Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice

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Rheumatoid arthritis is one of the most critical diseases that impair the quality of life of patients, but its pathogenesis has not yet been fully understood. Osteopontin (OPN) is an extracellular matrix protein containing Arg-Gly-Asp (RGD) sequence, which interacts with $\alpha v\beta 3$ integrins, promotes cell attachment, and cell migration and is expressed in both synovial cells and chondrocytes in rheumatoid arthritis; however, its functional relationship to arthritis has not been known. Therefore, we investigated the roles of OPN in the pathogenesis of inflammatory process in a rheumatoid arthritis model induced by a mixture of anti-type II collagen mAbs and lipopolysaccharide (mAbs/LPS). mAbs/LPS injection induced OPN expression in synovia as well as cartilage, and this expression was associated with joint swelling, destruction of the surface structures of the joint based on scanning electron microscopy, and loss of toluidine blue-positive proteoglycan content in the articular cartilage in wild-type mice. In contrast, OPN deficiency prevented the mice from such surface destruction, loss of proteoglycan in the articular joint cartilage, and swelling of the joints even when the mice were subjected to mAbs/LPS injection. Furthermore, mAbs/LPS injection in wild-type mice enhanced the levels of CD31-positive vessels in synovia and terminal deoxynucleotidyltransferase-mediated UTP end labeling-positive chondrocytes in the articular cartilage, whereas such angiogenesis as well as chondrocyte apoptosis was suppressed significantly in OPN-deficient mice. These results indicated that OPN plays a critical role in the destruction of joint cartilage in the rheumatoid arthritis model in mice via promotion of angiogenesis and induction of chondrocyte apoptosis.

Although rheumatoid arthritis is one of the major musculoskeletal disorders that cause destruction of joints and lead to serious loss of the quality of life, the pathogenesis is not totally understood (1, 2). Although it has been suggested that external stimuli and the genetic background could be involved, no full pictures have been obtained in this respect (3). The clinical features of rheumatoid arthritis include chronic inflammation in multiple joints associated with accelerated proliferation of synovial cells, which eventually leads to the destruction of cartilage and bone in the joint (4, 5). Such inflammation has been thought to be because of the activation of the cytokine system regulated by inflammatory cells (6). It is assumed that synovial cells, chondrocytes, and osteoblasts as well as bone marrow cells are also involved in the production of abnormal levels of cytokines (7, 8). During the course of inflammation, activated macrophages produce tumor necrosis factor α (TNF-α), IL-1, and IL-6, and these cytokines, in turn, stimulate the proliferation of synovial cells to form a mass of synovial tissue, called pannus, that degrades bone via the activation of osteoclasts (9–13).

OPN has been implicated in the mediation of inflammatory process, especially in response to external stimuli or stress to the body (14, 15). For instance, OPN is required for T helper 1-mediated cellular immunity (16), and it is also required for the process of rapid bone resorption resulting from osteoclastic activity after depletion of estrogen (17). Because expression of OPN has been induced by many inflammatory cytokines such as IL-1, IL-6, and TNF-α and this protein is produced by macrophages in response to the activation by inflammatory stimuli (15), OPN could be involved in arthritis (14, 15, 18). Recent reports indicated that in rheumatoid arthritis patients, OPN is expressed in the proliferating synovial cells and at the interface of cartilage and invading synovium (19). Most of these OPN-expressing cells appear to be fibroblastic synovial cells rather than macrophage-type cells, and the produced OPN may stimulate collagenase expression and secretion in human articular chondrocytes (19). These observations suggested that OPN could be involved in synovial cell attachment to chondrocytes or cartilage matrix in addition to its involvement in the cartilage matrix destruction by the production of collagenases in articular chondrocytes.

Because OPN is produced by synovial cells, chondrocytes, and osteoblasts as well as osteoclasts, this protein has been suggested to play a role in bone diseases. However, its function in rheumatoid arthritis has not yet been known. We injected a mixture of mAbs raised against type II collagen to induce rheumatoid arthritis in wild-type and OPN-deficient mice and examined the role of OPN in the process of arthritis. We found that OPN-deficiency significantly suppressed articular cartilage destruction, chondrocyte apoptosis, and synovial angiogenesis.

Material and Methods

Animals. Wild-type and OPN-deficient mice with a C57Bl6/129sv F2 background were produced as described by Rittling and Denhardt (20). A total of 22 male OPN-deficient mice and wild-type mice (6 to 7 weeks old) were used. All animal experiments were approved by the animal welfare committee of our institute.

