Endochondral Ossification in Cartilage Repair Tissue Hampers Bone Marrow Stimulating Techniques

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Abstract

Bone marrow-stimulating techniques are frequently applied to induce cartilage repair. Apart from insufficient chondrogenesis of the ingrowing bone marrow stem cells (BMSCs), these techniques are hampered by excessive ossification with formation of intralesional osteophytes, in which the ingrowing BMSCs tend to undergo the inherent programme of endochondral ossification. Within this programme, the chondrocyte phenotype only represents a transient state that is followed by terminal chondrocyte differentiation and replacement of the cartilaginous tissue by osseous tissue. The transcription factor Runx2 is considered the driving force for endochondral ossification, which integrates signals from growth factors that are released from the bone marrow, including bone morphogenetic proteins (BMPs), fibroblast growth factor-2 and members of the Wnt-family among others. Anti-hypertrophic factors such as PTHrP or anti-angiogenic proteins including Chondromodulin-I or Thrombospondin-1 can inhibit the endochondral ossification. In addition, antagonists of BMP- and Wnt-signalling can stabilize the non-hypertrophic chondrocyte phenotype. The generation of sizable cartilage tissue, however, does not only depend on extracellular factors but also on the fate of the originating cell population. Regardless of the spectrum of specific stimuli, BMSCs are prone to finally become osteocytes rather than chondrocytes. Since there is increasing evidence for epigenetic regulation including DNA methylation and histone modification for cartilage-relevant genes, future studies will have to explore the role of genomic imprinting of adult BMSCs. However, as long as tools that stabilize a chondrocyte-specific phenotype of adult BMSCs are not available in clinical routine, the transplantation of differentiated chondrocytes may remain the method of choice for cartilage repair.

Basic Problems of Cartilage Defects Treated by Bone Marrow-Stimulating Techniques

Articular cartilage has only limited capacities for spontaneous healing in case of injury or degeneration, since the adjacent chondrocytes are largely nonmotile and remain entrapped within the surrounding matrix and, in the nonvascularized tissue, mesenchymal stem cells (MSC) have limited access to cartilage lesions. Thus, the introduction of a new cell population seems necessary to generate cartilage repair tissue. Bone marrow-stimulating techniques, such as microfracturing (MFX) or abrasion of the subchondral bone plate within the defects areas, are simple, minimally-invasive and cost-effective cartilage repair approaches that are frequently applied in clinical settings [1,2]. These techniques allow stem or progenitor cells from the bone marrow to enter the cartilage lesions. Captured within a blood clot, these bone marrow-derived stem cells (BMSCs) proliferate and produce a repair tissue that may completely fill up the defect and contribute to relieve in symptoms of the patient [1-3]. However, the forming repair tissue lacks the biomechanical properties of hyaline articular cartilage and often fails in the long run, which results in deterioration of function and recurrence of clinical symptoms [2]. The inferior quality of the repair tissue results from improper cellular differentiation of the BMSCs, which is confronted with two basic problems:

a. In upper zones of the repair tissue, the chondrogenic differentiation appears to be incomplete. Thus, the ingrowing progenitor cells fail to fully differentiate into chondrocytes, which leads to the formation of fibrous or fibrocartilaginous tissue characterized by inferior biomechanical stiffness (Figure 1a) [4-8].

b. In the deeper zones of the repair tissue, the ingrowing progenitor cells tend to pass through the cascade of chondrogenic differentiation beyond the status of the mature chondrocyte and undergo terminal differentiation. The resulting chondrocyte hypertrophy is typically followed by endochondral ossification (Figure 1b). The forming osseous tissue often exceeds the original level of the subchondral bone plate in terms of intralesional osteophytes (Figure 1 and 2) [8-10].

While incomplete chondrogenesis and the formation of fibrocartilage is a well-recognized problem, the formation of intralesional osteophytes has just recently awakened more interest. Several studies have shown that excessive bone formation occurs in up to 70% of all lesions treated by MFX [2,3,9-13]. In experimental studies on minipigs, the volume of excessive osseous tissue accounted for more than 20% in relation to the total volume of the repair tissue [8,9]. Intralesional osteophytes affect the biomechanical properties of the repair tissue by increasing the overall stiffness [4], which might interfere with the durability of the overlying thinned cartilaginous layer. Our review will discuss the mechanisms of the undesirable endochondral ossification within cartilage repair tissue.

