that are essential to the development of osteophytes. Our primary goal was to identify whether the absence of osteophytes in RA is due to the presence of the inflammatory we wanted to identify if cytokine TNF-α via the potent osteophyte inhibitor Dickkopf-1. Secondarily, we wanted to determine the potential inhibitory effect of IL-1β and TNF-α on growth factor induced osteophyte formation.

In scientific literature, BMP2 has been described as an important factor in chondrogenic differentiation and cartilage formation during EBF [25]. BMP2 increases Col2 and aggrecan expression and induces formation of hypertrophic chondrocytes in rat periosteum-derived cells during in vitro chondrogenesis in an aggregate culture after 14 days [25]. We found that BMP2 is not capable of the induction of chondrogenesis or osteophyte formation in bovine explant periosteum, considering histology and Q-PCR results. Our Q-PCR results revealed that BMP2 down-regulated chondrogenesis-marker Col2 in our experiments. The effect of IL-1β and TNF-α on osteophyte formation could not be determined, since no osteophytes were formed in BMP2 stimulated explants.

In previous rabbit experiments, TGF-β stimulates chondrogenesis of periosteal explants cultured in vitro [14, 26]. Furthermore, TGF-β has the ability to induce Col2 expression by mesenchymal cells [27]. In our research, we observed that TGF-β alone, or when combined to BMP2 was able to induce chondrogenesis. TGF-β or TGF-β plus BMP2 induced Col2 and aggrecan expression. BMP2 alone could not induce chondrogenesis, but when combined to TGF-β chondrogenesis was enhanced, which can most likely be attributed to the fact that BMP2 is more important in the later phases of this process [8]. Thus, TGF-β alone was able to induce chondrogenesis and this effect was enhanced by BMP2.

IL-1β is a potent inhibitor of Col2 and proteoglycans synthesis [12], which are essential to the chondrogenic phase of osteophyte formation. IL-1β has a suppressive effect on chondrocyte proteoglycan synthesis and it also stimulates the chondrocyte to release destructive proteases, which mediate the breakdown of cartilage [4].

Unlike IL-1β as a destructive mediator in both OA and RA [4], cartilage-like cells without cartilage matrix were seen after 6 weeks of stimulation in our experiments. We observed that IL-1β inhibited Col2 and aggrecan expression. Explants stimulated with TGF-β alone or combined to BMP2, showed less chondrogenesis when they were exposed to IL-1β. These results were consistent with literature that IL-1β inhibits chondrogenesis [4, 12]. We also observed that explants exposed to IL-1β had a fibrous layer. IL-1β inhibited chondrogenesis, but did not block the production of the fibrous layer on the explant that is also observed in osteophyte development. Thus IL-1β only fully blocks the chondrogenic/osteogenic part of osteophyte formation.

TNF-α is a potential osteophyte inhibitor by inducing the breakdown of extracellular matrix molecules of articular cartilage [11]. Moreover, TNF-α is suggested to have a negative effect on osteophyte formation via DKK-1 [5]. In earlier mouse experiments, we found that osteophyte formation did not increase during inflammatory arthritis in TNF-α knockout mice [13]. Furthermore, we observed osteophytes are formed through IBF in RA models [2], whereas in our model, IBF was not induced, but we induced chondrogenesis instead. Therefore, we could not determine the effect of TNF-α in RA on osteophyte formation. The effect of TNF-α on Col2 expression in BMP2 stimulated explants is irrelevant, since BMP2 was not able to induce chondrogenesis. TNF-α blocked osteophyte formation and down-regulated Col2 and aggrecan expression in TGF-β stimulated explants. However, TNF-α did not fully block osteophyte formation completely in TGF-β plus BMP2 stimulated explants. The addition of BMP2 to TGF-β clearly overruled the inhibitory effect of TNF-α. TNF-α down-regulated aggrecan expression in TGF-β only stimulated explants, but when TGF-β was combined with BMP2, TNF-α was not longer able to down-regulate aggrecan expression. TNF-α did not upregulate DKK-1 expression remarkably. We found that TNF-α was able to inhibit TGF-β induced chondrogenesis. The addition of BMP2 to TGF-β stimulation overruled the effect of TNF-α.

Conclusion

TNF-α may block osteophyte formation, but only in TGF-β stimulated explants and not when TGF-β was combined with BMP2. Since only marginal effects were found on RNA levels, we could not confirm a possible blocking effect of TNF-α on osteophyte formation. We have found that IL-1β was a potent osteophyte inhibitor by blocking the chondrogenic/osteogenic part of osteophyte formation.

Osteophytes are present in OA an absent in RA. It is suggested that osteophytes are absent in RA due to the presence of TNF-α . We did not see chondrogenesis in BMP-2 stimulated explants. No chondrogenesis was observed when TGF-β stimulated explants were exposed to TNF-α, but chondrogenesis was observed when TGF-β plus BMP2 stimulated explants were exposed to TNF-α. It remains unclear whether TNF-α blocks osteophyte formation in RA via DKK-1 since no significant effects of TNF-α on the expression of DKK1 were found.

Considering these findings, TNF-α may have an inhibiting effect on osteophyte formation, but most likely not via DKK-1. To determine via which signalling pathway TNF-α blocks osteophyte formation in RA, further research is needed.

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CORRECT ANSWERS TO THE EXAM QUESTIONS

Question 1: Natural killer cells (Answer B, 67% answered correctly)
Natural killers cells are a type of cytotoxic lymphocyte. They are a part of the innate immune system and can be found in the bone marrow, spleen, blood and liver. NK cells are known for their rapid response and direct targeting of cancer and virus-infected cells. In addition they are the main producers of interferon-gamma, which among other things, activates macrophages.

Question 2: The cost of health care (Answer C, 42% answered correctly)
According to the recent announcement of next year's national budget, the Netherlands will spend 74.6 billion euros on health care in the coming year. This is the equivalent of about 200 million euros a day.