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Osteoclast Differentiation is Associated with Transient Upregulation of Cyclin-Dependent Kinase Inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$

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Abstract Osteoclasts, bone-resorbing multinucleated cells, develop from monocyte-macrophage lineage cells in the presence of osteoclast differentiation factor (ODF, also called RANKL/TRANCE/OPGL) and macrophage colony-stimulating factor (M-CSF). M-CSF-dependent bone marrow macrophages (M-BMMΦs) from mouse bone marrow cells have been shown to differentiate into osteoclast-like multinucleated cells (OCLs) in the presence of soluble ODF/RANKL (sODF/RANKL) and M-CSF within 3 days. In this study, we found that stimulation of M-BMMΦs with sODF/RANKL induced a transient expression of cyclin-dependent kinase inhibitors (CDK inhibitors) $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ by 24 h. The CDK inhibitor proteins disappeared by 48 h. Tumor necrosis factor alpha (TNF-α), which is reported to stimulate OCL differentiation, stimulated $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression in M-BMMΦs as well. However, M-CSF alone did not stimulate the expression of the two CDK inhibitors. To clarify the role of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in osteoclastogenesis, accumulation of these CDK inhibitors was aborted by antisense oligonucleotides. Treatment with $p21^{WAF1/CIP1}$ antisense oligonucleotide alone, or $p27^{KIP1}$ antisense oligonucleotide alone, showed a limited inhibitory effect on OCL formation. However, treatment with a mixture of these two antisense oligonucleotides strongly inhibited OCL formation. These results suggest that a combined modulation of the CDK inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ may be involved in osteoclast differentiation induced by ODF/RANKL. J. Cell. Biochem. 80:339–345, 2001.

Key words: osteoclast; differentiation; cyclin-dependent kinase inhibitor; p21 WAF1; p27 KIP1; ODF; RANKL

Osteoclasts are multinucleated cells with bone-resorbing activity that play a crucial role in bone remodeling [Roodman, 1997]. Osteoclast differentiation factor (ODF, also called RANKL/TRANCE/OPGL) is a newly discovered member of the tumor necrosis factor alpha (TNF-α) superfamily of cytokines that induces osteoclast differentiation from osteoclast precursor cells in the presence of macrophage colony-stimulating factor (M-CSF) [Suda et al., 1999; Takahashi et al., 1999]. The receptor for ODF/RANKL, RANK, transduces signals to activate NF-κB (nuclear factor kappa B) and JNK (c-Jun *N*-terminal kinase) pathways in osteoclast progenitors. TNF-receptor-associated factors (TRAFs) interact with RANK, and TRAF6 knockout mice become osteopetrotic, with defects caused by impaired osteoclast function [Lomaga et al., 1999; Wong et al., 1997]. However, the molecular mechanisms of osteoclast differentiation have yet to be elucidated.

Abbreviations used: ODF, osteoclast differentiation factor; RANKL, receptor activator of NF-κB ligand, M-CSF, macrophage colony-stimulating factor; OCL, osteoclast-like multinucleated cell; M-BMMΦ, M-CSF-dependent bone marrow macrophage; TNF-α, tumor necrosis factor alpha; TRAP, tartrate-resistant acid phosphatase; CDK, cyclin-dependent kinase.

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Differentiation of normal cells is regulated by a combination of stimulatory and inhibitory factors that can respond to external signals in a cell cycle-dependent manner. Increased expression of cyclin-dependent kinase (CDK) inhibitors is a general mechanism for the arrest of cell division [Sherr and Roberts, 1995]. Two families of CDK inhibitors, CIP/KIP ($p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$) and INK-4 (p15, p16, p18 and p19), have been previously described [Sherr and Roberts, 1995]. These CDK inhibitors control the progression of cells from one phase of their division cycle to the next. Recent studies have suggested that these CDK inhibitors may be involved in the control of differentiation, proliferation, and apoptosis of a number of cell types [Chellappan et al., 1998].

