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Osteopontin as a positive regulator in the osteoclastogenesis of arthritis

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Abstract

We examined the role of osteopontin (OPN) in the osteoclastogenesis of arthritis using collagen-induced arthritis (CIA). Cells from arthritic joints of wild-type (OPN +/+) mice spontaneously developed bone-resorbing osteoclast-like cells (OCLs). The cultured cells showed an enhanced expression of receptor activator of nuclear factor κB ligand (RANKL) and a decreased expression of osteoprotegerin (OPG). The addition of OPG reduced the number of OCLs, indicating that the osteoclastogenesis depends on the RANK/RANKL/OPG system. The cells also produced OPN abundantly and anti-OPN neutralizing antibodies suppressed the development of OCLs. Moreover, the addition of OPN increased the expression of RANKL and augmented differentiation of OCLs from OPN-deficient (OPN −/−) cells. OPN, like the combination of 1α,25-dihydroxyvitamin D_3 and dexamethasone, also enhanced the RANKL expression and decreased OPG expression in a stromal cell line, ST2. These results suggest that OPN acts as a positive regulator in the osteoclastogenesis of arthritis through the RANK/RANKL/OPG system.

Keywords: Osteopontin; RANKL; Osteoprotegerin; Collagen-induced arthritis; Stromal cell; Osteoclast

Rheumatoid arthritis (RA) is a major systemic autoimmune disease. Although its pathogenesis is unknown, RA is characterized by chronic inflammation in multiple joints, which develops into erosion of marginal bone and cartilage, juxta-articular bone loss, and a general reduction in bone mass [1]. It is widely accepted that inflammatory cells, especially lymphocytes and macrophages, are crucial players in the pathogenesis of RA, and that cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and IL-6, are also involved [2]. In addition, recent findings have shown that osteoclasts play a key role in joint destruction and osteoporosis in RA [3–7].

Osteoclasts are bone-resorbing multinucleated cells derived from macrophage–monocyte lineage progenitors [8]. In RA patients and in animal models of arthritis, increased numbers of osteoclasts have been detected in pannus and in inflammatory regions of arthritic joints [5–7]. Differentiation of osteoclasts is regulated by receptor activator of nuclear factor κB ligand (RANKL), its receptor RANK, and osteoprotegerin (OPG), which is a non-signaling decoy receptor for RANKL. The ligand and receptors have recently been identified as new members of the TNF ligand-receptor family [9–12]. RANKL is essential for osteoclastogenesis and is also important in the development of lymph nodes and in the activation of dendritic cells [10,11]. A possible relationship between the RANK/RANKL/OPG system and RA has recently received considerable

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attention. RANKL is expressed in cultured synovial cells of RA patients [13], and an increase in messenger RNA (mRNA) transcripts of RANKL has been detected in synovial lymphocytes of RA patients and in murine arthritis models [14]. RANK, along with RANKL, is also reported to have enhanced expression in arthritic mice [3,4,15]. According to other reports, RANKL-deficient mice are resistant to arthritis [16] and OPG prevents arthritis in animal models [14,17,18].

On the other hand, other than collagen, OPN is the major extracellular bone matrix protein, and it is believed to contribute to the balance between osteoblasts and osteoclasts in bone metabolism [19,20]. OPN is also able to regulate certain inflammatory reactions as a T helper 1 (Th1) cytokine [21]. RA is a typical bone-resorbing inflammatory disease, the pathogenesis of which is thought to stem from Th1 hyperactivation. We have previously reported that OPN increases in arthritic joints of both RA patients and collagen-induced arthritis (CIA) mice [22,23]. In addition, it has been reported that OPN-deficient (OPN −/−) mice resist postmenopausal osteoporosis [24], disuse bone atrophy [25], and anti-type II collagen antibody-transfer induced arthritis (CAIA) [26]. From the prospect of suppressing osteoclasts and regulating inflammation, osteopontin (OPN) is a possible therapeutic target in RA patients.

In this study, we examined the role of OPN in the osteoclastogenesis of arthritis using an in vitro osteoclast differential model of CIA [6].

Materials and methods

Mice. The animal experiments in this study were performed according to the guidelines of the Animal Experimentation Committee of Osaka University. OPN −/− mice were generated elsewhere [27]. We backcrossed these mice with DBA/1J mice (Nippon Charles River, Kanagawa, Japan) for six generations to introduce CIA susceptibility. After the sixth backcrossing, loss of OPN mRNA was confirmed in OPN −/− mice by reverse transcriptase-polymerase chain reaction (RT-PCR). Additionally, the absence of OPN protein in the serum of OPN −/− mice was demonstrated using an enzyme-linked immunosorbent assay (ELISA) as described below. Six-week-old OPN −/− mice and their wild-type (OPN +/+) littermates were used. The mice were bred in filter-top cages under standard pathogen-free conditions.

Cell line. A murine bone marrow stromal cell line, ST2, was obtained from RIKEN Cell Bank (Ibaragi, Japan). ST2 cells were maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) with 2×10^5 U/ml penicillin (PC) and 200 μg/ml streptomycin (SM) as previously described [28].

Induction of CIA. CIA was induced by established methods, as previously described [29]. Briefly, 18 male mice (10 OPN +/+ and 8 OPN −/−) were immunized by intradermal injection of 100 μg bovine collagen type II (MCK, Tokyo, Japan) in 0.1 M acetic acid, emulsified with an equal proportion of Freund's complete adjuvant (Difco, Detroit, MI). After a 3-week period, the mice were boosted using the same method. Six littermate OPN +/+ male mice without immunization were used as controls. At one time, two to four mice from each group were used and these experiments were repeated three times.

Preparation of cells from CIA mice. Cells from arthritic joints of CIA mice were prepared as previously described [6]. Briefly, 35 days after the first immunization, the immunized mice were anesthetized with pentobarbital and sacrificed. After removal of skin, entirely swollen arthritic paws were minced up including bone. Normal paws of the non-immunized mice were used as controls. The minced tissues were digested with 500 U/ml dispase (Godo Shusei, Tokyo, Japan) and 1 mg/ml collagenase S1 (Nitta Gelatine, Osaka, Japan) for 6 h at 37 °C in complete medium (10% FCS in α-minimal essential medium (α MEM; Nacalai Tesque, Kyoto, Japan), with 2×10^5 U/ml PC and 200 μg/ml SM). After the enzymatic reaction, the dispersed cells were harvested. By this method, $1–5 \times 10^6$ live cells were obtained per mouse.

Cell culture. The harvested cells were diluted to 1.5×10^5/ml and cultured in 100 μl of complete medium in 96-well plastic plates in octuplicate at 37 °C in a humidified 5% CO_2 incubator from day 0 to day 7. The same experiments were repeated three times. Every 2 to 3 days the culture medium was totally replaced. On day 7, the cells were fixed in citrate acetone fixative and stained for tartrate-resistant acid phosphatase (TRAP) with a staining kit (Sigma, St. Louis, MO). TRAP-positive osteoclast-like cells (OCLs) per well, defined as dark red cells with 3 or more nuclei, were counted under a microscope. In order to determine the capacity of generated OCLs to resorb bone, the harvested cells from arthritic joints were cultured on BD BioCoat Osteologic (BD Biosciences, San Jose, CA). After a 10-day cultivation period, the cells were removed by washing with hypochlorous acid, according to the manufacturer's protocol. Pits were determined as bright spots on slides using a microscope (Axioscop 2, Zeiss, Baden-Wuerttemberg, Germany).

Measurement of OPN produced by the cultured cells. The concentrations of OPN in the culture supernatants of OPN +/+ cells were measured using an OPN-ELISA kit (IBL, Gunma, Japan). The supernatants were prepared and stored at −80 °C until used. Color reaction was detected by an Immunoreader NJ-2300 (Nihon InterMed, Tokyo, Japan).

Treatment of the cells with OPG or anti-OPN neutralizing antibodies. Recombinant human OPG (PeproTech, London, England), polyclonal rabbit anti-OPN neutralizing antibodies, control normal rabbit IgG (Sigma, St. Louis, MO), or phosphate buffered saline (PBS) was applied to the culture medium of OPN +/+ cells at the following concentration ranges: OPG; 1.5–100 ng/ml, anti-OPN antibodies; 10–300 μg/ml, and control IgG; 300 μg/ml. The cells were cultured in octuplicate for 7 days. On day 7, TRAP staining was performed. TRAP-positive OCLs were counted in a similar manner to that described above. The anti-OPN neutralizing antibodies were generated in our laboratory [30].

Quantitative RT-PCR for RANKL and OPG in the cultured cells. Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) to measure the levels of mRNA for RANKL and OPG. As an internal standard, β-actin was used. Briefly, using an RNeasy kit (Qiagen, Hilden, Germany) with DNase treatment, RNA was extracted from the harvested cells incubated in complete medium with or without control antibody, anti-OPN neutralizing antibodies, or PBS for 24 h. One milligram of total RNA was transcribed to synthesize cDNA using SuperScript II reverse transcriptase and poly(T) primer (Invitrogen, Carlsbad, CA). Primers and TaqMan probes for real-time PCR were designed using primer design software.

The sequences of the primers and probes were as follows: β-actin, forward (5′-GCTCTGGCTCCTAGCCACCAT), reverse (5′-CTGCTTGCTGATCCACATCTG), and probe (5′-AAGATCATTGCTCCTCCTGAGCGCAA); RANKL, forward (5′-GCTCCGAGCTGGTGAAGAAAT), reverse (5′-CCCAAAGTACGTCGCATCTTG), and probe (5′-ATTCAGGTGTCCAACCCTTCCCTGCT); and OPG, forward (5′-ATCTCGGCCACTCGAACCT), reverse (5′-CTGCTCGCTCGATTTGCA), and probe (5′-CTTCTTGCCTTGATGGAGAGCCTGCCT).

