Table 1. Candidates for anti-RANKL therapy

OPG, OPG-Fc (23, 129, 144, 188, 189, 195, 196)
RANK-Fc (191–194)
Anti-RANKL monocloncal antibody (197)
OPG-like peptidemimetics (OP3-4) (190)
RANKL vaccine (199)
Interferon-β, γ (201, 202)
p38 inhibitor (SB203580, FR167653) (203, 204)
JNK inhibitor (SB600125) (59)
IKK inhibitor (NBD peptide) (209)
NF-κB inhibitor (NF-κB decoy) (206)
Calcineurin inhibitor (Cyclosporin A, FK 506) (59, 64, 65)
NFAT inhibitor (VIVIT peptide) (59)
PI3K inhibitor (wortmannin, LY290442) (85, 86, 205)

stage. Lymphotoxin β signaling is indispensable for both lymph node and Peyer's patch organogenesis, and the RANK signaling regulates lymphotoxin β expression in lymph nodes (208). Therefore, RANKL/RANK pathways may be required for lymph node genesis in the developmental stage but not for lymph node function in adulthood.

OPG binds to TNF-related apoptosis-inducing ligand (TRAIL) with the similar affinity to RANKL (209), and therefore, OPG treatment may affect the function of TRAIL. Mice with TRAIL gene deletion were more susceptible to experimental and spontaneous tumor metastasis, and they were more sensitive to chemical carcinogens, indicating the importance of TRAIL in the host defense against transformed cells (210). Although neither the increase in the overall risk of malignancy nor the exacerbation of the metastatic bone tumors has been reported in the clinical trials of Fc-OPG or anti-RANKL antibody, these data warrant a careful observation on the patients receiving anti-RANKL therapy.

The experience of biological agents for the treatment of RA patients displayed the potential risk of their immunogenicity, i.e. stimulation of the production of antibodies against themselves. Patients treated with mouse : human chimeric antibodies can develop human anti-mouse antibodies, which may reduce the clinical effectiveness of the drug, and patients treated with fully human antibodies can develop human anti-human antibodies. This recognition is particularly critical in case of Fc-OPG treatment, because antibodies against Fc-OPG have potential risk of cross-reacting with and neutralizing endogenous OPG. In fact, generation of anti-OPG antibodies was observed in one subject in a phase 1 study with an Fc-OPG fusion molecule (197).

Another potential concern about anti-RANKL therapy includes its adverse effects on mammary gland. RANKL or RANK knockout mice fail to form lobuloalveolar mammary gland structures during pregnancy and show a complete block in the formation of a lactating mammary gland (211).

Conclusion

The accumulating knowledge on the molecular mechanism regulating osteoclast development has opened a new era of therapeutic approaches against pathological bone disorders. In spite of the overwhelming scientific evidence that the RANKL/RANK/OPG axis plays a central role in the pathological bone destruction, which makes it an ideal therapeutic target, clinical application of anti-RANKL therapy has just begun. Intense basic and clinical studies will further uncover the potential advantages and disadvantages of anti-RANKL therapy.

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Regulation of Osteoclast Apoptosis and Motility by Small GTPase Binding Protein Rac1*

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ABSTRACT: The role of Rac1 in osteoclast survival and bone-resorbing activity was examined using adenovirus vector expression systems. Rac1 is critically involved in M-CSF receptor signaling and mediates survival signaling primarily through PI3K/Akt pathways. Rac1 also plays a significant role in bone resorptive activity, probably by regulating the motility of osteoclasts.

Introduction: Rac1 is a member of Rho family small G-proteins, and recent studies have revealed that it mediates anti-apoptotic signals in some types of cells. Rac1 is reported to be required for the cytoskeletal organization and bone-resorbing activity of osteoclasts, but their roles in osteoclast survival and function are not fully elucidated.

Materials and Methods: We constructed the adenovirus vector carrying cDNA of either the *dominant negative Rac1* (*Rac1^{DN}*) or *constitutively active Rac1* (*Rac1^{CA}*) gene, and osteoclast-like cells (OCLs) generated in mouse co-culture system were infected with these viruses. To examine the role of Rac1 in osteoclast survival and function, we performed pit formation assays, survival assays, and Western blotting, including an activated-Rac1 pull-down assay using adenovirus-infected OCLs. To further clarify the mechanism of Rac1 regulation in osteoclast survival, some specific inhibitors and adenovirus vectors of signal transduction molecules were used. To quantify membrane movement before and after macrophage colony-stimulating factor (M-CSF) treatment, OCLs expressing either enhanced green fluorescent protein (EGFP) or Rac1^{DN} were recorded with a time-lapse video microscope.

Results: Adenovirus vector-mediated dominant negative Rac1 (Rac1^{DN}) expression significantly reduced pit formation, and promoted their apoptosis. M-CSF rapidly activated Rac1, and the prosurvival effect of M-CSF for OCLs was abrogated by Rac1^{DN} overexpression. Constitutively active Rac1 enhanced OCL survival, which was completely suppressed by phosphatidylinositol 3'-kinase (PI3K) inhibitors, whereas a Mek inhibitor had only partial effect. Rac1^{DN} also partially blocked the activation of Akt induced by the overexpressing catalytic subunit of PI3K. Using time-lapse video microscopy, we found that Rac1^{DN} expression reduced membrane ruffling and the spreading of OCLs in response to M-CSF.

Conclusions: Small guanosine triphosphatase (GTPase) Rac1 is critically involved in M-CSF receptor signaling and mediates survival signaling of osteoclasts primarily by modulating PI3K/Akt pathways. Rac1 also plays a significant role in the bone resorptive activity of cells, probably by regulating the motility of osteoclasts.
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INTRODUCTION

OSTEOCLASTS ARE PRIMARILY responsible for bone resorption and play essential roles in maintaining skeletal homeostasis. They are terminally differentiated cells with a short life span and undergo rapid apoptosis in the absence of trophic factors, such as macrophage colony-stimulating factor (M-CSF) and RANKL.[1,2] Although recent findings suggest that osteoclast survival is regulated through interactions of various hormones and cytokines,[3,4] the underlying molecular mechanism is not fully understood. Bisphosphonates are widely used in the management of osteoporosis and are known to suppress pathological bone resorption by directly suppressing osteoclast activity. Numerous studies have shown that one of the principle mechanisms of bisphosphonate action is to induce osteoclast apoptosis both in vitro and in vivo. The remarkable success of bisphosphonates as an anti-osteoporotic treat-

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ment has led us to believe that the osteoclast apoptosis can be a good therapeutic target to develop efficient drugs for pathological bone loss, and therefore, the molecular mechanism of the osteoclast apoptosis has attracted a great deal of attention.

Guanosine triphosphate (GTP)-binding proteins (G-proteins) regulate cellular function by interconverting between the GTP-binding (active) form and the guanosine diphosphate (GDP)-binding (inactive) form. Small G-proteins are monomeric G-proteins with molecular weight of 20–30 kDa, and to date, >100 members have been identified. Recent studies have revealed that small G-proteins can be targets of nitrogen-containing bisphosphonates.[5] It has been shown that these bisphosphonates inhibit post-translational prenylation of small G-proteins, which may be a mechanism of their action to induce osteoclast apoptosis. Rho, Rac, and Cdc42 are members of Rho family small G-proteins,[6] and accumulating evidence has shown that they mediate growth factor receptor signaling and regulate the cytoskeletal organization in various types of cells.[7,8] Recent studies, however, have revealed that some members of Rho family small G-proteins, especially Rac1, also mediate anti-apoptotic signals in some types of cells, such as hematopoietic cells, cerebellar granule neurons, and COS7 cells.[9–11] RhoA and Rac1 are reported to be required for the cytoskeletal organization and bone-resorbing activity of osteoclasts, but their roles in the osteoclast survival and function are not fully elucidated.

