**Table 3. Therapeutic Options in the Treatment of APS**

| |
|---|
| **I. Current treatment** |
| • Anticoagulants: Heparin, LMWH and warfarin |
| • Low dose aspirin |
| • IVIG in pregnancy morbidity refractory to aspirin and heparin combination |
| • Antiplatelet agents rather than aspirin |
| • Corticosteroids* |
| • Plasma exchange* |
| • Vasodilators* |
| **II. Drugs currently used for other diseases with potential to be effective in APS** |
| • Hydroxychloroquine |
| • Statins |
| • Angiotensin-converting enzyme inhibitors |
| • Recombinant human activated protein C |
| • Recombinant human thrombomodulin |
| • New anticoagulants |
| • Tumor necrosis alpha antagonists |
| • Anti CD20$^+$ B cells antibody (Rituximab) |

LMWH: low molecular weight heparin, IVIG: intravenous immunoglobulins.
*Those drugs are currently used in case of catastrophic APS or refractory APS.

Aspirin showed a prophylactic role for primary prevention of thrombosis in aPL-positive patients [114], but those results are no supported by data from a prospective controlled study [115]. In addition, long-term oral anticoagulation at a relative high intensity is associated with an increase risk of bleeding and under-anticoagulation with a risk of recurrences [110]. Given these concerns, new, safer and more-efficient modalities for prevention and treatment of thrombosis are needed.

Antiplatelet agents, other than aspirin, such as dipyridamole, ticlopidine, clopidogrel bisulfate and cilostazol had been empirically used in selected patients with APS and may represent an alternative to warfarin to prevent further aPL-related arterial thrombosis. Those drugs could offer benefit in short-term secondary prevention after non-cardioembolic strokes or transitory ischemic attacks [116]. Dilazep and dipyridamole block the monocyte-TF expression induced by purified IgG from APS patients [117], but there are no clinical trials evaluating their efficacy on primary or secondary thrombosis prevention in patients with aPL.

In case of a life-threatening condition such as catastrophic APS, the combination of intravenous anticoagulation, corticosteroids and IVIG or plasma exchange with fresh frozen plasma showed the highest survival rates [118]. Plasma exchange can remove, not only pathologic aPL, but also activated complement and other inflammation mediators. The fresh frozen plasma provides natural intact anticoagulants, mainly antithrombin and protein C.

Rituximab, a monoclonal antibody that selectively depletes CD20$^+$ B cells, has been successfully employed in a small number of patients with resistant APS including catastrophic APS. This biological reagent reduces the aPL titers, but its effect on the prevention of thrombotic recurrence has not been proven [119-121].

Prostacyclin and prostaglandin E1 treatment result in vasodilatation and inhibition of platelet aggregation. The use of those drugs in patients with aPL is anecdotal and further studies are necessary to evaluate the effectiveness and the risk of re-thrombosis [122].

## POTENTIAL THERAPIES FOR APS

In this section, some drugs with anti-thrombotic and immunomodulatory effects currently used to treat other disease are discussed. Those agents potentially can be used as additional drugs for the treatment and the prevention of aPL-related complications.

### 1. Hydroxychloroquine

Hydroxychloroquine is considered an integral component of the treatment of APS in patients with concomitant SLE, and has been associated with a decreased thrombotic risk in aPL-positive SLE patients [123]. In addition to anti-inflammatory and immunomodulatory effects, hydroxychloroquine has anticoagulant potential which may be due to the ability to inhibit platelet aggregation and the release of arachidonic acid from stimulated platelets. Hydroxychloroquine repressed aPL- induced platelet activation *in vitro* [124], and reduced both thrombus size and time of thrombus persistence in an APS mouse model [125]. This drug may be of benefit in the management of patients with aPL, but its effectiveness for thromboprophylaxis should be determined by controlled studies [126].

### 2. Statins

Statins are cholesterol-lowering drugs that act as competitive inhibitors of 3-hydroxy-3-ymethylglutaryl-coenzyme A (HMG-CoA) which catalyzes the cholesterol synthesis in the mevalonate pathway. Beside cholesterol lowering activity, statins have anti-inflammatory effects modifying the function of endothelial cells, platelets and monocytes/macrophages. Statins down-regulated the production of cytokines in endothelial cells, interfered with leukocyte-endothelial interaction, inhibited TF expression by mononuclear cells, increased fibrinolytic activity and hampered platelet function [127]. Fluvastatin interfered with the expression of adhesion molecules and IL-6, and reversed the up-regulation of TF mediated by aPL in endothelial cells [93, 128, 129]. Moreover, in aPL-treated mice, fluvastatin diminished thrombus size [130].

Statins are widely used drugs known to be effective for the prevention and treatment of atherosclerosis and cardiovascular diseases [131], but still there are not data regarding its potential on thrombosis prevention in aPL-positive patients. In an animal model of mice treated with aPL, simvastatin and pravastatin decreased the expression of TF and protease-activator receptor 2 on neutrophils and prevented fetal loss, suggesting that statins may be a favorable treatment in woman with aPL-induced pregnancy complications [132].

### 3. Angiotensin-Converting Enzyme Inhibitors

Angiotensin-converting enzyme (ACE) inhibitors have beneficial effects in vascular diseases and significantly improved survival in patients with atherosclerosis. ACE inhibitors reduced TF levels and TF expression on monocytes in patients with myocardial infarction [133, 134]. Experimental studies demonstrated the inhibitory effect of ACE inhibitors on NFkB with consecutive down-regulation of TF [135-137]. Therefore, ACE inhibitors have the potential to reduce TF expression. Recently, angiotensin receptor blockers (ARB) are taking the place of ACE inhibitors as antihypertensive drugs. Clinical trials for ACE inhibitors or ARB in aPL-positive patients are warranted.

### 4. Recombinant Human Activated Protein C and Recombinant Human Thrombomodulin

Activated protein C is one of the major regulators of thrombin generation, and also possesses anti-thrombotic and anti-inflammatory properties reducing cytokine production and expression of adhesion molecules on endothelial cells [138]. Recombinant human activated protein C (Drotecrogin alpha) has been demonstrated beneficial in patients with severe sepsis where microvascular thrombi are the major feature. *Administration* of recombinant human activated protein C may restore the dysfunctional anticoagulant mechanisms, prevent amplification and propagation of thrombin generation and formation of microvascular thrombosis, and may simultaneously modulate the systemic pro-inflammatory response [139].

Thrombomodulin, a receptor of thrombin and thrombin-thrombomodulin complex, is a natural activator of protein C. Thrombomodulin works as anticoagulant only when thrombin is excessive. Therefore, thrombomodulin is the ideal anticoagulant with minimal risk of bleeding complications. The use of recombinant human soluble thrombomodulin has been approved for the treatment of disseminated intravascular coagulation [140].

Impairment of protein system C by aPL have an important role in the pathogenesis of APS. Those drugs are promising for APS when infection events trigger the development of thrombosis.

### 5. New Anticoagulants

Anticoagulants such as warfarin and heparin act on a number of targets, whereas the newer anticoagulants have been designed to selective inhibit one specific target in the coagulation system. Therefore, those new agents might be more effective and safer than current anticoagulants with a promising role in the management of APS. Some of these emerging anticoagulants agents are already approved and used in clinical practice but others are still in development phase.

New anticoagulants that selectively inhibit thrombin either directly through binding to thrombin and inhibiting its interaction, or indirectly through antithrombin, have been developed. Direct thrombin inhibitors include lepirudin and argatroban, approved for patients with heparin-induced thrombocytopenia [141]. Bivalirudin was approved for percutaneous coronary interventions in patients with acute coronary syndrome [142]. Ximelagatran, an oral direct thrombin inhibitor, showed very promising data and entered in late phase clinical development. However, there were many concerns in safety, especially with liver toxicity. In 2004, the Food and Drug Administration rejected the drug license in the United States, and in 2006 it was finally withdrawn from the world market [143].

Fondaparinux is a synthetic pentasaccharide, which binds to antithrombin thereby indirectly inhibiting activated factor X and thrombin generation. This drug has little effect on platelet aggregation. Fondaparinux is subcutaneously administrated and has been approved for the prevention and treatment of venous thromboembolism [144].