All PCRs were performed in triplicate and repeated three times. Quantitation was achieved using the comparative threshold cycle (C_t) method according to the manufacturer's protocol.

Treatment of the cells from OPN −/− mice with OPN. Recombinant murine OPN was added to the culture medium of OPN −/− cells at a concentration range of 2.5–30 μg/ml. The cells were cultured in octuplicate for 7 days. On day 7, TRAP staining was performed. TRAP-positive OCLs were counted in a similar manner to that described above. The recombinant mouse OPN was generated in our laboratory [30].

Quantitative real-time PCR was performed to measure the levels of mRNA for RANKL and OPG in the harvested OPN −/− cells incubated with or without OPN as described above.

Murine stromal cell line, ST2, was treated with OPN, 1α,25-dihydroxyvitamin D_3 and/or dexamethasone. ST2 cells were incubated for 24 h in complete medium with or without 30 μg/ml of recombinant OPN, 10^{-8} M 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and/or 10^{-7} M of dexamethasone (Dex; Sigma, St. Louis, MO). Similarly, the levels of mRNA for RANKL and OPG from the cells of each group were quantified.

Statistical analysis. Values are given as means ± SD. Significant differences were determined using an unpaired Student's t test. A p value <0.05 was considered to be significant.

Results

Cells isolated from arthritic joints developed activated OCLs

Cells from entirely swollen arthritic paws were harvested after the enzymatic reaction and cultured for 7 days. Cells from normal paws of non-immunized mice were used as controls. On day 7, TRAP staining was performed.

The cells from arthritic joints differentiated into TRAP-positive OCLs, while the cells from non-immunized mice did not as previously reported [6] (Figs. 1A and B). The numbers of OCLs from the arthritic joints and from the normal joints were 96.9 ± 21.7/well and 0/well, respectively. The bone resorption activity of the generated OCLs was confirmed by a pit formation assay (Fig. 1C).

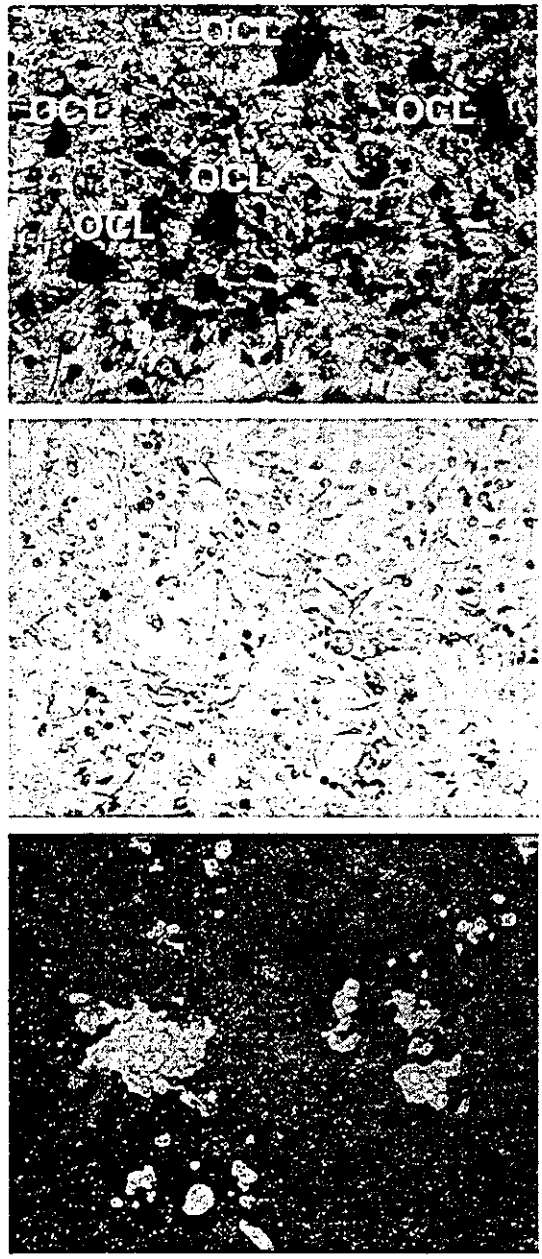

Fig. 1. Cells from the joints of OPN +/+ mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) for 7 (A,B) or 10 days (C). To evaluate the development of OCLs, TRAP staining of the cultured cells was performed as described in the Materials and methods. Cells from the arthritic joints of immunized mice (i.e., CIA mice) developed into OCLs, but those from the normal joints of non-immunized mice failed to. (A) Cells from the arthritic joints of CIA mice. (B) Cells from the normal joints of mice without immunization. (C) Pits formed by OCLs derived from cells of arthritic mice. The 'OCL' labels identify TRAP-positive osteoclast-like cells. (Original magnification; 100×.)

Osteoclastogenesis of arthritic joints was dependent on the RANK/RANKL/OPG system

After 24-h cultivation, mRNA was extracted from the harvested OPN +/+ cells. Quantitative real-time PCR was performed to measure the RANKL and OPG mRNA levels. In the cells from arthritic joints, RANKL mRNA levels increased, while OPG mRNA levels decreased compared to those in the cells from normal joints (Fig. 2, $p < 0.01$). In addition, to confirm that OPG inhibits the differentiation of osteoclasts, we applied OPG at a concentration range of 1.5–100 ng/ml to the culture medium of the arthritic cells and incubated them for 7 days. Application of OPG significantly reduced the numbers of OCLs in a concentration-dependent manner (Fig. 3, $p < 0.01$).

Cells isolated from arthritic joints produced OPN

The levels of OPN in the culture supernatants of the cells from arthritic joints increased during incubation, in contrast to the cells from normal joints. As for the arthritic cells, OPN levels were 1.50 ± 0.43 at day 3 versus 7.35 ± 1.84 at day 6, $p = 0.0008$ (Fig. 4). These results

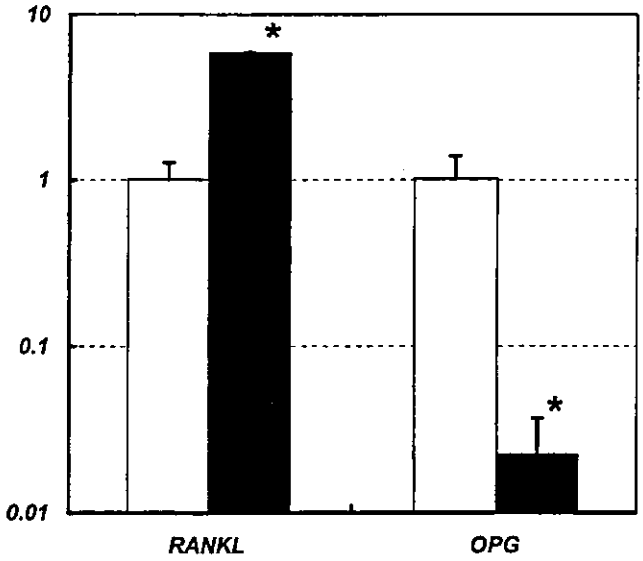

Fig. 2. By real-time PCR, mRNA for RANKL and OPG in the cells from joints of OPN +/+ mice was quantified. Quantitation was achieved from the C_t ratios of the mRNA either for RANKL or OPG to that for β-actin, an internal standard. The values were compared to the levels of mRNA from cells of the non-immunized normal joints. In the cells from the arthritic joints, expression of RANKL mRNA was enhanced while that of OPG mRNA decreased. White bar; in cells from the normal joints, and black bar; in cells from the arthritic joints. $^*p < 0.01$.

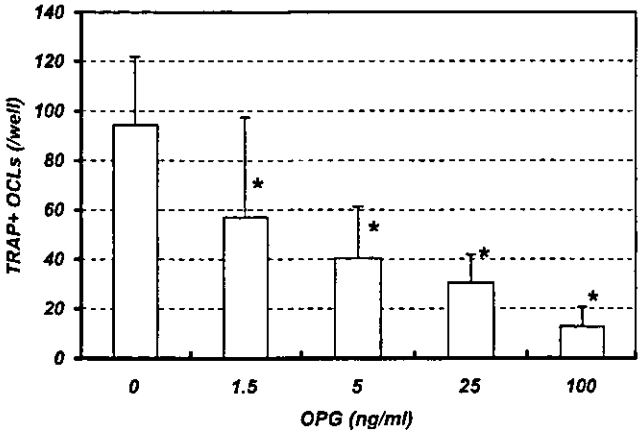

Fig. 3. Numbers of OCLs in the presence or absence of OPG. Cells from arthritic joints of OPN +/+ mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) in octuplicate for 7 days with or without OPG applied to the culture medium. After TRAP staining, TRAP-positive multinucleated cells in each well of a 96-well culture plate were counted. Adding OPG prevents the development of OCLs in a concentration-dependent manner. $^*p < 0.01$.

are consistent with our previous report that the serum concentration of OPN is enhanced in CIA mice during development of arthritis and that activated osteoclasts in bone erosive lesion express OPN [22].