In this study, with the adenovirus vector expression system, we investigated the role of RhoA, Rac1, and Cdc42 in osteoclast survival and function. Among them, we found that Rac1 is critical for both osteoclast survival and bone resorption, and we showed that Rac1 lies downstream of M-CSF receptor signaling, mediates the survival signaling of osteoclasts through phosphatidylinositol 3-kinase (PI3K)-Akt pathways, and plays an important role in bone-resorbing activity, probably by regulating osteoclast membrane movement.

MATERIALS AND METHODS

Animals and chemicals

Treatment of each animal was conducted in accordance with the Guide for Animal Experimentation established at our institute. Newborn ddY mice and 5-week-old male ddY mice were purchased from Sankyo Laboratories Animal Center. α-MEM and DMEM were purchased from GIBCO BRL and Life Technologies (Rockville, MD, USA), and FBS was purchased from Sigma Chemical (St Louis, MO, USA). Bacterial collagenase was purchased from Wako Pure Chemical (Tokyo, Japan) and dispase from Godo Shusei Co. (Tokyo, Japan). Prostaglandin E_2 (PGE$_2$) was obtained from Sigma Chemical, and 1α,25-dihydroxy-vitamin D$_3$ [1α,25(OH)$_2$D$_3$] was purchased from Calbiochem (La Jolla, CA, USA). Type I collagen gel was purchased from Nitta Gelatin (Osaka, Japan). MEK inhibitor PD98059 was purchased from Cell Signaling Technology (Beverly, MA, USA), PI3K inhibitor LY294002 was purchased from Sigma Chemical, and wortmannin and

rapamycin were obtained from Calbiochem. Recombinant mouse M-CSF was obtained from R&D Systems (Minneapolis, MN, USA). Anti-GFP antibody (JL-8) was obtained from Clontech (Palo Alto, CA, USA), anti-Rac1 and anti-ERK were from Transduction Laboratories (Lexington, KY, USA), anti-phospho-ERK was from New England Biolabs (Beverly, MA, USA), and anti-phospho Akt (S473) and anti-Akt were from Cell Signaling Technology. Other chemicals and reagents used in this study were of analytical grade.

Osteoclast culture

Osteoclast-like cells (OCLs) were generated in the mouse co-culture system as described previously.[12,13] Briefly, mouse primary osteoblastic cells from 1-day-old ddY mouse calvaria and bone marrow cells from tibias of 5-week-old male ddY mice were co-cultured on 10-cm plastic dishes or collagen gel-coated dishes with 10% FBS containing α-MEM in the presence of 10 nM 1α,25(OH)$_2$D$_3$ and 1 mM PGE$_2$. On day 4 or 5, when OCLs began to appear, mouse co-cultures were incubated with a small amount of α-MEM containing adenovirus vectors for 1 h at 37°C. The cells were washed twice with PBS and further incubated with α-MEM/10%FBS at 37°C. Twenty-four hours after adenovirus infection, collagen gel was digested with 0.2% collagenase, and co-cultured cells were reseeded onto dentin slices or plastic dishes. For Western blotting and the survival assay, OCLs were purified following a modified method originally reported by Tezuka et al.[14] In brief, osteoblasts and stromal cells were removed with α-MEM containing 0.1% collagenase and 0.2% dispase 4–8 h after reseeding.

Adenovirus construction

Every cDNA of fusion protein of enhanced green fluorescent protein (EGFP) and dominant negative mutant of RhoA (T19N, RhoA^{DN}), Rac1 (T17N, Rac1^{DN}), and Cdc42 (T17N, Cdc42^{DN}), or constitutively active Rac1 (G12V, Rac1^{CA}) gene cloned in pCAGGS vector was a kind gift from Dr Michiyuki Matsuda (Research Institute for Microbial Diseases, Osaka, Japan).[15] Adenovirus vectors carrying these cDNA was constructed using the in vitro ligation technique with a commercially available kit from Clontech. The adenovirus vector carrying only EGFP cDNA was used as a control vector. Adenovirus vector carrying the dominant negative mutant of Rac1 with CAG promoter was kindly provided by Yoh Takuwa (Kanazawa University, Japan). Adenovirus vector carrying cDNA of the myristoylated form of Akt (Akt^{CA}), which contains a Src myristoylation signal that promotes association with the plasma membrane causing constitutive activation through phosphorylation by Akt-activating kinases, was a generous gift from Dr Hideki Katagiri (Tohoku University).[16] Adenovirus vector carrying cDNA of a catalytic subunit p110α of PI3K was also kindly provided by Dr Hideki Katagiri.[17] To determine the multiplicity of infection (MOI) of the viruses, we used a modified endpoint cytopathic effect assay as previously described.[18]

Pit formation assay

The pit formation assay was carried out as reported.[19] Briefly, OCLs obtained on a collagen gel co-culture system were recovered by digesting the gel as described above. An aliquot of the crude OCL preparation was transferred onto dentine slices (Wako Pure Chemical) and cultured for an additional 8 h. To prevent the effect of OCL survival on the pit formation assay, the assay was performed after 8 h. After the 8-h incubation, the medium was removed, and 1 M NH_4OH was added to the wells for 30 minutes. Adherent cells were removed from the dentine slices by ultrasonication, and the resorption pits were visualized by staining with 1% toluidine blue. The resorbed area was measured using an image analysis system (System Supply, Nagano, Japan) linked to a light microscope (Nikon, Tokyo, Japan).

Osteoclast survival assay

The survival rate of OCLs was measured as reported.[1,12] Briefly, OCLs were subjected to TRACP staining at 0, 12, and 24 h after purification. Cell viability/survival rate was expressed as the proportion of morphologically intact TRACP$^+$ multinucleated cells. The number of viable cells remaining at the different time-points was shown as a percentage of the cells at time 0. To determine the effect of M-CSF or various inhibitors of signal transduction pathways on cell survival, each reagent was added to OCL cultures at time 0 after purification.

Western blotting

All extraction procedures were performed at 4°C or on ice. Cells were washed with ice cold PBS and lysed by adding TNE buffer (1% NP-40, 10 mM of Tris-HCl [pH 7.8], 150 mM of NaCl, 1 mM of EDTA, 2 mM of Na_3VO_4, 10 mM of NaF, and 10 μg/ml of aprotinin). The lysates were clarified by centrifugation at 15,000 rpm for 20 minutes. An equal amount of protein was subjected to 10% SDS-PAGE, transferred electrophoretically onto a nitrocellulose membrane, and probed sequentially with an appropriate primary antibody followed by a secondary antibody coupled with horseradish peroxidase (Promega, Madison, WI, USA). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Arlington Heights, IL, USA) following the procedure recommended by the supplier. The blots were stripped by incubating for 20 minutes in stripping buffer (2% SDS, 100 mM of 2-mercaptoethanol, and 62.5 mM of Tris-HCl [pH 6.7]) at 50°C and reprobed with other antibodies.