Rivaroxaban a selective direct inhibitor of activated factor X with high oral bioavailability, and dabigatran, an oral direct thrombin inhibitor, are approved for the prevention of venous thromboembolism following orthopaedic surgery [145, 146]

Other emerging anticoagulant is recombinant nematode anticoagulant protein c2 (rNAPc2), an 85-amino acid protein that is a potent inhibitor of the activated factor VII/ TF complex, the key physiological initiator of blood coagulation. Recombinant NAPc2 might be effective in thrombosis prophylaxis by attenuating the initiation and propagation of thrombin generation. This drug was effective in reducing the postoperative venous thromboembolism in patients undergoing total knee replacement [143], and suppressed thrombin generation in patients undergoing coronary angioplasty [147]. Because of its unique mechanism of inhibition of activated factor VII/TF, rNAPc2 should be considered as future therapeutic options for APS

### 6. TNFα Antagonists

In an animal model of APS, TNFα was a critical mediator of aPL-induced fetal loss and was released in response to complement activation. TNFα DNA vaccination prevented clinical manifestations of experimental APS [148], and TNFα deficiency provided fetal protection in aPL-treated mice. In addition, TNFα was released from monocytes treated with antiβ2GPI antibodies [100]. Those observations identify TNFα as a potential target for the therapy of the pregnancy complications of APS. However, TNFα blockade does not completely protect pregnancy problems, thus it is likely that other effector pathways contribute to the fetal death [149].

On the other hand, TNFα blockers therapy has been associated with increase in the frequency of aCL or antiβ2GPI antibody positivity [150-152]. No consensus exists on the merit of TNF blockade in patients with APS.

## NEW TARGETED TREATMENTS FOR APS

New targeted therapies with potential to be effective for the management of APS are reviewed and summarized in Table 4.

### 1. Inhibitors of p38MAPK and NFkB

The p38MAPK pathway is essential in mediating the effect of aPL. Both p38MAPK phosphorylation and NFkB

translocation are required for aPL-mediated TF up-regulation on endothelial cells and monocytes. Treatment with an specific p38MAPK inhibitor, SB203580, inhibits platelet aggregation and thromboxane A2 production induced by aPL [97]. Moreover, SB203580 reversed the effect of aPL on TF expression and IL-6 and IL-8 up-regulation [98].

**Table 4. New Therapeutic Strategies for APS Treatment**

| |
|---|
| **I. Specific targeted therapies** |
| ▪ p38MAPK pathway and NFkB inhibitors |
| ▪ Tissue Factor inhibitors |
| ▪ Platelet activation inhibitors: - Receptor specific antagonists<br>- Thromboxane A2 inhibitors |
| ▪ Specific complement inhibitors |
| **II. Immunomodulation** |
| ▪ Bone marrow transplantation |
| ▪ T cell blockage |
| ▪ Specific B cell molecular targeting |

p38 MAPK: p38 mitogen activated protein kinase, NFkB: nuclear factor kappa B.

The specific NFkB-inhibitor MG132 significantly reduced up-regulation of TF and enhancement of thrombosis mediated by aPL *in vivo* [153]. The activation of NFkB by aPL was also inhibited by SN50, a specific inhibitor of NFkB translocation [154]. Those findings represent innovative modalities of targeted therapies for the treatment of thrombosis in APS, but there is still many questions which need to be answered such a long-term effect and safety.

## 2. Inhibitors of Tissue Factor expression

Tissue factor is a transmembrane protein normally found in sub-endothelial structures of blood vessels and expressed upon activation on the surface of monocytes, endothelial cells and smooth muscle cells. Following its exposure to blood, TF binds specifically to activated factor VII. The formation of the activated factor VII/TF complex is critical for the initiation of the proteolytic reactions leading to the generation of thrombin, and eventually to clot formation. [155]. Increased TF expression is widely recognized as one the aPL-mediated mechanism of hypercoagulability [69, 70, 156, 157].

TF is one the major potential target for pharmacological interventions and blockage of TF activity is one of the promising therapies in APS. Therapeutic approaches targeting TF include dilazep dipyridamole, pentoxifyllines, defibrotide and ACE inhibitors such as captopril [117, 133, 158]. Those agents suppressed the monocytes TF expression induced by aPL or lipopolysaccharide. However, there are no clinical studies on patients with APS

## 3. Inhibitors of Platelet Activation

### *a) Receptor Specific Antagonists*

Antiphospholipid antibodies can cause platelet activation and aggregation in the presence of low concentration of platelet agonists [159]. Also, aPL can enhance the expression of platelet membrane GP particularly GPIIb/IIIa, a receptor involved in platelet aggregation [124]. Infusions of a GPIIb/IIIa antagonistic monoclonal antibody (1B5) abrogated the aPL-induced thrombosis *in vivo*. Further, aPL-mediated thrombosis is not observed in GPIIb/IIIa deficient mouse [127]. Double heterozygosity polymorphisms for platelet GPIa/IIa and GPIIb/IIIa increase the risk of arterial thrombosis in patients with APS [160].

Platelet GPIIb/IIIa antagonists abciximab, tirofiban and eptifibatide, have been used in the treatment of myocardial infarction and acute ischemic stroke, showing its efficacy in acute coronary syndrome in combination with other therapies. However, results have been less consistent in ischemic stroke. While phase I and II trials of abciximab as the sole agent were promising, the phase II trial was abandoned because of unfavorable benefit to risk ratio. Presumably, the benefit of these drugs for patients with APS is limited [161].

The effects of aPL in GPIIb/IIIa expression are significantly reduced by hydroxychloroquine and this inhibitory effect may be one of the mechanisms by which this drug prevent thrombosis [124].

The blockage of the platelet receptor ApoER2' using a receptor-associated protein (RAP) abrogated the increased adhesion of platelet to collagen induce by β2GPI-antiβ2GPI antibody complex [162]. Thus, inhibition of ApoER2' might contribute to the prevention of thrombosis in APS patients.

Recently, the binding of β2GPI to platelet factor 4 has been reported [163]. β2GPI forms stable complexes with platelet factor 4, leading to the stabilization of β2GPI dimeric structure that facilitates the antibody recognition. The β2GPI-platelet factor 4 complex is strongly recognized by serum of patients with APS. Moreover, platelets may be activated by β2GPI-antiβ2GPI antibody-platelet factor 4 or β2GPI-platelet factor 4 complexes. Almost every cell type can be a source of platelet factor 4 especially under some stimulation. Both, β2GPI I and platelet factor 4 are abundant in plasma, thus the preformed β2GPI-platelet factor 4 complexes may prime several pro-coagulants cells culminating in coagulation. The blockage of the interaction between β2GPI-platelet factor 4 might be a novel approach in the targeted therapies in APS.

The other putative receptor proteins described before may be potential target therapies to reduce the aPL-mediated effects, but there are not yet data available to suppose this therapeutic strategy.

### *b) Thromboxane A2 Inhibitors*

Thromboxane $A_2$ is one of the most powerful agonist for platelet activation and exerts a vasoconstrictor effect by serving as agonist of the thromboxane receptor. Clinical and experimental data suggest that inhibition of thromboxane production may be effective to prevent thrombotic complication in patients with aPL. Patients with APS had elevated urinary excretion of thromboxane $A_2$ metabolites [80]. Preincubation with β2GP-aCL complexes results in production by platelets of higher levels of thromboxane $B_2$ which is a stable metabolite of thromboxane $A_2$ [164]. Indomethacin, a thromboxane $A_2$ inhibitor, and theophylline, a phosphodi-

esterase inhibitor, abrogated aPL-mediated thromboxane $A_2$ production [165, 166]. On experimental APS, a long-term actin thromboxane receptor antagonist, BMS180,201, was effective to reduce the fetal resorption rate [167]. No data exist in patients who have APS regarding thromboxane receptor antagonists.

### 4. Specific Complement Inhibition

Inhibition of the complement cascade *in vivo*, using the C3 convertase inhibitor complement receptor 1-related gene/protein y (Crry)-Ig, blocks aPL-mediated fetal loss [168]. In addition, C3 and C5 knock-out mice less frequently developed aPL-related complications and antiC5-antibody reverse the thrombogenic properties of aPL [169]. Finally, treatment with heparin prevented aPL-induced complement activation *in vivo and in vitro*, and protected mice from aPL-related pregnancy complications [101]. Specific complement inhibitors are attractive therapies for APS. Potential targets for the therapies are drawn in Fig. (1).

## IMMUNOMODULATION

Bone marrow transplantation (BMT) is currently used for the treatment of some autoimmune diseases based on the fact that autoimmunity can be either transferred or eliminated by BMT. In APS, both transfer of APS and induction of tolerance to disease has been reported [170, 171], thus BMT may have some future application for APS. On the other hand autologous stem-cell transplanted patients with scleroderma developed APS [172].