Neutralization of OPN suppressed osteoclastogenesis

In order to investigate whether OPN is involved in the differentiation of osteoclasts, we added anti-OPN

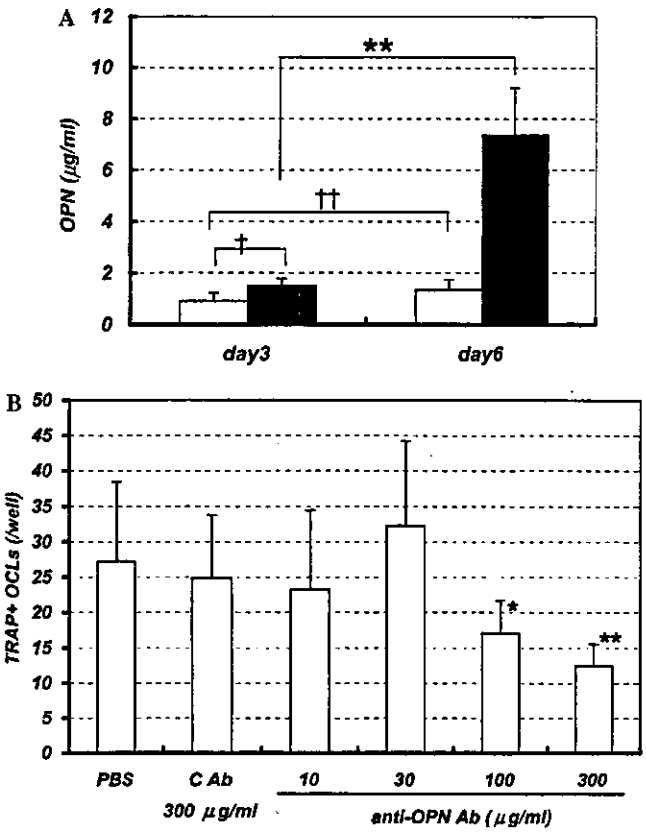

Fig. 4. (A) The concentration of OPN in the culture supernatant of OPN +/+ cells. Each measurement was performed three times in quadruplicate by ELISA. The levels of OPN secreted from cells from arthritic joints of CIA mice increased during incubation. White bar; the values of normal joints from non-immunized mice, black bar; the values of arthritic joints from CIA mice. †Day 3 OPN levels in the supernatant of cells from non-immunized versus immunized mice, $p > 0.05$. †† OPN levels in the supernatant of cells from non-immunized mice, day 3 versus day 6, $p > 0.05$. *OPN levels in the supernatant of cells from immunized mice, day 3 versus day 6, $p = 0.0008$. (B) Numbers of OCLs in the presence or absence of anti-OPN neutralizing antibodies. Cells from arthritic joints of OPN +/+ mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) in octuplicate for 7 days with polyclonal anti-OPN neutralizing antibodies, control antibody, or PBS applied to the culture medium. After TRAP staining, TRAP-positive multinucleated cells in each well of a 96-well culture plate were counted. Anti-OPN antibodies prevent development of OCLs from OPN +/+ cells. $^*p < 0.05$ and $^{**}p < 0.01$.

neutralizing antibodies to the culture medium of arthritic cells and incubated them for 7 days. The numbers of OCLs decreased in a concentration-dependent manner in the presence of the anti-OPN antibodies, whereas the control antibody had no effect (Fig. 4B).

Addition of OPN enhanced the osteoclastogenesis of OPN −/− cells

We examined the effect of recombinant OPN on OPN −/− cells. We added OPN to the culture medium of arthritic OPN −/− cells and incubated them for 7 days. In

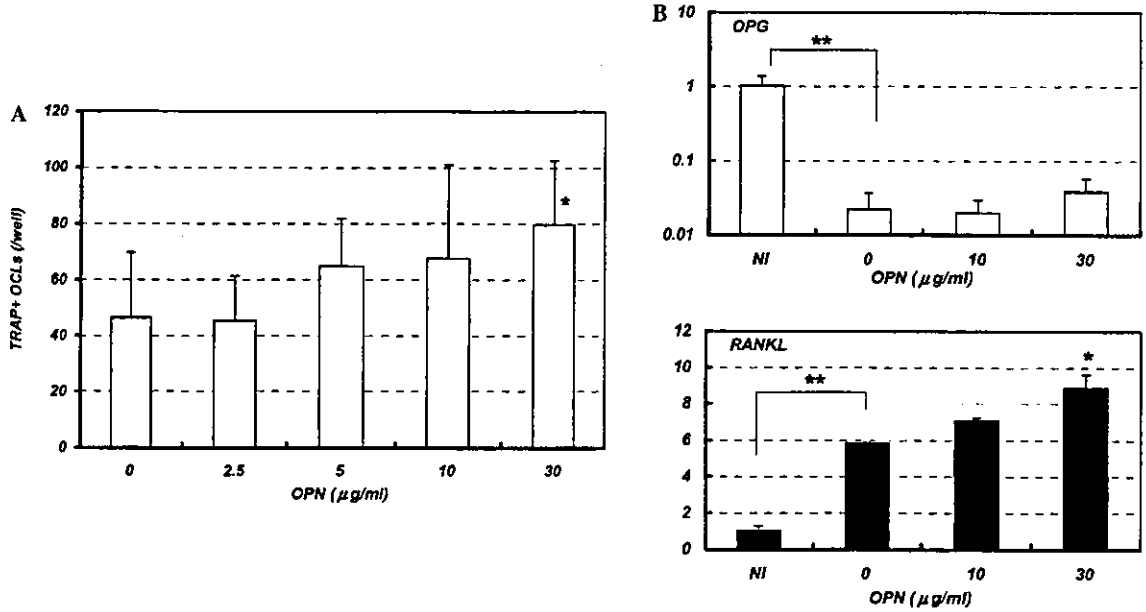

Fig. 5. (A) Numbers of OCLs in the presence or absence of recombinant OPN in OPN −/− cells. Cells from arthritic joints of OPN −/− mice were harvested and incubated in complete medium (1.5×10^4 cells/0.1 ml/well) in octuplicate for 7 days with OPN applied to the culture medium. After TRAP staining, TRAP-positive multinucleated cells in each well of a 96-well culture plate were counted. OPN enhances the development of OCLs while their development from OPN −/− cells. $^{**}p < 0.01$. (B) Quantitation of mRNA for RANKL and OPG of OPN −/− cells in the presence of OPN. We investigated whether OPN has a role in regulating expression of mRNA for RANKL and OPG. Using real-time PCR, mRNA from OPN −/− cells was quantified. Quantitation was achieved from the C_t ratios of the mRNA for RANKL and OPG to that for β-actin, an internal standard. The values were compared to the levels of mRNA from cells of the non-immunized normal joints. Upper panel: addition of OPN to OPN −/− cells did not reduce the expression of OPG mRNA Bottom panel; Addition of OPN to OPN −/− cells slightly enhanced the expression of RANKL mRNA. NI means values of cells from non-immunized mice without reagents. $^{**}p < 0.01$. *Addition of 0 μg/ml OPN versus 30 μg/ml, $p < 0.05$.

the presence of 30 μg/ml OPN, the number of developed OCLs was significantly increased (Fig. 5A).

To demonstrate whether OPN is involved in the enhanced expression of RANKL in arthritis, the levels of mRNA for RANKL and OPG were quantified in OPN −/− cells incubated with or without OPN. As is the case with OPN +/+ mice shown in Fig. 2, in OPN −/− mice the expression of RANKL mRNA was enhanced and that of OPG mRNA was reduced in arthritic joints compared to non-immunized normal joints (NI). Addition of 30 μg/ml OPN to OPN −/− cells increased the expression of RANKL mRNA, although not significantly affecting that of OPG mRNA (Fig. 5B).

OPN enhances the RANKL expression and suppresses the OPG expression on stromal cells

Because the harvested cells from joints were a heterogeneous mixture of cells derived from multiple sources, such as stromal cells, osteoclast precursors, and lymphocytes, etc., there are limitations in evaluating mRNA expression. To clarify the effect of OPN on the expression of RANKL and OPG, we used a murine stromal cell line, ST2. As positive control, we used $1,25(OH)_2D_3$ and Dex, which are well-known agents that induce stromal cells to express RANKL and reduce their expression of OPG [3,4,8,12,19]. While the combination of

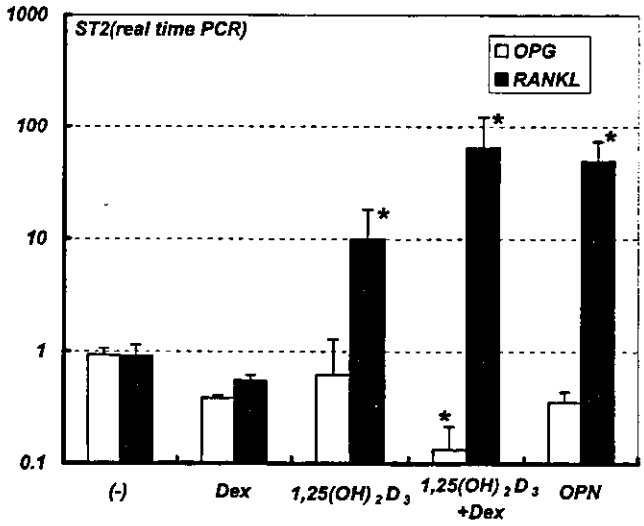

Fig. 6. Quantitation of mRNA for RANKL and OPG of a stromal cell line, ST2, in the presence of several reagents. We investigated whether OPN has a role in regulating expression of mRNA for RANKL and OPG by comparison with other reagents. ST2 cells were incubated with or without OPN, $1,25(OH)_2D_3$, and/or Dex. After 24-h cultivation, mRNA was extracted from ST2 cells and the mRNA levels of both RANKL and OPG were quantified by real-time PCR. OPN and $1,25(OH)_2D_3$ increased the level of RANKL mRNA and reduced the level of OPG mRNA. The values were compared to the levels of mRNA from ST2 without any reagent. OPN, $1,25(OH)_2D_3$, and Dex represent 30 μg/ml of recombinant OPN, 10^{-8} M of $1,25(OH)_2D_3$, and 10^{-7} M of Dex, each. $^{*}p < 0.01$.

