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Receptor Activator of Nuclear Factor κB Ligand (RANKL) Is a Key Molecule of Osteoclast Formation for Bone Metastasis in a Newly Developed Model of Human Neuroblastoma[1]

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ABSTRACT

Neuroblastoma originates from neural crest cells and is the most common extracranial solid tumor in childhood. Bone metastasis in neuroblastoma is an unfavorable prognostic factor even with intensive therapy. In the present study, we screened four cell lines of human neuroblastoma (NB-1, NB-16, NB-19, and NH-6) for tumorigenicity and metastatic capacity in nude mice and found that NB-19 cells caused osteolytic lesions after s.c. injection into mice. To detect micrometastases in the host tissue, we performed two kinds of PCR-based metastasis assays: (*a*) genomic PCR assay using the primers for human genome-specific Alu sequence; and (*b*) reverse transcription-nested PCR assay that detects the expression of tyrosine hydroxylase, a marker specific for neuroblastoma. The results of these PCR assays revealed the colonization of human neuroblastoma cells in the bone marrow of the mice that had received the s.c. injection of NB-19 cells. Because osteoclastic bone resorption has been reported to play important roles in osteolysis in some cancers such as breast cancer, we next examined the osteoclast (OC)-inducing activity of NB-19 cells using a coculture system in which NB-19 cells were cultured with murine bone marrow cells containing OC precursors and stromal cells. NB-19 cells induced tartrate-resistant acid phosphatase-positive multinucleated OC-like cells without requirement of 1,25-dihydroxyvitamin D_3 or other osteoclastogenic stimulators. To investigate the factors involved in the osteoclastogenesis in the coculture of mouse marrow cells and NB-19 cells, we performed reverse transcription-PCR analysis and revealed the increased expression of receptor activator of nuclear factor κB ligand (RANKL) in the coculture compared with the culture of bone marrow cells alone. Interleukin-1α and cyclooxygenase-2 expression in the murine marrow cells was also increased in the presence of NB-19 cells. To further study the role of RANKL in the OC-like cell formation in the coculture of NB-19 cells and murine marrow cells, an expression vector encoding the active portion of the murine osteoprotegerin, which is the native inhibitor of RANKL action, was constructed and introduced into COS-7 cells. The conditioned media of the COS-7 cells transfected with the osteoprotegerin expression vector effectively blocked OC-like cell formation in the coculture of the bone marrow cells and NB-19 cells. These results suggested that in the bone microenvironment of NB-19-bearing mice, the stimulated expression of RANKL plays an important role in OC formation, leading to osteolytic bone metastasis.

INTRODUCTION

Neuroblastoma originates in cells of the neural crest and is the most common extracranial solid tumor in childhood (1–4). Prognosis in neuroblastoma patients is strongly related to patient age, tumor stage, site at diagnosis, and biological features of tumor cells (5–8). Patients over 1 year of age often present with advanced disease, and bone marrow metastases are associated with a poor prognosis despite high-dose chemotherapy and autologous hematopoietic stem cell transplantation. Bone is one of the target organs of metastasis in advanced neuroblastoma, and the presence of bone metastasis is responsible for a poor prognosis. One possible reason for this is the difficulty in treating cancer cells that are colonizing the bone/bone marrow microenvironment. According to the staging system of neuroblastoma proposed by the Children's Cancer Study Group, patients with bone metastasis are classified as stage IV without regard to other clinical manifestations and have a survival rate of ~7% (9). In contrast, the patients with stage IV-S disease, where the tumor does not cross the midline despite the presence of metastasis to liver, skin, or bone marrow, show a rather favorable prognosis, and the survival rate has been reported to be ~75% (10, 11). These data suggest the urgent need to develop an effective bone-targeting therapy in neuroblastoma.

In some cancers such as breast cancer and myeloma as well, bone metastasis causes clinical complications including pain, pathological fractures, and nerve compression (12). In the case of breast cancer, the role of osteoclastic bone resorption enhanced by the secretion of PTHrP[3] has been demonstrated in the development of bone metastasis (13), and adjuvant therapy with inhibitors of the osteoclastic bone resorption including bisphosphonates was proven to be beneficial (14–17). In neuroblastoma, however, the mechanisms involved in the bone metastasis have not been elucidated. In addition, because treatment with bisphosphonates may have adverse effects on bone growth in infants and children, other therapies may be required to treat childhood patients with neuroblastoma, although bisphosphonates have proven to be effective in treating bone complications in breast cancer patients and myeloma patients (14, 18).