Determination of Rac1 and Cdc42 activation by M-CSF

Activation of Rac1 and Cdc42 in response to M-CSF was examined with a glutathione S-transferase (GST) pull-down assay using a commercially available Rac/Cdc42 activation assay kit (Upstate, Charlottesville, VA, USA). In brief, after adding 100 ng/ml M-CSF, total cell lysates of OCLs from 10-cm dishes were collected as described above at indicated time-points and incubated with p21-binding domain of PAK1 and GST fusion protein immobilized on

glutathione agarose beads for 1 h at 4°C. Precipitates were subjected to 10% SDS-PAGE and immunoblotted with anti-Rac1 or anti-Cdc42 antibody.

Actin ring formation

Cells were first stained for TRACP to identify osteoclasts and then incubated for 30 minutes with rhodamine-conjugated phalloidin solution (Molecular Probes, Eugene, OR, USA).[20] The actin rings formed by osteoclasts were detected with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Quantification of osteoclast membrane movement with time-lapse video microscopy

The effect of Rac1^{DN} expression on the dynamic cytoskeletal organization of OCLs was evaluated and quantified using time-lapse video microscopy as follows. After confirming the gene transduction to OCLs on collagen gel by detecting green fluorescence under a fluorescent microscope, the gel was digested, and OCLs infected by either EGFP or Rac1^{DN} adenovirus were reseeded on serum-coated glass coverslips placed in 35-mm dishes. Three to 8 h later, when OCLs had fully spread, 50 ng/ml M-CSF was applied to the cultures. Recording of OCLs started 30 minutes before the M-CSF treatment and continued for 90 minutes using a phase contrast time-lapse video microscope (LVR-3000N and pxc930; Sony, Tokyo, Japan). Resulting moving images were transferred to a computer. Pairs of the first and the second cell images with a 5-minute interval in between were selected at 30 minutes or longer after M-CSF treatment, and the contours of the cell pairs were traced with photo-retouch software (Photoshop; Adobe). Each pair's second image was subtracted from its first image. Total number of pixels remaining after the subtraction of two serial static images were counted on the image analysis software (NIH image) and called the motile area. Motility was expressed as a percentage of the motile area to the first image. The measurement was performed on 20 pairs from four OCLs in Rac1^{DN} and control virus.
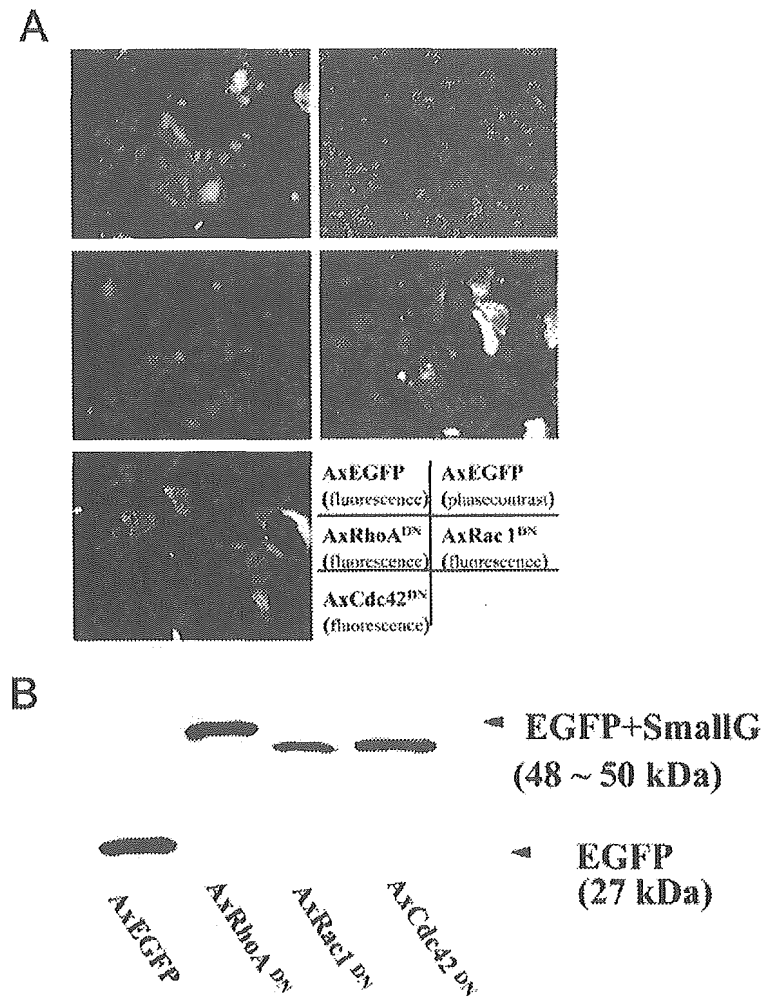
Statistical analysis

Each series of experiments was repeated at least three times. The results obtained from a typical experiment were expressed as the means ± SD. Significant differences were determined using an unpaired t-test, the Mann-Whitney test, or a factorial ANOVA. Fisher's protected least significant difference (PLSD) or Dunnett test was used as a posthoc test.

RESULTS

Adenovirus vector-mediated gene transduction into OCLs

To analyze the role of Rho family small G-proteins in mature OCLs, we constructed adenovirus vectors carrying constitutively active Rac1 or dominant negative RhoA, Rac1, and Cdc42 fused with EGFP and infected OCLs with these viruses. First, we confirmed the efficiency of adenovirus vector-mediated gene transduction into OCLs 36 h

A

AxEGFP (fluorescence)	AxEGFP (phasecontrast)
AxRhoA^{DN} (fluorescence)	AxRac1^{DN} (fluorescence)
AxCdc42^{DN} (fluorescence)	

B

EGFP+SmallG (48 ~ 50 kDa)

EGFP (27 kDa)

FIG. 1. Adenovirus vector-mediated gene expression in OCLs. The expression of EGFP, EGFP-RhoA^{DN}, EGFP-Rac1^{DN}, and EGFP-Cdc42^{DN} introduced into OCLs was confirmed 36 h after infection by (A) fluorescence microscopy and (B) Western blot analysis using anti-EGFP antibody. Most OCLs expressed EGFP or EGFP-fusion protein 36 h after infection. On Western blotting using anti-EGFP antibody, EGFP-Rho family small G-protein fusion protein was expressed as a molecule with molecular weight about 48–50 kDa.

after infection by fluorescence microscopy to detect EGFP fluorescence in situ and Western blot analysis using anti-EGFP antibody. Clear EGFP fluorescence was detected with a fluorescence microscope in almost 100% of the infected OCLs (Fig. 1A). Expression of fusion proteins of each dominant negative mutant and EGFP was observed as ~48-kDa molecular weight bands by Western blotting (Fig. 1B).
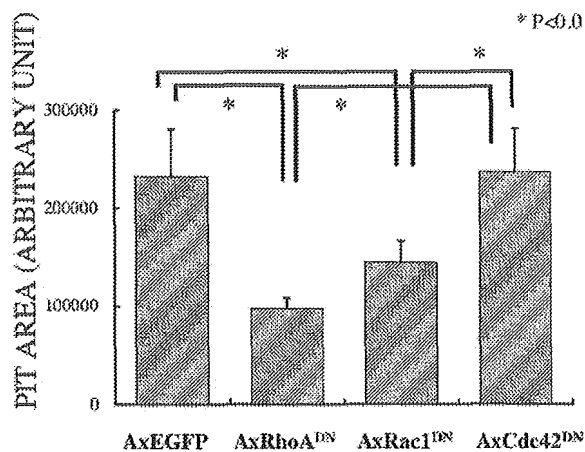
Effects of dominant negative mutants of Rho family small G-proteins on the activity and survival of OCLs

We examined the effect of Rho family small G-proteins mutants on pit-forming activity of OCLs and their survival. As shown in Fig. 2, RhoA^{DN} and Rac1^{DN} virus-infected OCLs showed a remarkable decrease in their bone-resorbing activity, whereas Cdc42^{DN} overexpression had no observable effect. In contrast, as shown in Fig. 3A, only Rac1^{DN} virus could significantly decrease their survival rate compared with the control virus, and RhoA^{DN} and Cdc42^{DN} viruses had no effect on their survival (Fig. 3).

