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Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis

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Summary

Objective: In human osteoarthritis (OA), various forms of pathology are observed. Besides cartilage damage and fibrosis, neogenesis of bone, osteophyte formation, also occurs. Osteophytes are thought to limit joint movement and cause pain. The objective of this study was to investigate whether synovial macrophages are involved in osteophyte formation in experimental OA, and if they are, to study which mechanism may be involved.

Design: Experimental OA was induced by two intra-articular injections of collagenase on alternate days into murine knee joints. The role of synovial lining macrophages in this model was studied by selective removal of these cells prior to OA induction using clodronate liposomes. After 1 and 2 weeks, knee joints were dissected and examined (immuno)histologically.

Results: At days 7 and 14 after induction of OA, osteophyte formation and fibrosis were observed. Depletion of synovial macrophages resulted in spectacular reduction of osteophyte formation, 84% and 66%, respectively, at days 7 and 14. Fibrosis and synovial activation, measured by MRP8/14 expression, were also ameliorated (40–60%). In addition, production of growth factors (TGFβ, BMP-2 and BMP-4) in the lining was largely prevented but production of these growth factors in deeper layers of the synovium and the periosteum did not differ from controls.

Conclusions: These results indicate the synovial macrophage to be a pivotal cell in the synovium mediating osteophyte formation and other OA-related pathology, like fibrosis, during experimental OA. Production of growth factors and induction of synovial activation by these cells may play a crucial role in this event.

Key words: Osteoarthritis, Macrophage, Osteophytes, Growth factors.

Introduction

During osteoarthritis (OA), many pathological changes are observed in the affected joints. These changes include loss of articular cartilage, chondro/osteophyte formation (new formation of ectopic cartilage and bone in the joint, hereafter called osteophyte formation) and sclerosis of subchondral bone. In addition, the synovium is involved. Activation of the synovial layer (synovitis) is seen in many OA joints[1] and formation of osteophytes at the junction of periosteum and synovium is a common feature[2,3]. It is commonly thought that these osteophytes develop from mesenchymal cells in or near the periosteum[2,4]. Although it is thought that osteophyte formation may be a repair mechanism to help stabilize joints, osteophytes also cause obvious negative effects, such as pain and loss of movement.

Growth factors like transforming growth factor (TGF)-β and bone morphogenetic protein (BMP)-2 have been implicated in osteophyte formation[5–8]; however, the mechanisms via which this occurs have to be elucidated. These growth factors have been described to induce chondro- and osteogenesis *in vivo* by direct injection into the knee joint[5,9,10], and *in vitro* in mesenchymal cells[11,12]. However, little is known about their endogenous *in vivo* role in models for OA, although our recent work suggests an essential role for TGFβ in this[13].

In experimental models for chronic arthritis, osteophyte formation is frequently found, which suggests that the presence of synovitis can induce formation of osteophytes[14]. Macrophages from the synovial lining have been shown to determine inflammation and cartilage damage in several experimental arthritis models[15–17]. Both synovial inflammation and cartilage damage are ameliorated, when synovial macrophages are selectively depleted from the synovial lining layer in several models of experimental arthritis. In addition, selective depletion of macrophages from the synovial lining also prevents osteophyte formation in these experimental arthritis models [unpublished observations]. Once activated, these macrophages produce a plethora of mediators that are involved in inflammatory, anabolic and catabolic processes. Activated macrophages can be detected using specific markers, the myeloid related proteins MRP8 and MRP14[18]. When macrophages are activated,

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the S100 proteins MRP8 and MRP14 are expressed by the cell. Performing immunohistochemistry on these proteins can be a helpful tool in demonstrating macrophage activation in the synovium of knee joint specimens[19].

Apart from mediating synovial activation, macrophages can produce several growth factors in high quantities (e.g. TGFβ, BMP-2 and BMP-4)[20–22]. Therefore, these cells may have direct effects on chondro- and osteogenesis that occurs in osteophyte formation. In a recent study it was shown that in osteophyte formation that occurs when TGFβ is injected into murine knee joints, macrophages are involved[23].

The objective of this study was to investigate whether macrophages are involved in osteophyte formation in early experimental OA and whether synovial inflammation and growth factors play a role in this. In the present study we used an experimental murine model for OA, based on the generation of instability of the knee joint[24], which leads to many of OA-like changes, and studied the role of synovial macrophages in osteophyte formation and synovial activation.

Methods

ANIMALS AND INDUCTION OF EXPERIMENTAL OA

In all experiments, male C57BL/6 mice were used, which were obtained from the Charles River Institute (Sulzfeld, Germany). The animals used were between the ages of 8 and 12 weeks and received a standard diet and tap water ad libitum. For the time points day 7 and day 14, respectively, 14 and 8 animals were used per group. In addition, for immunohistochemistry, six naïve, untreated animals were used. All animal experiments were approved by the ethics committee of the animal care facility in Nijmegen.

Experimental OA was induced as described before[24]. Briefly, 1 unit of collagenase type VII from *Clostridium histolyticum* (Sigma Chemical Co., St. Louis, MO, USA) was injected twice on alternate days into the right knee joint of mice. To study early osteoarthritic changes, at days 7 and 14, knee joints were dissected, fixed in 4% formalin and processed for histological examination.