Endochondral Ossification – the Endpoint of the Chondrogenic Differentiation Cascade

In growing BMSCs, the cellular key players of bone marrow-stimulating techniques, are multipotent progenitor cells. Upon
Apart from soluble mediators, the micro-environment which includes other cells, blood vessels, or matrix components, may play an important role for cellular differentiation of BMSCs. For example, a dense capillary network providing high oxygen levels may interfere with the maintenance of the chondrocyte phenotype following ectopic transplantation into subcutaneous tissue pouches. By contrast, low oxygen levels, contact to a synovial environment and the impact of certain biomechanical forces may favour chondrogenic differentiation [21,22].

Basically, adult MSCs recapitulate differentiation processes that are analogous to those within the embryonal limb bud or the growth plate of the growing skeleton, or to those within fracture callus or growing osteophytes of the adult organism [23-26]. Chondrogenesis and the subsequent endochondral ossification is a spatio-temporal process. As seen in the growth plate, the cellular differentiation stages simultaneously occur within the repair tissue. Thus, repair cartilage tissue induced by bone marrow-stimulating techniques is typically characterized by a stratified pattern.

In superficial and middle zones, the chondrogenic differentiation usually remains incomplete resulting in the formation of fibrocartilage that is typically positive for collagen type I but negative for collagen type II. Underneath, a zone of varying extent may contain differentiated chondrocytes that produce a hyaline-like matrix positive for collagen type II. In deeper zones, the cellular differentiation process typically progresses towards the terminal state in which the cells undergo hypertrophy characterized by expression of collagen type X [8,27]. Similar to the growth plate, these cells are putatively prone to undergo apoptosis and to be replaced by endochondral ossification [28]. Development of bone trabeculae within the deepest repair tissue zones results in formation of bone marrow spaces and the invasion of blood vessels [8,9].

Bone marrow-stimulating techniques significantly affect the integrity of the subchondral bone plate depending on the applied method [29]. In contrast to healthy articular cartilage, the repair tissue typically lacks a definite tide-mark and a plane subchondral bone plate. By contrast, the repair tissue may show signs of bone resorption and cyst formation, or even more often, excessive endochondral ossification leading to bone overgrowth above the projected tide-mark (Figure 1 and Figure 2) [2,3,10-13,29]. Such outgrowths alter the biomechanical properties of the joint surface [4] and interfere with the integrity of the rather thin and soft overlying fibrocartilaginous repair tissue, a process that may result in cartilage degeneration.

In view of the long-term outcome of bone marrow-stimulating techniques, it is important to mention that there is a point of no return once the irreversible endochondral differentiation programme has started and that excessive bone formation will not be remodelled into cartilage tissue again.

**Mediators of Terminal Differentiation and Endochondral Ossification**

Excessive endochondral ossification of cartilage repair tissue upon bone marrow-stimulating techniques is a process that basically occurs independently from application of exogenous growth factors. By contrast, terminal differentiation of invading BMSCs is driven by endogenous cell programs and is supported by factors that are incidentally released from bone marrow by penetration of the subchondral bone plate. Indeed, the bone marrow stores and releases a multitude of growth factors including BMPs, FGFs, VEGF, TGFβ, IGF-1 or PDGF (Figure 3) [30-32].
In particular, members of the TGFβ-superfamily, including TGFβ, BMP-2, -4 and -7 and GDF-5 (CDMP-1) are potent chondroinductive growth factors [14,24,33-35]. For cartilage repair, these proteins have been considered promising tools to promote chondrogenic differentiation of MSCs. The exogenous application of these factors in experimental settings could significantly improve the quality of cartilage repair tissue that was characterized by a hyaline matrix instead of fibrocartilaginous tissue [8,36-39].