Antigen uptake, processing and presentation are the first steps following the exposure of antigen to the immune system. If we could artificially control this procedure to reduce β2GPI-reactive $CD4^+$ T cell response, the subsequent reactions, including antiβ2GPI antibody production, would not occur, thus curing the disease. Therapeutic strategies should target interrupting the continuous autoimmune loop carried out by macrophages and β2GPI-reactive $CD4^+$ T cells and B cells [173, 174].

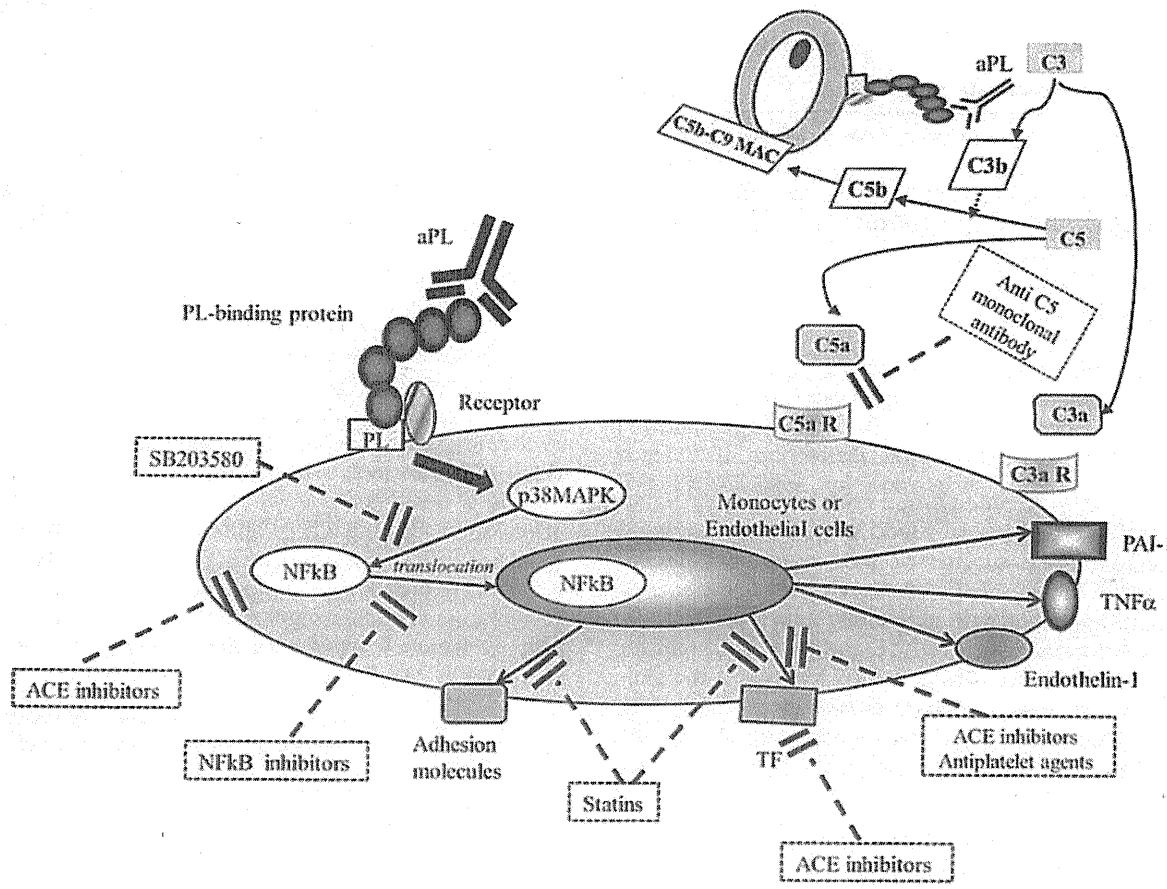


**Fig. (1). Targets for novel therapies in APS.**
Stimulation of procoagulant cells (monocytes or endothelial cells) by antibodies against phospholipid-binding proteins induce phosphorylation of p38MAPK which leads to the nuclear translocation of NFkB, and to the up-regulation of procoagulant substances. Specific inhibitors of p38MAPK (SB203580) or NFkB may block those processes. ACE inhibitors exert inhibitory effect on NFkB with consecutive down-regulation of TF. TF expression mediated by aPL can be abrogated by statins, and by some antiplatelet agents. aPL induce the production of cellular adhesion molecules by endothelial cells which may be reduced by statins. Finally, aPL may activate complement through the classical pathway. C3a, C5a and C5b-9 (MAC) may bind to specific receptors on endothelial cells and enhance the effects of aPL on cells. AntiC5-monoclonal antibody can reduce this binding.
aPL: antiphospholipid antibodies, PL: phospholipid, p38MAPK: p38 mitogen activated protein kinase; NFkB: nuclear factor Kappa B, ACE: angiotensin-converting enzyme, TF: tissue factor, TNFα: tumor necrosis factor alpha. PAI-1: plasminogen activator inhibitor-1, C5a R: C5a receptor, C5b R: C5b receptor, MAC: membrane attack complex.

The induction of immune tolerance at B-cell level is another future therapeutic approach for the management of APS. β2GPI-induced oral tolerance showed immunomodulatory effect in experimental APS [175]. A β2GPI-specific B cell toleragen, LJP 1082, was developed. This drug is a tetravalent conjugate of recombinant human domain 1 of β2GPI that has been shown to reduce domain I specific antibodies and levels of antigen-specific antibodies producing B cells [176]. Results from a phase I/II clinical trial in patients with antibody-mediated thrombosis showed that the drug was well tolerated and no differences on safety were found between patients receiving LJP1082 or placebo [177]. Further development is needed to assess the effect of this drug in the reduction of thrombotic events in APS.

## CONCLUSIONS

Ongoing research focused on the thrombotic mechanisms mediated by aPL has significantly advanced the understanding of the pathophysiology of the APS. Those novel discoveries opened new insights into the management of APS leading to the investigation of specific target therapy.

Data from animal models suggested attractive and novel therapeutic approaches for the prevention and treatment of aPL-related complications. However, there is still not enough information to warrant the use of those agents in APS patients. In the coming years, studies are required to validate the data in humans and to evaluate the efficacy and security of new targeted therapies in APS.

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ACE | = | Angiotensin-converting enzyme |
| aCL | = | Anticardiolipin antibodies |
| aPL | = | Antiphospholipid antibodies |
| ApoER2' | = | Apolipoprotein E receptor 2 |
| APS | = | Antiphospholipid syndrome |
| ARB | = | Angiotensin receptor blockers |
| β2GPI: | = | Beta 2Glycoprotein I |
| BMT | = | Bone marrow transplantation |
| ELISA | = | Enzyme-linked immunosorbent assay |
| GP | = | Glycoprotein |
| IL | = | Interleukin |
| IVIG | = | Intravenous immunoglobulins |
| LA | = | Lupus anticoagulant |
| LDA | = | Low dose of aspirin |
| LMWH | = | Low molecular weight heparin |
| Lp(a) | = | Lipoprotein a |
| MAPK | = | Mitogen activated protein kinase |
| NFkB | = | The nuclear factor kappa B |
| PAI | = | Plasminogen activator inhibitor |
| SLE | = | Systemic lupus erythematosus |
| TF | = | Tissue factor |
| TLR | = | Toll like receptor |
| TNFα | = | Tumor necrosis- α |
| tPA | = | Tissue-type plasminogen activator |

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# Human Mesenchymal Stem Cells Inhibit Osteoclastogenesis Through Osteoprotegerin Production

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*Objective.* **Mesenchymal stem cells (MSCs) have been proposed to be a useful tool for treatment of rheumatoid arthritis (RA), not only because of their multipotency but also because of their immunosuppressive effect on lymphocytes, dendritic cells, and other proinflammatory cells. Since bone destruction caused by activated osteoclasts occurs in RA, we undertook the present study to investigate the effect of MSCs on osteoclast function and differentiation in order to evaluate their potential use in RA therapy.**

*Methods.* **Human MSCs and peripheral blood mononuclear cells were cultured under cell–cell contact–free conditions with osteoclast induction medium. Differentiation into osteoclast-like cells was determined by tartrate-resistant acid phosphatase staining and expression of osteoclast differentiation markers.**

*Results.* **The number of osteoclast-like cells was decreased and expression of cathepsin K and nuclear factor of activated T cells c1 (NF-ATc1) was downregulated by the addition of either MSCs or a conditioned medium obtained from MSCs. Osteoprotegerin (OPG) was constitutively produced by MSCs and inhibited osteoclastogenesis. However, osteoclast differentiation was not fully recovered upon treatment with either anti-OPG antibody or OPG small interfering RNA, suggesting that OPG had only a partial role in the inhibitory effect of MSCs. Moreover, bone-resorbing activity of osteoclast-like cells was partially recovered by addition of anti-OPG antibody into the conditioned medium.**