DEPLETION OF SYNOVIAL MACROPHAGES

Macrophages were depleted from the synovial lining layer by injection of clodronate (dichloromethylene bisphosphonate, Cl_2MBP) encapsulated by liposomes. Clodronate was a gift of Roche Diagnostics (GmbH, Mannheim, Germany). Other reagents for the production of clodronate liposomes were phosphatidylcholine, obtained from Lipoid GmbH, Ludwigshafen, Germany. Cholesterol was purchased from Sigma Chemical Co., USA. The method of clodronate liposome production was described by van Rooijen *et al.*[25]. Seven days after injection of 6 μl of clodronate liposome suspension, depletion of synovial macrophages is maximal, which means more then 95%[15–17]. Liposomes are specifically phagocytosed by macrophages and after the liposome enters the cell, clodronate is set free, which causes the macrophage to undergo apoptosis[26,27]. As a negative control for lining depletion, animals were injected intra-articularly with 6 μl PBS.

HISTOLOGY

Dissected knee joints were fixed in 4% formalin, decalcified in 10% formic acid, and subsequently embedded in paraffin. Then frontal total joint sections were made at 7 μm thickness. After this, sections were mounted on superfrost glass slides (SuperFrost ® Plus, Menzel-Glaser, Germany) and deparaffinized. Both haematoxylin and eosin (H&E) and safranin-O (Saf-O) staining were performed. H&E slides were used to evaluate synovial activation by scoring thickening of the synovial lining and cellular influx into joint cavity and synovium. Of each knee joint, three sections were scored which were spaced 140 μm apart. Synovial activation was scored blindly by two observers as follows: 0, no changes compared to normal joints; 1, thickening of the synovial lining and some influx of inflammatory cells; 2, thickening of the synovial lining and intermediate influx of inflammatory cells; and 3, profound thickening of the synovial lining (more than four cell layers) and maximal observed influx of inflammatory cells. Inflammatory cells were discriminated based on their morphology. In addition, fibrosis was scored on H&E stained sections. Fibrosis was defined as cellularity of fibroblast-like cells (based upon morphology) in the synovial sublining and was scored blindly and independently by two observers as follows: 0, no increased cellularity; 1, some increased cellularity; 2, moderately increased cellularity; and 3, maximal increased cellularity.

Saf-O stained sections were used to assess osteophyte size and number. Osteophyte size was scored in each joint by measurement of three sections, each spaced 140 μm apart. Of each section, the surface areas of osteophytes were measured using an image analysis system (Leica Qwin, Leica Microsystems B.V., Rijswijk, The Netherlands) at the seven following sites in the joint: (1) lateral from the patella, (2) medial from the patella, (3) lateral from the femur adjacent to the patella, (4) medial from the femur adjacent to the patella, (5) lateral from the lateral condyle of the femur, (6) medial from the medial condyle, and (7) medial from the tibial plateau. The surface area of the osteophytes was indicated by hand and defined as cartilaginous and osseous structures that develop outside the normal margins of bone. The mean area per knee joint (three sections) was calculated and expressed in μm^2.

IMMUNOHISTOCHEMICAL DETECTION OF MACROPHAGE ACTIVATION MARKER MRP14 AND GROWTH FACTORS

Formalin-fixed sections of knee joints were prepared as described above. Rabbit antiserum against recombinant murine MRP14 (α-MRP14) was produced as described earlier[28]. Monospecificity of the antibody was analysed by immunoreactivity against recombinant MRP14 and Western blot analysis of lysates of granulocytes. Sections of knee joints that were isolated at days 7 and 14 were stained as described earlier using a final antibody concentration of 1 μg/ml. Primary antibodies were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova). An isotype-matched antibody without relevant specificity was used as negative control (Dianova). Finally, sections were counterstained with Mayer's haematoxylin (Merck, Germany). MRP14 positive cells present in the joint cavity and synovium were indicated by red staining.

TGFβ, BMP-2 and BMP-4 were detected immunohistochemically on cryostat sections on knee joints that were dissected at day 7 after the first collagenase injection. In order to preserve epitopes that are sensitive to formic acid, tissue was decalcified in 0.5% EDTA/PVP and subsequently frozen in OCT compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands). Sections were cut in

a cryostat (Microm, Walldorf, Germany). The sections were mounted on superfrost slides (Menzel-Glaser, Germany) and fixed in 4% paraformaldehyde. Endogenous peroxidase was blocked using 1% hydrogen peroxide in methanol. Subsequently, sections were stained for TGFβ3 (R&D Systems, Abingdon, UK), BMP-2 and BMP-4 (Santa Cruz, Biotechnology, Inc, California, USA) using specific polyclonal goat antibodies at a concentration of 1 μg/ml. Hereafter, sections were incubated with biotinylated secondary antibodies which were made in rabbit and directed to goat IgG (Vector Laboratories, Burlingame, CA, USA). Then peroxidase labelled avidin–biotin complexes were incubated on the sections using an ABC-kit (Vector Laboratories). Sections were developed using di-amino-benzidine (Sigma Chemical Co.) and subsequently counterstained using haematoxylin. To quantify the expression of growth factors, positive staining of the synovial lining, sublining and the periosteum was scored separately. Per knee joint, three sections were used that were spaced 140 μm apart. An arbitrary score was used and scoring was done by two observers in a blinded manner as follows: 0, no positive cells; 1, less then 10% positivity; 2, between 10% and 50% positivity; and 3, more than 50% positivity.