In this study, we found that ODF/RANKL stimulated $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression in osteoclast precursor cells. Furthermore, we found that disruption of the expression of the two CDK inhibitors by antisense oligonucleotides strongly inhibited osteoclast differentiation. These results suggest a crucial role of the two CDK inhibitors in osteoclast differentiation.

MATERIALS AND METHODS

Mice and Reagents

Female mice, ddY strain, were obtained from Japan SLC (Hamamatsu, Japan). Recombinant human soluble ODF/RANKL (sODF/RANKL) were kindly supplied by Kaken Pharmaceutical Co. (Kyoto, Japan). Recombinant mouse TNF-α, monoclonal anti-granulate macrophage colony-stimulating factor (GM-CSF), and anti-interferon gammma (IFN-γ) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Human M-CSF were obtained from Yoshitomi Pharmaceutical Co. (Osaka, Japan).

Oligonucleotides

Oligonucleotides tethered with 10-mer poly (G) stretch were used in the antisense experiments [Prasad et al., 1999]. Antisense oligonucleotides were synthesized based on the mouse $p21^{WAF1/CIP1}$and $p27^{KIP1}$ coding sequences according to previous reports [Coats et al., 1996; Poluha et al., 1996]. Antisense $p21^{WAF1/CIP1}$ is complementary to the region of the initiation codon (5′-ACATCACCAGGATTGGACAT-GGGGGGGGG-3′). Antisense $p27^{KIP1}$ (5′-ATCCTGGCTCTCCTGCGCC-GGGGGGGGGG-3′) is complementary to the target base pairs 301–320 of mouse $p27^{KIP1}$. Sense oligonucleotides (sense $p21^{WAF1/CIP1}$; 5′-ATGTCCAATCCTGGTGATGTGGGGGGGGGG-3′, and sense $p27^{KIP1}$; 5′-GGCGCAGGAGAGCCAGGAT-GGGGGGGGG-3′) targeted to the same sequences, were used as control oligonucleotides. All oligonucleotides were purchased from Greiner Japan Co. (Tokyo, Japan).

Mouse M-BMMΦ Cultures

Bone marrow cells were suspended in α minimum essential medium (αMEM; GIBCO BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; GIBCO BRL) and cultured in 48-well flat-bottomed culture plates (1.5×10^5 cells/0.3 ml per well) in the presence of M-CSF (100 ng/ml). After being cultured for 3 days, nonadherent cells were removed from the culture by pipette. More than 90% of the adherent cells (M-BMMΦs) were Mac-1-positive macrophages [Kobayashi et al., 2000]. M-BMMΦs were cultured with sODF/RANKL (100 ng/ml), M-CSF (50 ng/ml), and monoclonal anti-GM-CSF (2 μg/ml), and anti-IFN-γ (1 μg/ml) antibodies in the presence or absence of oligonucleotides. The two antibodies were involved in the culture medium, because GM-CSF and IFN-γ have been reported to inhibit osteoclast differentiation [Roodman, 1997; Takahashi et al., 1991; Udagawa et al., 1997]. The cells were replenished on Day 2 with fresh medium. On Day 4, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) as described previously [Suda et al., 1997]. TRAP-positive cells containing three or more nuclei were counted as OCLs. The results were expressed as the mean ± SD of four cultures. Statistical differences were analyzed by Student's *t*-test.

Western Blot Analysis

The cells were dissolved in 50 mM Tris–HCl containing 0.2% SDS (pH 6.8) and then centrifuged. The protein concentration of the supernatants was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA), and 50 μg of extracted proteins were separated in 12.5% SDS-polyacrylamide gels and then electroblotted on polyvinyldine fluoride membranes. Monoclonal anti-mouse $p21^{WAF1/CIP1}$ (F-5; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and

anti-mouse $p27^{KIP1}$ (F-8; Santa Cruz) antibodies were used for immunodetection of the $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ proteins, respectively. Antibodies for c-Fms and c-Src (Santa Cruz) were also used. Detection was performed using an ECL Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

Flow Cytometric Analysis

M-BMMΦs were suspended in hypotonic solution (0.1% Triton X-100, 1 mM Tris–HCl (pH 8.0), 3.4 mM sodium citrate, and 0.1% EDTA), stained with 5 μg/ml of propidium iodide, and then analysed by a FACScan (Beckton Dickinson, San Jose, CA, USA) [Perandones et al., 1993]. The population of cells in each cell-cycle phase was determined by CELLFIT software (Becton Dickinson).