$1,25(OH)_2D_3$ and Dex most effectively induced RANKL mRNA and reduced OPG mRNA, OPN also increased the expression of RANKL mRNA over 10-fold (Fig. 6).

Discussion

In this study, we used an in vitro osteoclast differentiation model of CIA as previously reported [6]. In this model, we confirmed that OCLs are inducible without additional agents or exogenous cytokines and that the generated OCLs have the ability to resorb hydroxyapatite. On the grounds that the cells from non-arthritic joints fail to differentiate into OCLs, we speculate that the factors induced or enhanced during the progression of arthritis are crucial. As expected from previous reports [3,13,15–17], the expression of RANKL mRNA was enhanced and that of OPG mRNA was reduced in arthritic joints. In addition, in vitro osteoclastogenesis from arthritic joints was inhibited by OPG in a concentration-dependent manner. Putting these observations together, we conclude that the RANK/RANKL/OPG system is the major contributor to the activation of osteoclasts in CIA.

On the other hand, we have previously reported that the expression of OPN is enhanced locally in arthritic joints [22], and in this study, we confirmed that cells from arthritic joints secrete OPN. Hence, we investigated if OPN could augment osteoclastogenesis. Although in vivo we did not discern any significant differences in either the incidence or severity of CIA induced in OPN −/− and OPN +/+ mice [31], in vitro application of the anti-OPN neutralizing antibodies to OPN +/+ cells prevented the development of OCLs in a concentration-dependent fashion. Moreover, the addition of OPN to OPN −/− cells increased the number of OCLs as a function of OPN concentration. Because of the facilitatory effect of OPN on the generation of OCLs, and the inhibitory effect of anti-OPN neutralizing antibodies akin to that of OPG, we suspected that OPN might play a role in regulating the RANK/RANKL/OPG system in arthritis. Real-time quantitative PCR showed that the presence of OPN increases the level of RANKL mRNA in OPN −/− cells. Besides, adding OPN to the culture medium of a stromal cell line, ST2, which is a typical cellular source of RANKL, enhanced expression of RANKL mRNA much more clearly. These results suggest that OPN affected stromal cells in arthritic joints and enhanced the expression of RANKL. This effect of OPN can contribute to activation of osteoclasts that ultimately results in the joint destruction seen in arthritis.

It has been reported that OPN activates osteoclasts through integrin-$\alpha v\beta 3$ by regulating cell adhesion and controlling cytoskeleton [32]. In addition, several studies, including ours, have already suggested that OPN plays an important role in the bone destruction of arthritis by promoting osteoclastogenesis through acting on osteoclast precursor cells stimulated by RANKL [22,23,26,30]. This study raises the possibility that OPN could act not only on the osteoclast precursor cells but also on the stromal cells and directly induced RANKL and suppress OPG, although to confirm this further experiments are needed.

OPN −/− mice have been reported to be resistant to experimental autoimmune encephalomyelitis (EAE) [33] and CAIA [26]. However, a conflicting report suggests that EAE, CIA, and CAIA are fully inducible without OPN in strain with B10Q allele, which is susceptible to CIA [34]. And we have reported that CIA can be fully induced with DBA/1J OPN −/− mice, which is highly susceptible to CIA [31]. As previous reports of IL-1β and IL-1 receptor antagonist, susceptibility to cytokines differs among strains. The efficacy of OPN deletion can be decided by genetic backgrounds. Additionally, in knockout mice, it is understood that the influence of the deleted gene can sometimes be compensated or substituted for by other similar genes.

OPN may not be an essential factor in the development of osteoclasts, considering that the effects of OPN deletion were not clear on CIA in vivo. However, inhibition of OPN may be an effective treatment for bone destruction, not only from the prospect of inhibiting the activation of osteoclasts, but also in prevention of osteoclast differentiation.

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CONCISE COMMUNICATIONS

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Mice with osteopontin deletion remain predisposed to collagen-induced arthritis

Osteopontin (OPN), also known as Eta-1 (early T lymphocyte activation protein 1), is a secreted phosphoglycoprotein that has a wide range of functions. Other than collagen, OPN is the major extracellular matrix protein in bone, and it has been shown to act as an activator in osteoclasts (1). OPN also works as a Th1 cytokine (2,3). Rheumatoid arthritis (RA) is a typical bone resorption inflammatory disease, the pathogenesis of which is thought to stem from Th1 hyperactivation (4). Moreover, we have demonstrated that expression of OPN is enhanced in arthritic joints of both RA patients (5) and mice with collagen-induced arthritis (CIA) (6). In addition, it has been reported that $OPN^{-/-}$ mice are resistant to experimental autoimmune encephalomyelitis (EAE) (3) and to anti–type II collagen (CII) antibody–induced arthritis (CAIA) (7). However, a conflicting report suggests that EAE, CIA, and CAIA are fully inducible without OPN (8), and therefore, the role of OPN in the above inflammatory diseases remains controversial. In order to investigate the role of OPN in the pathogenesis of RA, we examined its clinical and immunologic effects on CIA, a murine model for RA, using $OPN^{-/-}$ mice.

$OPN^{-/-}$ mice were generated (9) and were backcrossed with DBA/1J mice (Nippon Charles River, Kanagawa, Japan) for 6 generations to introduce CIA susceptibility. After backcrossing, the loss of OPN messenger RNA in the $OPN^{-/-}$ mice was confirmed by reverse transcriptase–polymerase chain reaction. Additionally, the absence of OPN protein in the serum of $OPN^{-/-}$ mice was confirmed by enzyme-linked immunosorbent assay (ELISA). CIA was induced by established methods, as previously described (6). Briefly, 30 male mice (17 $OPN^{+/+}$ and 13 $OPN^{-/-}$ littermates between 6 and 10 weeks of age) were immunized by intradermal injection of 100 μg of bovine CII (MCK, Tokyo, Japan), emulsified with Freund's complete adjuvant (Difco, Detroit, MI). On day 21, a booster injection was given using the same method.

Each week during a followup period of 13 weeks after the first immunization 3 independent observers assessed the mice for signs of arthritis. Severity of arthritis was graded on a 1–4 scale as follows: 0 = normal; 1 = swelling and/or redness in 1 joint; 2 = swelling and/or redness in >1 joint; 3 = swelling and/or redness in the entire paw; 4 = deformity and/or ankylosis. Each paw was graded, and the mean scores of the 4 paws were summed, such that the maximum possible score per mouse was 16. In both groups, of mice, signs of arthritis began to appear ~3 weeks after the first immunization, and the final incidence rates reached 100%. Arthritis scores did not differ significantly between the $OPN^{-/-}$ and $OPN^{+/+}$ mice ($P > 0.1$) (Figure 1A).

The mice were killed 13 weeks after the first immunization. Anteroposterior radiographs of all 4 limbs were obtained with a cabinet soft x-ray apparatus (MX-20; Faxitron, Wheeling, IL). Radiologic changes were evaluated by 3 independent judges under blinded conditions and were graded from 0 to 3 as follows: 0 = normal; 1 = slight erosion; 2 = bone resorption; 3 = joint destruction. The mean scores of the 4 paws were then summed, such that the maximum possible score per mouse was 12. Radiologic scores did not differ significantly between the 2 groups (mean ± SD 9.3 ± 2.1 in the $OPN^{-/-}$ mice, 8.3 ± 1.5 in the $OPN^{+/+}$ mice; $P = 0.13$).

Serum samples from 10 $OPN^{-/-}$ and 10 $OPN^{+/+}$ mice were obtained every 2 weeks from week 0 (before immunization) to week 12 after the first immunization. The serum levels of total IgG and of specific antibodies to CII (total IgG and subclasses IgG1 and IgG2a) were measured by ELISA as previously described (10), with minor modifications. Ninety-six–well plates were coated with anti-mouse IgG (Caltag, Burlingame, CA) or bovine CII antigen solution (2 μg/ml). Nonspecific binding was blocked with phosphate buffered saline containing 1% bovine serum albumin. Serially diluted serum samples were incubated for 2 hours at room temperature. Alkaline phosphatase–conjugated horse anti-mouse IgG heavy and light chain (Vector, Burlingame, CA) or alkaline phosphatase–conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was then added, followed by incubation for 2 hours at room temperature. Color development of *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) was monitored at 405 nm with an ImmunoReader NJ-2000 (Nihon InterMed, Tokyo, Japan). To establish a standard curve, serial dilutions of sera from $OPN^{+/+}$ mice with CIA were added to each plate. The standard was defined as 100 units, and antibody titers of samples were estimated relative to the standard curve.

The levels of total IgG did not show significant changes during the course of the study (data not shown). Levels of anti-CII antibodies became elevated beginning at week 4 and reached a peak between weeks 6 and 8 after the first immunization. The differences between the levels of anti-CII antibodies (subtypes IgG, IgG1, and IgG2a) in $OPN^{+/+}$ and $OPN^{-/-}$ mice during this period were not statistically significant (Figure 1B).