As negative control, polyclonal normal goat IgG was used. In none of the negative controls using irrelevant antibodies, background staining was observed.

Results

PATHOLOGICAL CHANGES DURING EXPERIMENTAL OA

Because the kinetics of osteophyte formation in collagenase induced OA has not been extensively described, we first studied the stages of osteophyte development that are found during the early phase of collagenase induced experimental OA. Osteophytes developed from cells in the periosteum, mostly at sites in the joint that are adjacent to cartilage surfaces. The first changes that are found using light microscopy are proliferation and subsequently some enlargement of cells in the periosteum at these sites in the joint (Fig. 1B) compared to naïve joints (Fig. 1A) and this is found already at day 7 after OA induction. Gradually, the matrix surrounding these cells stains red with Saf-O, indicating that already in this phase proteoglycans are deposited in the matrix (Fig. 1C). Hereafter, the structure increases in size, now maintaining a cartilage-like morphology, with (hypertrophic) chondrocytes and strong red staining of proteoglycans (Fig. 1D). These cartilage-like structures are called chondrophytes. Already at day 14 most of these chondrophytes are changing into bone-like structures (osteophytes) by endochondral ossification (Fig. 1E). In this stage, also bone marrow cavities are formed within the chondrophyte/osteophyte structure, which connect to the bone marrow cavity of the adjacent bone.

Apart from osteophyte formation, also synovial pathology developed within 2 weeks after induction of experimental OA. These changes in the synovium underline synovial activation, as indicated by proliferation of fibroblasts, also called fibrosis (Fig. 1F), influx of macrophages leading to thickening of the synovial lining (Fig. 1G) and even inflammatory cells (Fig. 1H) are clearly present in the deep synovial layer, although cell influx was very low compared to arthritis models (not shown). However, pathology that is found in late stage OA, like cartilage matrix erosion, was not yet demonstrable at this time point.

SYNOVIAL EXPRESSION OF GROWTH FACTOR PRODUCTION AND MACROPHAGE ACTIVATION MARKERS DURING EXPERIMENTAL OA

We further studied specific changes in the synovium, such as production of growth factors that have been implicated in new formation of cartilage and bone.

The growth factors TGFβ3, BMP-2 and BMP-4 were detected in total knee joint sections of mice in which experimental collagenase OA was induced 7 days earlier and in naïve knee joints. All three growth factors were produced by cells of the synovial lining (30% positive cells for TGFβ, 10% positive cells for both BMPs), but also by 20–30% of cells in the sublining and periosteum (Fig. 2D–F). BMP-2 and -4 were hardly detectable in knee joints of naïve mice (Fig. 2B, C), whereas TGFβ was already detectable in these joints and was only mildly increased in knee joints with OA (Fig. 2A, D).

We next set out to determine which cell type in the synovium was activated in such a way that growth factors were produced. Immunohistochemical detection of the macrophage activation marker MRP14 in knee joints demonstrated positive staining, both in the lining and in the sublining of knee joints in which experimental OA was induced, both 7 and 14 days previously (Fig. 2H, I). This indicated that macrophages, both those in the lining and in the sublining are activated up to 14 days after induction of experimental OA. Naïve knee joints were negative for MRP14 staining (Fig. 2G).

ACTIVATED SYNOVIAL MACROPHAGES ARE INVOLVED IN OSTEOPHYTE FORMATION IN EXPERIMENTAL OA

To investigate the role of these activated macrophages during experimental OA, we next studied the effect of depletion of lining macrophages on development of early OA pathology. Macrophages were depleted prior to induction of OA, by intra-articular injection of clodronate liposomes. Strikingly, the absence of synovial lining macrophages in the first phase of OA resulted in a very strong reduction of osteophyte size, at day 7 (mean reduction 84±16%) and at day 14 (66±13%). Reduction of osteophyte size was significant at all measured locations in the knee joint (Fig. 3). In addition, in chondrophytes/osteophytes that did occur in the lining depleted group, less ossification was observed and the cartilaginous transformation of the structures was less pronounced and in many cases did not develop at all, indicating that the entire process of chondrophyte/osteophyte formation was inhibited. Joint stability itself was not affected by the clodronate treatment, as dislocation of the knee joint occurred equally both in macrophage depleted and non-depleted groups (not shown).

Besides the effects of macrophage depletion on the formation of chondro/osteophytes, also an effect was found on fibrosis, lining thickening and influx of inflammatory cells. Fibrosis and influx of inflammatory cells were both significantly reduced by about 50% (Figs. 4 and 5) by depletion of synovial macrophages prior to OA induction, as was thickening of the lining (Fig. 5A, B).