RESULTS

ODF and TNF-α Transiently Increase the Protein Levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in M-BMMΦs

M-BMMΦs differentiated to TRAP-positive mononucleated cells by 24 h and then became multinucleated in the presence of sODF/RANKL (100 ng/ml) and M-CSF (50 ng/ml) (data not shown) [Kobayashi et al., 2000]. Multinucleated cells appeared after 48 h, and 30–40% became OCLs after 72 h. The protein levels of c-Fms and c-Src were elevated during the differentiation (Fig. 1). To examine the role of CDK inhibitors in osteoclast differentiation, protein levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ were assesed by Western blotting. The protein levels of these two CDK inhibitors increased after 24 h, but decreased after 48 h (Fig. 1). TNF-α is reported to induce differentiation of osteoclasts from M-BMMΦs [Kobayashi et al., 2000]. Treatment of M-BMMΦs with TNF-α (20 ng/ml) in the presence of M-CSF (50 ng/ml) caused upregulation of the protein levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ similarly (Fig. 2B). M-BMMΦs cultured in the presence of M-CSF alone showed limited levels of the two CDK inhibitors (Fig. 2). To test the effect of sODF/RANKL on the cell cycle progression of M-BMMΦ, M-BMMΦs cultured with M-CSF alone, and those cultured with both sODF/RANKL and M-CSF for 24 h, were analyzed for the cell population in each cell cycle phase by flow cytometry

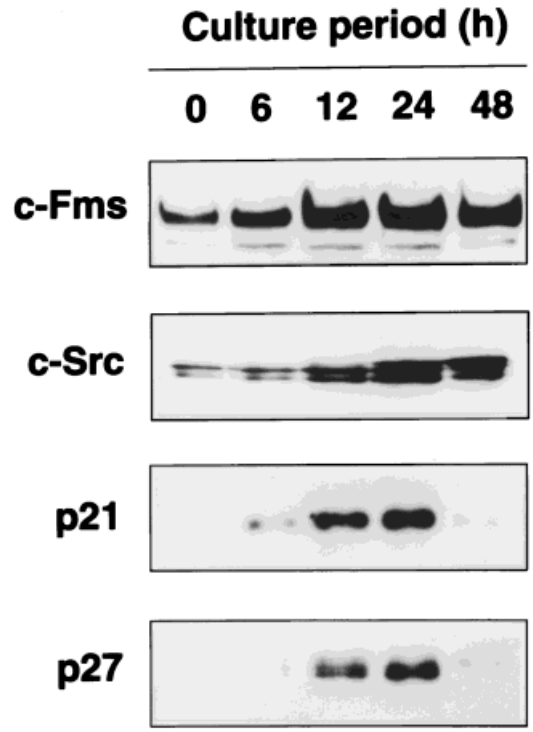

Fig. 1. Effect of sODF/RANKL and M-CSF on expression of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, c-Fms, and c-Src proteins. M-BMMΦs were treated for various periods (0–48 h) with sODF/RANKL (100 ng/ml) and M-CSF (50 ng/ml). Expression of each protein was analyzed by Western blotting. TRAP staining revealed that TRAP-positive multinucleated cells (OCLs) appeared in the cultures by 48 h, and 30–40% of the cells became OCLs by 72 h.

(Fig. 2C). sODF/RANKL decreased cells in the G1 phase from 83 to 62% and increased those in the S phase from 12 to 22%, showing that sODF/RANKL stimulated the cell cycle progression from the G1 phase to S phase. This stimulation of cell cycle progression was transient, and the population of the cells in the S phase decreased after 48 h.