In the present study, we did not discern any significant differences in either the incidence or the severity of CIA induced in $OPN^{-/-}$ and $OPN^{+/+}$ mice. Hence, we observed no effects of OPN deletion on CIA, as suggested by Blom et al (8) and in contrast to the findings reported by Yumoto et al (7), using another arthritis model, the CAIA model. There are several conceivable reasons for this contradiction. First, it may be due to differences in pathogenesis between CAIA and CIA, regarding their immunologic backgrounds. CAIA occurs independently of activation of lymphocytes, and anti-CII antibodies directly induce only acute inflammation. In fact, no infiltration of activated lymphocytes is observed in the arthritic joints of animals with CAIA (11). In contrast, CIA is a well-known model of chronic arthritis that is dependent on both humoral and cellular immunity specific for CII, and is especially dependent on Th1 activation (4,10). In this study, there were no notable differences in the serum levels of anti-CII IgG antibodies (total IgG and subclasses IgG1 and IgG2a) between the 2 groups of mice, which suggests that the Th1/Th2 balance in CIA is not changed by OPN deletion. Therefore, deletion of the OPN gene may not affect lymphocyte function in CIA,

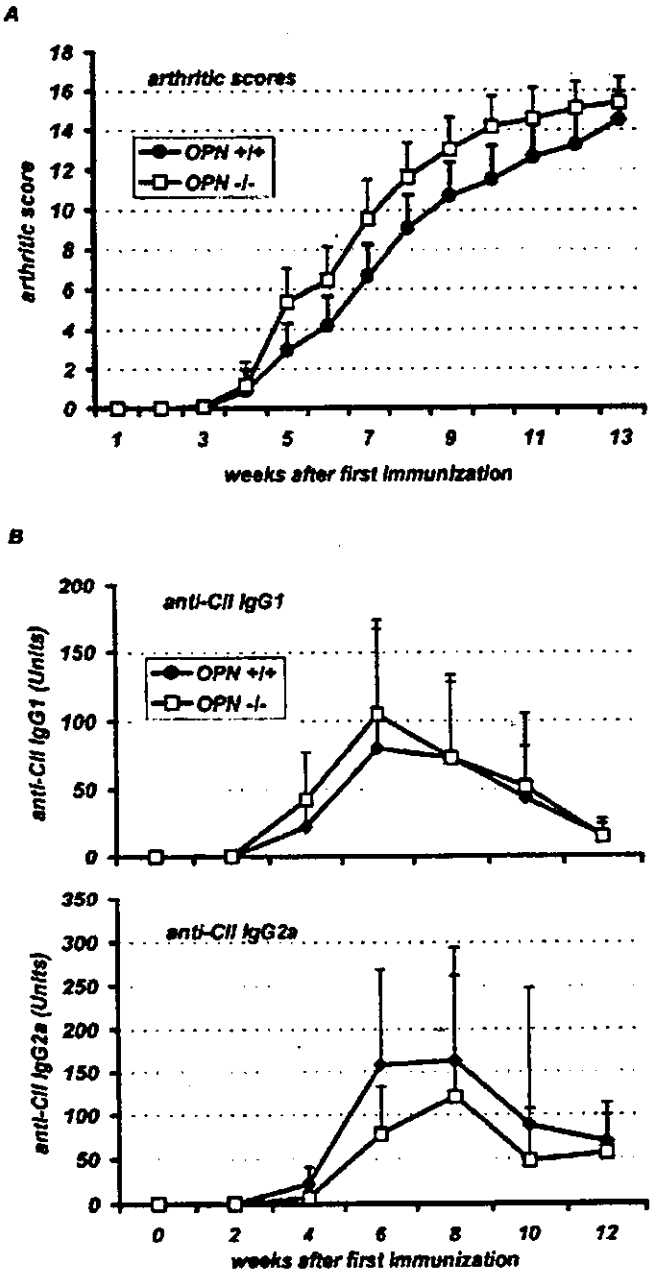

Figure 1. A, Arthritis scores in osteopontin-deleted ($OPN^{-/-}$) and $OPN^{+/+}$ mice. Male $OPN^{+/+}$ mice (n = 17) and their male $OPN^{-/-}$ littermates (n = 13) were immunized with type II collagen (CII) in complete Freund's adjuvant. Mean scores estimated by 3 observers for each paw were summed. Values are the mean and SD. The mean arthritis score did not differ significantly between the $OPN^{-/-}$ and $OPN^{+/+}$ mice at any time point. B, Changes in serum anti-CII antibody levels. Serum samples were obtained from $OPN^{+/+}$ mice (n = 10) and $OPN^{-/-}$ mice (n = 10) every 2 weeks from before the first immunization (0 weeks) to 12 weeks after the first immunization. Serum levels of total IgG and IgG anti-CII (data not shown) and of IgG1 and IgG2a anti-CII were measured by enzyme-linked immunosorbent assay. Values are the mean and SD. Mean levels did not differ significantly between the $OPN^{-/-}$ and $OPN^{+/+}$ mice at any time point.

allowing arthritis in $OPN^{-/-}$ mice to develop and reach the same severity as in $OPN^{+/+}$ mice.

Second, the differences may depend on the murine genetic backgrounds. DBA/1J mice, used in the present study, are highly susceptible to CIA. Blom et al induced CIA in a strain with the B10Q allele, which is also susceptible to CIA (8), whereas Yumoto and colleagues used mice of the C57BL6/129 background (7), which are normally resistant to CIA. As shown in studies of CIA with addition of interleukin-1β (IL-1β) and of IL-1 receptor antagonist–deficient mice (12,13), susceptibility to cytokines differs among strains. The role of OPN deletion could be determined by genetic backgrounds.

Third, the lack of an effect of OPN deletion may be due to compensation or substitution of the OPN gene function by 1 or more other gene(s): in knockout mice, it is understood that the influence of the deleted gene can sometimes be compensated or substituted for by other similar genes. Although surrogates for OPN have not been described, other bone matrix proteins or cytokines might fulfill this function.

In conclusion, OPN is not indispensable in the induction of CIA. However, considering the limitations in studies using knockout mice and previous reports on OPN as a cytokine and a factor in osteoclast activation (1,2,9), the role of OPN in RA and other human inflammatory diseases remains open to dispute.

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Chronic Active EBV Infection and Hypersensitivity to Mosquito Bites: Pathophysiology and Pharmacology

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Abstract: Hypersensitivity to mosquito bites (HMB) is characterized clinically by intense skin reactions at mosquito bite sites with severe systemic symptoms. Another important feature is the high mortality rate due to complications, such as malignant NK cell-lineage granular lymphoproliferative disorder (NK-GLPD) and hemophagocytic syndrome (HPS). Previous studies have indicated that chronic active Epstein-Barr virus infection (CAEBV) is closely associated with HMB and its malignant complications. We and other groups have recently shown the abnormal oligoclonal expansion of EBV-infected NK cells in the periphery of HMB patients, which contributes to the pathogenesis of pleiotropic symptoms in HMB. To explore the therapeutic possibility, we have examined the anti-viral drugs on the symptoms, and some drugs have been emerging as the candidates for the treatment for HMB. In this brief review, we show the recent progresses in the studies elucidating the intricate web among CAEBV, NK-GLPD and HMB. The pathophysiology and pharmacology regarding CAEBV and HMB should also be generally important in viral-associated rheumatic diseases and their therapeutics.

Keywords: Chronic active Epstein-Barr virus infection (CAEBV), NK cell-lineage granular lymphoproliferative disorder (NK-GLPD), hypersensitivity to mosquito bites (HMB), anti-viral therapy.

INTRODUCTION

Epstein-Barr virus (EBV), belonging to herpesviridae, is a ubiquitous virus in humans, and most individuals are affected by their early adulthood [1-3]. Whereas the primary infection with EBV in infancy is usually asymptomatic, approximately 50% of primary infections in early adulthood exhibit acute inflammatory diseases known as infectious mononucleosis (IM). IM is characterized by fever, sore throat, cervical lymphadenopathy with a diagnostic elevation of atypical lymphocytes in the periphery. On the other hand, in rare cases, EBV causes chronic active EBV infection (CAEBV) in apparently immunocompetent hosts. CAEBV is characterized by chronic or recurrent IM-like symptoms, an abnormal pattern of anti-EBV antibodies (increased anti-viral capsid antigen (VCA) and early antigen (EA), in the absence of anti-Epstein-Barr virus nuclear antigen (EBNA)). CAEBV is a high-mortality, high-morbidity disease with life-threatening malignant complications, such as lymphomas and hemophagocytic syndrome, and the patients have poor prognosis.

Whereas, in IM, EBV commonly infects B lymphocytes via a receptor designated CD21 or complement receptor 2 (CR2), predominant population infected with EBV in CAEBV are T or NK cells, but not B cells, with unknown receptors/mechanisms [4]. CAEBV patients can be classified into two groups, *i.e.*, T-cell type and NK-cell type, and each group has distinct clinical features [5]. Among them, interestingly, NK-cell type CAEBV is known to be closely associated with a characteristic allergy reaction to mosquito, called "hypersensitivity to mosquito bites (HMB)" [6].