The survival rate of OCLs in EGFP virus–, RhoA^{DN} virus–, Rac1^{DN} virus–, and Cdc42^{DN} virus–infected cultures at 24 h was $35.7 \pm 5.0\%$, $37.3 \pm 3.5\%$, $24.0 \pm 1.0\%$, and $41.7 \pm 4.7\%$, respectively. These results clearly show that Rac1 signaling promotes osteoclast survival. Similar results were obtained using Rac1^{DN} adenovirus vector with CAG promoter (data not shown).

Rac1 lies downstream of M-CSF receptor and mediates an anti-apoptotic signal

M-CSF markedly enhances the survival of osteoclasts and causes their spread in vitro.[1,2] Because Rac1 is also known to regulate the cytoskeletal organization and induce cell spreading in some types of cells, we hypothesized that Rac1 might lie downstream of M-CSF receptor pathways and mediate signaling pathways essential for the survival and the cytoskeletal organization of osteoclasts. We first examined whether Rac1 was activated in OCLs in response to M-CSF treatment using the GST pull-down assay. As expected, Rac1 was activated immediately after application of M-CSF, and the activation was sustained for at least 10
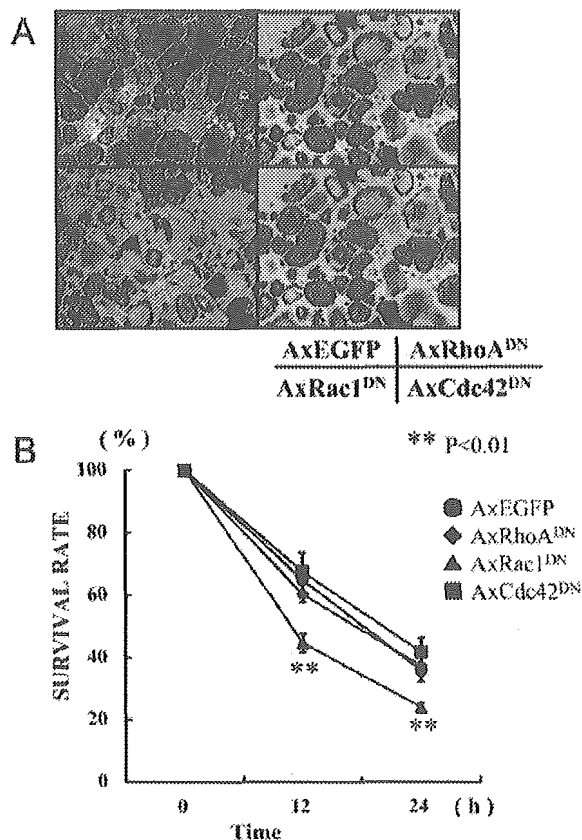
FIG. 2. Effect of dominant negative mutant of RhoA, Rac1, and Cdc42 expression on bone-resorbing activity of OCLs. Twenty-four hours after adenovirus infection, OCLs were collected and reseeded onto dentin slices. OCLs were removed 12 h later, and pits were visualized with 0.5% toluidine blue. Pit area was quantified with an image analysis system. Overexpression of RhoA^{DN} and Rac1^{DN} decreased bone resorptive activity of OCLs, whereas Cdc42^{DN} had no significant effect.

minutes (Fig. 4A). Next we studied the effect of Rac1^{DN} overexpression on M-CSF–induced promotion of OCL survival. As shown in Fig. 4B, M-CSF clearly increased the survival of control virus–infected OCLs. Rac1^{DN} overexpression not only suppressed the survival rate of OCLs at the basal level but also completely abrogated the pro-survival effects of M-CSF (Fig. 4B). In addition, the survival rate of Rac1^{CA}-infected OCL at 24 h after purification was significantly higher than that of control virus–infected OCLs, further confirming the role of Rac1 in OCL survival (Fig. 4C).

Rac1^{CA} induced osteoclast survival and its survival signal of osteoclasts was mediated mainly through the PI3K/Akt pathway

To further clarify the role of Rac1 in the survival signal, we studied the downstream cascade of Rac1 signaling. We previously reported that the Ras/Erk pathway promotes osteoclast survival, whereas other groups described the anti-apoptotic function of the PI3K/Akt signaling pathway.[3,12] To determine whether these pathways contribute to the effects on cell survival by Rac1, we used specific inhibitors to these molecules. Figure 5A shows the effects of the inhibitors on OCL survival at 12 h after purification and addition of each reagent. The effect of Rac1^{CA} on the promotion of OCL survival was blocked by either LY294002 or wortmannin but not significantly by PD98059 at this time-point, indicating the essential role of PI3K/Akt pathways downstream of Rac1. Mandatory activation of Akt pathways by overexpressing Akt^{CA} remarkably enhances OCL survival as shown in Fig. 5B, further confirming the anti-apoptotic role of these pathways. Consistent with these results, M-CSF–induced Akt phosphorylation was markedly suppressed by Rac1^{DN}, whereas RhoA^{DN}


FIG. 3. Effect of dominant negative mutant of RhoA, Rac1, and Cdc42 expression on survival of OCLs. Twenty-four hours after adenovirus infection, OCLs were purified and cultured for an additional 24 h. (A) The survival of the cells after 24 h of purification was evaluated by TRACP staining. (B) After TRACP staining, viable OCLs were counted after 12 and 24 h of purification and expressed as a percentage of the cells at time 0. Rac1^{DN}-infected OCLs died significantly earlier than those in the control group, whereas Rho^{DN} and Cdc42^{DN} had no effect on OCL apoptosis. Results represent the mean ± SD for a typical experiment among three independent experiments.

and Cdc42^{DN} had no effect (Fig. 5C). On the other hand, the activation of Erk as determined by anti-phospho-Erk antibody blotting was not affected by any of the mutants of three small G-proteins, as shown in Fig. 5D.