Effects of Antisense Oligonucleotides to $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ on Osteoclast Differentiation

To examine whether the expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ is related to sODF/RANKL-induced OCL formation, we used antisense oligonucleotides specific for the two CDK inhibitors. As shown in Figure 3, the induction of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ proteins was largely abrogated in M-BMMΦs exposed to antisense oligonucleotides targeted at $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, respectively. Treatment of M-BMMΦs with $p21^{WAF1/CIP1}$ antisense alone (2 μM), or $p27^{KIP1}$ antisense alone

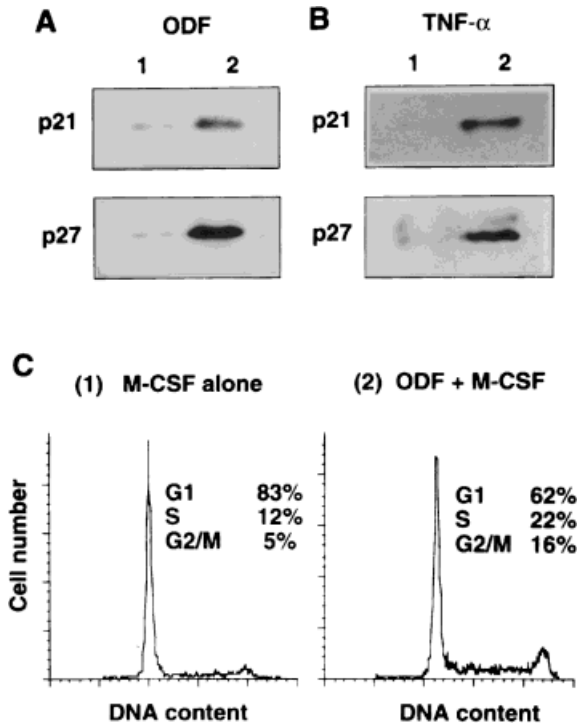

Fig. 2. Effect of sODF/RANKL (**A**) and TNF-α (**B**) on expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ proteins in M-BMMΦs. **Lane 1**: M-BMMΦs were cultured for 24 h in the presence of M-CSF (50 ng/ml) alone. **Lane 2**: M-BMMΦs were cultured in the presence of sODF/RANKL (100 ng/ml) and M-CSF 50 ng/ml) (**A**), or in the presence of TNF-α (20 ng/ml) and M-CSF (50 ng/ml) (**B**). Expression of each protein was analyzed by Western blotting. **C**: Cell-cycle analysis of M-BMMΦs cultured with M-CSF (50 ng/ml) alone (**1**), or with sODF/RANKL (100 ng/ml) and M-CSF (50 ng/ml) (**2**) for 24 h. DNA content was analyzed by flow cytometry.