*Conclusion.* **The present results indicate that human MSCs constitutively produce OPG, resulting in inhibition of osteoclastogenesis and expression of NF-ATc1 and cathepsin K in the absence of cell–cell contact. Therefore, we conclude that human MSCs exert a suppressive effect on osteoclastogenesis, which may be beneficial in inhibition of joint damage in RA.**

Rheumatoid arthritis (RA) is a prototypical autoimmune inflammatory disease characterized by chronic inflammation. The major inflamed milieu is the synovium, where the presence of proinflammatory cells and cytokines leads to damage to cartilage and bone, resulting in deformity of the joints and decreased quality of life. Progression of bone destruction in RA involves abnormal activation of osteoclasts by interaction with synovial fibroblasts and T helper cells expressing RANKL (1,2). In addition, osteoblast function is known to be compromised at sites of focal bone erosion (3). Treatment of RA with biologic agents targeting tumor necrosis factor (TNF) and interleukin-6 (IL-6) improves

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disease activity and inflammation, leading to remission (4,5), and also has been associated with repair of bone erosions (6), presumably due to suppression of inflammatory cytokine–dependent activation of osteoclasts. However, only a small proportion of patients exhibit repair.

Human mesenchymal stem cells (MSCs) are multipotent cells that are able to differentiate to osteoblasts, chondrocytes, and adipocytes and can be isolated from bone marrow, adipose tissue, and other mesodermal tissues (7). This multipotency, especially the ability to differentiate to osteoblasts and chondrocytes, is attractive for tissue engineering of bone and cartilage in various disorders, including RA, osteoarthritis, and osteoporosis. Intriguingly, MSCs have also been shown to exert immunosuppressive effects. Although elucidation of the immunosuppressive mechanism has been limited, several in vitro and in vivo studies indicate that MSCs strongly suppress effector T cell responses and activation of dendritic cells and natural killer (NK) cells (8–13), suggesting the potential use of human MSCs as a novel cell therapy for autoimmune diseases, especially RA. Moreover, MSCs are known to secrete a variety of cytokines and growth factors that exert paracrine activities on various cells, i.e., inhibition of tissue fibrosis and cell apoptosis, enhancement of angiogenesis, and modulation of cell differentiation (referred to as trophic effects of MSCs) (14,15).

The above-described reports prompted us to postulate that MSCs may inhibit osteoclastogenesis from osteoclast precursors, namely peripheral blood mononuclear cells (PBMCs). In particular, we were interested in elucidating how MSCs act on osteoclasts, which play an important role in bone resorption and destruction in RA. We assessed the effects of human MSCs on osteoclastogenesis and found that MSCs constitutively produce osteoprotegerin (OPG), a decoy receptor for RANKL, leading to suppression of osteoclastogenesis.

## MATERIALS AND METHODS

**Cells.** Human MSCs were purchased from Lonza Walkersville. The source of the human MSCs was bone marrow obtained from the posterior iliac crest of the pelvic bone of healthy volunteers. MSCs were cultured as recommended by the manufacturer. Briefly, cells were cultured in MSC growth medium including 10% fetal bovine serum (FBS; Lonza Walkersville) at 37°C in a 5% $CO_2$ atmosphere and were subcultured every 6–7 days. MSCs from passages 2–10 were used in this study.

Human PBMCs were isolated from peripheral blood obtained from healthy volunteers, using LSM lymphocyte separation medium (MP Biomedicals). PBMCs ($1 \times 10^6$) were seeded into wells of 24-well plates (Corning) and cultured in minimum essential medium $\alpha$ (MEM$\alpha$; Invitrogen) with 10% FBS (Tissue Culture Biologicals) and penicillin/streptomycin (Invitrogen) supplemented with osteoclast induction medium (OCIM) (50 ng/ml human macrophage colony-stimulating factor [M-CSF; GenScript], 50 ng/ml human soluble RANKL [sRANKL; PeproTech], and 100 nmoles/liter 1$\alpha$,25-dihydroxyvitamin $D_3$ [1$\alpha$,25$(OH)_2D_3$; Sigma]) at 37°C in a 5% $CO_2$ atmosphere.

**Coculture assay and MSC-conditioned medium assay.** A coculture assay with PBMCs and MSCs was performed to evaluate the effects of MSCs on osteoclast differentiation. PBMCs ($1 \times 10^6$) in OCIM were seeded in the lower wells and human MSCs in the upper wells of 24-well Transwell plates (Corning) with 0.4-$\mu$m–diameter pore. To determine cell viability, PBMCs were stained with fluorescein isothiocyanate–labeled annexin V antibody (BD Biosciences) and positive cells were detected as dead cells by flow cytometry. MSC-conditioned medium was obtained by collecting supernatants of MSCs cultured in MEM$\alpha$ for 3 days at the confluent phase and subsequently adding 50 ng/ml M-CSF and 50 ng/ml sRANKL. PBMCs ($1 \times 10^6$) or peripheral blood CD14+ cells ($5 \times 10^5$) were plated into 24-well plates and cultured in MSC-conditioned medium or OCIM. Anti-human OPG neutralizing monoclonal antibody or isotype control IgG1 (both from R&D Systems) was added as indicated.

**Tartrate-resistant acid phosphatase (TRAP) staining.** To confirm osteoclastogenesis from PBMCs or peripheral blood CD14+ cells, cultured cells were stained using a leukocyte acid phosphatase kit according to the instructions of the manufacturer (Sigma). TRAP-positive and multinuclear cells were counted under brightfield microscopy. TRAP-positive cells with ≥3 nuclei were regarded as osteoclast-like cells.

**Measurement of cathepsin K, nuclear factor of activated T cells c1 (NF-ATc1), and OPG expression.** PBMCs from healthy donors and OCIM-treated PBMCs were collected and lysed. Total messenger RNA (mRNA) was purified with an RNeasy Mini Kit (Qiagen), and complementary DNA was obtained by reverse transcription. Real-time polymerase chain reaction was performed using cathepsin K– and NF-ATc1–specific primers and TaqMan probes (Hs01080388_m1 and Hs00542678_m1, respectively; Applied Biosystems) with a Step One Plus instrument (Applied Biosystems). Cathepsin K and NF-ATc1 mRNA expression levels were normalized to levels of GAPDH (TaqMan probe Hs99999905_m1; Applied Biosystems) as an endogenous control, and relative quantity compared to a healthy control PBMC sample as a reference was calculated using the $\Delta\Delta C_t$ method. OPG mRNA expression was similarly determined by real-time polymerase chain reaction using TaqMan probes targeting OPG (Hs00900360_m1) and $\beta$-actin (Hs99999903_m1) (both from Applied Biosystems) as an endogenous control. Relative quantity compared to a reference sample of normal human MSCs was calculated by the $\Delta\Delta C_t$ method.

MSC culture supernatants were collected at the con-

fluent phase after MSC growth medium was exchanged for MEM$\alpha$ with 10% FBS for 3 days to exclude the effect of growth factors in MSC growth medium. Coculture supernatants were collected at intervals of 3 or 4 days. OPG concentration was measured with a DuoSet enzyme-linked immunosorbent assay development system for human OPG/TNF superfamily 11B (R&D Systems).

**Transfection of small interfering RNA.** Small interfering RNA (siRNA) targeting OPG mRNA (OPG siRNA; sequence 5′-UGAUCUUCUUGACUAUAUCUUGGUC-3′) was purchased from Invitrogen. Stealth RNAi Low GC Duplex (Invitrogen) was used as negative control siRNA. MSCs ($1 \times 10^3$) were seeded into upper wells of Transwell plates in MSC growth medium supplemented with 10% FBS without antibiotics. After 24 hours, siRNA was transfected into MSCs using Lipofectamine RNAiMAX in Opti-MEM I according to the protocol recommended by the manufacturer (Invitrogen).

**Pit formation assay.** To evaluate bone-resorbing activity in osteoclast-like cells, CD14+ cells ($1 \times 10^5$) isolated from PBMCs were plated onto dentin slices in 96-well plates and cultured in OCIM or MSC-conditioned medium for 14 days. Recombinant human OPG, anti-OPG antibody, or isotype control IgG1 was added to the culture medium. After culturing, dentin slices were stained with Mayer's hematoxylin (Wako) and analyzed by microscopy (Biorevo BZ-9000; Keyence).