Hypersensitivity to mosquito bites (HMB), also referred as 'severe' hypersensitivity to mosquito bites, is not a common disease mostly affecting Asians. When an individual possessing HMB is bitten by a mosquito, the skin around the bitten sites form deep ulcers with systemic high-grade fever. Another important feature of HMB is its frequent malignant complications known as NK cell-lineage granular lymphoproliferative disorder (NK-GLPD), which is now suggested to be related to NK-cell type CAEBV, complicated with HMB patients [6, 7].

To date, a number of excellent reviews have already been published regarding the clinical manifestations and etiology of CAEBV [1, 2, 8]. In this brief review, we highlight the association between HMB and CAEBV from the patho-physiological aspects, and try to elucidate how CAEBV forms the characteristic clinical manifestations in HMB. Recent trials for pharmacological treatments for this illness are also described.

OLIGOCLONAL EXPANSION OF EBV-INFECTED NK CELLS IN HMB

In the periphery of HMB, the population of CD56-positive, CD3-negative large granular lymphocytes (LGL), which is suggested to be NK cells, is remarkably increased up to 30 - 60% in total PBMC (Fig. 1) [6, 9]. PCR amplification of EBV-DNA as well as *in situ* hybridization of EBV-encoded small nuclear RNA (EBER)-1 indicate that these NK cells are infected with EBV (Fig. 2) [9]. Southern blot hybridization using a probe detecting EBV terminal repeat detects multiple bands, indicating that these NK cells

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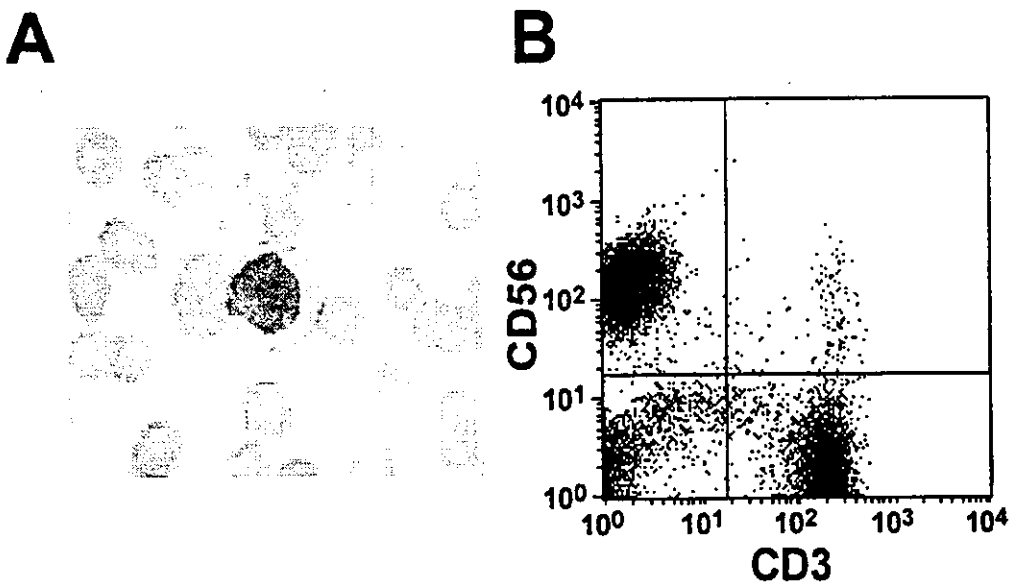

Fig. (1). Abnormal increase in CD56(+)- CD3(-)- NK cells in HMB. (A) LGL morphology in HMB patients. May-Gruenwald-Giemsa staining shows the morphology of large granular lymphocytes (LGL) with azurophillic granules and abundant cytoplasm under light microscopy (X1, 000). [Reproduced with permission from Ohshima *et al.* (2002)]. (B) The expression of CD3 and CD56 in peripheral blood mononuclear cells (PBMC) from patients with HMB/CAEBV was assessed by two-color FACS analysis. [Reproduced with permission from Ishii *et al.* (2003)].

lack the expression of CD21 (Ishii *et al.*, unpublished observation), an EBV receptor for infecting B cells [11], it still remains unsolved how EBV infects NK cells, although alternative routes and mechanisms for viral entry have been proposed [12, 13]. Recent it has been indicated that the formation of immunological synapses during early steps of NK cell attack on EBV-infected B cells leads to the trans-synaptic acquisition of CD21 on a membrane patch, which is causative of EBV infection in NK cells [14].

LATENCY OF EBV INFECTION IN HMB

Three distinct forms of EBV latent gene expression in B cells have been demonstrated and designated as Latency I, II, and III (Table 1) [3]. Burkitt's lymphoma and gastric carcinoma are classified into "latency I", which is characterized by the expression of EBV-encoded RNA (EBER)-1, -2, and EBNA-1. Additional expression of latent membrane protein (LMP)-1 is observed in "latency II", which contains nasopharyngeal carcinoma, Hodgkin's

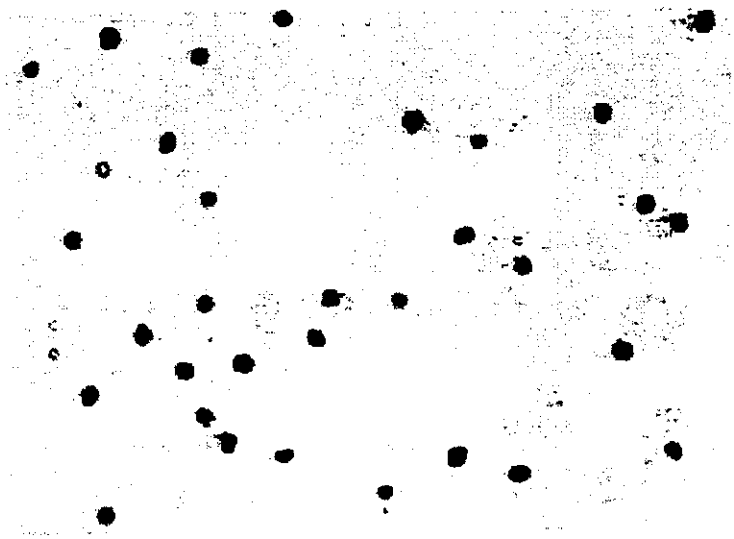

Fig. (2). Detection of EBER1 expression in NK cells from HMB/CAEBV patients by *in situ* hybridization. In situ hybridization was performed using the DIG-labeled antisense EBER1 oligoprobe. Strong signals, indicated as stained cells, were observed in 90% of cytospinned NK cells (X200). [Reproduced with permission from Ohshima *et al.* (2002)].

Table 1. Latent Gene Expression in EBV-Iinfected cells

		EBER	EBNA-1	EBNA-2	LMP-1	LMP-2
Latency I		+	+	-	-	-
	Burkitt's lymphoma					
	Gastric carcinoma					
Latency II		+	+	-	+	±
	Nasopharyngeal carcinoma					
	Hodgkin's disease					
	T/NK-GLPD					
	HMB					
Latency III		+	+	+	+	+
	Infectious mononucleosis (IM)					
	B-cell lymphoproliferative disorders					

disease and T-/NK-GLPD. Latency III (IM, B cell lymphoproliferative disorders, etc) is characterized by expression of all viral latent genes.

As described above, in HMB the infection of EBV is observed in NK cells, but not in B cells nor in T cells [6, 9]. Infected NK cells show the expression of EBER, EBNA-1 and LMP-1 in the absence of LMP-2, suggesting that the EBV infection on these NK cells in HMB is latency II. The viral-associated membrane proteins, such as LMP-1, can serve as a target antigen for EBV-specific cytotoxic T lymphocyte (CTL) activity, and the question remained how the infected NK cells escape from the attack by CTL and maintain the abnormal proliferations.

Possible Mechanisms For Abnormal Expansion of NK Cells

How EBV-infected NK cells can abnormally proliferate in HMB periphery? They must escape from the immunological surveillance by CTL response. It was reported that, in HMB patient, CTL response is greatly reduced not only against EBV-infected patients' NK cells but also against B-lymphoblastoid cell lines (B-LCL), suggesting that CTL itself is impaired for unknown mechanisms [15]. We have previously revealed the augmented expression of Fas-ligand (Fas-L) in EBV-infected NK cells and resultant increase in secreted soluble Fas-L (sFas-L) in the sera [9]. It is well-known that T cells, including CTL, constitutively express Fas, and therefore EBV-infected NK cells might make a counterattack against CTL by means of Fas-L on their cell surface as well as secreted sFas-L, which leads to the vulnerability of CTL response in HMB. Such mechanisms for evading CTL response can be observed in the tumorigenesis [16]. We can suppose that the abundant expression of membrane-bound Fas-L as well as sFasL may contribute to various organ and tissue damages, such as skin ulcer and liver dysfunction, often observed in HMB or CAEBV.

Although the expression of Fas on the NK cell membrane is not altered, these EBV-infected NK cells show resistance against Fas-induced apoptotic cell death [9]. This is caused by the enhanced expression of Bcl-2, a major anti-apoptotic regulator, in EBV-infected NK cells (reviewed in Kirkin *et al.*, 2004 [17]). This result seems to be reasonable because LMP-1, expressed in these NK cells, is reported to induce the expression of Bcl-2 [18].

Another important feature in EBV-infected NK cells is augmented proliferation response to interleukin (IL)-2 [9, 19]. CD25, a high affinity IL-2 receptor, is detected to be highly expressed in these EBV-infected NK cells, whereas normal NK cells only express a low affinity IL-2 receptor, composed from β and γ chains, and does not essentially possess CD25 [9]. This observation is supported by a previous report showing that LMP-1 leads to the enhanced expression of CD25 via NF-κB dependent pathway in Hodgkin cell lines [20, 21]. This aberrant response to IL-2 may be also responsible for the abnormal proliferation of EBV-infected NK cells.