Anticollagen Antibody-Induced Arthritis. Arthritis antibody kits were obtained from Immuno-Biological Laboratories (Gunma, Japan), and arthritis was induced according to the manufacturer's instructions (21). Briefly, mice were injected i.p. with a mixture of four anti-type II collagen mAbs (2 mg each) on day 0, followed by i.p. injection with 50 μg lipopolysaccharide (LPS) (0111:B4) 3 days later. Severity of the macroscopic levels of arthritis was graded up to 14 days after mAb injection in each of the four limbs per mouse on a 1–4 scale as described previously (22). The criteria for the grading were as follows: 0, normal; 1, swelling and/or redness in one joint; 2, swelling and/or redness in more than one joint; 3, swelling

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Abbreviations: OPN, osteopontin: LPS, lipopolysaccharide: D-Pyr, deoxypyridinoline; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; TNF-α, tumor necrosis factor α.

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and/or redness in the entire paw; and 4, maximal swelling. Maximum value of the sum of the scores obtained from the four limbs of each mouse was 16 (4×4).

Histology. On day 18, mice were killed, and the fore and hind paws of each mouse were removed, fixed in 4% paraformaldehyde in PBS, decalcified in EDTA, embedded in paraffin, sectioned, and stained with hematoxylin/eosin or toluidine blue.

Immunohistochemistry. OPN protein expression was examined by immunohistochemistry with anti-mouse OPN antibody according to the manufacturer's instructions (Immuno-Biological Laboratories). Briefly, sections were treated with microwaves for 10 min at 90°C in 10 mM citrate buffer, pH 6.0. The sections then were incubated overnight at 4°C with rat anti-mouse OPN antibody diluted at 1: 20. The sections then were incubated at room temperature for 30 min with biotinylated goat anti-rat IgG antibody and then visualized by peroxidase-conjugated avidin and diaminobenzidine with a Vectastain kit (Vector Laboratories). The expression of PECAM-1 (CD31) was examined by using MEC 13.3 rat monoclonal anti-mouse PECAM-1 (CD31) antibody (PharMingen; ref. 23). Sections were incubated at 4°C overnight with anti-mouse PECAM-1 (CD31) antibody. The sections then were incubated at room temperature for 1 h with biotinylated goat anti-rat IgG antibody (BioSource International, Camarillo, CA), and then the signal was visualized by peroxidase-conjugated avidin and diaminobenzidine.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay. TUNEL assay was conducted by using a TUNEL-detection kit according to the manufacturer's instruction (Takara Shuzo, Kyoto). Briefly, sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-FITC horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

Scanning Electron Microscope. Samples were prefixed with 2.5% glutaraldehyde in PBS, rinsed with PBS, postfixed with 1% osmium tetroxide in PBS for 2 h, and dehydrated in a series of ethanol, followed by critical-point drying with an HCP-2 apparatus (Hitachi, Tokyo), employing CO_2 as the transitional fluid. The specimens mounted on stubs were coated with platinum and were examined with a scanning electron microscope (S-4500; Hitachi).

Serum TNF-α Levels. Serum samples were obtained 1 h after i.p. injection with LPS (0111:B4; 50 μg per body), and TNF-α levels was measured with an ELISA system kit according to the manufacturer's protocols (Amersham Pharmacia).

Urinary Deoxypyridinoline (D-Pyr) Levels. Mouse urine was collected by using metabolic cages during the last 24 h on day 14, and urinary D-Pyr levels were measured by using an ELISA system kit according to the manufacturer's protocols (Metra Biosystems, Mountain View, CA).

Statistical Analysis. Data were expressed as means ± SEM, and statistical evaluation was performed based on Student's *t* test, by using a statistical software package for Macintosh, STATVIEW v.4.5 (Abacus Concepts, Berkeley, CA).

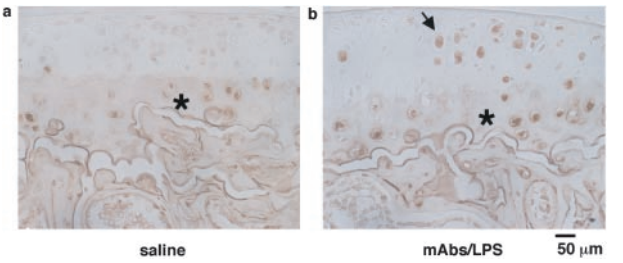


Fig. 1. OPN protein expression in the knee joints of wild-type mice with arthritis. OPN protein expression in the knee joints was detected by OPN immunostaining in saline (*a*) and mAb/LPS-injected, wild-type mice (*b*). OPN expression was enhanced in the superficial layer (arrow) and deep layer (*) of cartilage by injection with mAb/LPS (*a* vs. *b*).

Results

Expression of OPN Protein in Cartilage Was Enhanced in Arthritis-Induced Mice. Immunohistological examination revealed low levels of OPN protein expression in the chondrocytes in the deep layer (Fig. 1*a*) but not in the superficial layer of articular cartilage in saline-injected wild-type mice (Fig. 1*a*). mAbs/LPS injection into wild-type mice induced OPN expression in chondrocytes located in the superficial layer of articular cartilage (Fig. 1*b*) and also enhanced OPN expression in deep layer of articular cartilage (Fig. 1*b*).