Rac1 and PI3K synergistically act downstream of M-CSF receptor signaling

These results suggest that Rac1 lies upstream of PI3K pathways, but there is a controversy with regard to the hierarchy of Rac1 and PI3K. Therefore, we further analyzed the relationship between Rac1 and PI3K activation downstream of M-CSF receptor pathways using specific inhibitors and adenovirus vectors. The activation of Rac1 in response to M-CSF treatment was not suppressed by LY294002 (Fig. 6A). Overexpression of a catalytic subunit of PI3K, p110, promoted the downstream effector Akt phosphorylation even in the absence of M-CSF, which was

A


M-CSF 100 ng/ml

B

C
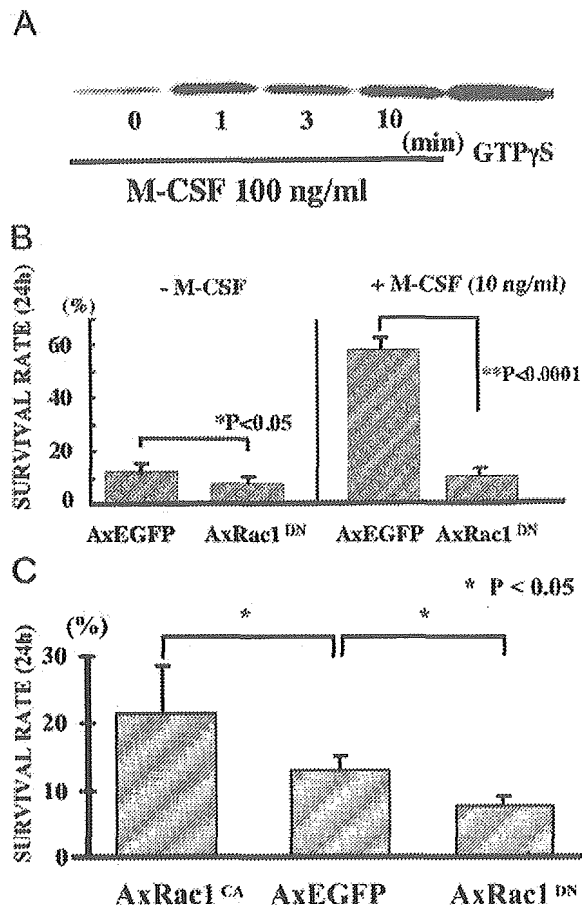
FIG. 4. Rac1 lies downstream of M-CSF signaling in OCLs. (A) After application of 100 ng/ml M-CSF, total cell lysates of OCLs from 10-cm dishes were collected at indicated time-points and incubated with p21-binding domain of PAK1 and glutathione S-transferase (GST) fusion protein immobilized on glutathione agarose beads for 1 h at 4°C. Precipitates were subjected to 10% SDS-PAGE and immunoblotted with anti-Rac1 antibody. (B) Purified adenovirus-infected OCLs were incubated for 24 h with or without 10 ng/ml M-CSF, and cell survival was assessed. Whereas M-CSF clearly increased OCL survival in control virus-infected OCLs, Rac1^{DN} completely abrogated the M-CSF-induced survival. (C) The survival rate of Rac1^{CA} virus-infected OCLs was significantly higher than that of control virus-infected cells, whereas Rac1^{DN} expression promoted their apoptosis. Results represent the mean ± SD for a typical experiment among three independent experiments.
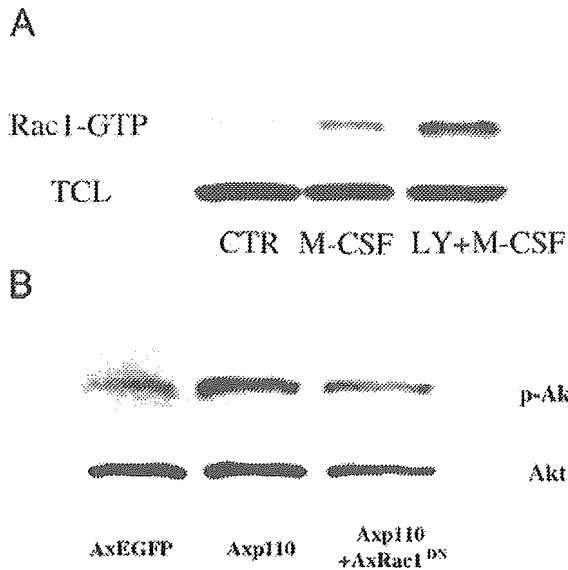
partially blocked by co-expression of Rac1^{DN} (Fig. 6B). These results suggest that Rac1 serves as both upstream and downstream effector of PI3K and that interaction between Rac1 and PI3K is important for the survival signaling of OCLs.

Rac1 overexpression does not affect actin ring formation in OCLs, but reduces M-CSF–induced cell spreading

We next examined the effect of Rac1 activation or inactivation on the cytoskeletal organization of OCLs. Unex-

A

B

C

D
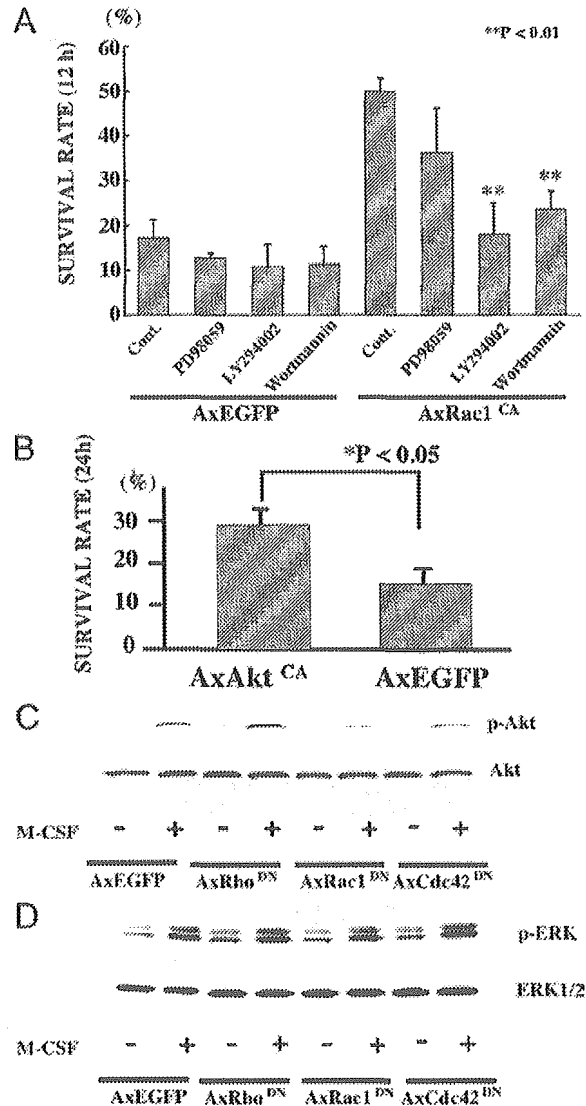
FIG. 5. Involvement of PI3K/Akt pathways on OCL survival. (A) Purified EGFP adenovirus- or Rac1^{CA} adenovirus-infected OCLs were incubated for 12 h in the presence of one of the following inhibitors: PD98059 (40 μM), LY294002 (4 μM), or Wortmannin (100 nM). After TRACP staining, viable OCLs were counted, and the survival rate was expressed as a percentage of the cells at time 0. The effect of Rac1^{CA} on the promotion of OCL survival was blocked by LY294002 and Wortmannin, but not significantly blocked by PD98059. (B) The active form of Akt (Akt^{CA}) was overexpressed in OCLs by adenovirus, and OCL survival was assayed. Akt^{CA} increased OCL survival about 2-fold to control virus-infected OCLs. (C and D) The effect of RhoA^{DN}, Rac1^{DN}, and Cdc42^{DN} adenovirus on Akt and ERK activation was examined by Western blotting with anti-phospho-Akt or ERK antibody. M-CSF treatment stimulated both PI3K and Mek-Erk pathways within 5 minutes. The activation of Akt was abrogated only by Rac1^{DN} (C), whereas these dominant negative mutants did not affect the phosphorylation of Erk (D). Results represent the mean ± SD for a typical experiment among three independent experiments.
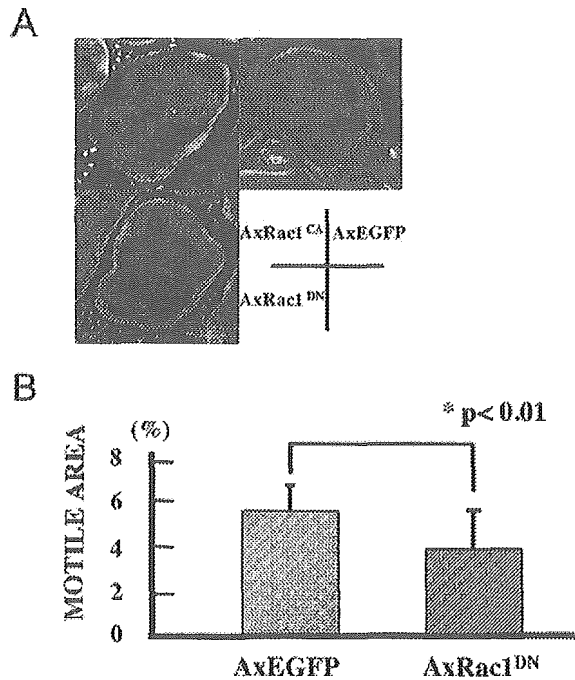
A

