Resveratrol Inhibits Myeloma Cell Growth, Prevents Osteoclast Formation, and Promotes Osteoblast Differentiation

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Abstract

Multiple myeloma is characterized by the accumulation of clonal malignant plasma cells in the bone marrow, which stimulates bone destruction by osteoclasts and reduces bone formation by osteoblasts. In turn, the changed bone microenvironment sustains survival of myeloma cells. Therefore, a challenge for treating multiple myeloma is discovering drugs targeting not only myeloma cells but also osteoclasts and osteoblasts. Because resveratrol (trans-3,4,5-trihydroxystilbene) is reported to display antitumor activities on a variety of human cancer cells, we investigated the effects of this natural compound on myeloma and bone cells. We found that resveratrol reduces dose-dependently the growth of myeloma cell lines (RPMI 8226 and OPM-2) by a mechanism involving cell apoptosis. In cultures of human primary monocytes, resveratrol inhibits dose-dependently receptor activator of nuclear factor-κB (NF-κB) ligand–induced formation of tartrate-resistant acid phosphatase (TRACP)–positive multinucleated cells, TRACP activity in the medium, up-regulation of cathepsin K gene expression, and bone resorption. These inhibitions are associated with a down-regulation of RANK expression at both mRNA and cell surface protein levels and a decrease of NFATc1 stimulation and NF-κB nuclear translocation, whereas the gene expression of c-fms, CD14, and CD11a is up-regulated. Finally, resveratrol promotes dose-dependently the expression of osteoblast markers like osteocalcin and osteopontin in human bone marrow mesenchymal stem cells (hMSC-TERT) and stimulates their response to 1,25(OH)2 vitamin D3 [1,25(OH)2D3]. Moreover, resveratrol up-regulates dose-dependently the expression of 1,25(OH)2D3 nuclear receptor. Taken together, these results suggest that resveratrol or its derivatives deserve attention as potential drugs for treating multiple myeloma. (Cancer Res 2005; 65(21): 9943-52)

Introduction

Multiple myeloma is an incurable disease characterized primarily by the accumulation of plasma cells in the bone marrow (for a review, see ref. 1). This accumulation alters dramatically the activity of neighboring cells, which leads to a fatal disorder associated with severe bone complications. Current treatments, such as chemotherapy, radiotherapy, bisphosphonates, and autologous peripheral stem cell transplantation, allow a median survival of ∼5 to 7 years. Patients who respond to treatments eventually relapse and become resistant. There is thus an urgent need for more efficient treatment and for an identification of the pathways leading to the major pathologic events associated to myeloma.

Interactions of myeloma cells with the bone marrow microenvironment seem to be a major driving force for disease development (2). Through interactions with bone marrow stromal cells, myeloma cells stimulate production of survival/proliferation factors as well as osteoclast-activating cytokines, such as interleukin (IL)-6, IL-1, macrophage inflammatory protein-1α, tumor necrosis factor-α, macrophage colony-stimulating factor (M-CSF; refs. 2, 3), and the tumor necrosis factor family member receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL; ref. 4). In contrast, osteoprotegerin, the decoy receptor of RANKL, is down-regulated (4). M-CSF, RANKL, and osteoprotegerin play key functions in osteoclastogenesis: M-CSF acts as a growth and survival factor for the common monocytic precursor shared between macrophages and osteoclasts (5), and RANKL stimulates osteoclast differentiation (6), activation (7), and migration (8). In multiple myeloma, the high RANKL/osteoprotegerin ratio leads to an increase of the number of osteoclasts and enhanced bone resorption (9). In turn, increased osteoclastic activity promotes myeloma cell proliferation and survival (10, 11). Enhancement of bone resorption is normally associated with increased bone formation, allowing rebuilding the bone that was removed, but a striking feature of myeloma is that bone lesions are not repaired, because osteoblast function is deficient (12). Recent studies suggest that this deficiency is due to the production of DKK1 by myeloma cells, a soluble factor that can antagonize the Wnt signaling pathway during osteoblast differentiation (13).

Resveratrol (trans-3,4,5-trihydroxystilbene) is a natural compound present in various plant species and relatively abundant in wine (up to 25 μmol/L; ref. 14). Resveratrol is raising a lot of interest because of a series of observations suggesting that it has cancer chemopreventive properties (for a review, see ref. 15). Resveratrol was found to inhibit tumorigenesis in rodent cancer models (16); to inhibit proliferation and induce apoptosis in several human cancer cells, including B-cell malignancies (17); and to affect a series of critical events associated with tumor initiation and progression, including up-regulation of p53 and p21 levels, induction of nitric oxide, inhibition of cyclooxygenase, protection against reactive oxygen intermediates, down-regulation of survival factors, and down-regulation of proteinases (18, 19). Effects of resveratrol have been documented in a wide variety of cell types, including macrophages, polymorphonuclear cells, platelets, osteoblasts, neurons, and adrenal cells, and it was suggested that resveratrol has therapeutic potential for allergy and cardiovascular and neurologic disorders (19, 20). In the present study, we have investigated the effect of resveratrol on...
three major events associated with multiple myeloma and found that resveratrol inhibits myeloma cell growth, prevents osteoclastogenesis and bone resorption, and promotes osteoblast differentiation.