The interaction between cancer cells and the bone marrow microenvironment plays an important role in bone metastasis. In the case of breast cancer, it is reported that estrogen-independent MDA-MB-231 cells respond to produce more PTHrP in the bone microenvironment when exposed to transforming growth factor β, which is stored in bone matrix and released by osteoclastic bone resorption (19). The increased production of PTHrP may accelerate the osteoclastic destruction of bone, creating the space for cancer proliferation in bone. Another line of studies using the murine mammary tumor cell line MMT060562 has demonstrated that PGE_2 plays a role in *in vitro* osteoclastogenesis in a coculture system of tumor cells and bone marrow cells, although this tumor cell line does not possess metastatic potential *in vivo* (20).

Very recently, RANKL, a novel cytokine also known as OPG ligand, OC differentiation factor, and TNF-related activation-induced cytokine, has been identified as being critical to OC recruitment (21–24). RANKL, expressed in bone marrow stromal cells that support OC differentiation, encodes a 316-aa type II transmembrane protein and belongs to the TNF ligand family. RANKL exerts its

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[3] The abbreviations used are: PTHrP, parathyroid hormone-related protein; OC, osteoclast; IL, interleukin; RT-PCR, reverse transcription-PCR; COX, cyclooxygenase; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase; TRAP(+) MNC, TRAP-positive multinucleated cell; OPG, osteoprotegerin; PG, prostaglandin; TNF, tumor necrosis factor; aa, amino acid(s); CMV, cytomegalovirus; M-CSF, macrophage colony-stimulating factor.

Table 1 *Primers used in the RT-PCR analysis*

Primers		Sequences (5′→3′)	Product size (bp)
β-Actin	Sense	GTGGGGCGCCCCAGGCACCA	548
	Antisense	CTCCTTAATGTCACGCACGATTTC	
Murine IL-1α	Sense	CTCTAGAGCACCATGCTACAGAC	308
	Antisense	TGGAATCCAGGGGAAACACTG	
Human IL-1α	Sense	ATGGCCAAAGTTCGAGACATG	816
	Antisense	CTACGCCTGGTTTTCCAGTATCTGAAAGTCAGT	
Murine IL-1β	Sense	TTGAAGAAGAGCCCATCCTCTG	320
	Antisense	GATCCACACTCTCCAGCTGC	
Human IL-1β	Sense	ATGGCAGAAGTACCTAAGCTC	810
	Antisense	TTAGGAAGACACAAATTGCATGGTGAACTCAGT	
Murine IL-6	Sense	TGGAGTCACAGAAGGAGTGGCTAAGG	155
	Antisense	TCTGACCACAGTGAGGAATGTCCAC	
Human IL-6	Sense	ATGAACTCCTTCTCCACAAGC	639
	Antisense	CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG	
Murine TNF-α	Sense	AAGGGATGAGAAGTTCCCAA	249
	Antisense	AGAACCTGGGAGTAGACAA	
Murine TNF-β	Sense	AGGGATGAGAAGTTCCCAA	271
	Antisense	GGTATGAGATAGCAAATCGG	
Murine COX-2	Sense	ACTCAGTTTGTTGAGTCATTC	579
	Antisense	TTTGATTAGTACTGTAGGGTTAATG	
Human COX-2	Sense	ATGAGATTGTGGGAAAATTG	820
	Antisense	GATACTTTCTGTACTGCGGG	
Murine RANKL	Sense	ATCAGAAGACAGCACTCACT	750
	Antisense	ATCTAGGACATCCATGCTAATGTTC	
Murine OPG	Sense	GGAAGCTTGAAACCCTTCCTCCAAAG	560
	Antisense	CCAGATCTTCACAGGGTGACATCTATTCC	