**Statistical analysis.** Mean ± SEM or mean ± SD values from triplicate samples from 1 of at least 3 independent experiments were calculated. Statistical significance was ascertained by Student's *t*-test or one-way analysis of variance. *P* values less than 0.05 were considered significant.

## RESULTS

**Inhibition of osteoclast differentiation by human MSCs.** Although human and murine MSCs have immunosuppressive potency, their effects on osteoclast differentiation and activation have not been well elucidated. Therefore, we first investigated the effect of human MSCs on osteoclastogenesis. PBMCs isolated from healthy donors were cultured with human MSCs under cell–cell contact–free conditions. Medium was supplemented with M-CSF, sRANKL, and $1\alpha,25(OH)_2D_3$ (OCIM) to induce osteoclast differentiation. After 16 days, ~300 osteoclast-like cells per square centimeter were observed when PBMCs were cultured alone (Figures 1A and B). However, the number of osteoclast-like cells was significantly decreased after coculture with human MSCs. The suppressive effect was dependent on the number of human MSCs and did not depend on apoptosis, as assessed by the presence of annexin V–positive cells (Figure 1C). Accordingly, viable cells

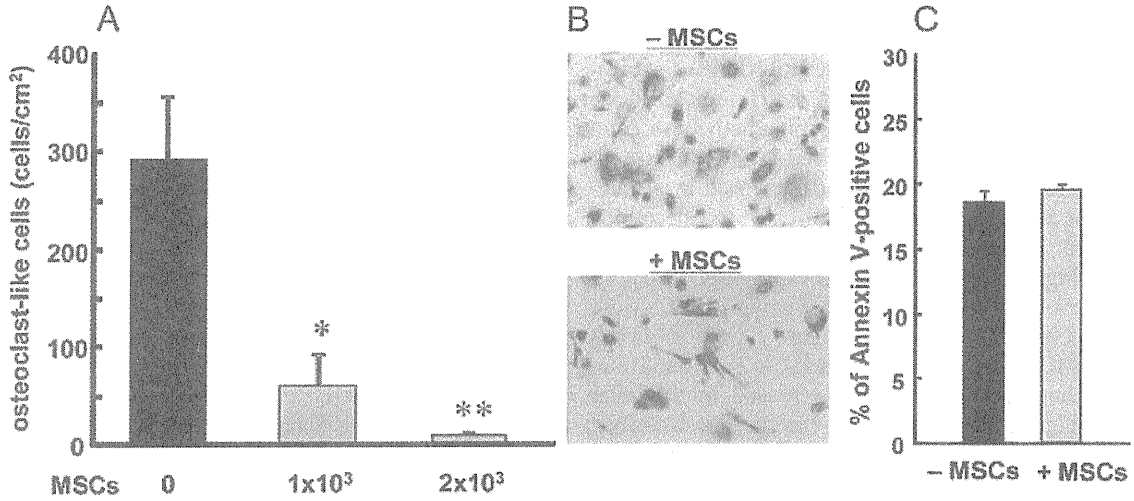


**Figure 1.** Human mesenchymal stem cells (MSCs) inhibit osteoclastogenesis in a cell–cell contact–free system. Peripheral blood mononuclear cells (PBMCs; $1 \times 10^6$/well) and human MSCs ($2 \times 10^3$/well) were cocultured in osteoclast induction medium (OCIM), using Transwells. **A,** Number of osteoclast-like cells, counted by microscopy after tartrate-resistant acid phosphatase (TRAP) staining. **B,** TRAP-positive osteoclast-like cells observed on day 16 in PBMCs cultured with or without human MSCs. Original magnification × 100. **C,** Proportion of apoptotic and dead PBMCs (annexin V positive), determined by fluorescence-activated cell sorting, in PBMCs cultured for 3 days in OCIM with or without MSCs. Values in **A** and **C** are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$ versus wells without MSCs, by Tukey's test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

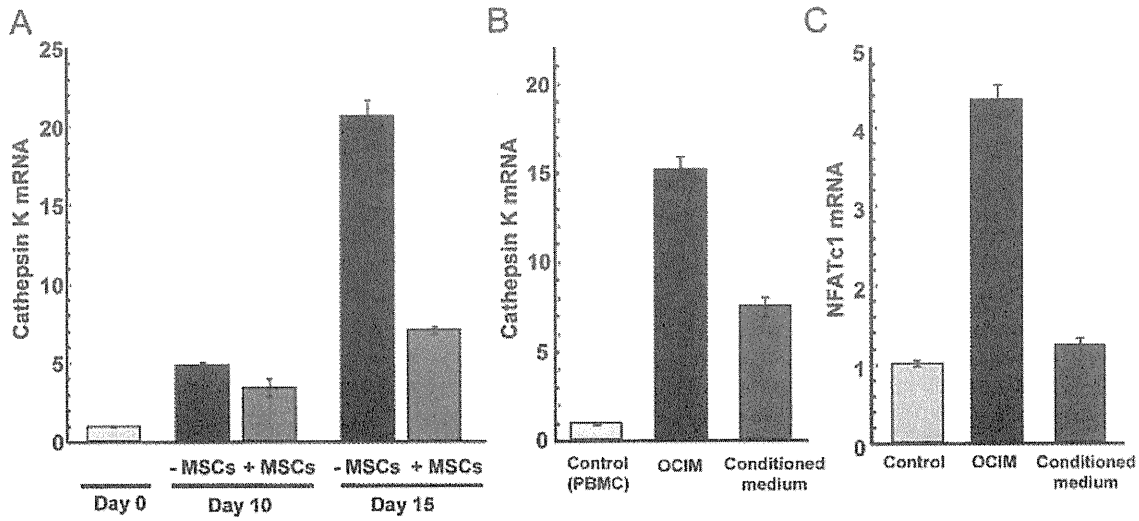


**Figure 2.** Human MSCs suppress induction of cathepsin K and expression of nuclear factor of activated T cells c1 (NF-ATc1). **A,** PBMCs ($1 \times 10^6$/well) were cocultured for 10 or 15 days with human MSCs ($2 \times 10^3$) in OCIM, using Transwells. After coculture, PBMCs in OCIM were lysed and total RNA was isolated. Cathepsin K mRNA expression compared with GAPDH was determined by real-time polymerase chain reaction (PCR). **B,** PBMCs ($1 \times 10^6$/well) were cultured in OCIM or MSC-conditioned medium. After 15 days, cathepsin K mRNA expression was analyzed by real-time PCR. **C,** CD14+ cells isolated from PBMCs were cultured in OCIM or MSC-conditioned medium. After 7 days, NF-ATc1 mRNA expression was analyzed by real-time PCR. Levels of mRNA expression in control samples (day 0) were set at 1. Values are the mean ± SD of triplicate samples from 1 of 3 independent experiments. See Figure 1 for other definitions.

were observed in coculture wells on day 16, but osteoclast-like cells were not (Figure 1B).

**Suppression of cathepsin K and NF-ATc1 expression by human MSCs.** Cathepsin K is a cysteine protease that is essential for the bone-resorbing activity of osteoclasts. When PBMCs were cultured in OCIM, cathepsin K expression was induced and increased in a time-dependent manner, ultimately (on day 15) becoming 15–20-fold higher than expression in unstimulated PBMCs (Figure 2A). Cathepsin K expression on day 15 was, however, markedly suppressed when PBMCs were cocultured with human MSCs (Figure 2A). In addition, when PBMCs were cultured in MSC-conditioned medium, cathepsin K expression decreased in a similar manner (Figure 2B). To confirm the suppressive effect of MSCs on osteoclastogenesis, expression of NF-ATc1, known as the master transcription factor for osteoclast differentiation, was measured. NF-ATc1 mRNA expression increased in CD14+ cells cultured for 7 days with OCIM, whereas MSC-conditioned medium completely suppressed its expression (Figure 2C). These results therefore suggest that a soluble mediator from human MSCs plays an important role in inhibition of osteoclastogenesis.

**Inhibition of osteoclastogenesis by OPG produced from human MSCs.** The key role of RANKL in osteoclastogenesis has been established. In order to identify the soluble factor that inhibits osteoclastogenesis, we first considered the possibility of OPG, a decoy receptor for RANKL. Interestingly, human MSCs constitutively produced OPG, at both the mRNA and the protein levels (Figures 3A and B), which was also detectable in coculture wells but not in wells without human MSCs (Figure 3C). In addition, although neither TNF$\alpha$ nor prostaglandin $E_2$ ($PGE_2$) was detected in coculture wells (data not shown), IL-6, known as a modulator of osteoclastogenesis, was produced (~10–15 ng/ml) (Figure 3D).