Destruction of Cartilage Was Suppressed in OPN-Deficient Mice. Articular cartilage surface was smooth in saline-injected mice based on scanning electron microscopy (Fig. 2*a*), whereas such smooth appearance was lost on the joint surface in wild-type mice after induction of arthritis by injection with mAbs/LPS (Fig. 2*b*). Examination at higher magnification revealed a smooth surface in saline-injected wild-type mice (Fig. 2*e*), whereas destruction of the lacunae of articular chondrocytes was observed in mAb/LPS-injected wild-type mice (Fig. 2*f*). In contrast, no such surface destruction was observed in OPN-deficient mice before (Fig. 2 *c* and *g*) or after the mAbs/LPS injection (Fig. 2 *d* and *h*). Quantification of the erosion area indicated that about 30% of the articular surface was eroded in wild-type mice, whereas no major erosion was observed in saline-injected wild-type mice or in OPN-deficient mice regardless of mAbs/LPS injection (Fig. 2*i*).

The levels of toluidine blue staining in the articular cartilage was markedly reduced in wild-type mice injected with mAbs/LPS (Fig. 3*b*), indicating loss of proteoglycan in the cartilage matrix compared with saline-injected mice (Fig. 3*a*). In contrast, the levels of toluidine blue staining in OPN-deficient mice injected with mAbs/LPS (Fig. 3*d*) were similar to those in saline-injected mice (Fig. 3*c*).

OPN Deficiency Suppressed Apoptosis in Chondrocytes in Articular Cartilage in Arthritis-Induced Mice. To examine how the presence of OPN but not its absence could lead to joint destruction in arthritis in mice, we examined the levels of apoptosis in chondrocytes. The levels of apoptosis in chondrocytes in articular cartilage were low in saline-injected, wild-type mice (Fig. 4 *a* and *e*); however, they were enhanced in the joints of mice subjected to mAbs/LPS injection (Fig. 4 *b* and *e*). The basal levels of apoptosis in saline-injected, OPN-deficient mice were similar to those in saline-injected, wild-type mice (Fig. 4 *c* and *e*). In contrast to wild-type mice, OPN-deficient mice revealed no major enhancement in the levels of apoptosis even after mAbs/LPS injection (Fig. 4 *d* and *e*).

Osteopontin Deficiency Reduced Joint Swelling. To address OPN effects on the time course of arthritis, the levels of joint swelling were scored as a function of time and were compared between the wild-type and OPN-deficient mice. The suppression of joint destruction in OPN-deficient mice was associated with the reduction in the levels of joint swelling. The arthritis

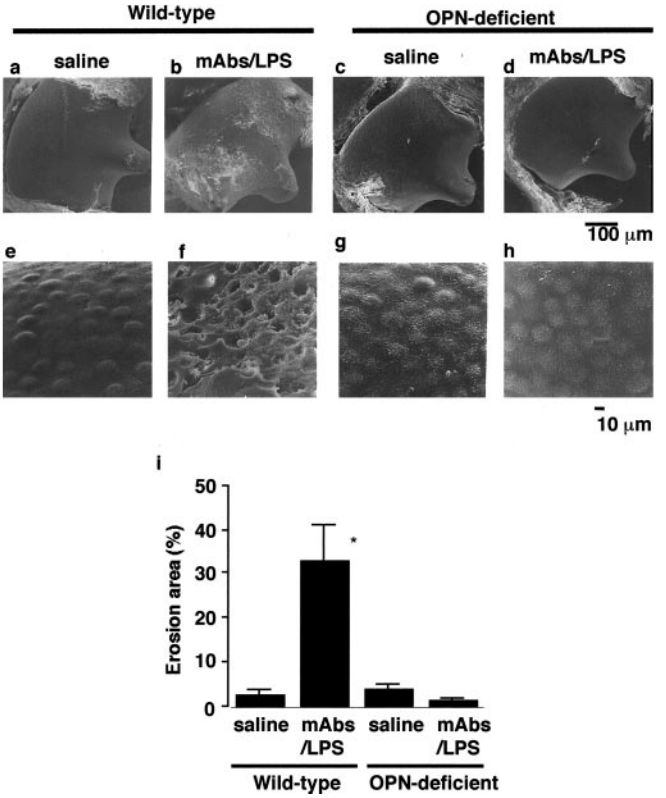


Fig. 2. Surface morphology of articular cartilage examined by scanning electron microscopy. Surface of articular cartilage of finger joints of wild-type (*a*, *b*, *e*, and *f*) or OPN-deficient (*c*, *d*, *g*, and *h*) mice was examined by scanning electron microscopy on day 18 after saline (*a*, *e*, *c*, and *g*) or mAb (*b*, *f*, *d*, and *h*) injection. (*i*) Quantification of the destructed area in articular cartilage surface. The rough areas of cartilage in saline-injected, wild-type ($n = 5$); mAb/LPS-injected, wild-type ($n = 5$); saline-injected, OPN-deficient ($n = 5$); and mAb/LPS-injected, OPN-deficient ($n = 6$) mice were measured with an image analyzer equipped with the Luzex system (Nireco, Tokyo). Data are expressed as means ± SEM. *, $P < 0.05$; asterisk indicates that the difference between saline vs. mAb/LPS-injected, wild-type mice was statistically significant.