effects through its cognate receptor, RANK, which is expressed on the OC precursors (25). OPG is a soluble OC-inhibitory molecule that is a member of the TNF receptor family, along with RANK (23). OPG binds to RANKL as a decoy receptor and inhibits OC recruitment by interrupting the interaction between RANKL and RANK. There is no doubt that RANKL is a key regulator of osteoclastogenesis because RANKL-knock out mice show severe osteopetrosis and a defect of tooth eruption as a result of an inability of osteoblasts to support osteoclastogenesis (26). It has also been revealed that a variety of extracellular factors including 1,25-dihydroxyvitamin D_3, parathyroid hormone, PGE_2, IL-1, and IL-6 induce osteoclastogenesis through the stimulation of RANKL expression in bone marrow stromal cells. It is likely that increased cytokine production may cause the stimulated RANKL expression and accelerate osteoclastic bone destruction in the bone marrow microenvironment where cancer cells are colonized.

To study the mechanisms of metastasis and develop a new strategy for treating patients with cancer, appropriate animal models are required. However, there are few good models of bone metastasis; one example is the breast cancer model by intracardiac inoculation of MDA-MB-231 cells (15, 27). The number of human cancer cell lines that are heterotransplantable to animals is limited, and only a few of them metastasize to distant organs (28, 29).

In the present study, we screened several human neuroblastoma cell lines for tumorigenicity and metastatic capacity in immunocompromised nude mice and developed a new bone metastasis model of human neuroblastoma. We then examined whether the bone-metastasizing cell line NB-19 exhibited OC-inducing activity, and we found that the presence of NB-19 cells in murine bone marrow cells induced OC-like cell formation in culture through increased expression of IL-1α, COX-2, and RANKL.

MATERIALS AND METHODS

Cell Culture. Human neuroblastoma cell lines NB-16 and NB-19 were provided by Riken Cell Bank (Saitama, Japan). NB-1 and NH-6 were obtained from Human Science Research Resources Bank (Osaka, Japan). NB-16, NB-19, and NB-1 cells were maintained in RPMI 1640 (Nikken, Kyoto, Japan) supplemented with 10% FCS (Life Technologies, Inc., Grand Island, NY) and antibiotics (Life Technologies, Inc.), and NH-6 cells were cultured in α-MEM (Nikken) with 10% FCS and antibiotics under a 5% CO_2 atmosphere.

Tumor Cell Injection into Nude Mice. Animal protocols were approved by the Institutional Animal Care and Use Committee at Osaka Medical Center and Research Institute for Maternal and Child Health. Four-week-old female nude mice (BALB/cA Jcl-nu, *nu/nu*) were supplied by Clea Japan (Tokyo, Japan) and maintained under pathogen-free conditions. For heterotransplantation, NB-16 and NB-1 cells were mechanically detached from the culture dish, and NB-19 and NH-6 cells were harvested by treatment with 0.25% trypsin-0.5 mM EDTA solution (Life Technologies, Inc.) following the manufacturer's instructions. Then the cells were washed once with PBS and suspended in PBS at a concentration of 1×10^8 cells/ml. One hundred μl of the cell suspension were mixed with the same volume of Matrigel (Becton Dickinson Collaborative Biomedical Products, Bedford, MA) and injected s.c. into the right back of each mouse. Tumorigenicity of each cell line in mice was evaluated as the mass size 8 weeks after tumor cell injection.

X-ray of Mice. Animals in a prone position against the film were X-rayed using SOFTEX CMB-2 (SOFTEX Co. Ltd., Tokyo, Japan) while under anesthesia.

Histological Examination. Bones of tumor-bearing mice and control mice were fixed in 10% buffered formalin, followed by decalcification in 14% EDTA solution for 2 weeks at room temperature with gentle stirring. Sections were made by the conventional method and stained with H&E.