To exclude the possible effect of $1\alpha,25(OH)_2D_3$ on osteoblast differentiation and OPG production, experiments without $1\alpha,25(OH)_2D_3$ were performed. Irrespective of the presence of $1\alpha,25(OH)_2D_3$, osteoclastogenesis was suppressed in a similar manner, resulting in reduced RANKL expression, alkaline phosphatase activity, and alizarin red staining (results not shown). In addition, OPG production levels were comparable between MSC culture supernatants and coculture supernatants (Figures 3A and C), indicating that constitutive

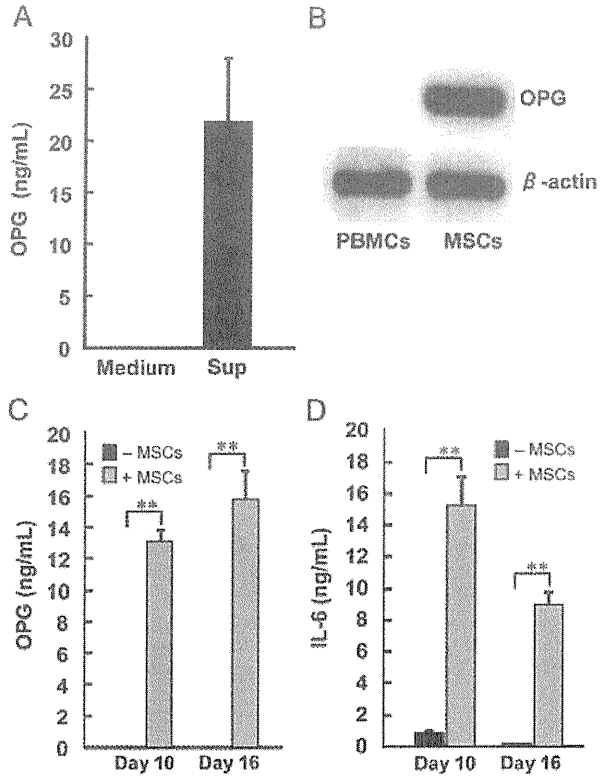


**Figure 3.** Constitutive production of osteoprotegerin (OPG) by human mesenchymal stem cells (MSCs). **A,** Concentrations of OPG protein in culture supernatant (Sup) of human MSCs in the confluent growth phase compared with concentrations in MSC growth medium were measured by enzyme-linked immunosorbent assay (ELISA). **B,** OPG mRNA expression in human MSCs and peripheral blood mononuclear cells (PBMCs) was determined by polymerase chain reaction (PCR). PCR products analyzed by electrophoresis are shown. **C** and **D,** PBMCs ($1 \times 10^6$/well) and human MSCs ($1 \times 10^3$/well) were cocultured in OCIM, using Transwells. Coculture supernatants were collected on days 10 and 16. Concentrations of OPG (**C**) and interleukin-6 (IL-6) (**D**) were measured by ELISA. Values in **A, C,** and **D** are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test.

OPG production by MSCs inhibited osteoclastogenesis. As expected, addition of anti-OPG neutralizing monoclonal antibody to the coculture system increased the number of osteoclast-like cells compared to that obtained with control antibody (Figure 4).

To further confirm the inhibitory effect of OPG, human MSCs were transfected with OPG siRNA (OPG-knockdown [OPG-KD] MSCs). The production of OPG was reduced by 86% at both the mRNA level (data not shown) and the protein level (Figure 5B). When PBMCs were cocultured with OPG-KD MSCs, the number of osteoclast-like cells significantly recovered compared to negative siRNA–transfected MSCs (Figure 5A). Additionally, cathepsin K expression recovered when PBMCs were cocultured with OPG-KD MSCs (Figure 5C). However, recovery of osteoclast-like cell numbers and cathepsin K expression was only partial. These results show that OPG partially contributes to the inhibition of osteoclastogenesis by human MSCs, leaving open the possibility that other soluble factors are also involved. Accordingly, recovery of cathepsin K expression was lower compared to the recovery of osteoclast-like cells.

**Offset of MSC-mediated inhibition of osteoclastogenesis by its specific blockade.** To evaluate the function of bone-resorbing activity of osteoclast-like cells, a pit formation assay was performed. When CD14+ cells isolated from PBMCs were cultured in OCIM for 14 days, >200 osteoclast-like cells per square centimeter were observed in the 96-well plates, with pit formation making up 15.6% of the surface of dentin slices (Figure 6). In contrast, a mean of only 43

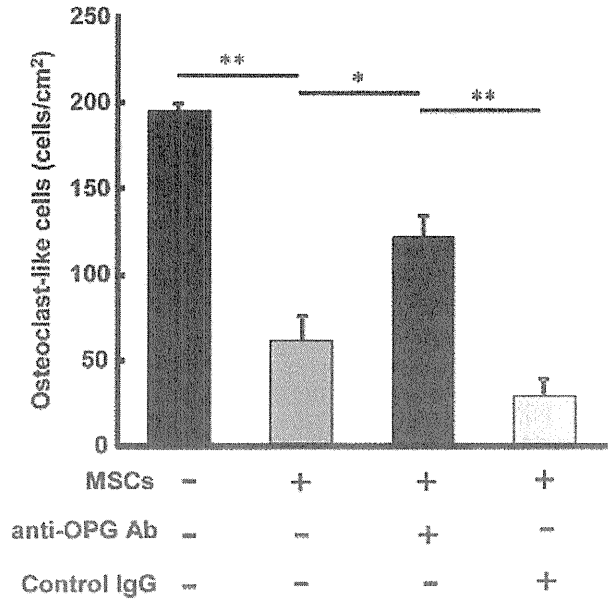


**Figure 4.** OPG blocking induces recovery of osteoclastogenesis. PBMCs ($1 \times 10^6$/well) and human MSCs ($1 \times 10^3$/well) were cocultured for 16 days in OCIM with anti-osteoprotegerin (anti-OPG) neutralizing monoclonal antibody (Ab) or mouse IgG1 isotype control. Osteoclast-like cells (recognized by TRAP positivity) and multinuclear cells were counted by microscopy. Values are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$, by Tukey's test. See Figure 1 for other definitions.

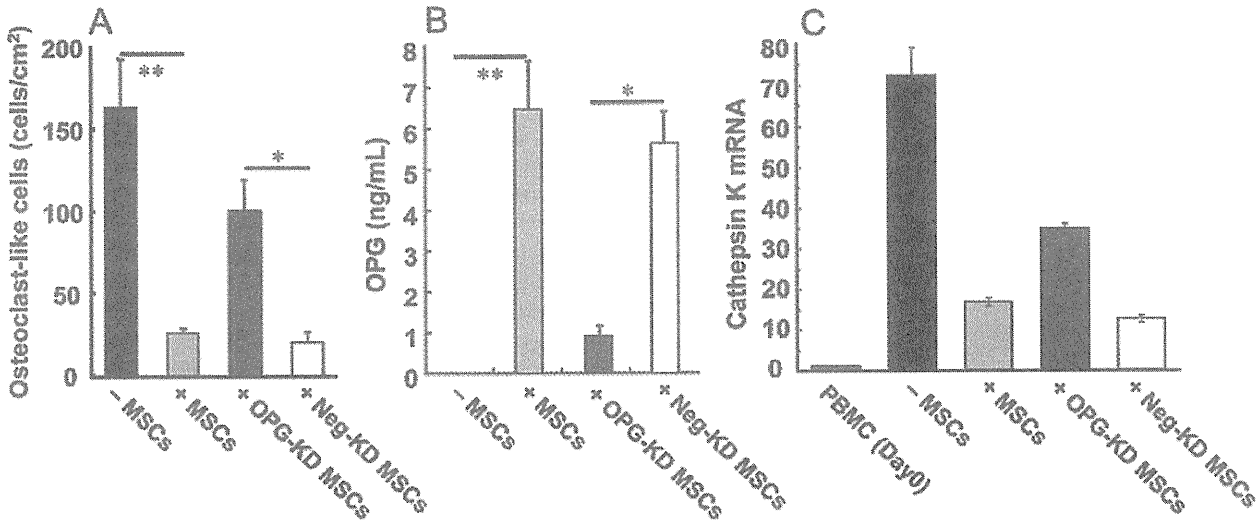


**Figure 5.** OPG small interfering RNA (siRNA)–transfected human MSCs induce partial recovery of osteoclastogenesis. Human MSCs ($1 \times 10^3$/well) were transfected with OPG siRNA (OPG-knockdown [OPG-KD]) or negative control siRNA (Neg-KD), and PBMCs ($1 \times 10^6$/well) were then cocultured for 14 days with OPG-KD or negative control MSCs ($1 \times 10^3$/well) in OCIM. **A,** Tartrate-resistant acid phosphatase (TRAP)–positive and multinuclear cells were counted by microscopy. **B,** OPG concentrations in coculture supernatant were measured by ELISA. **C,** On day 14, PBMCs with OCIM were lysed and cathepsin K mRNA expression was quantified by real-time PCR. Values are the mean ± SEM (**A** and **B**) or the mean ± SD (**C**) of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$, by Tukey's test. See Figure 3 for other definitions.