Materials and Methods

Reagents and cell lines. Recombinant human M-CSF (rhM-CSF) and soluble recombinant RANKL (rhRANKL) were obtained from Peprotech (London, United Kingdom), resveratrol from Sigma (Brondby, Denmark), and 1,25(OH)2 vitamin D3 (1,25(OH)2D3) from Leo Pharma (Ballerup, Denmark). Human myeloma cell lines RPMI 8266 and OPM-2 were obtained from the cell line bank DSMZ (Braunschweig, Germany) and grown in RPMI 1640 (Life Technologies/Invitrogen, Tastrup, Denmark) supplemented with 10% FCS. hMSC-TERT is a well-characterized cell line established from normal human bone marrow mesenchymal stem cells (hMSC) that stably expresses human telomerase reverse transcriptase gene (TERT) and thus its name hMSC-TERT (21). All cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

Cell growth assay. Myeloma cell lines were cultured in microtiter plates in serum-free medium for 3 days at 37°C. The number of cells per well was assessed with 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) kit (Boehringer Mannheim, Roche Molecular Biochemicals, Hvidore, Denmark) at different time points according to the manufacturer's instruction. XTT was proven comparable with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide but is water soluble.

In vitro osteoclast differentiation and resorption assays. Human osteoclast precursors were prepared from the blood of healthy donors provided anonymously by the blood bank of Vejle Hospital. Peripheral blood mononuclear cells (PBMC) were first separated by centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Hillerod, Denmark); then, monocytes were isolated by magnetic cell sorting according to the manufacturer's instruction. Briefly, PBMCs resuspended in 2% FCS in PBS were incubated first with biotinylated anti-human CD14 goat antibody (R&D Systems, Abingdon, United Kingdom) for 15 minutes at 4°C and then with Magnectel streptavidin ferrofluid (R&D Systems) for 15 minutes at 4°C. After incubation, tagged cells were retained by a magnetic device, whereas negative cells were discarded by extensive washes in 2% FCS in PBS. Finally, sorted cells were cultured in α-MEM (Life Technologies/Invitrogen) supplemented with 10% FCS (full medium) and 30 ng/ml rhM-CSF for 3 days at 37°C. For osteoclast differentiation, adherent monocytes were trypsinized and reseded at defined cell densities in cell culture plates in full medium supplemented with 30 ng/ml rhM-CSF. The day after, supernatants were replaced by osteoclast differentiation medium containing both 30 ng/ml rhM-CSF and 30 ng/ml rhRANKL with different concentrations of resveratrol. Cells were cultured for 6 to 7 days with replacement of medium every second day.

To monitor osteoclast differentiation, both tartrate-resistant acid phosphatase (TRACP) activity and the number of TRACP multinucleated cells were examined. Conditioned media were collected at different times, and TRACP activity was colorimetrically measured by adding p-nitrophenyl phosphate (Sigma) in the presence of 25 mmol/L sodium tartrate (pH 5.5; absorbance, 405-645 nm). At the end of the experiment, cells were fixed with 4% formaldehyde and stained for TRACP using the Leukocyte Acid Phosphatase kit (Sigma). The number of TRACP-positive multinucleated cells was counted using Metavue software (Universal Imaging Corp., Brock & Michelson, Birkerød, Denmark) after taking randomly pictures in different areas of each well.

For resorption assay, cells were handled like described above but seeded on dentine discs (Immunodiagnostic System Ltd., Boldon, United Kingdom). Cells were cultured at 37°C up to 12 days and medium was replaced every second day. At the end, cells were scraped off in water containing 1% Triton X-100 and resorption pits were detected using peroxidase-conjugated wheat germ agglutinin (WGA)-lectin according to Selander et al. (22). 3,3′-Diaminobenzidine-based staining of peroxidase activity (Sigma) was enhanced with ammonium nickel sulfate (Sigma), which gives a black precipitate. The resorbed areas on the dentine discs were measured with Metavue software, and results were presented as the total resorbed area in percentage of the whole disc surface.

Osteoblast differentiation. hMSC-TERT cells were grown in phenol red-free MEM supplemented with 10% FCS. At 60% to 70% cell confluence, cells were incubated with fresh complete medium containing different concentrations of resveratrol in the absence or presence of 10−9 mol/L 1,25(OH)2D3.

Real-time PCR analysis of osteoclast cultures. Cultures of monocytes taken at different time points were lysed and homogenized by centrifugation through QIAShredder columns (Qiagen, Hilden, Germany). Total RNA was purified from the homogenate using the RNaseasy Mini kit (Qiagen) and then reverse transcribed into single-stranded cDNA using the Iscript cDNA synthesis kit (Bio-Rad Laboratories, Herts, United Kingdom) according to the manufacturer's instructions.