Micrometastasis Assay Based on Genomic PCR for Alu Sequence and RT-PCR for Tyrosine Hydroxylase. To detect the micrometastases of human neuroblastoma cells in the bone marrow of the host mice, we performed a PCR-based metastasis assay using the amplification of Alu sequences that are specific to the human genome (30). Genomic DNA was isolated from the bone marrow of tumor-bearing mice and control mice using Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. Specific primers for human Alu sequences were Alu-sense (5′-ACGCCTGTAATCCCAGCACTT-3′) and Alu-antisense (5′-TCGCCCAGGCTGGAGTGCA-3′), which produced a band of 224 bp (30). PCR was carried out using 100 ng of genomic

Table 2 *Tumorigenicity of human neuroblastoma cell lines in nude mice*

Cells (1×10^7 cells/mouse) of each cell line were injected s.c. into the right dorsal area of five nude mice, and the size of the formed mass was evaluated 8 weeks after the inoculation. Mean diameter indicates the mean of the major axis and minor axis of the formed mass, and the data are described as the mean ± SE.

Cell line	Mean diameter (mm; mean ± SE)	Mice with mass (%)
NB-1	6.4 ± 0.9	80
NB-16	15.6 ± 2.4	100
NB-19	19.6 ± 1.1	100
NH-6	9.7 ± 0.4	100

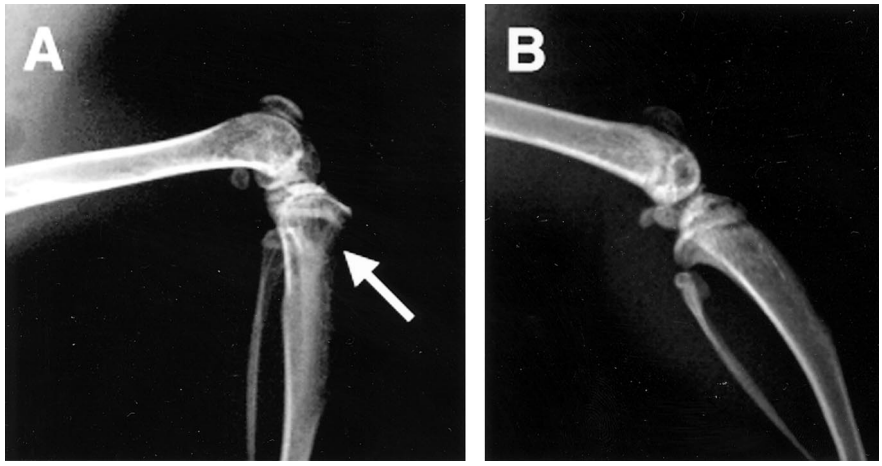

Fig. 1. *A*, X-ray of the knee of a nude mouse that had received the s.c. injection of NB-19 cells. Cells (2×10^7) were injected s.c. into the right dorsal area of 4-week-old female nude mice, and the mice were X-rayed 8 weeks later. The *arrow* indicates the osteolytic lesion. *B*, X-ray of the knee of an age-matched control mouse that did not receive the tumor cell injection.

DNA as template and recombinant Taq DNA polymerase (Takara Syuzo, Kyoto, Japan) under the following thermocycling conditions: an initial denaturation step of 94°C for 4 min; 25 cycles comprised of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s; and a final extension step of 72°C for 7 min.

We also performed another micrometastasis assay using RT-PCR that amplified cDNA of tyrosine hydroxylase, a specific marker for neuroblastoma (31). Total RNA was extracted from the bone marrow of the mice bearing each cell line using Trizol reagent at the same time as the genomic DNA was obtained. Two μg each of total RNA were then reverse transcribed using random hexamers (Promega, Madison, WI) and SuperScript II reverse transcriptase (Life Technologies, Inc.). For PCR, the following primers were used: (*a*) hTH340F (sense), 5′-AGCCAAAATCCACCATCTAG-3′; (*b*) hTH528F (sense), 5′-TGTCAGAGCTGGACAAGTGT-3′; (*c*) hTH826R (antisense), 5′-GATATTGTCTTCCCGGTAGC-3′; and (*d*) hTH866R (antisense), 5′-TGCGCTCCTTCAGGAAGCGG-3′. The first-round PCR was performed using the primer set of hTH340F and hTH866R, which was expected to amplify a 526-bp fragment in the presence of tyrosine hydroxylase mRNA. Thirty cycles of amplification were carried out with 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. For the second-round PCR, the primer set of hTH528F and hTH826R was used with 2-μl aliquots of the first-round PCR products (20 μl) as template, amplifying a 298-bp fragment. The thermocycling program for the second-round PCR was the same as that used for the first-round PCR.