score in wild-type mice began to rise on day 4 and peaked to exceed the values of 10 by day 7 (Fig. 5*a*). On the other hand, the arthritis score in OPN-deficient mice started to rise later on day 5, and the value never exceeded 4 (Fig. 5*a*). Swelling levels of the joints (Fig. 5*b*) also were quantitatively evaluated by taking soft x-ray photographs of the paws (Fig. 5*c*). Thick-

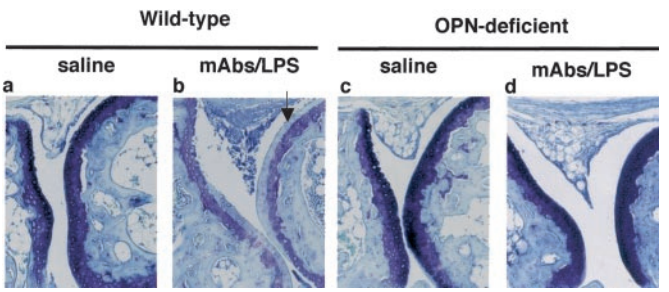


Fig. 3. Proteoglycan-staining levels in articular cartilage. The sections of articular cartilage of finger joints in hind paws of wild-type (*a* and *b*) or OPN-deficient (*c* and *d*) mice were stained with toluidine blue to visualize proteoglycan levels after injection with saline (*a* and *c*) or mAbs/LPS (*b* and *d*). Significant loss of proteoglycan staining (arrows) was observed in mAb/LPS-injected, wild-type mice (*b*), but such loss was not observed in mAb/LPS-injected, OPN-deficient mice (*d*).

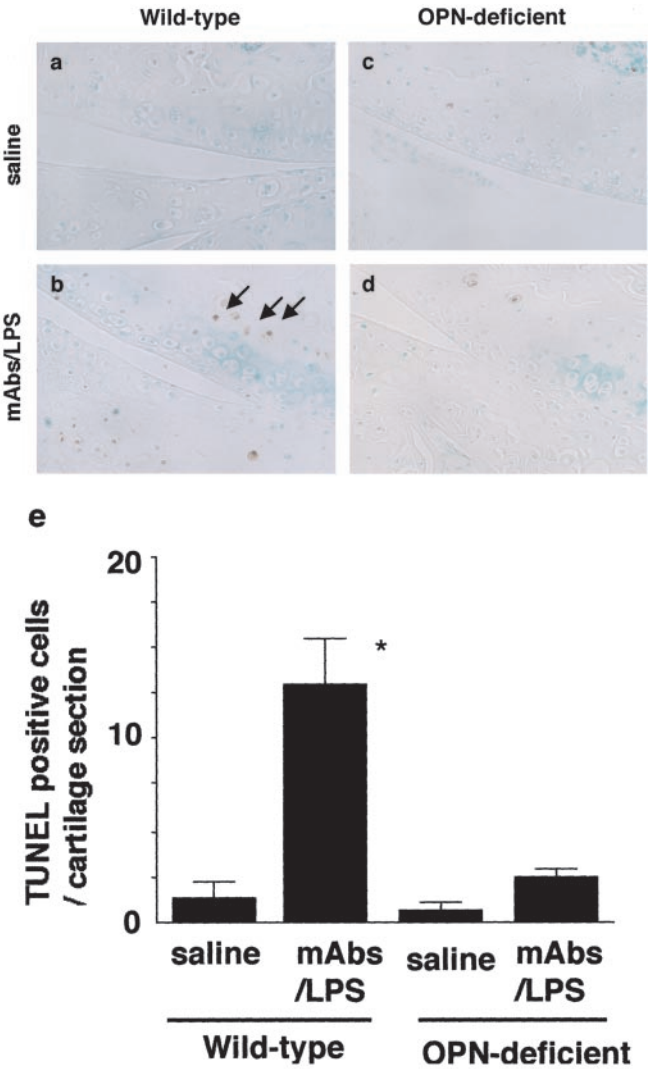
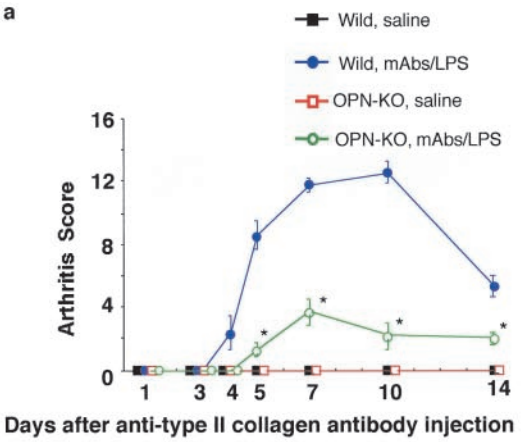
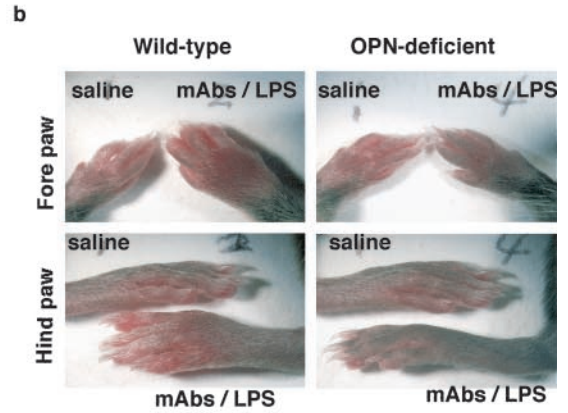