Relative gene expression was analyzed by Taqman-based quantitative real-time PCR as described previously (23) with a ABI PRISM 7900HT sequence detection system and software (PE Applied Biosystems, Inc., Naerum, Denmark). Primers and probes for the Taqman system were obtained as an Assay-on-Demand (PE Applied Biosystems) or designed to meet the specific criteria by using Primer Express 2.0 software (PE Applied Biosystems) and subsequently synthesized by MWG (Risskov, Denmark). The Assays-on-Demand, which contained MGP probes labeled with the fluorescence reporter FAM, were used for the analysis of cathepsin K (Hs00355865_m1), β-actin (4337362F), RANK (Hs00187199_m1), c-fms (Hs00234617_m1), CD14 (hs0169122_q1), and CD11a (Hs00158218_m1). NFATc1 primers and probe were designed according to the NFATc1 Genbank accession no. U90815. NFATc1-fp TGGAGATCCCTGCATCGA, NFATc1-rp CATCGTGAAACACCTGTATTGT and NFATc1-tp: ATACCT-TGTGCTTGGGGCTGTCAC.

After PCR reactions in standard conditions, Ct values were converted into relative gene expression according to an arbitrary standard curve and normalized to the relative gene expression of an endogenous control using the relative standard curve method according to the PE Applied Biosystems instructions. When testing different endogenous control genes using the primers/probe sets described previously by Beillard et al. (24), β-actin was found to be appropriate, showing no difference between different culture conditions.

Real-time PCR analysis of osteoblastic markers. Total RNA was isolated from cultured hMSC-TERT cells using single-step method with TRIzol according to the manufacturer's instructions (Invitrogen A/S, Tastrup, Denmark). The integrity and purity of total RNA was verified by spectrophotometry and gel electrophoresis on 0.8% Seakem agarose (BMA, Hellerup, Denmark). Total RNA (5 μg) was then reverse transcribed into DNA using RevertAid H Minus first-strand cDNA synthesis kit (Fermentas, Copenhagen, Denmark) according to the manufacturer's instructions. Quantitative PCR was done with an iCycler IQ detection system (Bio-Rad Laboratories) by using SYBR Green I as a double-strand DNA-specific binding dye. Thermocycling was done in a final volume of 20 μL containing 3 μL cDNA sample (diluted 1:30), 20 pmol of each primer, 2 mmol/L MgCl2, 0.2 mmol/L deoxynucleotide triphosphate mixture, 1× Taq reaction buffer, 0.5 units HotStart Taq DNA polymerase (Qiagen), 0.5 μL of 1:3,000 dilution of SYBR Green I (Roche Molecular Biochemicals), and 10 mmol/L fluorescein calibration dye (Bio-Rad Laboratories). The gene expression analysis of the target gene and the endogenous control β-actin was done in separate tubes using the primers used previously by Abdallah et al. (25). After initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute were carried out. Each reaction was run in triplicates and the fluorescence data were selected for collection at the end of the extension step in every cycle.

To ensure specific amplification, a melting curve was made for each PCR reaction by increasing the temperature from 60°C to 95°C with temperature increment rate of 0.5°C/10 s.

Fold induction and expression levels for each target gene and endogenous control were calculated using the comparative Ct method [ΔΔCt] formula [Peirkin-Elmer (Hvidore, Denmark) User Bulletin No. 2]. Data were analyzed using the optical system software version 3.1 (Bio-Rad Laboratories).
Flow cytometry. Flow cytometry analyses were done on a FACSCalibur (BD Biosciences, Bredby, Denmark) using CellQuest Pro software. For detection of cell surface RANK, monocytes were washed in PBS and detached with 5 mmol/L EDTA in bovine serum albumin (BSA)/PBS. Resuspended cells were incubated with a biotinylated goat anti-RANK polyclonal antibody (R&D Systems) or control antibody (biotinylated normal goat immunoglobulin; R&D Systems) in BSA/PBS for 30 minutes at 4°C, washed several times, and then incubated with FITC-conjugated streptavidin (DAKO, Glostrup, Denmark) in BSA/PBS for 30 minutes at 4°C. After several washes, cells were fixed in 1% paraformaldehyde.

For apoptosis analysis, myeloma cells were washed twice in PBS and then subjected either to FITC-conjugated Annexin V staining according to the manufacturer’s instruction (BD Biosciences) or propidium iodide (PI) staining for measuring cell DNA content. For the latter method, cells were fixed in 70% ethanol at -20°C overnight and then incubated with PBS containing 100 µg/mL RNAse and 40 µg/mL PI for 30 minutes at room temperature. The data were measured on a linear scale and apoptotic cells were evaluated as a sub-G1 hypodiploid cell population.

In situ detection of nuclear factor-κB nuclear translocation. Monocytes were incubated with 100 µmol/L resveratrol for 1 hour or 3 days in full medium containing 30 ng/mL rM-CSF and then exposed to 100 ng/mL rhRANKL for 30 minutes at 37°C. Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature followed by a second fixation/permeabilization with cold methanol for 15 minutes at -20°C. Cells were incubated with a mouse monoclonal anti-p65 antibody (Santa Cruz Biotechnology, AH Diagnostics, Aarhus, Denmark) in BSA/PBS overnight at 4°C, washed several times in PBS, and then incubated with a secondary Alexa Fluor-568 goat anti-mouse IgG antibody (Molecular Probes, Invitrogen, Taastrup, Denmark) in BSA/PBS for 1 hour at room temperature. After several washes, the nucleus of cells was counterstained with Hoechst 33258 (Sigma). Resuspended cells were incubated with a biotinylated goat anti-RANK polyclonal antibody (R&D Systems) or control antibody (biotinylated normal goat immunoglobulin; R&D Systems) in BSA/PBS for 1 hour at room temperature. After several washes, the nucleus of cells was counterstained with Hoechst 33258 (Sigma). Cells showing a bright staining for p65 in the nucleus were scored, and the results were presented as the number of cells with NF-κB nuclear translocation in percentage of the total number of cells scrutinized.