EFFECT OF LINING DEPLETION ON MACROPHAGE ACTIVATION AND GROWTH FACTOR PRODUCTION

We next set out to investigate whether these effects of macrophage depletion on the neogenesis of chondrophytes and osteophytes could be due to a decrease in activation of

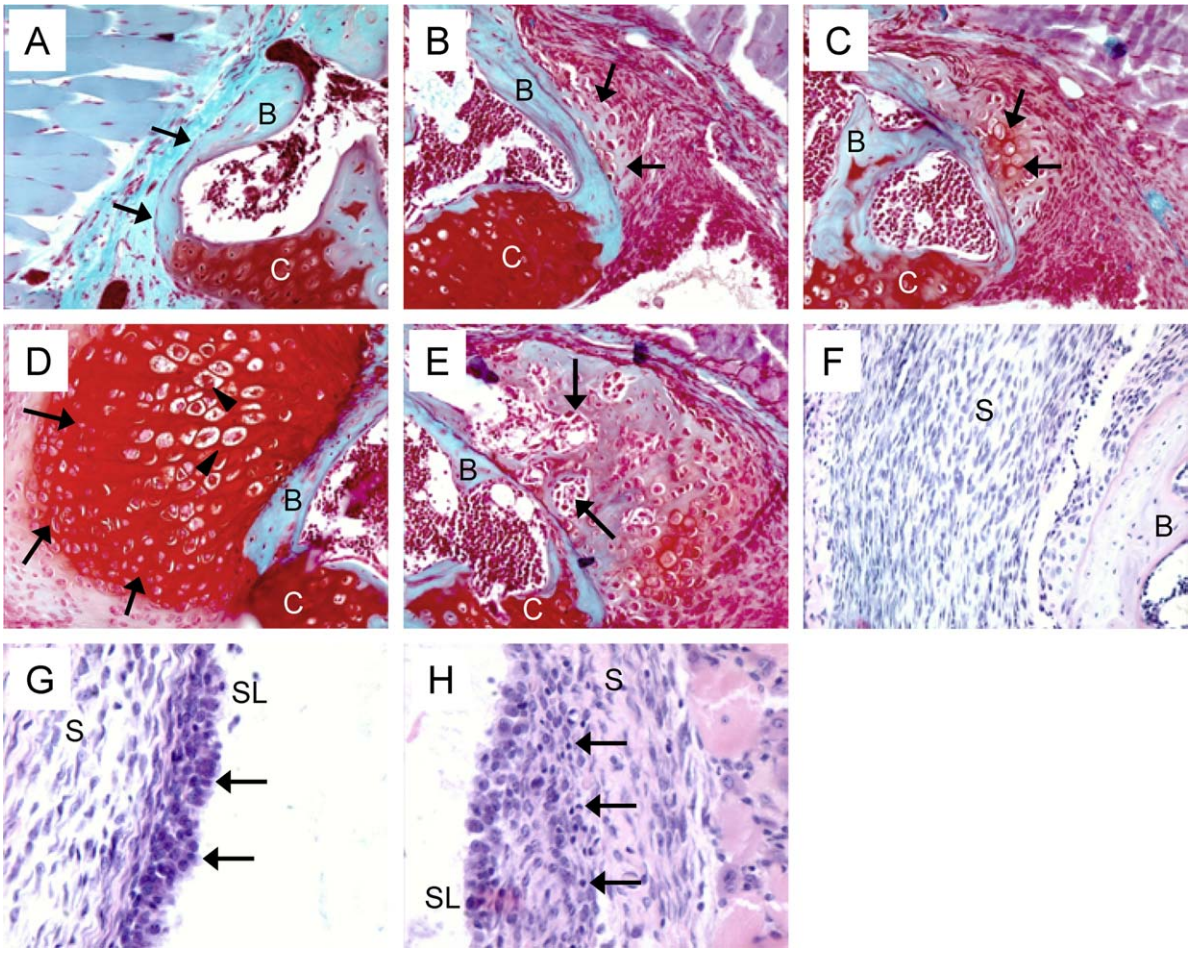

Fig. 1. Photomicrographs describing OA-like pathology that is found in murine experimental OA. Total knee joint sections were made and stained using safranin-O (Saf-O) (A–E) or haematoxylin and eosin (F–H) staining. To study naïve joints, six mice were used, for days 7 and 14, 2×8 mice were used. (A) In naïve knee joints, one thin cell layer, the periosteum, covers the bone (arrows). (B) Osteophyte formation starts with proliferation and subsequently enlargement of cells in the periosteum at sites near the cartilage–bone junction (arrows). (C) The tissue that is formed by proliferating cells from the periosteum begins to stain positive with Saf-O (arrows), indicating that deposition of proteoglycans is already taking place (day 7). (D) Hereafter, days 7–14, larger structures develop consisting of cartilage-like tissue, chondrophytes (arrows), that contain chondrocyte-like cells and in the centre even hypertrophic chondrocytes (arrowheads). The matrix stains highly positive with Saf-O. (E) These chondrophytes then turn into bone-like structures (osteophytes) by endochondral ossification. Note that in this stage, bone marrow cavities are already formed (arrows). Besides osteophyte formation also other pathology, like fibrosis, as indicated by high numbers of fibroblasts (F), thickening of the synovial lining layer (G; arrows) and influx of inflammatory cells (arrows) in deeper layers (H), is found. B = bone, C = cartilage, S = synovium, SL = synovial lining (original magnification A–F: 200×, G and H: 400×).

synovial macrophages and whether the production of growth factors was affected.