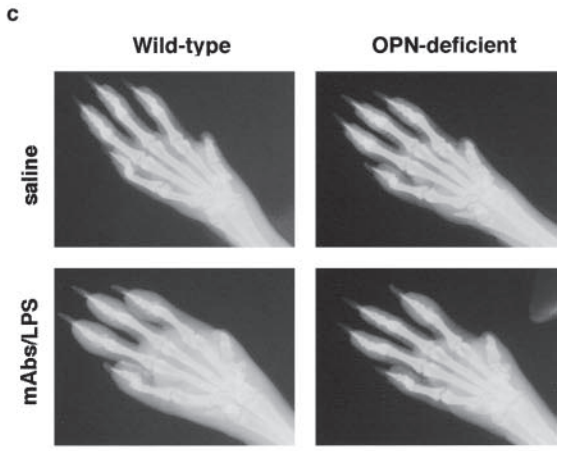
Fig. 4. TUNEL signals in cartilage. The levels of TUNEL-positive cells were low in the knee joint cartilage in saline-injected mice (*a*), but they were enhanced significantly by the injection of mAbs/LPS (*b* arrows) in wild-type mice. In contrast, such up-regulation was not observed in OPN-deficient mice even after mAb/LPS injection (*c* and *d*). (*e*) Quantification of TUNEL-positive cells ($n = 4$). Data are expressed as means ± SEM. *, $P < 0.05$, saline vs. mAb/LPS-injected, wild-type mice.

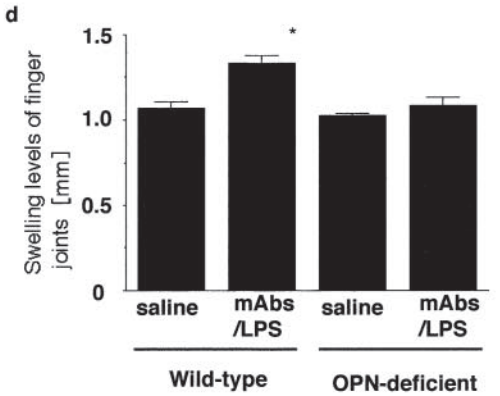
ness of the soft tissues surrounding the interphalangeal finger joints in wild-type mice was increased by about 30% after mAbs/LPS injection (Fig. 5*d*). In contrast, thickness of the soft tissue of the finger joints in mAb/LPS-injected, OPN-deficient mice was similar to that in saline-injected, OPN-deficient mice (Fig. 5 *c* and *d*).

OPN Deficiency Suppressed Angiogenesis in Synovial Tissue of Arthritis-Induced Mice. Swelling in the joints of arthritis patients has been known to be associated with enhanced infiltration of the inflammatory cells and enhancement in angiogenesis in synovial tissues (24, 25). Furthermore, our recent data suggest that OPN is involved in angiogenesis *in vivo* (23); therefore, we examined the effect of OPN deficiency on the levels of inflammatory cell infiltration and vascularity in the synovial tissues. Histological examination (Fig. 6*a*) indicated that inflammatory cells infiltrated into the synovial tissue in wild-type mice injected with mAbs/LPS (Fig. 6*b*), but such inflammatory cell infiltration