Rac1-GTP

TCL

CTR   M-CSF   LY+M-CSF

B

p-Akt

Akt

AxEGFP   Axp110   Axp110
                         +AxRac1 ^{DN}

FIG. 6. Relationship between Rac1 and PI3K activation. The activation of Rac1 after 100 ng/ml M-CSF treatment with or without 20 μM LY294002 was examined by GST pull-down assay mentioned in Fig. 4A. The M-CSF–induced Rac1 activation was not suppressed by LY294002 (A), whereas enhancement of Akt phosphorylation by overexpression of catalytic subunit p110α of PI3K was partially blocked by coinfection of Rac1 ^{DN} (B).

A

AxRac1 ^{CA} AxEGFP

AxRac1 ^{DN}

B

*$p < 0.01$

MOTILE AREA (%)

AxEGFP   AxRac1 ^{DN}

FIG. 7. Rac1 regulates the motility of OCLs. (A) EGFP or Rac1 mutant adenovirus-infected OCLs were incubated for 12 h after purification, and actin ring formation was visualized by rhodamine-phalloidin staining. No obvious difference in cytoskeletal organization was observed between mutated Rac1-expressed OCLs and control OCLs. (B) Before and after 50 ng/ml M-CSF treatment, OCLs expressing either EGFP or Rac1 ^{DN} were recorded with a time-lapse video microscope, and membrane movement was quantified with image analysis software. Results represent the mean ± SD of a typical experiment.

pectedly, however, as shown in Fig. 7A, we could not detect any obvious difference in actin ring formation between mutant Rac1 adenovirus-infected and control vector–infected OCLs in a static condition. All the OCLs were almost the same size and showed apparently normal actin ring formation. On the other hand, using time-lapse video microscopy, we found that overexpression of Rac1 ^{DN} dramatically reduced the membrane ruffling and spreading of the cells in response to 50 ng/ml M-CSF application as shown in Fig. 7B. Motile area of control virus– and Rac1 ^{DN}-infected OCLs was 5.7 ± 1.1% and 4.0 ± 1.7%, respectively ($p < 0.01$).

DISCUSSION

Rho family members are known to mediate various growth factor receptor signaling pathways and to regulate cytoskeletal organization of the cells.[8,22] Rac1 is a member of Rho family small G-proteins and is known to be a potent activator of actin polymerization and induce lamellipodia formation and surface membrane ruffling.[7] In addition to its role in the cytoskeletal organization, Rac1 is also known to be involved in the apoptosis signal. Whereas its proapoptotic function has been shown in some types of cells through JNK activation,[23–26] anti-apoptotic effects of Rac1 signaling have also been reported in other types of cells.[9–11,27] Moreover, the Rac1$^{-/-}$ embryos showed numerous programmed cell deaths in the space between the embryonic ectoderm and endoderm, leading to early embryonic lethality.[28] These results clearly indicate that Rac1 is implicated in the survival signals in various types of cells.

Nitrogen-containing bisphosphonates such as alendro-

nate and risedronate are potent therapeutics of osteoporosis and suppress bone-resorbing activity of osteoclasts and induce their apoptosis. It has been proposed that nitrogen-containing bisphosphonates act on osteoclasts by inhibiting post-translational prenylation of Rho family small G-proteins. Zhang et al.[29] first described the importance of RhoA in osteoclast cytoskeletal organization and function using clostridium botulinum–derived ADP-ribosyltransferase (C3 exoenzyme). Similarly, using dominant active and negative mutant proteins of RhoA, Chellaiah et al.[30] reported that integrin-dependent activation of phosphoinositide synthesis, actin stress fiber formation, podosome reorganization for osteoclast motility, and bone resorption require Rho stimulation. Razzouk et al.[31] revealed that both Rac1 and Rac2 are involved in actin ring formation and the bone-resorbing activity of the cells by introducing anti-Rac1 or anti-Rac2 antibody into permeabilized osteoclasts. Ory et al.[32] showed that Rho and Rac worked antagonistically in avian multinucleated giant cells and that Rac activation promoted spreading of the cells. More recently, Faccio et al.[33] showed that RhoA and Rac1 lie downstream of the β3 integrin and are involved in the cytoskeletal organization of osteoclasts. These results suggest that RhoA and Rac1 critically regulate the cytoskeletal organization and function of osteoclasts.

In this study, using adenovirus vector-mediated gene transduction systems, we showed the essential role of Rac1 in bone-resorbing activity, survival, and motility of OCLs. Two major pathways have been reported to be involved in osteoclast survival signaling (i.e., the Mek/Erk pathway and the PI3K/Akt pathway). The effect of Rac1^{CA} on the promotion of OCL survival was blocked by PI3K inhibitors but not by Mek inhibitors. M-CSF–induced phosphorylation of Akt was inhibited by Rac1^{DN} expression but not by Rho^{DN} or Cdc42^{DN} expression. In contrast, Rac1^{DN} expression did not affect M-CSF–induced Erk phosphorylation, indicating that Rac1 is specifically involved in Akt activation downstream of M-CSF receptor pathways (Figs. 5C and 5D). These results clearly show that the prosurvival action of M-CSF on OCLs is mainly mediated by Rac1 and that Rac1 is important for M-CSF–dependent PI3K/Akt activation in OCLs. Lee et al.[3] also showed that TNF-α prolonged the survival of osteoclasts, which was abrogated by PI3K inhibitor. They also revealed the involvement of Grb2 and ceramide in TNF-α–induced Erk activation in osteoclasts.[3] However, contrary to these observations, Sugatani and Hruska[34] recently reported that silencing of Akt1 and/or Akt2 by small interfering RNA suppressed osteoclast differentiation but did not affect osteoclast survival. The reason for this discrepancy remains unknown, and further study is required to clarify the exact role of PI3K/Akt pathways in osteoclast survival. Recent studies have shown the involvement of mTOR (mammalian target of rapamycin) in osteoclast survival.[34,35] We also found that rapamycin strongly suppressed OCL survival in both the presence and absence of M-CSF (data not shown). The role of Rac1 on mTOR activation remains elusive, and further studies will be required.