Obviously, depletion of macrophages from the synovial lining resulted in nearly complete removal of MRP14 staining from the superficial layer (not shown). However, at day 14 also in the deeper layers, a decrease of activated macrophages was found, indicating that selective removal of synovial lining cells during OA leads to a generalized reduction of macrophage activation throughout the synovium (Fig. 6). Note that in the deeper layers of knee joints that were not depleted of synovial lining macrophages, MRP14 staining increases almost 30% from day 7 to day 14.

In contrast, this decrease in synovial activation did not result in a decrease of growth factor expression (TGFβ, BMP-2, BMP-4) in the deeper synovial layers (Table I). In addition, no change was found in expression of these growth factors by cells in the periosteum (not shown). Production of growth factors by the synovial lining itself was of course reduced after depletion of macrophages. This suggests that growth factors produced by the synovial lining macrophages themselves are essential in the induction of early OA changes.

Discussion

In this study, three important findings are described. The first being the observation that collagenase induced experimental OA is a very useful model to study osteophyte formation in OA. The second observation is that during this experimental model for OA, as in human OA, activation of synovial macrophages is observed, which indicates the potential involvement of these cells in the OA process. Finally, as the third and most astonishing finding we demonstrated that synovial macrophages indeed are involved

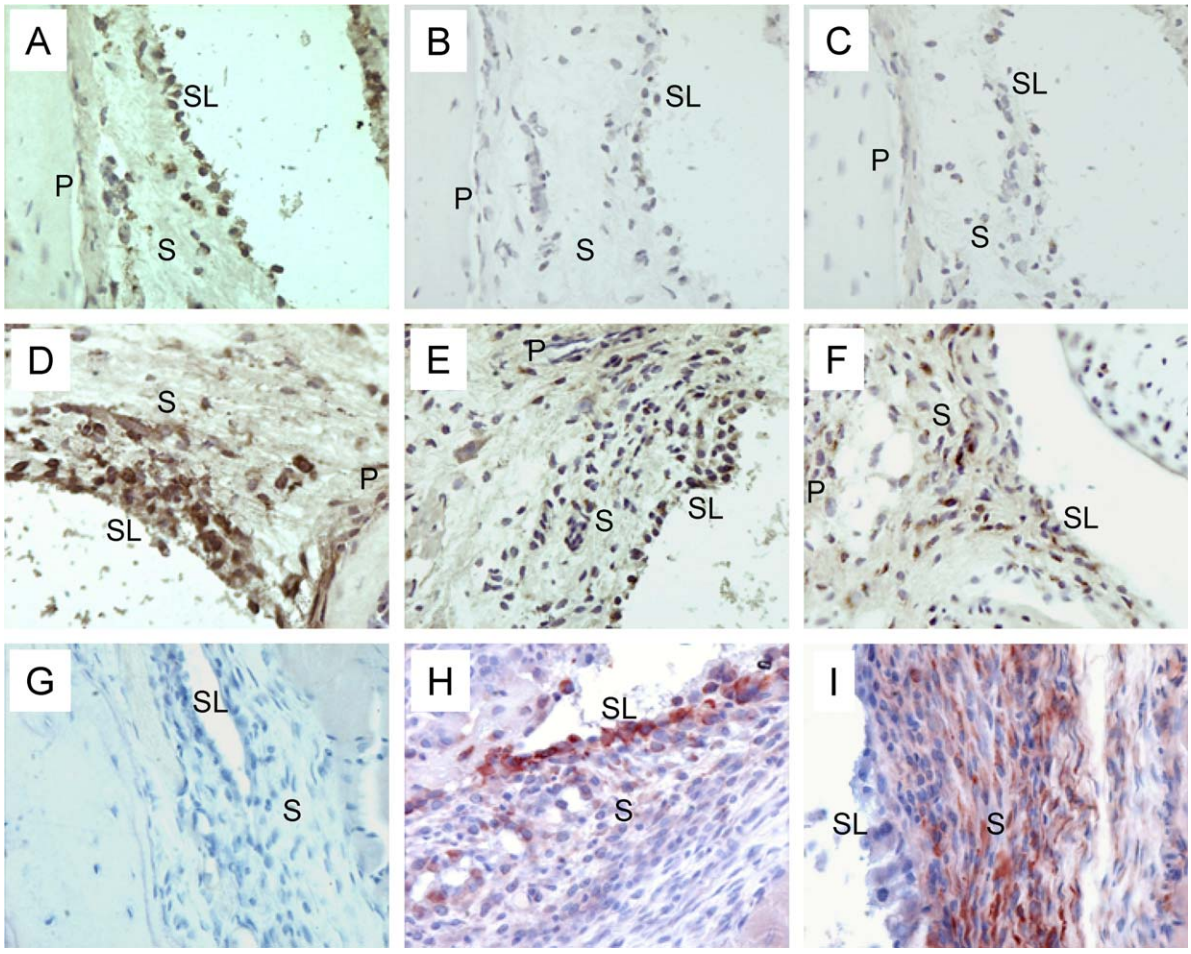

Fig. 2. Immunohistochemical detection of various growth factors (day 7, $n = 6$) and a marker for macrophage activation (day 7, $n = 8$; day14, $n = 8$) in total knee joint sections during the early phase of experimental OA development. The growth factors TGFβ (A), BMP-2 (B) and BMP-4 (C) were detected in naïve knee joints. Note that a higher number of cells are positive for TGFβ, compared to BMPs. Seven days after induction of experimental OA, large numbers of cells still stain positive for TGFβ3, in synovial lining, sublining and periosteum (D). BMP-2 (E) and BMP-4 (F) expression is increased especially in the synovial lining, but also in sublining and periosteum. Please note that TGFβ3 staining was the most abundant of the three studied growth factors. To study whether macrophages are activated in early experimental OA, MRP14 staining was performed in naïve knee joints (G), and in knee joints 7 (H) and 14 (I) days after induction of OA. Note that naïve knee joints do not show any MRP14 expression, whereas at days 7 and 14 after OA induction, activated macrophages could be clearly detected. S = synovium, SL = synovial lining, P = periosteum (original magnification 400×).

in several pathological processes that occur in experimental OA, particularly in osteophyte formation.