Statistics. Statistical differences between groups were analyzed using one-way ANOVA followed by Dunnett’s or Bonferroni’s post-test. Data presented are from a single experiment and representative for at least three independent experiments.

Results
Resveratrol inhibits growth of myeloma cells and induces apoptosis. Two myeloma cell lines OPM-2 and RPMI 8226 were maintained in culture for 2 or 3 days in the presence of increasing concentrations of resveratrol. In absence of resveratrol, RPMI 8226 and OPM-2 cells showed a high proliferation rate reflected by a 3- to 4-fold increase of the total cell metabolic activity after 2 to 3 days of culture (Fig. 1A and B). Addition of resveratrol decreased dose-dependently the number of living myeloma cells the second day and onwards. The effect was significant above 25 µmol/L resveratrol. After a 3-day culture in the presence of 100 µmol/L resveratrol, the net metabolic activity was equal to or lower than at day 0, showing that high resveratrol concentrations can prevent completely myeloma cell growth.

To understand further how resveratrol mediated myeloma cell growth inhibition, we examined the incidence of apoptosis in our cell cultures. Treating OPM-2 cells for 2 days with 25 or 100 µmol/L resveratrol strongly increased the population of Annexin V–positive (Fig. 1C) and sub-G1 hypodiploid cells (Fig. 1D), two characteristics of cells undergoing apoptosis.

Taken together, our results show that resveratrol reduces significantly myeloma cell growth by a mechanism involving cell apoptosis.

Resveratrol prevents receptor activator of nuclear factor-κB ligand–induced osteoclast differentiation. Because myeloma cells stimulate the recruitment of osteoclasts, we investigated whether resveratrol might affect osteoclast differentiation. Therefore, human PBMC-isolated monocytes were cultured with 30 ng/mL M-CSF and 30 ng/mL RANKL for 3 and 7 days. When compared with cultures with M-CSF alone, the presence of RANKL induced numerous TRACP multinucleated cells (Fig. 2A), increased strongly TRACP activity in the medium (Fig. 2B), and stimulated the expression of cathepsin K mRNA (Fig. 2C), all hallmarks of osteoclast differentiation.
osteoclast differentiation. Addition of resveratrol to the cultures inhibited the RANKL-induced formation of multinucleated cells in a dose-dependent manner (significant at ≥12.5 μmol/L) with a complete abrogation at 100 μmol/L resveratrol (Fig. 2A as well as Fig. 3B, 6-day treatment). This resveratrol effect was concomitant with a strong decrease of TRACP activity in the culture medium (Fig. 2B) and of cathepsin K expression (Fig. 2C). These decreases in TRAP activity and cathepsin K expression were both dependent on the same range of resveratrol concentrations with full inhibition of RANKL-induced up-regulation at 100 μmol/L.

To examine whether resveratrol affects the growth/survival of monocytes during their differentiation into osteoclasts, we monitored the number of cells with a XTT assay. After 3 days of culture in the presence of M-CSF and RANKL, no significant difference was found between cell cultures without or with resveratrol at concentrations ranging from 12.5 to 100 μmol/L (data not shown). At day 7, resveratrol concentrations of 50 and 100 μmol/L brought the cell numbers down to 68.10 ± 5.13% and 68.10 ± 6.19%, respectively (mean ± SD; control, 100 ± 3.58%; n = 4). Cells incubated for 7 days with M-CSF plus RANKL and 100 μmol/L resveratrol exhibited a striking elongated morphology, which is not all typical of cells undergoing apoptosis. Furthermore, DNA staining with Hoechst dye did not reveal any increase of DNA condensation/fragmentation in these cells compared with cells cultured in the absence of resveratrol (data not shown).

To determine which stage of osteoclast differentiation is impaired by resveratrol, we followed RANKL-induced TRACP activity and number of TRACP multinucleated cells in cultures treated with resveratrol either from days 0 to 3 or from days 3 to 6, which is the period when cells start fusing. We found that resveratrol was inhibitory both during the RANKL-mediated early activation (Fig. 3, days 0-3) and during the cell fusion period (days 3-6) but to a lesser extent than in the full-course inhibition (days 0-6). Although an effect of resveratrol on osteoclast differentiation was visible in all treatment periods, the elongated cell morphology observed at 100 μmol/L resveratrol was lost 3 days after resveratrol withdrawal (Fig. 3C, resveratrol during days 0-3 versus days 3-6 or days 0-6), showing a reversible effect on cell morphology.