Beyond their chondroinductive properties, however, in particular BMPs were originally considered bone-inducing factors [40]. Thus, they also strongly promote chondrocyte hypertrophy and endochondral ossification [8,24,34-43]. Intraarticular administration or overexpression of BMP-2 or TGFβ, which resulted in the formation of significant mature osteoarthropathies [44-46], highlighted the osteoinductive capability of these mediators even within the synovial joint. We have recently examined the repair of cartilage lesions within osteochondral defects could be improved and stabilize the chondrocyte phenotype. Indeed, the formation of therapeutically significant osteophytes [8,9]. In addition to its pro-angiogenic effects, VEGF and its receptors are also expressed by both articular chondrocytes and growth plate chondrocytes [56,57]. In cartilage, VEGF acts as a mitogen and also propagates the terminal differentiation of ingrowing vascular structures within the osseous tissue of intralesional osteoarthropathies [8,9]. In addition to its pro-angiogenic effects, VEGF and its receptors are also expressed by both articular chondrocytes and growth plate chondrocytes [56,57]. In cartilage, VEGF acts as a mitogen and also propagates the terminal differentiation of ingrowing vascular structures within the osseous tissue of intralesional osteoarthropathies [8,9].

Indeed, in MFX-induced cartilage repair tissue, we could identify ingrowing vascular structures within the osseous tissue of intralesional osteoarthropathies [8,9]. In addition to its pro-angiogenic effects, VEGF and its receptors are also expressed by both articular chondrocytes and growth plate chondrocytes [56,57]. In cartilage, VEGF acts as a mitogen and also propagates the terminal differentiation of chondrocytes. It inhibits the expression of cartilage-specific genes like collagen type II and aggrecan [58] and increases the secretion of matrix-degrading MMPs, in particular MMP13 [59]. In this respect, therapeutic approaches focus on the inhibition of VEGF to protect and stabilize the chondrocyte phenotype. Indeed, the formation of cartilage repair tissue within osteochondral defects could be improved by intravenous administration of monoclonal antibodies against VEGF with formation of cartilaginous repair tissue that resisted endochondral hypertrophic cells was replaced by excessive bone tissue leading to the formation of impressive intralesional osteophytes [8]. Thus, our findings demonstrated both the chondrogenic and osteogenic effects of BMPs. The concentrations of endogenous BMPs released from the bone marrow may be lower than those applied experimentally, but a prolonged release from ample endogenous storages might have similar effects leading to the formation of intralesional osteoarthropathies as seen in many clinical cases. Two other important members of the TGFβ-superfamily, TGFβ and GDF5 (CDMP-1), also exert chondroinductive effects but have a less prominent impact on promoting chondrocyte hypertrophy [47-50], which explains the favorable effect of both factors observed in a number of experimental cartilage repair studies [51-54].

VEGF, a potent pro-angiogenic factor that exerts a crucial role in endochondral ossification, is also released from the bone marrow [31,32]. VEGF appears to promote the invasion of blood vessels, which is required for the replacement of cartilage by bone [55]. Indeed, in MFX-induced cartilage repair tissue, we could identify ingrowing vascular structures within the osseous tissue of intralesional osteoarthropathies [8,9]. In addition to its pro-angiogenic effects, VEGF and its receptors are also expressed by both articular chondrocytes and growth plate chondrocytes [56,57]. In cartilage, VEGF acts as a mitogen and also propagates the terminal differentiation of chondrocytes. It inhibits the expression of cartilage-specific genes like collagen type II and aggrecan [58] and increases the secretion of matrix-degrading MMPs, in particular MMP13 [59]. In this respect, therapeutic approaches focus on the inhibition of VEGF to protect and stabilize the chondrocyte phenotype. Indeed, the formation of cartilage repair tissue within osteochondral defects could be improved by intravenous administration of monoclonal antibodies against VEGF with formation of cartilaginous repair tissue that resisted endochondral...
ossification [60]. In line with these findings, transplantation of MSCs overexpressing sFlt-1, the soluble splice variant of VEGF receptor-1 which reduces free circulating levels of VEGF, prevented bone invasion into BMP-4-induced cartilage repair tissue [60].