Summarized with these facts, we can speculate that abnormal expansion of EBV-infected NK cells should be achieved by (I) enhanced expression of both membrane-bound Fas-L and soluble Fas-L, which may cope with CTL response, by (II) abundant expression of Bcl-2, which is responsible for the resistance of Fas-induced apoptosis, and by (III) aberrant expression of CD25, which contributes to IL-2 dependent abnormal proliferation of these cells (Fig. 3).

TRIALS FOR TREATMENT OF CAEBV AND HMB

Regarding treatment for CAEBV, several trials have been reported [22, 23]. However, these reports have been anecdotal and no definite treatments have been established so far. In our recent study, several anti-herpesvirus drugs have been tested to the proliferation both of oligoclonally expanding EBV-infected NK cells in HMB patient and of malignant EBV-infected NK cell lines [10]. In results, only two anti-viral drugs, vidarabine and foscarnet, but not

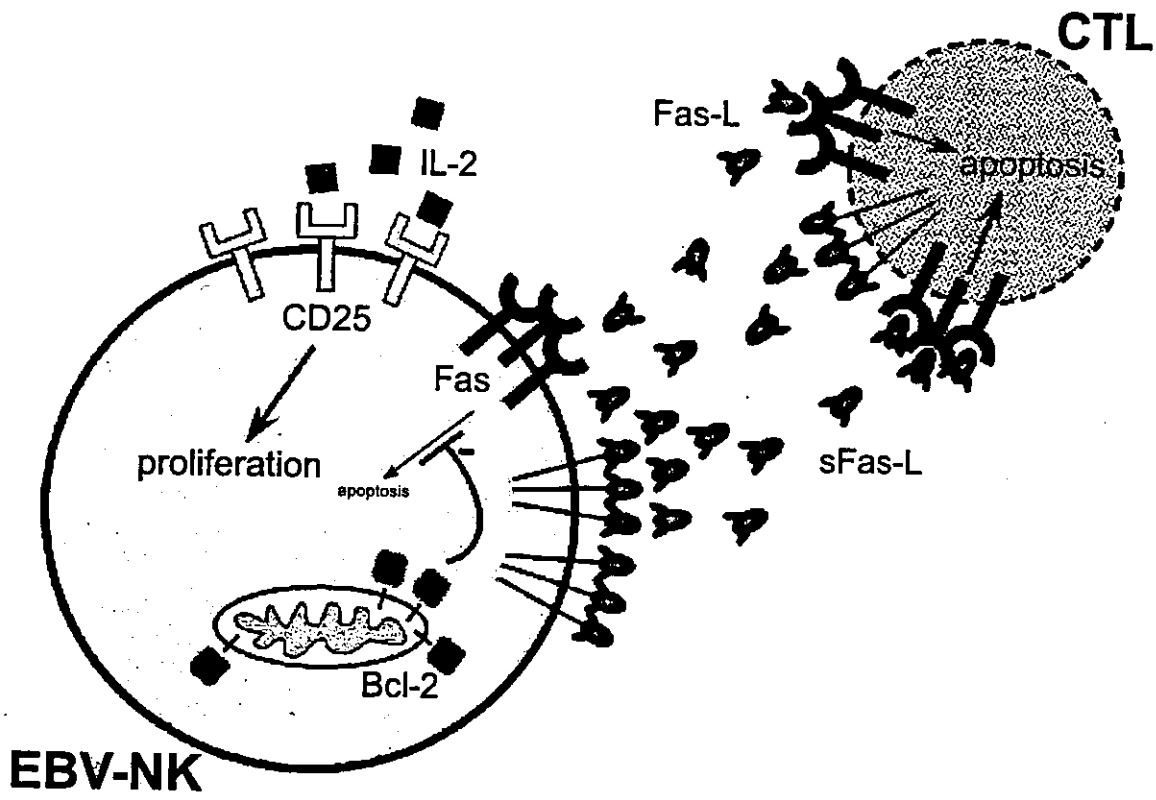

Fig. (3). Schematic representation showing the mechanisms underlying the abnormal expansion of EBV-infected NK cells in HMB. Three mechanisms are suggested here; (I) enhanced expression of both membrane-bound Fas-L and soluble Fas-L, coping with CTL response; (II) abundant expression of Bcl-2, inhibiting Fas-induced apoptosis; (III) aberrant expression of CD25 (a high-affinity IL-2 receptor), enhancing IL-2 dependent abnormal proliferation.

acyclovir nor gancyclovir, selectively and potently inhibit the proliferations of EBV-infected NK cells in HMB, which are oligoclonally expanding at a preneoplastic stage (Fig. 4). On the other hand, none of these drugs have suppressive effect on the proliferation of malignant EBV-infected NK cell lines, which have completely transformed into malignant stages. These results suggest that the proliferation of NK cells in HMB is still dependent on the EBV activity, whereas that of NK malignant cell line is independent of EBV. Therefore it is suggested that the anti-viral drugs might be useful for preventing CAEBV at the preneoplastic stage from the progression of the desperate clinical course.

Vidarabine is also reported to be effective for improving the general symptoms in CAEBV [24]. In our institute, we have successfully treated a CAEBV/HMB patient exacerbating her diseases (Ohshima *et al.*, manuscript in preparation). Her symptoms are dramatically improved and acutely increased NK cell count is restored to a steady level. Based on these laboratorial and clinical observations, vidarabine has been emerging as a good candidate for treating CAEBV/HMB diseases.

REMAINING QUESTIONS

Regarding CAEBV and HMB, plenty of significant progresses have recently been made thanks to the continuous efforts of many researchers. However, several questions have still remained to be solved. Among them, one of the most fundamental is how HMB is caused. Why can only the mosquito, but not other insects, cause severe hypersensitivity? Because EBV-infected NK cells are abnormally expanding in the periphery of HMB patients, one can assume that these NK cells are responsible for the pathogenesis in HMB. However, a recent study has suggested that CD4(+)-T cells, but not EBV-infected NK cells nor CD8(+)-T cells, infiltrate the bitten sites and contribute to primary skin reactions at the local areas as well as systemic symptoms [25]. Further investigations are necessary for elucidating how EBV-infection in NK cells is related to the characteristic clinical manifestation in HMB.

Abnormally expanding NK cells are generally oligoclonal. Once they transformed into malignancy (NK-GLPD), they showed monoclonal expansion. To date, the molecular switch of the clonality in NK cells have not been identified. Because HMB shows poor prognosis exclusively due to its malignant complications, it is highly desirable to detect the molecular identity of this switch. Controlling the switching in oncogenesis, combined with anti-viral therapy, must dramatically improve the prognosis of these diseases.

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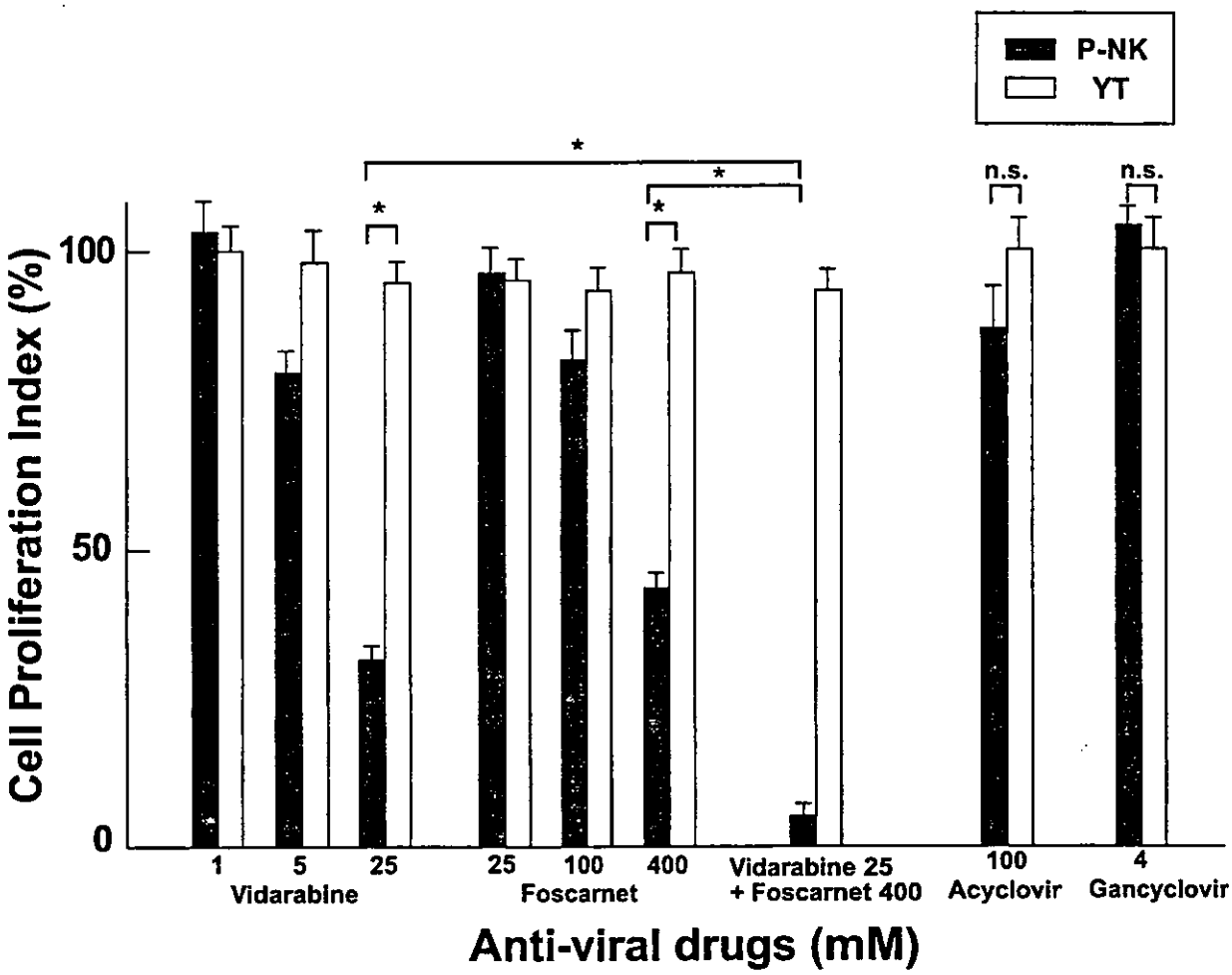