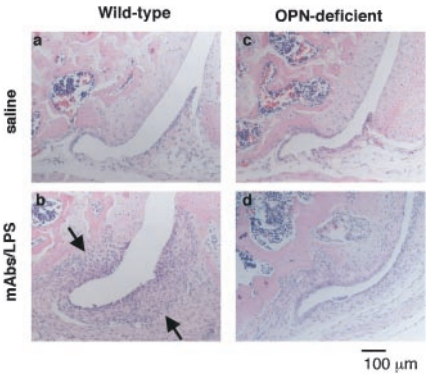


Fig. 6. Inflammatory cell infiltration in the knee joint. The sections of knee joints were stained with hematoxylin/eosin. Sections were prepared from wild-type (*a* and *b*) or OPN-deficient (*c* and *d*) mice injected with saline (*a* and *c*) or mAbs/LPS (*b* and *d*). Inflammatory cells accumulated in synovia were abundant (arrows) in mAb/LPS-injected, wild-type mice (*b*). In contrast, they were less severe in OPN-deficient mice even after injection with mAbs/LPS (*c* and *d*).

into the synovia was less severe in OPN-deficient mice even after injection of mAbs/LPS (Fig. 6 *c* vs. *d*).

Another feature associated with joint swelling is the development of microvasculatures in synovial tissues in arthritic joints. Therefore, we quantified the levels of angiogenesis in synovial tissues by using sections immunostained with an antibody raised against an endothelial cell specific marker, PECAM-1 (CD31). The levels of PECAM-1 (CD31)-positive cells in synovia in wild-type mice were significantly higher in the mAb/LPS-injected group than the control group in wild-type mice (Fig. 7 *a* vs. *b* and *e*). In contrast, the levels of PECAM-1 (CD31)-positive cells in the synovia of OPN-deficient mice were similar regardless of mAbs/LPS injection (Fig. *c* vs. *d* and *e*).

Serum TNF-α Production Induced by LPS in OPN-Deficient Mice Was Normal. Because OPN deficiency suppressed cartilage destruction and inflammatory response in the joints induced by systemic administration of mAbs/LPS, we examined the possibility that response in the TNF-α levels in the blood to LPS injection was impaired in OPN-deficient mice (26). Serum TNF-α levels were very low in saline-injected mice but were elevated within 1 h after LPS injection in wild-type mice (Fig. 8*a*). In OPN-deficient mice, similar elevation of TNF-α levels was observed after LPS injection, indicating that TNF-α response to LPS was not impaired in OPN-deficient mice.

OPN Deficiency Suppressed D-Pyr Levels Enhanced by mAbs/LPS Injection. Because bone destruction is also an important feature of arthritis, we examined the effects of OPN deficiency on the levels of bone resorption in arthritis-induced mice. D-Pyr is a bone-resorption marker, and high levels of urinary D-Pyr are

Fig. 5. The swelling of joints in wild-type and OPN-deficient mice after injection with mAbs/LPS. Wild-type ($n = 5$) or OPN-deficient ($n = 6$) mice ages 6 to 7 weeks were injected i.p. with mAbs on day 0, followed by i.p. injection of 50 μg of LPS on day 3. (*a*) Arthritis score was graded on a 1–4 scale as described in *Materials and Methods*. Data are means ± SEM. *, $P < 0.05$, saline vs. mAb/LPS-injected, wild-type mice. (*b*) Photographs of swelling in fore and hind paws in wild-type and OPN-deficient mice on day 9. (*c*) Soft x-ray photographs of fore paws on day 14. (*d*) Thickness of soft tissues at finger joints ($n = 3$); quantification of the thickness of the soft tissues at finger joints was conducted by using soft x-ray pictures. The levels of the thickness of the soft tissues at the phalangeal joints were expressed as averaged soft tissue width of middle three fingers (2nd, 3rd, and 4th) in fore paw of three mice. Data are expressed as means ± SEM. *, $P <$ 0.05; asterisk indicates that the difference between saline vs. mAb/LPS-injected, wild-type mice was statistically significant.

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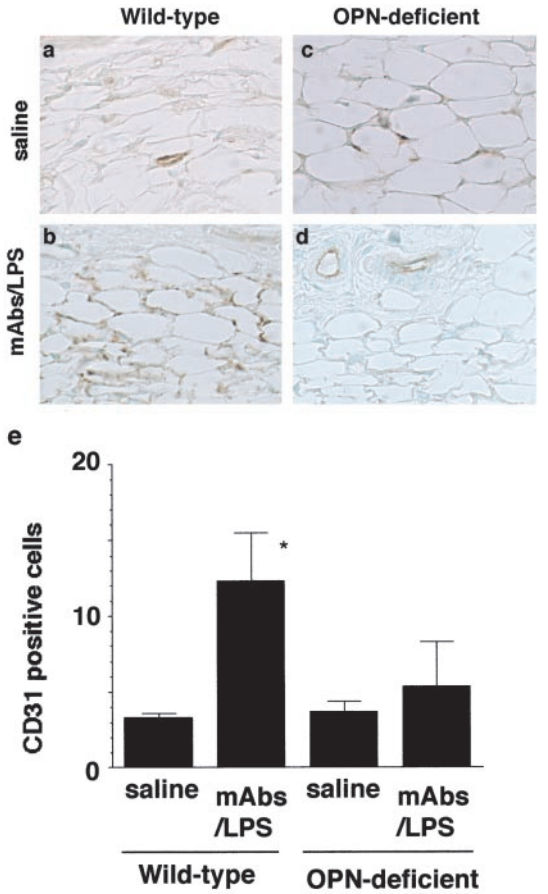