Finally, the effect of resveratrol on osteoclast resorption activity was investigated by culturing monocytes on dentine discs in the presence of M-CSF and RANKL (Fig. 4). Resveratrol inhibited dose-dependently RANKL-induced pit formation. Concentrations of ≥25 μmol/L were close to fully inhibiting (Fig. 4B).

We thus conclude that resveratrol is a strong inhibitor of osteoclast differentiation and resorption.

**Resveratrol prevents receptor activator of nuclear factor-κB ligand signaling and favors expression of myeloid markers.**

To determine whether resveratrol acts on RANKL signaling, we examined the gene expression of NFATc1, a key transcription factor regulated by RANKL and reported to be essential for the osteoclast differentiation program (26, 27). As expected, stimulation of monocytes with RANKL significantly increased NFATc1 mRNA level in a time-dependent way (Fig. 5A). This up-regulation was inhibited by resveratrol dose-dependently, at the same concentrations as those inhibiting osteoclast differentiation, and 100 μmol/L inhibited completely both events.

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**Figure 2.** Resveratrol inhibits RANKL-induced osteoclast differentiation. PBMC-isolated monocytes were cultured with 30 ng/mL rmM-CSF with or without 30 ng/mL hRANKL for 7 days. In conditions with RANKL, cells were treated for the entire culture period with 0, 1, 25, 50, or 100 μmol/L resveratrol. A, morphology of the cells at the end of the cell cultures after TRACP staining: culture with M-CSF alone or M-CSF + RANKL in the presence of 25 or 100 μmol/L resveratrol. B, relative level of TRACP activity detected in conditioned media after culture for 3 and 7 days. C, relative gene expression of cathepsin K measured by quantitative real-time PCR in cells cultured for 3 or 7 days with M-CSF or M-CSF + RANKL in the absence or presence of 100 μmol/L resveratrol (left) or in 7-day culture treated with M-CSF or M-CSF + RANKL in the presence of increasing concentrations of resveratrol (right). The levels of cathepsin K mRNA were normalized to β-actin mRNA and are represented as induction fold compared with cells cultured with M-CSF alone for 3 days (left) or 7 days (right). Columns, mean of four cultures; bars, SD. *, P < 0.01, statistically significant resveratrol effects versus cultures with M-CSF + RANKL. C, relative gene expression of cathepsin K measured by quantitative real-time PCR in cells cultured for 3 or 7 days with M-CSF or M-CSF + RANKL in the absence or presence of 100 μmol/L resveratrol (left) or in 7-day culture treated with M-CSF or M-CSF + RANKL in the presence of increasing concentrations of resveratrol (right). The levels of cathepsin K mRNA were normalized to β-actin mRNA and are represented as induction fold compared with cells cultured with M-CSF alone for 3 days (left) or 7 days (right). Columns, mean of four cultures; bars, SD. *, P < 0.01.
Because RANK is obligatory for transducing RANKL signaling into the cells, we examined the effect of resveratrol on its expression. Addition of 100 μmol/L resveratrol to cells in their differentiating medium resulted in a drop of RANK mRNA (Fig. 5B). After 3 days, the decrease reached already 50%, and RANK protein expression on the cell surface was also much lower (Fig. 5B).

Interestingly, pretreating the cells with 100 μmol/L resveratrol for 3 days reduced the stimulation by RANKL by >50% (9.7 ± 1.9%). On the other hand, pretreating the cells for only 1 hour with 100 μmol/L resveratrol had no effect on the level of RANKL-induced NF-κB translocation (22.8 ± 5.2%). It should be noticed that resveratrol (3 days or 1 hour) did not affect the NF-κB signal in the absence of RANKL (data not shown).

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Because RANKL-induced osteoclastogenesis involves the activation of NF-κB, we examined whether resveratrol affects this signaling pathway in our cultures. The level of NF-κB activation can be efficiently assessed in osteoclasts by visualizing its translocation from the cytosol to the nucleus after being released from IκB on RANKL stimulation (28). NF-κB nuclear translocation is transient and is detected within 30 minutes after RANKL exposure, whereas 1 hour later the factor becomes again predominantly localized in the cytoplasm. Similarly, stimulation of our monocytes with RANKL for 30 minutes strongly induced NF-κB nuclear translocation (21.7 ± 2.5%) compared with cultures without RANKL (0.5 ± 0.2%; Fig. 5C).

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Finally, to characterize further the phenotype of the monocytic cells treated with resveratrol in osteoclast differentiating medium, we examined the expression pattern of several genes related to monocyte lineage (Fig. 5D). In contrast with its effect on RANK and NFATc1, resveratrol up-regulated strongly the mRNA level of c-fms (M-CSF receptor) and CD14 (lipopolysaccharide/LPS-binding protein receptor), two genes that were not regulated during RANKL-induced osteoclast differentiation. In addition, we found that resveratrol also increased the level of CD11a mRNA (αL subunit of LFA1 integrin) that was significantly down-regulated during RANKL-induced osteoclast differentiation.

Taken together, our results show that resveratrol inhibits osteoclast differentiation at least in part by impairing RANKL signaling in osteoclast precursors and that resveratrol-treated cells exhibit a more pronounced expression of monocyte lineage markers.