Experimental OA based on generation of instability, which is induced by intra-articular injection of collagenase, has been shown to share some important features with human OA, like the development of osteophytes and cartilage erosions. Also during the early phase that was described in this study, similarities were found in respect to fibrosis and osteophyte formation. The positions in the joint where the new formation of bone first occurs (in the periosteum near the cartilage surface and at sites of bone–ligament junctions) are similar to those seen in human disease[3,6]. In addition, the whole process of new formation of bone is similar, with activation of periosteum as a first event, followed by the generation of cartilage-like tissue and subsequently endochondral ossification. These similarities underline that this model is very suitable to study fibrosis and osteophyte formation during OA.

During the first 2 weeks of experimental OA we found activation of the synovium, as was indicated by macrophage activation (MRP14 expression), influx of low numbers of inflammatory cells, expression of growth factors in the synovium and finally fibrosis. In human OA, it has already been proposed by several groups that inflammatory activation of the synovium is important in the development of OA-like pathology[1,29,30]. By using the MRP14 marker, we found that during experimental OA, macrophages in the synovial lining and in the deeper synovial layers were activated. Because an increase of MRP14 was demonstrated from day 7 to day 14, it is unlikely that MRP14 expression is a direct effect of collagenase injection. MRP14 is one of the S100 proteins, which are involved in calcium homeostasis and its expression characterizes an activated phenotype of macrophages. Why exactly these cells are activated during OA is still unclear. However, it is very well possible that matrix fragments that leak from the cartilage, which is damaged by changed loading of the joint or by enhanced enzyme activity, activate synovial cells, e.g. via Toll-like receptors, as was described for instance for fibronectin or proteoglycan fragments[31–33]. In addition, these fragments can cause

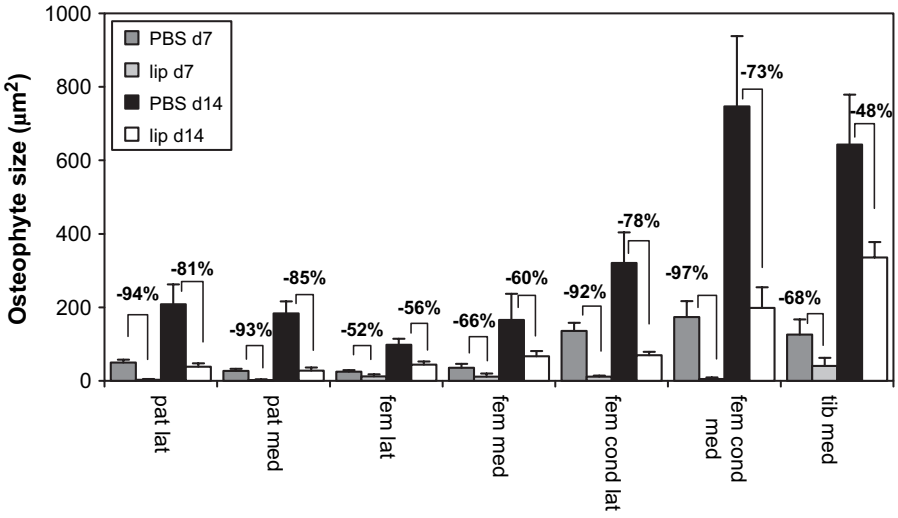

Fig. 3. Osteophytes developing during OA were measured using image analysis, 7 and 14 days after OA induction in macrophage depleted joints and compared to controls. The size was measured at seven different positions in the joint. pat lat = lateral from the patella, pat med = medial from the patella, fem lat = lateral from the femur adjacent to the patella, fem med = medial from the femur adjacent to the patella, fem cond lat = lateral from the lateral condyle of the femur, fem cond med = medial from the medial condyle, tib med = medial from the tibial plateau. Note that a significant decrease in osteophyte size is found in macrophage depleted joints. Data express the mean ± SEM ($n = 8$ per group) and were statistically analysed using a Student's t test with Bonferroni transformation. For each separate time point and position, the percentage of decrease in size is stated.

production of matrix degrading enzymes like matrix metalloproteinases (MMP) by different cell types[32,34,35]. The possibility that the leakage of these fragments is mediated by macrophages, for example by (induction of) production of MMPs, suggests a dual role for the macrophage, leading to a downwards spiral in which macrophages cause leakage from the cartilage of pathogenic fragments, which in turn activate macrophages causing further cartilage damage. However, since this model is based on induction of instability, it is likely that the initial changes lie within the cartilage itself.