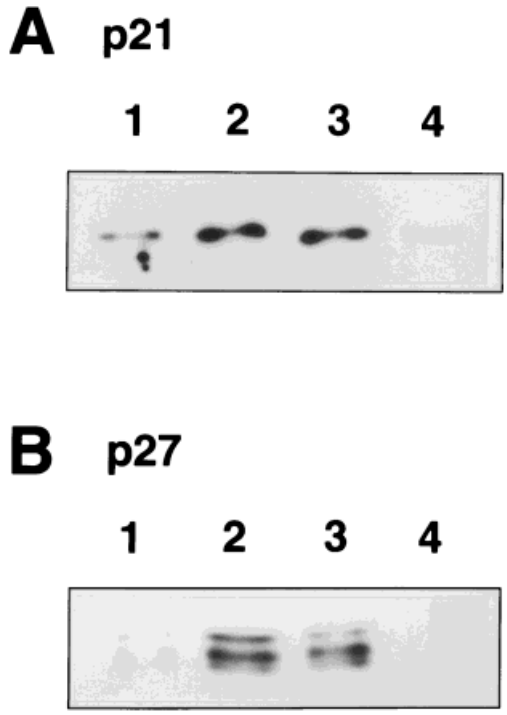

Fig. 3. Western blot analysis of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression in M-BMMΦs treated with antisense or sense oligonucleotides. **A**: M-BMMΦs were cultured in the presence of antisense or sense oligonucleotides (2 μM) targeted to $p21^{WAF1/CIP1}$. **B**: M-BMMΦs were cultured in the presence of antisense or sense oligonucleotides (2 μM) targeted to $p27^{KIP1}$. **Lane 1**: M-BMMΦs were cultured with M-CSF alone (50 ng/ml). **Lanes 2 to 4**: M-BMMΦs were cultured with sODF/RANKL (100 ng/ml) and M-CSF (50 ng/ml). **Lanes 1 and 2**: M-BMMΦs were cultured without oligonucleotide. **Lane 3**: M-BMMΦs were cultured with sense oligonucleotide. **Lane 4**: M-BMMΦs were cultured with antisense oligonucleotide.

(2 μM), resulted in a limited inhibition of sODF/RANKL-induced OCL formation (Fig. 4). However, a combination of the two antisense oligonucleotides (2 μM each) showed a strong inhibitory effect on OCL formation (Fig. 4). Dose-dependency of the inhibitory effect was observed in 0.5–3 μM of the mixture of the two antisense oligonucleotides (Fig. 5). Photomicroscopic observations of the cells after TRAP staining showed that most of the M-BMMΦs cultured in the presence of antisense oligonucleotides had differentiated to TRAP-positive cells but remained mononucleated (Fig. 6). M-BMMΦs cultured with M-CSF alone (Fig. 6A) were TRAP-negative and spindle-like in shape.

DISCUSSION

In the present study, we found that M-BMMΦs cultured in the presence of M-CSF expressed low levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. Stimulation of M-BMMΦs with sODF/RANKL or TNF-α triggered a transient increase of the two CDK inhibitors (Fig. 2). In contrast, c-Fms and c-Src proteins steadily increased after 48 h, suggesting that these two proteins, in particular c-Src, are associated with osteoclast differentiation. In fact, a previous report has demonstrated that c-Src is selectively induced during differentiation of monocytes to osteoclasts [Higuchi et al., 1999].

Since previous studies have suggested that CDK inhibitors may be a component of the cellular differentiation process, as well as cell cycle arrest, we further investigated the role of the two CDK inhibitors, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, with regard to differentiation of osteoclasts. Recent investigations have demonstrated that an antisense strategy is useful in

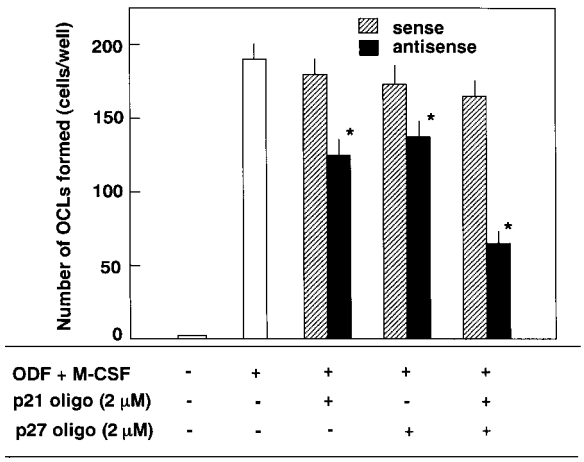

Fig. 4. Effects of antisense and sense oligonucleotides targeted to $p21^{WAF1/CIP1}$ or $p27^{KIP1}$ on OCL formation. M-BMMΦs were cultured for 4 days in the presence of sODF/RANKL (100 ng/ml) and M-CSF (50 ng/ml). Antisense or sense oligonucleotides (2 μM) were added during the entire culture period. The number of OCLs formed were counted after TRAP staining. Data are expressed as the means ± SD of quadruplicate cultures. $^*P < 0.01$ vs. control culture.