Fig. 7. Angiogenesis in knee joints. The levels of PECAM-1 (CD31)-positive cells were assessed by staining, using MEC 13.3 rat monoclonal anti-mouse PECAM-1 (CD31) antibody in saline-injected (*a* and *c*) or mAb/LPS-injected (*b* and *d*), wild-type (*a* and *b*), or OPN-deficient (*c* and *d*) mice. (*e*) PECAM-1 (CD31)-positive cells ($n = 3$) in the area (0.14×0.16 mm^2) of synovium were counted. Data are expressed as means ± SEM. *, $P < 0.05$; asterisk indicates that the difference between saline- and mAb/LPS-injected, wild-type mice was statistically significant.

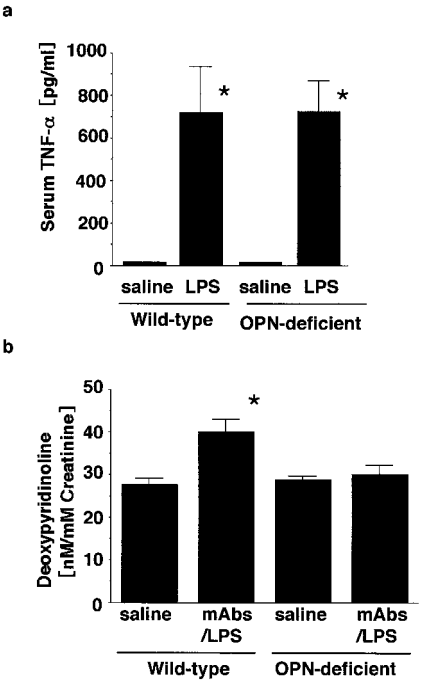

Fig. 8. Serum TNF-α levels in mice injected with LPS and urinary D-Pyr levels in mAb/LPS-injected mice. (*a*) Serum TNF-α levels 1 h after i.p. LPS (0111:B4; 50 μg per body) injection ($n = 5$) were examined. Serum TNF-α levels induced by LPS injection were similar in wild-type and OPN-deficient mice. (*b*) Urinary D-Pyr levels (nM/mM creatinine) were measured on day 14 after the mAb injection. Data are expressed as means ± SEM. *, $P < 0.05$; asterisk indicates that the difference between saline- and mAb/LPS-injected mice was statistically significant.

observed in arthritis patients. We measured the levels of urinary D-Pyr in mAb/LPS-injected mice. The levels of D-Pyr were low in the saline-injected, wild-type mice; however, they were markedly enhanced by mAbs/LPS injection (Fig. 8*b*). The basal levels of D-Pyr in OPN-deficient mice were similar to those in saline-injected, wild-type mice. In contrast to wild-type mice, OPN-deficient mice revealed no enhancement in the levels of D-Pyr even after mAb/LPS injection, indicating that bone resorption in the arthritic mice did not occur in OPN-deficient mice.

Discussion

In this study, we presented evidence that OPN is required for the progression of arthritis induced by injection with a mixture of anti-type II collagen mAbs followed by LPS treatment. It has been known that in the case of rheumatoid arthritis, even though the immune response is mediated by cells such as T and B cells, which are in the systemic circulation, tissues that are affected are located mostly within the joints. Articular cartilage is the main target of the pathological events, and, thus, joint destruction is the major feature of this disease that devastatingly impairs the musculoskeletal function because of the pain and loss of joint functions. OPN is expressed in cells in joints including chondrocytes. OPN is also present in cartilage matrix, and OPN mRNA expression has been observed in articular chondrocytes. Thus, we focused on the elucidation of the function of OPN within articular chondrocytes, which are one of the major sites of pathology in rheumatoid arthritis.

As we have shown, the absence of OPN protects chondrocytes on the surface of the articular joint against destruction resulting from arthritis. This was shown clearly by scanning electron microscopy analyses. Histological sections indicated further that the loss of proteoglycan in the surface layer of the articular cartilage because of arthritis was reduced in OPN-deficient mice that were challenged with mAbs/LPS. These features indicated that OPN is involved in the process of cartilage destruction. To elucidate whether this role of OPN in the progression of arthritis induced by mAb/LPS injection would be related to the apoptosis of chondrocyte, a TUNEL study was conducted. We found that arthritis-driven apoptosis in articular chondrocytes was suppressed by the absence of OPN, indicating that expression of OPN is required for the progression of joint cartilage destruction induced by stress such as arthritis in the body.