OC-like Cell Formation Assay. OC-like cell formation assay was carried out following the methods originally described by Takahashi *et al.* (32), with some modifications. Murine bone marrow cells were collected aseptically from C57BL/6N mice (Clea Japan). The collected cells were washed and resuspended in α-MEM supplemented with 10% FCS (Hyclone, Logan, UT), and the cell suspension was incubated in 10-cm culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) at 37°C for 2 h. After the incubation, the nonadherent cells were collected and plated onto 48-well plates at a density of 1×10^6 cells/well with various numbers of NB-19 cells ($0–1 \times 10^4$ cells/well) and cultured for 6 days in α-MEM supplemented with 10% FCS in the absence of 1,25-dihydroxyvitamin D or any other OC-inducing reagent. When the cultures were terminated, the cells were fixed and stained for TRAP activity using a commercial kit (Sigma, St. Louis, MO). The TRAP(+)MNCs containing two or more nuclei were counted as OC-like cells.

RT-PCR Analysis for Cytokines, COX-2, RANKL, and OPG. To examine what kind of cytokines were involved in the TRAP(+) MNC formation in the coculture of murine bone marrow cells and NB-19 neuroblastoma cells, RT-PCR analysis was performed. Murine bone marrow cells (1×10^7 cells/well) and NB-19 cells (1×10^5 cells/well) were plated together or separately to each well of 6-well plates and cultured for 6 days in α-MEM supplemented with 10% FCS in the absence of 1,25-dihydroxyvitamin D or other osteoclastogenic stimulators. When the cultures were terminated, total RNA was ex-

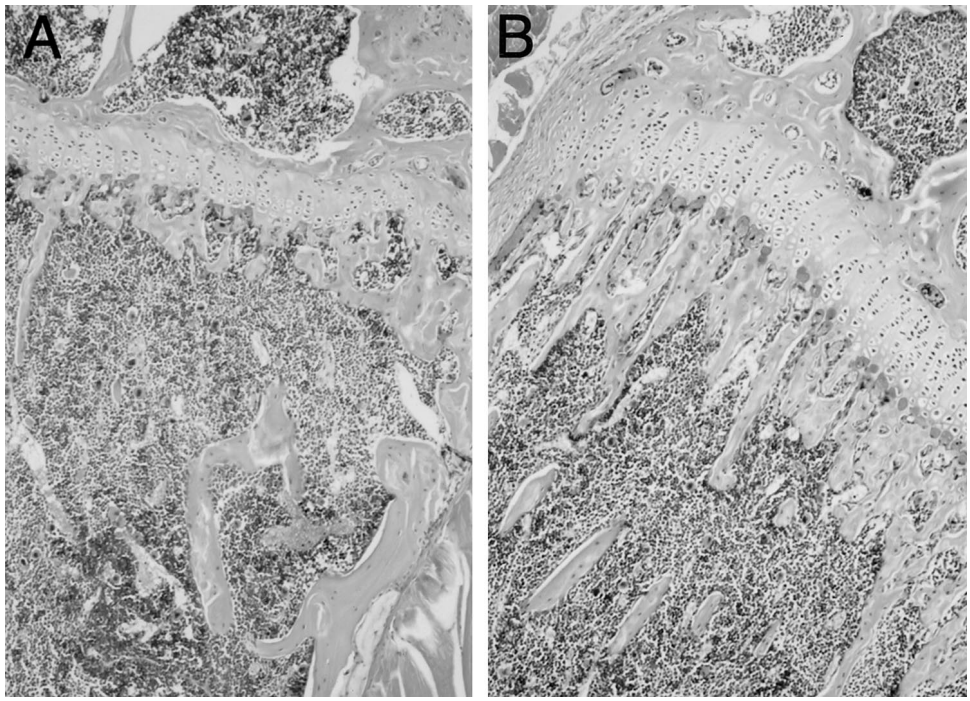

Fig. 2. H&E-stained sections of tibiae in NB-19-bearing mice (*A*) and non-tumor-bearing control mice (*B*). In NB-19-bearing mice, trabecular volume was decreased compared with that of control mice.