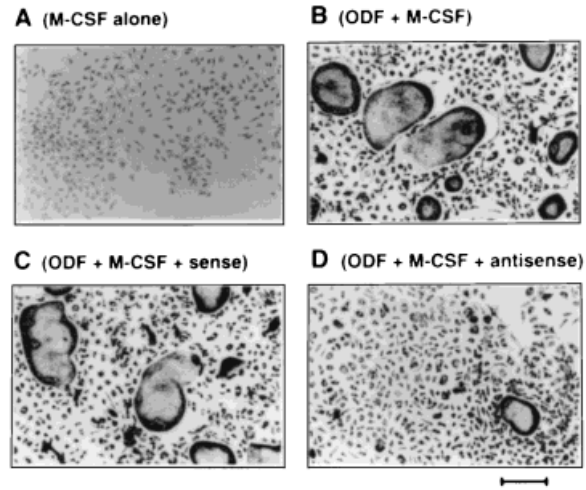

Fig. 6. Photomicroscopic observation of the effect of antisense oligonucleotides on OCL formation. **A**: M-BMMΦs were cultured for 4 days with M-CSF alone (50 ng/ml). **B**: M-BMMΦs were cultured with sODF/RANKL (100 ng/ml) and M-CSF (50 ng/ml). **C**: M-BMMΦs were cultured with sODF/RANKL (100 ng/ml), M-CSF (50 ng/ml), and mixed sense oligonucleotides $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (2 μM each) **D**: M-BMMΦs were cultured with sODF/RANKL (100 ng/ml), M-CSF (50 ng/ml), and mixed antisense oligonucleotides $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (2 μM each). M-BMMΦs treated with mixed antisense oligonucleotides did not differentiate to OCLs, but were TRAP-positive (**D**). Bar = 50 μm.

studying the functions of a wide variety of genes in osteoclast differentiation [Inui et al., 1997; Kukita et al., 1999; Laitala and Vaananen, 1994]; thus, we introduced antisense oligonucleotides, targeted at $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, into M-BMMΦs and compared them with M-BMMΦs treated with sense oligonucleotides. This antisense approach revealed that simultaneous treatment with both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ antisenses strongly suppressed the number of OCLs formed (Fig. 4), suggesting that these CDK inhibitors may be involved in the proccess of osteoclast differentiation. We noted that although the number of multinucleated OCLs was reduced, most mononucleated cells were TRAP-positive (Fig. 6), suggesting that the progression of differentiation still partially occurred in the cells treated with antisense oligonucleotides. Therefore, the down-regulation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ inhibited the formation of mature multinucleated osteoclasts, though partial differentiation also occurred. In a preliminary study, the INK-4 family of CDK inhibitors (p15, p16, p18 and p19) were not detected in M-BMMΦs treated with ODF/RANKL, when examined by Western blotting using polyclonal antibodies. Thus, we did not examine further the INK-4 family CDK inhibitors. In the present study, we emphasized the role of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in osteoclast differentiation; however,

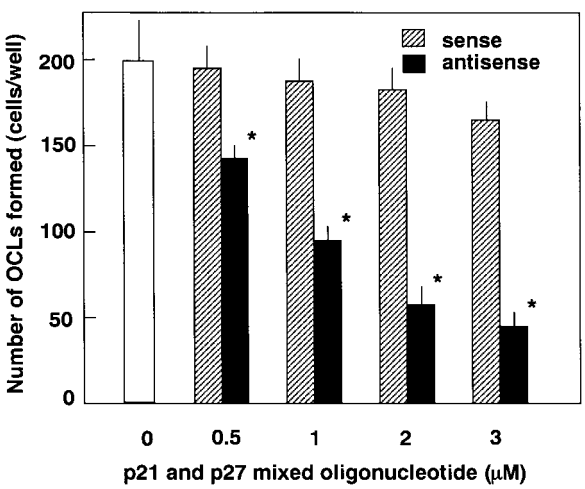

Fig. 5. Dose dependency of the inhibitory effect of mixed antisense oligonucleotides targeted to $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. M-BMMΦs were cultured in the presense of sODF/RANKL (100 ng/ml), M-CSF (50 ng/ml), mixed $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ antisense oligonucleotides of various concentrations (0–3 μM). The number of OCLs formed were counted after TRAP staining. Data are expressed as the means ± SD of quadruplicate cultures. $^*P < 0.01$ vs. control culture.

other cell cycle-associated proteins may also play critical roles.