Fibroblast Growth Factors (FGFs) are produced by fibroblasts within the bone marrow and released upon penetration of the subchondral bone plate [30]. The network effect of FGFs on chondrogenesis is complex and includes signals from 22 different ligands and three different receptors [61]. In the growth plate, FGF3 prevents chondrocyte hypertrophy and stabilizes the chondrocyte phenotype. A number of FGFs are capable of activating FGF3, but expression analyses suggest that FGF9 and FGF18 are the preferred ligands of this receptor [62]. By contrast, FGFRI is upregulated in the prehypertrophic and hypertrophic chondrocytes of the growth plate and its preferred ligand FGF2 (bFGF) promotes terminal chondrocyte differentiation and endochondral ossification [61-63]. Similar findings were shown for MSCs, in which FGFRI and its ligand FGF2 promoted their differentiation into a hypertrophic chondrocyte phenotype [64]. Since FGF2 is released from fibroblasts of the bone marrow, it may be an important factor to stimulate chondrocyte hypertrophy and excessive bone formation following opening of the bone marrow space by MFX [30].

Within the growth plate, Wnt/β-catenin signalling promotes chondrocyte hypertrophy and induces osteoblast differentiation with subsequent endochondral ossification [65,66]. Within the cell, both BMP- and Wnt/β-catenin signalling result in increased activity of the runt-domain transcription factor 2 (Runx2), which is the key transcription factor for osteoblast differentiation. In this context, Runx2 activity leads to the expression of proteins typical of hypertrophic cartilage, including Coll10a1, alkaline phosphatase (ALP), osteocalcin (BGLAP) and MMP13 [24,67-69]. Nevertheless, the transcriptional activation of these genes may also depend on factors other than Runx2. In this respect, GADD45β is considered an important co-factor that acts in synergy with Runx2 to induce transcription of Coll10a1 and MMP13 [70]. Interestingly, GADD45β itself was identified to be an early response gene of BMP/Smad-signalling [70].

Factors That Stabilize the Chondrocyte Phenotype and Inhibit Hypertrophy

Clinical experience shows that the osseous fusion of arthrodesees is interfered by interposed articular cartilage [71], since articular cartilage by itself exerts strong anti-angiogenic and anti-osteogenic effects. In this context, we could demonstrate that the transplantation of differentiated chondrocytes into microfractured lesions effectively inhibited excessive endochondral ossification within the repair tissue [9]. The cartilage matrix is rich in proteins with anti-angiogenic properties, including Chondromodulin-I (Chm-I), Thrombospondin-1 and -2 (TSP-1, -2) and Tissue Inhibitors of Metalloproteinases-2 and -3 (TIMP-2, -3) (Figure 3) [72]. Recently, we have demonstrated in a minipig model that the application of recombinant TSP-1 could significantly prevent chondrocyte hypertrophy and excessive ossification within cartilage repair tissue induced by MFX and supplemented with BMP-7/OP-1 [8]. In a similar model, the overexpression of Chm-I within MFX-treated lesions could also prevent terminal chondrocyte differentiation and the formation of intralesional osteophytes (Figure 4) [27].

Chm-I and TSP-1 may not only have anti-angiogenic effects on endothelial cells but also direct anti-hypertrophic activity on the repair cells. This dual action seems obvious, since the occurrence of hypertrophic chondrocytes significantly precedes the ingrowth of vessels [8,27]. The direct anti-hypertrophic effect of TSP-1 on chondrocytes may be at least in part mediated by inhibiting the expression of GADD45β, a crucial transcriptional enhancer for the expression of Coll10a1 and MMP13 [8,70]. The anti-angiogenic and chondrocyte-stabilizing effects of Chm-I have not been fully identified yet but may involve modulation of the cell cycle and cell proliferation by inducing the cell cycle inhibitor p21\(^{\text{up\text{-}regulated}}\) [27,73]. Cell cycle arrest or cellular quiescence with up-regulation of p21\(^{\text{up\text{-}regulated}}\) is a characteristic feature of post-mitotic articular chondrocytes and stabilizes the typical chondrocyte phenotype [74,75].