Once activated, macrophages are responsible, either directly or indirectly, for the production of several growth factors (TGFβ, BMP-2 and BMP-4). It has already been demonstrated that these cells are capable of producing these factors that are thought to be very important in generation of fibrosis and in osteophyte formation[21]. Unfortunately, due to different fixation and immunohistochemistry protocols, it was not possible to perform a double staining for MRP14 and the respective growth factors. Because of this technical problem, we cannot conclude that growth factors are exclusively produced by the activated macrophages.

The most important message in this study is the clear and first time demonstration of a crucial role for synovial lining macrophages in osteophyte formation and fibrosis during experimental OA. The finding that macrophages can have a potent osteoinductive effect has already been suggested in fracture healing[21,36]. In this process, infiltrating macrophages are thought to play an important active role, not only by removing debris but also by generation of inflammatory cytokines and growth factors. The essential role for macrophages in osteophyte formation was found using selective depletion of synovial macrophages by intra-articular injection of clodronate-containing liposomes. To reach a valid conclusion, the possibility of artifacts caused by the clodronate liposome technique has to be excluded. Due to

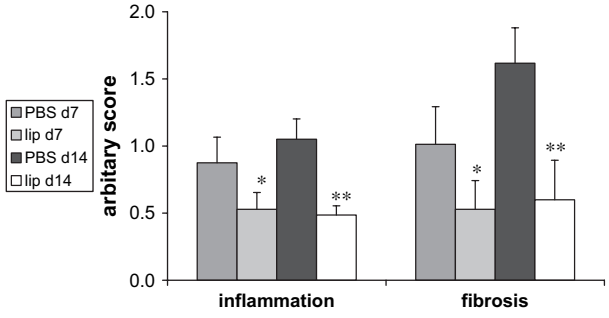

Fig. 4. Influx of inflammatory cell and fibrosis in the synovium were scored based upon the difference in morphology of fibroblast-like cells and inflammatory cells, using an arbitrary score as described in Methods section. Although only a low level of inflammation was found, both inflammation and fibrosis were significantly lower in knee joints from which the macrophages were depleted prior to induction of experimental OA. Data express the mean ± SEM of eight mice and were evaluated statistically using the Mann–Whitney U test. Significance: * <0.05, ** <0.02.

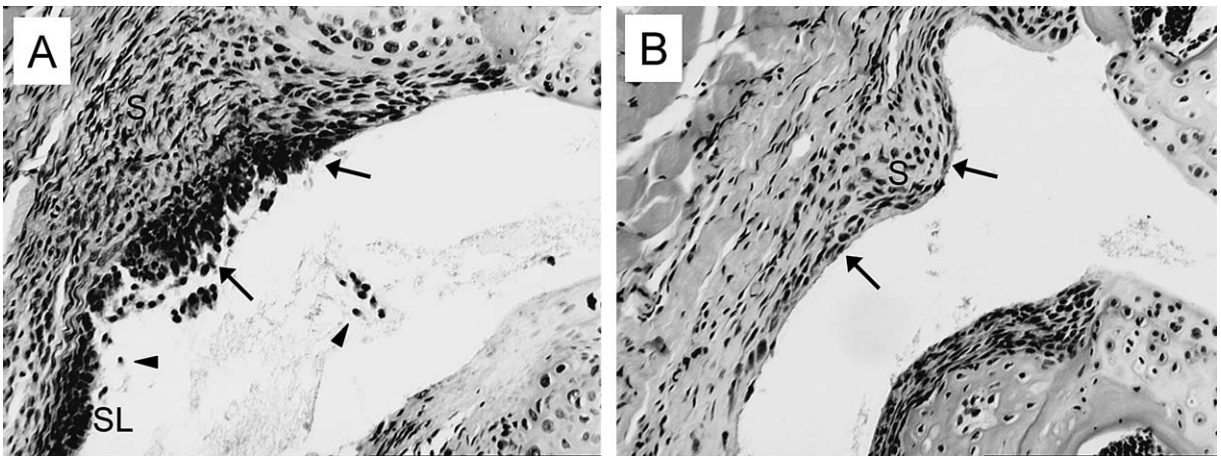

Fig. 5. Photomicrographs of H&E stained sections of knee joints of mice in which experimental OA was induced. Synovial activation, as indicated by a thickened synovial lining (arrows), and influx of inflammatory cells (arrowheads), were clearly visible in OA knee joints (A; $n = 8$), whereas in macrophage depleted OA knee joints, both synovial activation and influx of inflammatory cells were decreased (B; $n = 8$). Note that in these mice the synovial lining macrophages have been successfully removed (arrows). S = synovium, SL = synovial lining (original magnification 200×).