Although our results suggest that Rac1 seems to act upstream of PI3K in OCLs and is consistent with some reports,[36,37] other studies have shown that Rac1 serves as downstream effector of PI3K.[38,39] Because many guanine-nucleotide exchange factors (GEFs) for Rac1 have been identified, and among them, members of the Vav, Sos, Tiam, PIX, SWAP-70, and P-Rex families have been suggested to be regulated by PI3K.[40] Furthermore, phosphatidylinositol 3,4,5-triphosphate, the endproduct of PI3K, can bind directly to Rac1 in vitro. In this study, we showed that PI3K inhibitor did not affect M-CSF–induced Rac1 activation (Fig. 6A), which means Rac1 lies upstream of PI3K. However, our results also indicate that Rac1 seems to act downstream of PI3K, because p110α-induced Akt phosphorylation was partially blocked by Rac1^{DN} (Fig. 6B), and Rac1^{CA} could not activate Akt by itself (data not shown). One possible explanation for these contradictory results is that Rac1 and PI3K act synergistically on cell survival or there is a positive feedback loop between Rac1 and PI3K activation, which may play an important role in the survival signaling of OCLs.

Finally, we examined the effect of Rac1 on the cytoskeletal organization of OCLs. Unexpectedly, however, there was no remarkable difference in actin ring formation between control OCLs and Rac1^{DN} or Rac1^{CA}-infected cells (Fig. 7A). Because this is considered to be caused by the static condition in which we observed the cells, dynamic

cytoskeletal rearrangement of OCLs in response to M-CSF treatment was examined using a video microscope. Suppression of Rac1 pathways by dominant negative mutant overexpression induced less membrane movement in response to M-CSF treatment compared with EGFP adenovirus–infected cells (Fig. 7B). Based on these observations, we concluded that Rac1 plays a crucial role in membrane movement of OCLs and that decreased bone resorption in Rac1^{DN}-infected OCLs is probably caused by the reduced motility of the cells.

In conclusion, small GTPase Rac1 is critically involved in M-CSF receptor signaling and mediates survival signaling of osteoclasts primarily by modulating the PI3K/Akt pathways. Rac1 also plays a significant role in bone resorptive activity of the cells, probably by regulating the motility of osteoclasts.

ACKNOWLEDGMENTS

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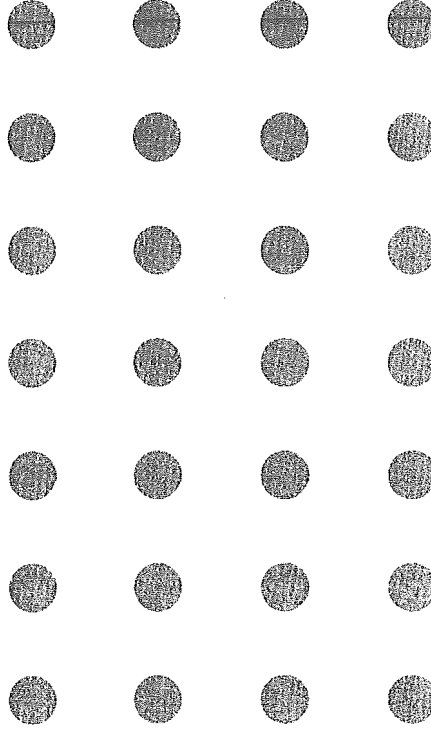
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改訂 第4版

疾患別 最新処方

■監修
矢崎義雄・菅野健太郎

MEDICAL VIEW

処方のポイント

Behçet病は、再発性口腔内アフタ性潰瘍、皮膚症状（結節性紅斑、毛嚢炎様皮疹、皮下の血栓性静脈炎）、外陰部潰瘍、関節炎、ぶどう膜炎、特殊病型（回盲部中心の潰瘍性病変を示す腸管Behçet、大小の動静脈の血栓症を示す血管Behçet、脳幹・小脳・大脳白質の病変を主体とする神経Behçet）が認められる。特殊な場合を除いて、一定の部位の炎症性病変が慢性に持続するのではなく、急性の炎症が反復し、増悪と寛解を繰り返し、遷延した経過をとるのが特徴である。

治療の原則としては、視力障害を残す眼病変や生命予後に影響を及ぼす特殊病型（腸管病変、血管病変、神経病変、皮膚病変）に対しては積極的な薬物療法を行うが、口腔内アフタ、陰部潰瘍、皮膚病変に対してはステロイドの外用を中心とした局所療法で対応する。しかし、これら軽症例でも、疼痛の強い場合や発作の頻度が高い慢性型のBehçetに対しては全身的薬物療法を行う。一般に、一部の患者でみられる進行性の痴呆を主徴とする慢性進行型の神経Behçetに対しては、メトトレキサートの少量パルス療法が有効であるが、ステロイドはあまり有効ではないが、メトトレキサートの少量パルス療法が有効である。

使用上の注意・禁忌

- コルヒチンは好中球機能を抑制することから、Behçet病の基礎治療薬としては繁用されるが、生殖系への影響が大きいので、十分な配慮が必要である。その他の副作用として肝障害、胃腸障害、催奇形性、筋症状（こむらがえり）に注意する。妊婦に対しては禁忌である。
- 難治性ぶどう膜炎に対してはネオーラル（シクロスポリン）の投与を行う。この際、血中トラフ値（服薬直前の最低値）が150ng/mLを超えないよう（100ng/mL前後）内服する。副作用として腎障害、髄膜炎様症状にとくに注意する。神経Behçetに投与は禁忌である。
- 腸管Behçet・血管Behçet・神経Behçet（急性型）などの特殊病型に対しては、コルヒチンに加えて中等量～大量のステロイドの全身投与が行われる。反応が不十分な場合は、免疫抑制剤を併用する。
- 慢性進行型の神経Behçetに対しては、メトトレキサートの少量パルス療法を行う。副作用としては、肝障害、骨髄抑制、間質性肺炎に注意する。

相互作用

コルヒチンとネオーラルを併用するとミオパチーをきたしやすいので注意が必要である。シクロスポリンの血中濃度を上昇させるものとしては、マクロライド系抗生物質、アゾール系抗真菌薬、アダラート、ヘルベッサーなどのCa拮抗薬、グレープフルーツジュースなどがあり、逆にリファンピシンやフェニトインは血中濃度を低下させる。

［広畑俊成］

Behçet病

Behçet's disease

[1] 各種症状が反復する場合の基礎治療
- ①コルヒチン(0.5mg)　1～2T、分1～2
- ②エパデールS(600mg)　3P、分3

[2] 各種症状に対する治療

■口腔粘膜のアフタ性潰瘍に対して：下記のいずれかを用いる。
- ①口腔用ケナログ　1T、1～2回、患部に付着　3回、患部へ塗布

または
- アフタッチ(25μg)　1T、1～2回、患部に付着
- ②アセゼチン(1mg)　2～4T、分2　患部へ塗布

■毛嚢炎・結節性紅斑・外陰部潰瘍などに対して：下記のいずれかを用いる。
- ①リンデロン-VG　数回、患部へ塗布
- ②デルモベート　数回、患部へ塗布

[3] 病態・重症度に応じた治療

■関節炎、副睾丸炎、結節性紅斑、皮下血栓性静脈炎などに対して：下記のいずれかを用いる。
- ①ロキソニン(60mg)　3T、分3
- ②オステラック(200mg)　2T、分2