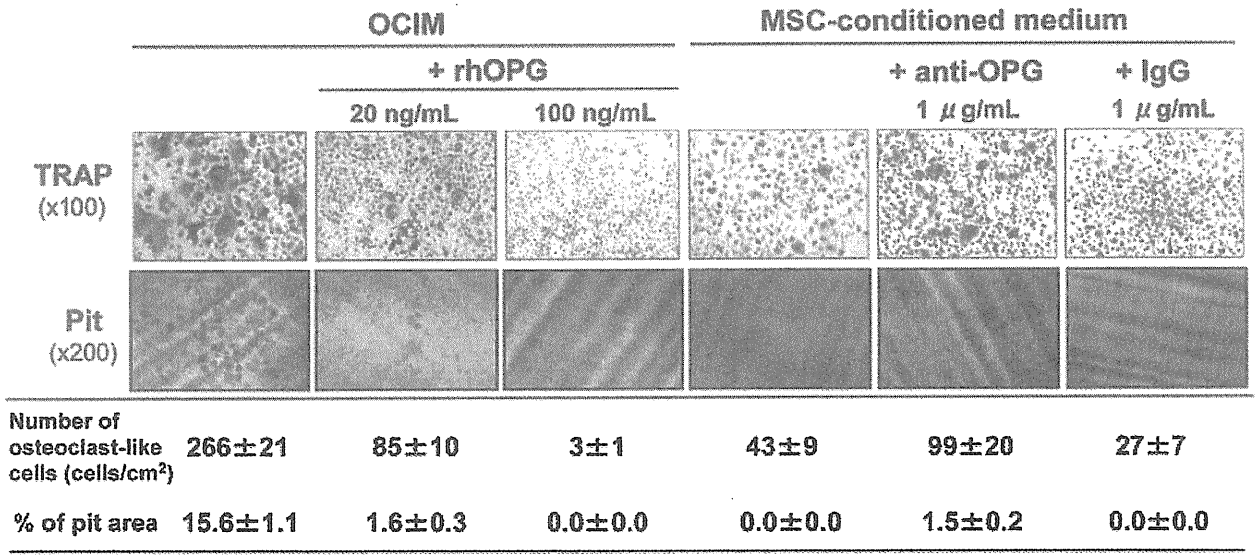


**Figure 6.** MSC-conditioned medium inhibits osteoclastogenesis and bone-resorbing activity of cultured osteoclast-like cells. Peripheral blood CD14+ cells were cultured in OCIM or MSC-conditioned medium in the presence of recombinant human OPG (rhOPG; 20 ng/ml or 100 ng/ml), anti-OPG antibody (1 μg/ml), or mouse IgG1 antibody (1 μg/ml). Pit formation assay was performed by culturing CD14+ cells on dentin slices. After 14 days, cells and dentins were stained with TRAP and hematoxylin, respectively. The number of osteoclast-like cells was counted, and the percentage of pit area in relation to the total surface area of dentin was calculated. Values are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. Figure 1 for other definitions.

osteoclast-like cells per square centimeter was observed when cells were cultured in MSC-conditioned medium, with complete inhibition of pit formation. Osteoclastogenesis was partly recovered with addition of anti-OPG neutralizing antibody to MSC-conditioned medium, but the size and the number of osteoclast-like cells remained smaller compared to those in experiments with OCIM. In addition, pit formation was recovered with addition of anti-OPG antibody, but pit area was no more than 1.5% of dentin surface.

This partial recovery of pit formation by OPG blocking correlated with the recovery of expression of cathepsin K (Figure 5C), an indispensable protease for bone-resorbing activity of osteoclasts. In OCIM, both osteoclast-like cells and pit formation decreased with addition of OPG, in a concentration-dependent manner. Interestingly, the suppressive effects on osteoclast-like cell differentiation and pit formation in the presence of 20 ng/ml recombinant OPG were less than those observed in MSC-conditioned medium containing ~20 ng/ml OPG (Figure 3A). These results suggested that human MSCs can inhibit bone-resorbing activity not only by OPG secretion but also via other soluble mediators.

## DISCUSSION

RA is a prototypical inflammatory disease that presents significant health and socioeconomic burdens. Even though biologic agents have made it possible to prevent joint destruction and also induce repair in some cases, improvement of physical function is still limited. MSCs are widely understood to exert trophic effects on various cells, which is considered to be a useful therapeutic avenue in a number of diseases. In fact, there are currently several ongoing clinical trials to evaluate their usefulness in graft-versus-host disease, autoimmune diseases, and autoinflammatory diseases (16). Given the known multipotency and immunosuppressive effects of MSCs, we considered that they might be a new source of RA treatment. Because the effect of MSCs on osteoclasts, which play an important role in bone resorption and joint destruction, remained unclear, we investigated the role of MSCs in osteoclastogenesis. We found that human MSCs markedly suppressed osteoclast differentiation and activation by constitutive production of OPG under cell–cell contact–free conditions. Our data suggest that a cell-based therapy using human MSCs would be expected to improve bone-erosive diseases including RA. In fact, blockade of RANKL has been shown to prevent progress of bone loss in an RA clinical study (17).

MSCs are able to differentiate into osteoblasts, chondrocytes, and adipocytes. Originally identified in bone marrow, MSCs can also be identified in adipose, synovium, umbilical cord, skin, and various other tissues (8,18). The MSCs used in the present study were derived from bone marrow donated by healthy donors. These cells have been characterized to differentiate into osteocytic, chondrocytic, or adipocytic lineages, and to be positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, and CD120a as surface proteins. On the other hand, hematopoietic markers, such as CD14, CD34, and CD45, are known to be negative on these cells (7). However, specific cell surface makers for MSCs have not been identified and moreover, differences between MSCs derived from different tissues remain unclear. As an example, although adipose-derived stem cells (ADSCs) are also known for their multipotency, of most interest is their powerful angiogenic potential rather than an immunosuppressive effect (8,19). Therefore, ADSCs may be useful for treatment of ischemic diseases rather than autoimmune diseases. Interestingly, OPG was constitutively produced not only by MSCs but also by ADSCs (data not shown), suggesting a common phenotype with MSCs.

MSCs are known for their strong immunosuppressive functions in vitro caused by soluble mediators such as antiinflammatory cytokines (20), and based on this intriguing property, they might be useful as a therapeutic application in autoimmune diseases, including RA. In fact, Augello et al have reported that a single intravenous injection of MSCs prevented the occurrence of severe, irreversible damage to bone and cartilage in experimental collagen-induced arthritis in mice (21). Although the effect of MSCs on T cells, dendritic cells, and NK cells has been reported by several groups, their effects on other immune cells and inflammatory cells remain to be fully elucidated. In particular, the effect of MSCs in differentiation and function of osteoclasts, which play an important role in bone resorption leading to joint destruction in RA, is still unclear. In the present study, we found that osteoclastogenesis was significantly inhibited by MSCs via constitutive production of OPG. However, experiments with anti-OPG neutralizing antibody and knockdown of OPG by its siRNA (Figures 4 and 5) suggested that not only OPG, but also other soluble mediators, are involved in inhibition of osteoclastogenesis by MSCs. Accordingly, MSC-conditioned medium containing ~20 ng/ml of OPG showed stronger

inhibition compared to that obtained with addition of 20 ng/ml recombinant OPG to OCIM (Figures 3 and 6).