Resveratrol stimulates vitamin D receptor expression in bone marrow osteoblast precursors and synergizes with 1,25(OH)2 vitamin D3 to induce expression of osteocalcin and osteopontin genes. Myeloma cells inhibit osteoblast differentiation (12, 13). We thus investigated whether resveratrol could favor bone formation by stimulating osteoblast differentiation. Therefore, we analyzed the effect of resveratrol on a human bone marrow mesenchymal stem cell line, hMSC-TERT. These cells have kept the potential to differentiate in vivo after s.c. implantation in immunodeficient-mice (21) or in vitro when treated for 3 days in cultures with the osteogenic hormone, 1,25(OH)2D3 (25). We first cultured hMSC-TERT cells for 3 days in the presence of increasing concentrations of resveratrol and analyzed by quantitative PCR the relative gene expression of two late markers of osteoblast differentiation, osteocalcin and osteopontin genes.

Figure 5. Resveratrol impairs RANK and NFATc1 expression as well as NF-κB translocation while up-regulating c-fms, CD14, and CD11a expression. A, relative gene expression of NFATc1 measured by quantitative real-time PCR in monocytes cultured for 3 or 7 days with M-CSF or M-CSF + RANKL in the absence or presence of 100 μM resveratrol (left) or in 7-day cultures treated with M-CSF or M-CSF + RANKL in the presence of increasing concentrations of resveratrol (right). Levels of mRNA expression were normalized to β-actin and are represented as induction fold compared with cells cultured with M-CSF alone for 3 days (left) and 7 days (right). Columns, mean of four cultures; bars, SD. *, P < 0.001. B, left, relative gene expression of RANK in monocytes cultured for 3 or 7 days with M-CSF or M-CSF + RANKL in the absence or presence of 100 μM resveratrol. Levels of mRNA expression were normalized to β-actin and are represented as induction fold compared with cells cultured with M-CSF alone at day 3. Columns, mean of four cultures; bars, SD. *, P < 0.001. Right, level of RANK expression on the cell surface analyzed by flow cytometry. Monocytes were cultured for 3 days with M-CSF + RANKL in the absence or presence of 100 μM resveratrol. Thin line, isotypic control; filled histogram, untreated cells; thick line, cells treated with resveratrol. C, detection by immunofluorescence of RANKL-induced NF-κB nuclear translocation in cultures of monocytes treated with 100 μM resveratrol for 1 hour or 3 days. In absence of RANKL, most of the cells showed a cytoplasmic localization of NF-κB (W/O), whereas after 30 minutes RANKL exposure ~ 20% cells displayed a strong nuclear staining (With RANKL). Asterisks, cell nuclei. Graphs show the number of cells with NF-κB nuclear translocation in percentage of the total number of scrutinized cells (counting >400 cells per culture). Columns, mean of four cultures; bars, SD. *, P < 0.01. D, relative gene expression of c-fms, CD14, and CD11a in cells cultured for 3 or 7 days with M-CSF or M-CSF + RANKL in the absence or presence of 100 μM resveratrol. Levels of mRNA expression were normalized to β-actin and are represented as induction fold compared with cells cultured with M-CSF alone at day 3. Columns, mean of four cultures; bars, SD. *, P < 0.001.
osteopontin. Resveratrol alone stimulated the mRNA expression of osteocalcin and osteopontin in a dose-dependent manner with a statistically significant effect above 25 μmol/L (Fig. 6A, inset, and B). At 100 μmol/L, resveratrol stimulated osteocalcin and osteopontin mRNA expression 3- and 18-fold, respectively. If osteoblast differentiation was induced with a suboptimal concentration of 1.25(OH)2D3 (10⁻⁹ mol/L), the effect of resveratrol was much greater and still dose dependent (Fig. 6A and B); whereas 10⁻⁹ mol/L 1.25(OH)2D3 alone induced a 36- and 3-fold increase of osteocalcin and osteopontin mRNA expression, respectively. Costimulation with 100 μmol/L resveratrol raised the level of osteocalcin and osteopontin mRNA up to 207- and 45-fold, respectively. Because 1.25(OH)2D3 is known to activate directly transcriptional promoters of osteocalcin and osteopontin genes (29), we examined the relative gene expression of the nuclear receptor of 1.25(OH)2D3 [vitamin D receptor (VDR); Fig. 6C]. Interestingly, we found that VDR mRNA expression was also up-regulated by resveratrol in a dose-dependent manner and that resveratrol displayed a synergistic effect with 1.25(OH)2D3 at ≥25 μmol/L. In summary, our results show that resveratrol stimulates the expression of two osteoblastic markers, osteocalcin and osteopontin, in bone marrow mesenchymal stem cells and has a synergistic effect with 1.25(OH)2D3 on their expression most likely because resveratrol also up-regulates VDR gene expression.