Why, then, is OPN present in a normal body? It appears that, to some extent, OPN is required for normal apoptosis so that the damaged chondrocytes could be induced to apoptotic dead and removed normally. This also could be true in the case of hypertrophic chondrocytes that express OPN. However, when an excess amount of stress was given, the balance of the positive and negative regulation of the maintenance of the chondrocytes may be shifted irreversibly to the negative side. If this is the case, normal functions of OPN may be abused to promote the loss of articular cartilage in arthritis.

In addition to the intrinsic action of OPN in the articular cartilage, OPN also is involved in the generation of the inflammatory process, which causes the swelling of the joint. The joint

swelling was suppressed significantly by the absence of OPN, and this was associated with the reduction in the number of the CD31-positive vessels in the synovial tissues, indicating that OPN may be involved in the inflammatory process via promoting a new vessel formations. In fact, we have observed in ectopic bone implantation experiments that OPN in the host and in the bone matrix promoted angiogenesis (23). Thus, the second role of OPN in the course of arthritis is its enhancement of angiogenesis triggered by inflammation.

Because our experimental system was examined within 2 weeks, we did not observe major destruction of the subarticular bone and trabecular bone in the metaphyseal and epiphyseal regions of the long bones; however, even with antibody-induced arthritis, bone loss has been observed in the long term (27). Because we have observed that OPN is involved in bone loss resulting from the increase in osteoclastic activity in estrogen-depleted mice (17), the presence of OPN also could promote bone loss induced by such antibody-induced arthritis in the long term. In fact, D-Pyr levels in urine were elevated in wild-type mice after induction of arthritis, indicating the presence of systemic bone loss in wild-type mice with arthritis. However, no such increase in D-Pyr was observed in OPN-deficient mice even after mAb/LPS injection, suggesting that OPN plays a role in bone loss in the course of arthritis.

Because OPN also has been called early T cell activator protein 1 (Eta-1), based on observations that it is expressed in T cells as one of the major genes expressed in response to LPS, OPN should be involved in the activation of T helper 1 cells (16). It is known that T cells activated by tax gene overexpression could transfer the phenotype of arthritis from the transgenic mice to the host wild-type mice by simply transferring these activated T cells (5, 28). Therefore, it is likely that T cell activation is one of the key steps in which OPN is acting. In addition, glucose-6-phosphate isomerase also could be involved in rheumatoid arthritis (29). Because OPN expression is induced by LPS in chondrocytes and T cells during the course of antibody-induced arthritis, LPS injection may fail to boost the immune response, which normally is the case for this protocol because of the lack of OPN in knockout mice. Our observation that LPS injection could enhance TNF-α levels in OPN-deficient mice similar to levels in wild-type suggests that the LPS Toll-like receptor system would be normal and, therefore, should not account for the lack of arthritic destruction of joint structures in OPN-deficient mice. Interestingly, in human osteoarthritis (OA) chondrocytes, OPN may inhibit IL-1-induced NO and prostaglandin production (30). Thus, OPN may act in an opposite way in aged, human OA articular cartilage.

Our data provide evidence that OPN is required for the onset of antibody-induced arthritis. Complete preservation of the chondrocytes in the absence of OPN even after antibody injection revealed that the treatment maneuvers that suppresses the function of OPN could protect the destruction of articular cartilage in rheumatoid arthritis patients. In fact, recent reports describe that anti-$\alpha v\beta 3$ drugs can protect arthritic joint destruction without blocking the inflammatory responses (31). In our case, deficiency of OPN not only protects articular cartilage destruction but also suppresses the inflammatory cell infiltration as well as the swelling of the joints. These observations indicate that OPN plays a role in multiple steps during the course of arthritis. Some of those functions of OPN would be blocked, in part, by the $\alpha v\beta 3$ antagonist whereas OPN could act through interfaces other than the $\alpha v\beta 3$-dependent process (30). Such interactions could be mediated by other types of receptors, including $\alpha 9$, $\alpha 4$, $\alpha 7$, and $\beta 1$ integrins as well as CD44. Thus, blocking the OPN ligand function rather than individual receptor function could be more efficacious to suppress the progression of arthritis, which depends on the functions of multiple types of osteopontin receptors. Recent development of biological drugs for the treatment of rheumatoid arthritis revealed outstanding effects on the suppression of the disease progression (31). However, the enormous cost of this type of treatment restricts the use of such biological drugs to a small number of selected patients with high severity. Development of nonpeptidyl antagonists against OPN actions could solve such problems.

In conclusion, we have shown that OPN is one of the key molecules in the promotion of arthritic destruction in cartilage tissues. Our data support the notion that the antagonists blocking the interaction between OPN and its receptors should be developed to entirely block joint destruction and the inflammatory response occurring in rheumatoid arthritis (32, 33).

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