It has been reported that IL-6, IL-8, $PGE_2$, and vascular endothelial growth factor are secreted from MSCs identical to the ones we used (8). Intriguingly, in our coculture experiments, IL-6 levels were increased to 10–15 ng/ml (Figure 3D). The role of IL-6 in osteoclastogenesis remains controversial. IL-6 has been indicated as a key molecule in driving osteoclastogenesis in various in vivo studies (22–24), and others have shown that IL-6 inhibited RANKL-dependent osteoclastogenesis (25,26). Our results suggest either that MSCs are able to inhibit osteoclastogenesis in the presence of IL-6 or simply that IL-6 did not induce osteoclastogenesis in our system. The incomplete recovery of osteoclast differentiation by inhibition of OPG can be related to an inhibitory effect of IL-6 on osteoclastogenesis. Further, although $PGE_2$ was previously reported to have an inhibitory effect on human osteoclast formation (27), the maximum concentration of $PGE_2$ in our coculture medium (on day 3) was only 1.5 ng/ml (data not shown). Based on previously reported findings (8), we speculated that $PGE_2$ production requires either interferon-$\gamma$ or TNF$\alpha$ stimulation. Therefore, we believe inhibition of osteoclastogenesis is unrelated to $PGE_2$ in our coculture system.

Given the known immunosuppressive effect of MSCs, it is possible that immunosuppressive soluble mediators contribute to inhibition of osteoclastogenesis. Murine MSCs are able to induce apoptosis and cell cycle arrest of splenocytes stimulated with anti-CD3 and anti-CD28 antibodies under cell–cell contact–free conditions, and this is thought to be mediated by IL-10 (28). In fact, differentiation of human osteoclasts is reported to be suppressed by IL-10 due to inhibition of RANK signaling (29). However, IL-10 was undetectable in our experiments (data not shown). In recent years, indoleamine 2,3-dioxygenase and nitric oxide have also been reported to be important molecules suppressing the immune response of NK cells and proliferation of T lymphocytes (11,28,30). Further evaluation is needed to clarify what factors other than OPG are involved in the suppressive effect on osteoclastogenesis.

OPG is known to be an inhibitory molecule for RANKL-dependent osteoclast differentiation and function (31). In fact, OPG-deficient mice exhibit severe osteoporosis due to excessive bone resorption by osteoclasts (32,33). Accordingly, loss of trabecular bone density in OPG-deficient mice was recovered by intravenous injection of recombinant human OPG-Fc fusion protein, which decreased the number of osteoclasts (34). Moreover, it has been reported that short-term treatment with soluble OPG protein or OPG-Fc fusion protein significantly reduced osteoclast numbers and prevented bone erosion in rats with experimental arthritis, without affecting synovitis (35,36). Surprisingly, those effects were evident after subcutaneous injection of OPG-Fc for only 5–7 days. These results indicate that MSCs are an OPG producer, exerting an antierosive effect by decreasing osteoclasts.

Although we have demonstrated here that inhibition of osteoclastogenesis by human MSCs is independent of physical cell contact, the effect of cell contact is not certain. In a previous study, human MSCs were shown to induce osteoclastogenesis from CD34+ bone marrow hematopoietic stem cells (HSCs) in the presence of cell–cell contact, by up-regulation of IL-6, IL-11, and leukemia inhibitory factor production from MSCs (37). Interestingly, this effect was observed in the absence of osteoclastogenic stimulation such as OCIM, and under cell–cell contact–free conditions as well (37).

The mechanism of this effect is considered to involve the altered nature of MSCs, including production of cytokines, growth factors, and surface molecules. Accordingly, it may be that OPG expression by MSCs is reduced by contact with HSCs rather than by up-regulation of RANKL, unless osteoclastogenesis is driven by a RANKL-independent pathway such as via IL-6 and IL-11 (38). Additionally, use of PBMCs or HSCs can be involved in the different effect of MSCs on osteoclastogenesis. Although alteration of the characteristics of MSCs under conditions of coculturing with PBMCs in OCIM remains to be confirmed, RANKL mRNA expression was not detected in MSCs at the end of coculture (data not shown). Therefore, MSCs were at least not differentiated into osteoblasts expressing RANKL in our coculture system. However, further investigation is necessary to elucidate the effects of human MSCs on osteoclastogenesis from PBMCs in OCIM in the presence of cell–cell contact.

In conclusion, it is clear that human MSCs inhibit osteoclastogenesis without cell–cell contact, partly due to constitutive secretion of OPG. OPG can block RANKL–RANK interaction, which is essential for differentiation and activation of osteoclasts. In the synovium of patients with RA, activated T cells and synovial fibroblasts express RANKL and induce maturation and activation of osteoclasts instead of osteoblasts, resulting in characteristic bone erosion (39). Accordingly, OPG-producing human MSCs may prevent progression of bone destruction in RA through the production of OPG and subsequent suppression of osteoclastogenesis.

Moreover, because human MSCs are well known to have strong immunosuppressive potential, they should be capable of ameliorating synovial tissue inflammation in RA. We therefore suggest that cell therapy using human MSCs may be a novel strategy for the treatment of RA, which would be fully expected not only to suppress the autoimmune response but also to prevent excessive bone resorption by osteoclasts. Furthermore, development of a therapy taking advantage of the multipotency of MSCs, by which they are able to differentiate into osteoblasts and chondrocytes (a component of joint tissue), should enable regeneration of damaged joints in RA.

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### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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# Dopamine Induces IL-6–Dependent IL-17 Production via D1-Like Receptor on CD4 Naive T Cells and D1-Like Receptor Antagonist SCH-23390 Inhibits Cartilage Destruction in a Human Rheumatoid Arthritis/SCID Mouse Chimera Model

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**A major neurotransmitter dopamine transmits signals via five different seven-transmembrane G protein-coupled receptors termed D1–D5. Several studies have shown that dopamine not only mediates interactions into the nervous system, but can contribute to the modulation of immunity via receptors expressed on immune cells. We have previously shown an autocrine/paracrine release of dopamine by dendritic cells (DCs) during Ag presentation to naive CD4$^+$ T cells and found efficacious results of a D1-like receptor antagonist SCH-23390 in the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis and in the NOD mouse model of type I diabetes, with inhibition of Th17 response. This study aimed to assess the role of dopaminergic signaling in Th17-mediated immune responses and in the pathogenesis of rheumatoid arthritis (RA). In human naive CD4$^+$ T cells, dopamine increased IL-6–dependent IL-17 production via D1-like receptors, in response to anti-CD3 plus anti-CD28 mAb. Furthermore, dopamine was localized with DCs in the synovial tissue of RA patients and significantly increased in RA synovial fluid. In the RA synovial/SCID mouse chimera model, although a selective D2-like receptor antagonist haloperidol significantly induced accumulation of IL-6$^+$ and IL-17$^+$ T cells with exacerbated cartilage destruction, SCH-23390 strongly suppressed these responses. Taken together, these findings indicate that dopamine released by DCs induces IL-6–Th17 axis and causes aggravation of synovial inflammation of RA, which is the first time, to our knowledge, that actual evidence has shown the pathological relevance of dopaminergic signaling with RA.** *The Journal of Immunology*, **2011, 186: 3745–3752.**

Dopamine is the major neurotransmitter in the CNS, and it is involved in the control of locomotion, emotion, cognition, and neuroendocrine secretion (1, 2). The CNS and the neurotransmitters have been previously reported to control the immune system and to regulate host defense (3–5). Receptors for various neurotransmitters are expressed on the cell surface of lymphocytes, indicating the importance of such neuroimmune interactions (6–12). Dopamine receptors are seven-transmembrane G protein-coupled receptors and five subtypes from D1 to D5 (2, 13). These subtypes are classified into two subgroups. D1 and D5 are D1-like receptors that coupled to Gαs, which increases intracellular cAMP. In contrast, D2, D3, and D4 are D2-like receptors that coupled to Gαi, which decreases intracellular cAMP (2, 13). Dopaminergic signaling via D2-like receptors coupled to the inhibition cAMP production in T lymphocytes often has an immunostimulatory effect (7, 8, 10). Conversely, signaling via D1-like receptors coupled to increasing cAMP promotes the inhibition of the immune response (8). Dopamine has been shown to inhibit proliferation of human lymphocytes, and even to induce apoptosis in peripheral mononuclear cells (14, 15). Studies carried out on human and murine lymphocytes have demonstrated that these cells express all subtypes of dopamine receptors (10, 14, 16–19). However, most of these results are still inconclusive and even contradictory. Lymphocytes are a mixture of different classes and functional subsets. Different lymphocyte classes and subsets may express different dopamine receptor subtypes. Furthermore, differences in ligand binding affinity, basal activity, efficacy, G protein activation, desensitization, and internalization rates may be expected to be discriminating properties of the dopamine receptors. Because these variations and differences of dopamine receptors sometimes make interpretation of data extremely difficult, immunomodulation by dopamine needs to be investigated with each lymphocyte subset.

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Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; OA, osteoarthritis; RA, rheumatoid arthritis; SCID-HuRAg, SCID mice engrafted with human RA synovium; Treg, regulatory T cell; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.



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