Discussion

In multiple myeloma, the accumulation of malignant plasma cells in the bone marrow cavity causes profound changes in their microenvironment. Serious consequences are increased osteoclast formation and bone resorption and absence of osteoblast recruitment and bone formation (30). Furthermore, bone resorption promotes myeloma cell growth (10, 11). The overall consequence is a vicious cycle with accelerated bone loss and progression of myeloma. The ideal treatment of multiple myeloma patients should thus aim at preventing myeloma cell growth, inhibiting bone resorption, and promoting bone formation. Our study shows that resveratrol exhibits precisely this set of properties: it prevents growth of myeloma cells, inhibits osteoclast differentiation and bone resorption, and favors osteoblast differentiation.

Our demonstration that resveratrol prevents osteoclast differentiation is based on several observations. In cultures of PBMC-isolated monocytes treated with M-CSF and RANKL, the same concentrations of resveratrol inhibit the formation of TRACP multinucleated cells; pit formation on dentine discs; release of TRACP, a well-established osteoclast marker, in the conditioned medium; and up-regulation of cathepsin K, the key protease for osteoclastic bone degradation (31). Furthermore, we provide insight in the mode of action of resveratrol on osteoclast differentiation and show that resveratrol interferes with RANKL signaling. This cytokine is indispensable for triggering the full osteoclast differentiation program and is the most important cytokine for osteoclast recruitment and activation in multiple myeloma (30). First, we show that resveratrol down-regulates mRNA and cell surface levels of RANK, the RANKL receptor that is expressed on the osteoclast precursors. Secondly, we show that resveratrol reduces the level of the RANKL-triggered NF-κB activation, a transcription factor that is essential for osteoclast differentiation (32). Interestingly, resveratrol was already reported to act on NF-κB activation in several cell types (19), but its effect in our cultures occurred only after several days, suggesting that resveratrol does not target directly this signaling cascade. Finally, we show that resveratrol prevents the induction of NFATc1 by RANKL, a downstream transcription factor reported recently to be dispensable for switching monocytes into osteoclasts (26, 27). It is noteworthy that the dose dependency of the inhibitory activity of resveratrol against NFATc1 and osteoclast differentiation is

Figure 6. Resveratrol induces gene expression of osteocalcin (OC) and osteopontin (OPN) in TERT cells, synergizes their response to 1.25(OH)2D3, and up-regulates expression of 1.25(OH)2D3 nuclear receptor. hMSC-TERT cells were cultured for 3 days with or without 10⁻⁹ mol/L 1.25(OH)2D3 in the presence of the indicated resveratrol concentrations. RNA was purified and the expression of osteocalcin (A), osteopontin (B), and VDR (C) was evaluated by quantitative real-time PCR. Levels of mRNA expression were normalized to β-actin mRNA and are represented as induction-fold compared with the condition without 1.25(OH)2D3 and without resveratrol. Columns, mean of three independent experiments; bars, SD. Note that the inset in (A) shows the values of the conditions without 1.25(OH)2D3 with another Y-scale. *, P < 0.01, statistically significant resveratrol effects versus cultures without resveratrol.
similar, suggesting a close relation with its effects on osteoclast differentiation. Thus far, it is not clear to what extent the effects of resveratrol on RANK, NF-κB, and NFATc1 are interrelated. For instance, it has been reported recently that inhibition of osteoclast differentiation obtained by using a NF-κB inhibitor correlate with a down-regulation of NFATc1, suggesting that NF-κB regulates NFATc1 (33). One may thus speculate that a low activation of NF-κB together with low RANK expression contributes to impaired NFATc1 stimulation. Additional targets of resveratrol can of course not be excluded. In addition to its action on RANK signaling, resveratrol may also affect cell changes occurring during the terminal differentiation of osteoclasts. Indeed, we found that the formation of osteoclasts was also inhibited when resveratrol was added 3 days after RANKL, at a time point where NFATc1 is already activated (Fig. 5A). Because cell spreading is a required step for monocytes to form multinucleated cells (34), one may speculate that the dramatic morphologic change induced by resveratrol impairs cell fusion process. Taken together, our findings show that resveratrol can inhibit osteoclastogenesis at several critical steps, including RANKL signaling. Because RANKL is a key factor in enhanced osteoclastogenesis in multiple myeloma, we propose that resveratrol could be an efficient osteoclast inhibitor in multiple myeloma.

The effects of resveratrol on osteoclastogenesis cannot be merely ascribed to a nonspecific toxic effect of resveratrol. After 3-day resveratrol treatment, when the effects exerted by resveratrol on gene expressions and NF-κB signaling were already significant, there was no reduction in the number of viable cells. Even if a 7-day treatment resulted in a 30% reduction in cell number, there was no indication of resveratrol-induced apoptosis. Cell densities remained high (Figs. 2A and 3C), and the most striking feature was the elongation of the cells. Furthermore, in the same cell cultures where resveratrol induced a decrease in RANK, NFATc1, cathepsin K, and TRACP, there was a concomitant increase in c-fms, CD14, and CD11a expression. The up-regulation of these genes by resveratrol suggests a relationship of these cells with the monocyte/macrophage lineage. Finally, our observations suggest that the effect of resveratrol may be reversible, because, after resveratrol withdrawal, elongated morphology started being lost and similarity to cells stimulated with M-CSF alone increased (Fig. 3C).