the pharmacokinetic qualities of the encapsulated drug, clodronate, and the delivery system, liposomes, it is highly unlikely that the cells from which osteophytes develop are targeted. Liposomes can only be phagocytosed by macrophages and are therefore not likely to target cells from which osteophytes originate[37]. In addition, clodronate in free form cannot be transferred into other cells. As was previously demonstrated, the periosteum was still intact after liposome treatment[23]. In line with this, our present results indicate that production of several growth factors by the cells in the periosteum is not impaired when clodronate liposomes are administered prior to OA induction. In the aforementioned study it was shown that C3H10T1/2 mesenchymal cells are not affected by *in vitro* addition of these clodronate liposomes. The above indicates that prevention of osteophyte formation by clodronate liposome treatment is not likely to be a mere effect of the treatment on osteophyte forming cells.

Then why does this macrophage depletion show such strong effects on osteophyte formation and fibrosis? The selective removal of macrophages from the synovium during experimental OA has a strong effect on synovial activation. It was already discussed that this macrophage activation may lead to production of several growth factors, TGFβ, BMP-2 and BMP-4. Production of these factors by the synovial lining cells was strongly diminished when macrophages were removed from the lining. However, diminished activation of macrophages in the deeper synovial layers did not result in a changed percentage of growth factor producing cells in these layers or in the periosteum. This suggests that the production of growth factors by the synovial lining layer contributes significantly to the total production of growth factors. Immunohistochemistry is at best a semiquantitative method (only the percentage of positive cells can be assessed, not the production per cell), and therefore at our laboratory,

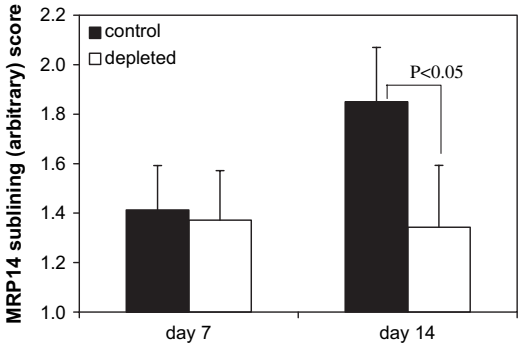

Fig. 6. MRP14 expression was detected in OA knee joints using immunohistochemistry and used as a marker for macrophage activation. MRP14 expression in the synovium was scored arbitrarily as described in Methods section, and control OA joints were compared to macrophage depleted OA joints. Note that MRP14 staining of the synovial lining layer was absent in macrophage depleted OA joints (not shown, $n = 8$). This is in contrast to normal OA joints, in which at day 7 the synovial lining layer stained positive for MRP14, indicating that the cells are activated ($n = 8$). At day 14 the amount of activated macrophages is even somewhat higher compared to day 7 ($n = 8$). Note that the activation of macrophages in the sublining is significantly decreased at day 14 in knee joints from which synovial lining macrophages were depleted prior to OA induction, indicating that macrophage activation is mediated by synovial lining macrophages ($n = 8$) (original magnification 200×).

Table I
Staining for TGFβ3, BMP-2 and BMP-4 in total knee joint sections of naïve, control and lining macrophage depleted mice was quantified blindly using an arbitrary scoring system. 0, no positive cells; 1, less then 10% positivity; 2, between 10% and 50% positivity; 3, more than 50% positivity

Growth factor	Knee joint	Lining	Sublining
TGFβ3	naïve	1.5±0.6	1.7±0.4
	control	2.2±0.5	2.2±0.5
	depleted	0.8±0.3*	2.1±0.3
BMP-2	naïve	0.3±0.2	0.2±0.1
	control	1.0±0.6#	1.1±0.5#
	depleted	0.3±0.3*	1.4±0.4#
BMP-4	naïve	0.1±0.2	0.1±0.3
	control	1.0±0.8#	1.2±0.4#
	depleted	0.1±0.2*	1.6±0.4#

Values that were significantly different in the macrophage depleted joints compared. *:$P<0.05$ if compared to control joints, #:$p<0.05$ if compared to naïve knee joints. Note that an effect of macrophage depletion was found only in the lining.

techniques are developed to assess total levels of these growth factors within the knee joint. One might also argue that other cell types, like chondrocytes, may be involved in production of growth factors and that macrophage may regulate this. However, we did not observe differences in growth factor expression in macrophage depleted OA joints compared to controls but the immunostaining technique was not optimized for cartilage staining.

Apart from effect on total growth factor levels, another explanation might be that, along with the studied growth factors, additional factors that are produced by synovial macrophages are necessary for osteophyte development and fibrosis. This is supported by our recent finding that synovial macrophages play a crucial role in TGFβ-mediated osteophyte formation[23].

This study identifies macrophages as key players in the generation of osteophytes and fibrosis during OA. This raises the question whether macrophages are involved in other OA-related pathology, like cartilage erosion. Because macrophages are also potent producers of enzymes that are involved in cartilage breakdown, such as MMPs, here might lay an additional role of macrophages in OA pathology. This is currently under investigation. Therapies in which specifically macrophages are targeted may be of use in the treatment of OA.

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