Fig. (4). Effect of anti-viral drugs on the proliferation of EBV-infected NK cells (P-NK) and an EBV-associated malignant NK cell line (YT). The cell proliferation index is a ratio of cell proliferation in the presence of several drugs (shown below) relative to that in the control condition. Results are shown as mean values obtained from three independent experiments and error bars represent the s.e.m. Astarisk: $p<0.05$. n.s.: not significant. [Reproduced with permission from Ishii *et al.* (2003)].

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骨吸収性疾患におけるオステオポンチン

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骨吸収性疾患におけるオステオポンチン

佐　伯　行　彦

要旨　オステオポンチン（OPN）の骨吸収性疾患に関連した最近の知見についてわれわれの研究成果をまじえて解説した．OPNは多彩な作用をもつ一種のサイトカインであるが，とくに，破骨細胞による骨吸収において必須の分子のひとつとして知られている．

最近の研究結果から，OPNは閉経後の骨粗しょう症，関節リウマチ，多発性骨髄腫などさまざまな骨吸収性疾患において重要な役割を果たしていることが明らかになってきた．OPNは，これらの骨吸収性疾患において診断や疾患活動性の指標，あるいは治療上の標的分子として注目されている．

（キーワード：オステオポンチン，破骨細胞，骨粗しょう症，関節リウマチ，多発性骨髄腫）

OSTEOPONTIN IN BONE-RESORBING DISEASES

Yukihiko SAEKI

Abstract I reviewed recent reports including our data regarding osteopontin (OPN) in various bone-resorbing diseases. OPN is categorized as a kind of cytokines with diverse biological functions and known as one of the key molecules for osteoclastic bone-resorption. Recent investigations have revealed that OPN plays a crucial role for the pathogenesis, especially bone destruction, in bone-resorbing diseases such as osteoporosis, rheumatoid arthritis, and multiple myeloma. OPN might be both a useful diagnostic biomarker and a potential therapeutic target for these bone-resorbing diseases.

(Key Words : osteopontin, osteoclast, osteoporosis, rheumatoid arthritis, multiple myeloma)

オステオポンチン（osteopontin；OPN）は，もともと骨の細胞外基質から単離された分泌型のリン酸化糖タンパク分子である[1)2)]．OPNは破骨細胞，マクロファージ，活性化T細胞，平滑筋細胞や上皮細胞などさまざまな細胞により産生され，骨，腎臓，胎盤，平滑筋，腺組織など多くの組織でその発現が認められる．また，OPNはアルギニン−グリシン−アスパラギン酸（RGD）配列を有し，$\alpha v\beta 1$，$\alpha v\beta 3$，$\alpha v\beta 5$などの複数のインテグリンと結合することができ，さまざまな細胞において接着，遊走やシグナル伝達に関与し，骨吸収，血管新生，創傷の治癒などの正常組織にみられるリモデリングに関与していることが知られている[3)]．さらに，最近，骨破壊，再狭窄，動脈硬化，腎疾患，腫瘍など病態／疾患とも関わりがあることがわかってきた．また，OPNはEta-1（early T cell activation-1）という分子と同一分子であり，TH1免疫の誘導やマクロファージの活性化など免疫系においても重要な役割を果たしていて，多彩な作用を有する一種のサイトカインと考えられている（Table 1）[4)−41)]．

本稿では，OPNの骨吸収性疾患に関連した最近の知見をわれわれの研究成果をまじえて紹介したい．

OPNの構造（Fig. 1）

OPNは約400個のアミノ酸からなる，分子量約32,000のポリペプチドを骨格にもつ分泌型リン酸化糖タンパク質分子である．リン酸化や糖付加の程度により分子量は44,000−75,000に変化する．OPNはグルタミン，グルタミン酸，アスパラギン，アスパラギン酸が総アミノ酸の半数以上を占める特徴的なタンパク分子である．また，中央部にはトロンビン切断部位が存在し，そのすぐN末

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Table 1 Diverse Biological Functions of Osteopontin

Cell／Tissue／Organ	Biological Functions	Reference
Bone	reguration of the deposition of minerals	4, 5,
	mechanical stress signal transduction	6
	osteoclast attachment	7, 8
	bone resorbing diseases (osteoporosis, rheumatoid arthritis, multiple myeloma)	9−15
Immunological System	early component of type-1 immunity	16
	augmentation of IL-12 and IFNg production	
	suppression of IL-10 production	
	macrophage chemoattractant	17, 18
	augmentation of antibody production	19, 20
	Th1 diseases (rheumatoid arthritis, multiple myeloma)	21
Cardiovascular System	restenosis	22, 23
	plaque formation, calcification	24, 25
	cardiomyopathy	26
Renal System	suppression of NO synthesis	27
	prevention of mineral precipitation	28
	glomerulonephritis	29−31
Brain	ischemia	32
Granulomatous Tissues	wound healing, tuberculosis, sarcoidosis	33−36
Tumors	transfomation, carcinoma	37−41

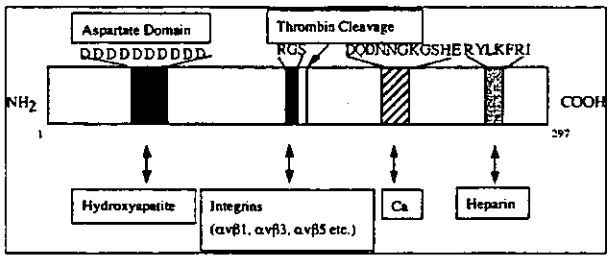

Fig. 1 A Schematic Structure of Osteopontin

端側には細胞接着ドメインと考えられるRGD配列をもつ．さらに，カルシウムやヒドロオキシアパタイトなどに親和性の高いドメインをもち，骨などの石灰化基質に高い親和性をもつ．

破骨細胞による骨吸収おけるOPNの役割

最近，主にノックアウトマウスを用いた研究により，破骨細胞による骨吸収の分子機構が明らかとなってきた[42]．破骨細胞による骨吸収の最終ステップである，骨への接着にはOPNと$\alpha v\beta 3$インテグリンとの結合が必須と考えられている．実際に$\alpha v\beta 3$インテグリンに対する抗体でこの結合を阻害すると破骨細胞による骨吸収は抑制される[43]．また，OPNノックアウトマウスにおいては軽度の大理石病が観察され，$\beta 3$インテグリンノックアウトマウスでも同様に軽度の大理石病が観察される．これらの事実から，OPNがインテグリンとの結合により破骨細胞による骨吸収において促進的な制御に関わっていることが推察される．

骨吸収性疾患

骨は，絶えず骨形成と骨吸収を繰り返しturn-overされ，維持されている．したがって，正常な（健康な）骨を維持するためには骨形成と骨吸収のバランスが保たれなければならない．そのバランスが破綻し，骨吸収へ傾くと病的な骨，骨吸収性疾患を生じることになる．骨吸収性疾患は，(1)非炎症性のもの，(2)炎症性のもの，(3)腫瘍性のものの，大きく3つに分けることができる．まず，非炎症性の骨吸収性疾患は加齢や女性ホルモンの減少などを原因とするもので，代表的なものとして閉経後の骨粗しょう症がある．次に炎症性のものとしては，関節リウマチ（RA）をはじめとするリウマチ性疾患がある．また，腫瘍性のものとしては多発性骨髄腫が挙げられる．

最近，これらの骨吸収性疾患において，OPNの関与を示唆する報告がみられる．

非炎症性骨吸収性疾患とオステオポンチン

OPNのノックアウトマウスは正常に生まれ，出産し，胎児数や寿命においても野生型とほぼ同等である．しかしながら，野田らのグループにより，閉経後骨粗しょう症の実験モデルである卵巣摘出マウスにおける骨吸収はOPNノックアウトマウスでは野生型に比べて有意に抑制されることが報告されている[9]．このことから，閉経後の骨粗しょう症における骨吸収の亢進にOPNの関与が示唆されている．

炎症性骨吸収性疾患とオステオポンチン

われわれは，実験的関節炎モデルである，コラーゲン誘導関節炎（CIA）において，破骨細胞がまさに骨吸収をしている骨びらん部位に限局して，OPNの発現がみられることをタンパク，mRNAレベルで報告した（Fig. 2）[10]．また，関節炎の進行と平行して血中のOPNレベルの上昇がみられる（Fig. 3）．一方，野田らのグルー