A peculiarity of multiple myeloma bone disease is that increased bone resorption is not compensated by bone formation (12). Formation of new bone requires mature osteoblasts producing extracellular matrix. Osteoblasts differentiate from bone marrow stromal cells, so-called mesenchymal stem cells in response to local factors as well as to systemic hormones like parathyroid hormone or 1,25(OH)2D3 (35). In multiple myeloma, osteoblast numbers are reduced (12). The mechanism is not well understood, but myeloma-stromal cell interactions and the soluble Wnt signaling antagonist DKK1 have been implicated in the inhibition of osteoblast differentiation (13, 36). Up-regulation of osteocalcin and osteopontin are well-established indicators of late-stage osteoblastic differentiation (37). Osteocalcin levels in blood allow monitoring bone formation and are decreased in multiple myeloma patients (38). It is thus of interest that our study shows that resveratrol can stimulate osteocalcin and osteopontin expression dose-dependently in the multipotent hMSC-TERT cell line, a cell line able to commit into osteoblasts (21, 25). Similar effects were observed while using normal human bone marrow stromal cells (data not shown). Moreover, resveratrol at concentrations between 25 and 100 μmol/L synergizes with 1,25(OH)2D3 to induce osteocalcin and, to a lesser extent, osteopontin expression. These effects of resveratrol suggest that resveratrol can commit early osteoprogenitor cells in bone marrow into the osteoblastic lineage. In agreement with our results, Mizutani et al. have reported that alkaline phosphatase, another marker of osteoblast differentiation, is also increased by resveratrol in mouse osteoblastic MC3T3 cells, which are cells at a later stage of osteoblast maturation (20). Taken together, these data support a stimulatory effect of resveratrol on osteoblast commitment and differentiation. With respect to the mode of action of resveratrol on osteoblast differentiation, we found that resveratrol regulates VDR, the nuclear receptor of 1,25(OH)2D3, dose-dependently. Because 1,25(OH)2D3 directly targets the osteocalcin and osteopontin gene promoters via the VDR (29), one could speculate that resveratrol-induced up-regulation of VDR may enhance greatly the responsiveness of bone marrow stromal cells to 1,25(OH)2D3 and thereby stimulate osteoblast differentiation.

Finally, we found that resveratrol reduces cell growth of two different myeloma cell lines in a dose-dependent manner by a mechanism involving cell apoptosis. Similarly to our observations, Jazirehi et al. (17) have shown that resveratrol induces cell cycle arrest and triggers apoptosis in non-Hodgkin's lymphoma, another B-cell–derived tumor. The authors also found that the combination of resveratrol and paclitaxel, a chemotherapeutic agent, enhanced cell death in non-Hodgkin's lymphoma and myeloma cells. Another recent study has shown that resveratrol can sensitize the myeloma cell line IM-9 to radiation when used at high concentrations (39). In addition, it is worthwhile noticing that concentrations of resveratrol reported to induce apoptosis in different blood cancer cell lines do not affect the survival of normal human peripheral mononuclear cells (17, 40, 41). Taken together, these results support that resveratrol is a proapoptotic agent in myeloma cells and can work in synergy with other anticancer treatments.

In conclusion, our study shows that resveratrol has a unique set of properties of interest for treatment of multiple myeloma patients, especially with respect to its different actions on myeloma cell growth, bone resorption, and bone formation. Our in vitro findings justify further in vivo studies to evaluate treatment of multiple myeloma with resveratrol or derivatives showing the same set of properties. The intake of resveratrol during daily moderate wine consumption (1.25 mg/glass of wine containing high resveratrol concentration, 25 μmol/L) is likely too low to induce in vivo the effects we observed in vitro (~0.02 mg/kg resveratrol for a 70 kg person). However, it has been reported that a daily oral administration of a dose as high as 20 mg/kg to rats for 28 days was not harmful with respect to final body weight and growth rate of animals, hematologic and biochemical variables, and histologic examination of vital organs (42). Such high doses of resveratrol showed significant chemopreventive and/or antitumor activities in vivo in breast cancer (43), skin cancer (16), liver cancer (44), colorectal and intestinal cancers (45, 46), lung cancer (47), and neuroblastoma (48). Regarding the effects of resveratrol on bone in vivo, two rat studies have shown that oral administration of resveratrol to ovarietomized rats prevents decrease in bone femur strength and increases epiphysis bone mineral density in the same manner as the bisphosphonate alendronate (49, 50). There are thus already in vivo studies suggesting, in accordance with our present data, that resveratrol may prevent increased bone degradation by osteoclasts or may stimulate compensative bone formation by osteoblasts. Although resveratrol has proven to be effective in vivo,
several pharmacokinetic studies have reported that its concentration in the plasma is rather low after administration due to its quick biotransformation into conjugate forms (51, 52). The plasma levels found to be efficacious may contribute to the overall pharmacologic activity (53). In addition, one should mention that the gain of interest for resveratrol for cancer treatment has encouraged the design and synthesis of resveratrol derivatives more stable and 100-fold more potent than the native molecule in inhibiting proliferation and inducing apoptosis in cancer cell lines (54, 55). It would be of great interest to determine whether these structural analogues act in the same manner on bone cells.

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**References**


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