Several groups have reported a possible role for $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in the differentiation of hematopoietic cells, as well as of myocytes and keratinocytes [Chellappan et al., 1998]. The upregulation of $p21^{WAF1/CIP1}$ is demonstrated in human bone marrow cells exposed to granulocyte colony-stimulating factor (G-CSF), in M1 myeloblasts exposed to interleukin-6 (IL-6), and in HL-60 leukemia cells and U937 myelomonocytic cells in response to chemical agents [Steinman et al., 1994]. Exposure of HL-60 cells to phorbol ester (PMA) or 1α,25-dihydroxyvitamin D_3 (1α,25 $(OH)_2D_3$) induces differentiation along the monocyte/macrophage pathway with a transient overexpression of $p21^{WAF1/CIP1}$ [Schwaller et al., 1995]. Furthermore, antisense-expressing HL-60 cells treated with PMA display reduced G1 phase arrest and expression of the monocytic maturation marker, suggesting that down-regulation of $p21^{WAF1/CIP1}$ in HL-60 cells interferes with PMA-related G1 arrest and differentiation [Freemerman et al., 1997]. The differentiation of U937 myelomonocytic cells induced by 1α, $25(OH)_2$ D_3 is associated with the upregulation of $p21^{WAF1/CIP1}$ protein, and the transient overexpression of $p21^{WAF1/CIP1}$ and/or $p27^{KIP1}$ in U937 cells results in the differentiation of the monocyte/macrophage phenotype [Liu et al., 1996]. Similar results have been obtained in UF-1 acute promyelocytic leukemia cells [Muto et al., 1999].

In the present study, we found that treatment with antisense $p21^{WAF1/CIP1}$ alone, or antisense $p27^{KIP1}$ alone, showed little effect, while treatment with a mixture of the two significantly inhibited OCL formation. Therefore, our results suggest some redundancy in the effect of the two CDK inhibitors on cellular differentiation. It may be possible that the two CDK inhibitors have an overlapping function in the control of cellular differentiation. In addition, as mentioned above, most studies using leukemia cell lines have shown that induction of myelocytic/monocytic differentiation is associated with accumulation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ along with a concomitant exit from cell-cycling, emphasizing the importance of these CDK inhibitors in cell differentiation. However, a recent study using primary human $CD34^+$ cells has demonstrated that $p21^{WAF1/CIP1}$ levels peaked concurrent with cellular proliferation and then decreased with terminal myelocytic differentiation [Yaroslavskiy et al., 1999]. We found that stimulation of M-BMMΦs with sODF/RANKL induced a transient cell cycle progression from the G1 phase into the S phase with simultaneous upregulation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Fig. 2). These results suggest that $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ may regulate cell differentiation without exerting the CDK inhibitory activity. In this regard, LaBaer et al. [1997] have demonstrated that $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ promote the association of CDK4 and the D-type cyclins, suggesting that in addition to their roles as inhibitors, the CIP/KIP family of CDK inhibitors may also have roles of adaptor proteins that assemble and program kinase complexes for specific functions. Precisely how these two CDK inhibitors are linked to the osteoclast differentiation process remains unclear and requires additional investigation.

In summary, ODF/RANKL transiently induced $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ during differentiation of M-BMMΦs to OCLs in the presence of M-CSF. The disruption of expression of these two CDK inhibitors by antisense oligonucleotides resulted in a significant inhibition of OCL formation. These results suggest that $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ may play an important role in osteoclast differentiation by regulating cell cycle progression.

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