■眼病変・血管病変・神経病変（急性型）
- ①プレドニン(5mg)　6～12T、分2～3

上記①で不十分なときに、下記②③いずれかを追加
- ②エンドキサン(50mg)　1～2T、分1～2
- ③イムラン(50mg)　1～2T、分1～2

腸管Behçetに対して、①に追加
- ④サラゾピリン(500mg)　4～6T、分2
- ⑤ペンタサ(250mg)　9T、分3

血管Behçetに対して、下記⑥～⑨のいずれかを①に追加
- ⑥パナルジン(100mg)　2～3T、分2～3
- ⑦バファリン(81mg)　1T、分1
- ⑧ワーファリン(1mg)　2～5T、分1(朝)（プロトロンビンINR値を2.0程度に保つ）
- ⑨ドルナー(20μg)　3～6T、分3

■進行性Behçetに対する治療
- ①プレドニン(5mg)　1～2T、分1(朝)
- ②メトトレキサート(2.5mg)　3～4T、週1回内服(分2～3)

Immunology handbook

免疫学ハンドブック

免疫学ハンドブック編集委員会　編

Ohmsha

一致率が二卵性双生児のそれと比べて高いこと，特定の標識遺伝子陽性者の頻度が健康対照集団と比較して患者集団で増加していること，遺伝的に規定された動物モデルの存在などが遺伝的要因の関与を示唆している．家族集積性に関与する因子には，感染の要素，貧富，環境要因などの影響もあり得るが，これらを考慮に入れても遺伝的要因の関与は確実であると考えられている．**一卵性双生児の疾患一致率**は，各自己免疫疾患でだいたい 15 〜 30% 程度であり，これらがそれほど高くない理由としては，**遺伝要因が弱いという解釈**よりも，**非遺伝的要因も疾患の表現に大きく関与する**と解釈したほうがよいと考えられている．

このように遺伝要因の重要性はわかっているが，従来から研究されている主要組織適合遺伝子複合体（MHC，ヒトでは HLA）クラス II 遺伝子との相関があることは確実であるが，それ以外の特定の遺伝子の同定が進んでいなかった．遺伝子の解析が進まない理由として，以下の問題点をあげることができる．① 自己免疫疾患では候補遺伝子が明確でなく，検索すべき遺伝子が特定できない．② メンデルの遺伝形式（優性か劣性か）がわからない，さらに多くの自己免疫疾患は単純なメンデルの法則に従って遺伝せず，複雑または不明の様式で遺伝する．③ 一卵性双生児の罹患一致率に比べ，同胞罹患一致率が極端に低下するので，多因子遺伝であることが想像される．④ 病態は多くの遺伝子座の多様性が複雑に影響しあって決定されていると考えられ，エピスタシスな遺伝子の相互作用（epistatic interaction）もあると考えられる．⑤ それぞれの遺伝子の浸透率が低く，検出感度が落ちる，すなわち疾患関連遺伝子と病気との関係は遺伝子と表現系が 1 対 1 対応するということはまずない．⑥ 環境の影響，確率的影響が大きい．⑦ RA などの比較的高齢発症の疾患では，診断確定時に両親の遺伝子型が決定できない，などである．

◎3.2　病態・診断および治療

本節においては，代表的自己免疫疾患である全身性エリテマトーデスの病態，および各種疾患の治療において用いられる免疫抑制薬について概説するとともに，近年進歩の著しい生物学的製剤についても触れる．

〔1〕　全身性エリテマトーデスの病態および診断

全身性エリテマトーデス（SLE）と**関節リウマチ**（RA）は自己免疫疾患を代表する二大疾患であり，何れの疾患においても種々の免疫異常が病態形成上重要な役割を果たすと考えられている．RA においてはリウマトイド因子や**抗 CCP 抗体**が特異的に上昇し，主たる病変が関節滑膜に集中する．これに対して，SLE では多彩な自己抗体が出現するとともに，全身の多臓器に病変が及ぶことが一つの特徴である．このような SLE における多臓器病変の起こる理由として，かつては免疫複合体の形成と各臓器への沈着があげられていた．しかし，近年になり，特定の自己抗体が特定の病変を惹起することが明らかとなってきている[1]．

SLE 血清中に検出される多彩な自己抗体のうち，主なものを**表 3.1** に示した．こ

表 3.1　SLE で見られる主な自己抗体

自己抗体	対応抗原	備　考
抗 ds-DNA 抗体	2 本鎖 DNA	ループス腎炎
抗 ss-DNA 抗体	1 本鎖 DNA	
抗ヒストン抗体	ヒストン	薬剤性ループス
抗 nRNP 抗体	U1-RNA タンパク	MCTD の疾患標識抗体
抗 Sm 抗体	U1, 2, 4, 5, 6RNA タンパク	
抗 SS-A/Ro 抗体	RNA タンパク	先天性心ブロック
抗 PCNA 抗体	DNA ポリメラーゼ δ 補助因子	
抗赤血球抗体（クームス抗体）	赤血球抗原	溶血性貧血
抗リンパ球抗体	CD45 など	
抗カルジオリピン抗体	β2-グリコプロテイン I	抗リン脂質抗体症候群
抗リボソーム P 抗体	リボソーム P タンパクの C 末端 22 アミノ酸	ループス精神病

れらのうち**抗 dsDNA 抗体**と**抗 Sm 抗体**は SLE に特異性の高いものであり，疾患標識抗体として診断的価値がある．さらに，**抗カルジオリピン抗体**も比較的 SLE に特異性が高いことから，新たに診断基準に加えられている．表 3.1 に示した自己抗体のうちには特定の臓器病変との関連が証明されているものが多い．例えば，抗 dsDNA 抗体とループス腎炎，**抗リボソーム P 抗体**とループス精神病はおのおの密接な関係を有する．このように，SLE における多様な臓器病変は出現する自己抗体の種類によって規定されていると考えられる．

　こうした自己抗体の産生機序として，かつては多クローン性 B 細胞活性化が考えられてきたが，近年は，こうした自己抗体の遺伝子に体細胞突然変異（somatic mutation）が見られることなどから，抗原刺激による誘導（antigen-driven）の機序の関与も示唆されている．こうして産生された各種自己抗体が種々の臓器病変を惹起するにあたっては，従来より指摘されているような免疫複合体の形成・沈着とは異なる機序が働いていることが考えられるが，不明な点も多く，今後の検討が必要である．

〔2〕　各種免疫抑制薬の作用機序

a)　副腎皮質ステロイド[2)]

　ステロイドはフォスフォリパーゼ A2（phospholipase A2）およびシクロオキシゲナーゼ（cyclooxygenase）阻害によるプロスタグランディンおよびロイコトリエンの産生抑制を介して抗炎症作用を発揮するが，今一つの重要な作用機序は免疫抑制作用である．これは，一般的に抗炎症作用よりも大量の抑制薬を必要とする．免疫抑制作用のうち重要であるのがサイトカイン産生抑制作用である．この作用は，各種サイトカイン遺伝子の転写を調節する**転写因子**（AP-1 や NF-κB など）のおのおのの DNA モチーフとの結合をステロイドが阻害することにより生じることが近年明らかにされてきている．一般的に T リンパ球の機能はステロイドにより著明に抑制されるが，B リンパ球の機能を抑制するためにはより高濃度のステロイドが必要と考えられている．