Other secreted factors that act in a paracrine manner may also mediate the anti-hypertrophic effect of transplanted chondrocytes within MFX-treated lesions. Among those, PTHrP may be the most relevant candidate. This peptide is involved in a negative feedback-loop described for the growth plate [76]. The expression of PTHrP in the periarticular perichondrium is induced by Indian hedgehog (Ihh) which is released from hypertrophic chondrocytes. PTHrP, in turn, acts in a gradient to prevent or delay premature hypertrophic differentiation of chondrocytes of the proliferating zone [76]. In the adult, we detected a significantly higher expression of PTHrP in articular chondrocytes compared with transiently differentiated chondrocyte-like cells within the cartilaginous cap of osteophytes [77]. In other cell culture experiments, PTHrP was secreted by articular chondrocytes and suppressed the hypertrophic differentiation of co-cultured MSCs in a paracrine manner [78]. The biological action of PTHrP is tightly coupled with the action of the transcription factor Sox9, the key element in inducing and stabilizing the chondrocyte phenotype. While Sox9 was shown to transactivate PTHrP gene expression [79], PTHrP itself phosphorylates Sox9 and thereby increases its transcriptional activity [80]. Thus both factors act in concert to inhibit chondrocyte hypertrophy and matrix calcification.

Osteophytes may represent a model for secondary cartilage formation and they share great similarities with MFX-induced cartilage repair tissue. Both tissues arise from mesenchymal progenitor cells that transiently differentiate into a chondrocyte-like phenotype. Particularly within the deeper zones of both tissues, the cells tend to undergo hypertrophy, which is followed by endochondral ossification.
Rheumatology

Because of these analogies, the development of osteophytes may be a useful model to study endochondral ossification in MFX-induced cartilage repair. In this context, we have recently performed a genome-wide microarray analysis comparing the gene expression of permanent articular cartilage with that of the cartilaginous cap of osteophytes of adult joints. In this comparative analysis, we identified GREM1 as one of the most differentially expressed genes with a more than 20-fold upregulation in articular chondrocytes compared with osteoarthritic chondrocytes [77]. GREM1, a functional BMP-antagonist, binds and blocks the action of BMP-2, -4 and -7 [81]. In early skeletal development, GREM1 is involved in a self-regulatory feedback system that maintains the chondrocyte phenotype during limb formation [82]. GREM1 also plays a central role in epiphyseal development with a significantly higher expression in the region of the epiphyses compared to the hypertrophic physical zone of developing bones of 7-day-old mice [83]. GREM1 is supposed to maintain the chondrocyte phenotype within the epiphyses, while the action of BMPs propagates endochondral ossification in the physes. Accordingly, a recent study based on an expression analysis of epiphyseal and growth plate cartilage of new-born rats revealed the presence of a gradient in BMP-activity that increases from the epiphyses towards the hypertrophic zone. Thus, epiphyseal cartilage, the origin of the developing articular cartilage, exhibited lower expression of BMPs-2, -4, -6 and -7 and higher expression of the BMP antagonists GREM1 and Noggin when compared with the hypertrophic zone [84]. This BMP-gradient of the growth plate may also be transferred to the adult articular cartilage, in which low levels of BMPs [85] help to maintain the cells in a quiescent, non-hypertrophic state.

Our recent genome-wide expression analysis revealed that two further inhibitors of growth factor signaling, WNT1 inducible signaling pathway protein-3 (WISP3) and Frizzled-related Protein (FRzb), were upregulated in stable permanent articular chondrocytes compared to transient osteoarthritic chondrocytes [77]. WISP3 is an inhibitor of both BMP- and Wnt-signaling and has stabilizing effects on the chondrocyte phenotype by inhibiting cellular maturation and hypertrophy [86-88]. FRzb predominantly antagonizes the signaling of Wnt ligands. In the developing skeleton, FRzb is prominently expressed within the epiphyses. Since Wnt-signaling promotes osteoblast differentiation and endochondral ossification, FRzb delays transformation to hypertrophy and inhibits trabecular bone development [83,89]. The lack of FRzb in FRzb(-/-) mice displayed increased periosteal appositional new bone formation and osteoarthritis-like changes [90]. Furthermore, variants of the FRzb were associated with hip osteoarthritis in humans associated with increased osteophyte formation [91,92].

Taken together, there is increasing evidence that the generation and the maintenance of a stable, non-hypertrophic chondrocyte phenotype does not only depend on mitogenic and anabolic factors but also on the modulating function of antagonistic and inhibitory factors.

**BmScs Differ from Articular Chondrocytes with Respect to Their Cellular Fate**

As discussed above, BMSCs adopt a transient rather than permanent chondrocyte phenotype and tend to undergo terminal differentiation, which is followed by endochondral ossification. In this context, the induction and, even more important, the maintenance of a chondrocytic phenotype does not occur spontaneously, but incessantly depends on the influence of certain exogenous factors. For example, we could recently demonstrate that BMSCs, indeed, adopted a non-hypertrophic chondrocytic phenotype by applying the prochondrogenic stimulus BMP-7/OP-1 and simultaneously the anti-angiogenic and anti-hypertrophic factor TSP-1 [8]. However, the follow-up period of this study was limited to 6 months and it may be speculated that, as soon as the influence of the exogenously applied therapeutic proteins declines, a fibroblastic dedifferentiation or terminal differentiation of the cells will occur. Such secondary dedifferentiation was obvious in another study, in which BMP-2 gene transfer only transiently induced a chondrocyte-like phenotype of mesenchymal repair cells and in course of a declining BMP-2 stimulus, the cells reverted back to a fibroblastic phenotype [37]. Thus, ever ongoing stimuli seem mandatory to maintain chondrocytic differentiation of BMSCs.

Since the physiological role of BMSCs in the adult is to serve for fracture healing, it appears obvious that the restorative response does not stop with cartilage formation but further progresses to bone formation. Of note, MSCs from other origins, such as the synovial membrane, adipose tissue, muscle, periosteiurn or perichondrium, also show chondrogenic potential, but permanently stable chondrogenic differentiation could not be demonstrated for any of these cell populations so far [18-20,93,94].

The use of differentiated articular chondrocytes for cartilage repair approaches might help to overcome this obstacle. Articular chondrocytes are known to spontaneously produce a cocktail of anti-hypertrophic factors, including PTHrP, TSP-1 and Chm-I [8,9,27,78]. In co-culture experiments, articular chondrocytes effectively inhibited hypertrophic differentiation of MSCs [78,95,96]. Furthermore, the simultaneous transplantation of differentiated chondrocytes into microfractured lesions could prevent excessive endochondral ossification [9]. Even the application of undigested cartilage tissue fragments into cartilage lesions reduced the formation of intralesional osteophytes [10]. Thus, the presence of differentiated chondrocytes or cartilage tissue within repair tissue seems to be beneficial.

Beyond that, there is increasing evidence that articular cartilage itself contains a progenitor cell population, which is located particularly within the superficial zones and which may be capable to restore smaller superficial lesions [97-102]. Although this endogenous repair capacity is commonly not sufficient to heal clinically symptomatic larger cartilage defects, this predetermined cell population may represent a favourable source for cartilage repair [96-99]. This cell population may clinically be utilized by the transplantation of allogeneic juvenile minced epiphyseal cartilage fragments [103], which may evolve into a promising alternative to autologous chondrocyte transplantation.

Based on the current knowledge, one might speculate that genetic imprinting during early joint formation may differentiate between the determination of a permanent articular chondrocyte originating from cap of the epiphyses and the determination of a transient growth plate chondrocytes originating from the resting zone. Epigenetic mechanisms include gene activation by histone acetylation and gene silencing by methylation of CpG-rich DNA regions. Recently, epigenetic regulation of the COL10a1 gene in chondrocytes has been demonstrated: While hypermethylation of the COL10a1 gene in articular chondrocytes results in silencing of this hypertrophic marker, demethylation in MSC is associated with induction of COL10a1 [104]. By contrast, the COL2a1 gene is sparsely methylated in chondrocytes [105]. Histone acetylation facilitates transcription by modulating the chromatin structure. In this respect, histone deacetylases (HDAC) exert a transcriptional repression. HDAC4, which is expressed in prehypertrophic chondrocytes, may have a central role in skeletal development by inhibiting the expression of Runx2 and thus regulating chondrocyte hypertrophy and endochondral ossification [106].
Outlook

Future therapeutic approaches, which have to focus on the stabilization of the chondrocytic phenotype and inhibition of chondrocyte hypertrophy, will have to consider the following challenges: 1. the identification of the appropriate repair cell population under consideration of genetic imprinting mechanisms that may predetermine a chondrogenic fate of progenitor cells. 2. the interplay of different signalling molecules, that will not only include anabolic growth factors, but also antagonists and inhibitory factors. Until these aspects have not completely been identified, the transplantation of differentiated chondrocytes may remain the method of choice to induce hyaline-like repair cartilage and to avoid excessive bone formation.

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