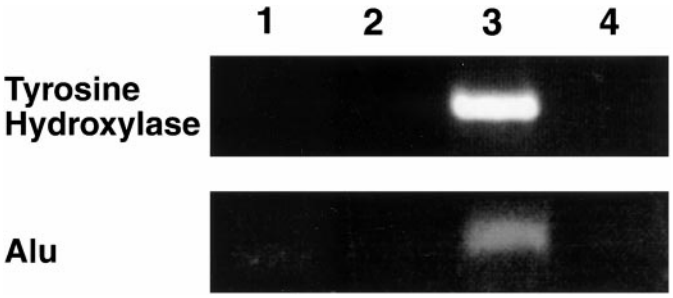

Fig. 3. PCR-based metastasis assay using the bone marrow obtained from NB-19-bearing mice. Genomic DNA and total RNA were extracted from the nude mice that had received a s.c. injection of neuroblastoma cells and subjected to genomic PCR for human-specific Alu sequence and reverse transcription-nested PCR for tyrosine hydroxylase, respectively. *Lane 1*, bone marrow from a NB-1-bearing mouse; *Lane 2*, bone marrow from a NB-16-bearing mouse; *Lane 3*, bone marrow from a NB-19-bearing mouse; *Lane 4*, bone marrow from a NH-6-bearing mouse.

tracted from each culture using Trizol reagent and treated with DNase (Takara Syuzo) to remove the contaminated genomic DNA. Two μg each of DNase-treated RNA were then reverse transcribed using SuperScript II. The cDNA samples were then subjected to PCR using the primers listed in Table 1. The amplification of the expected fragments was confirmed by sequencing of the PCR products using an automated sequencer (a 377 model; PE Applied Biosystems, Tokyo, Japan).

Construction of Recombinant RANKL and OPG. To construct the expression vectors for murine soluble RANKL and murine OPG, we first performed PCR-based cloning of the cDNAs for these genes using total RNAs extracted from mouse bone marrow cells and mouse kidney, respectively. Reverse transcription was carried out using random hexamers and SuperScript II, followed by PCR using the pairs of primers msRANKL-F (sense; 5′-GGAAGCTTGATCCTAACAGAATATCAG-3′) and msRANKL-R (antisense; 5′-CCTCTAGATCAGTCTATGTCCTGAAC) and mOPG-F (sense; 5′-GGAAGCTTGAAACCCTTCCTCCAAAG-3′) and mOPG-R (antisense; 5′-CCAGATCTTCACAGGGTGACATCTATTCC). These primer sets were designed to amplify the soluble form of RANKL (aa 76–316) and the active portion of OPG (aa 22–201), respectively (21, 33). The amplified PCR products corresponding to the soluble RANKL and the active portion of OPG were then cloned in frame into pFLAG-CMV-1 expression vector (Sigma) containing the FLAG® tag and the signal peptide of preprotrypsin after digestion with *Hin*dIII/*Xba*I and *Hin*dIII/*Bgl*II, respectively. After the nucleotide sequences were confirmed, the constructed expression vector for RANKL or OPG or the empty vector was introduced into COS-7 cells using the cationic lipid method (LipofectAMINE; Life Technologies, Inc.). The culture media were changed to serum-free media 36 h after the transfection, and the 24-h conditioned media were harvested to test for the biological activity in an osteoclastogenesis assay of murine bone marrow culture described above. The secretion of the expected fusion proteins into the media was confirmed by Western blotting using the anti-FLAG M2 monoclonal antibody (Sigma; data not shown).

Statistical Analysis. The data were analyzed by ANOVA. All values were represented as the mean ± SE.

RESULTS

Development of a Neuroblastoma Bone Metastasis Model. Table 2 summarizes the tumorigenicity of the four cell lines of human neuroblastoma examined as described in "Materials and Methods." NB-19 and NB-16, originating from the involved bone marrow of patients with advanced neuroblastoma, exhibited stronger tumorigenicity than NB-1 and NH-6, which were established from the primary sites of the disease. Interestingly, NB-19-bearing mice showed splenomegaly. Radiological examination of NB-19-bearing mice revealed the development of osteolytic lesions 8 weeks after tumor cell injection (Fig. 1). Of the 10 mice that had been inoculated

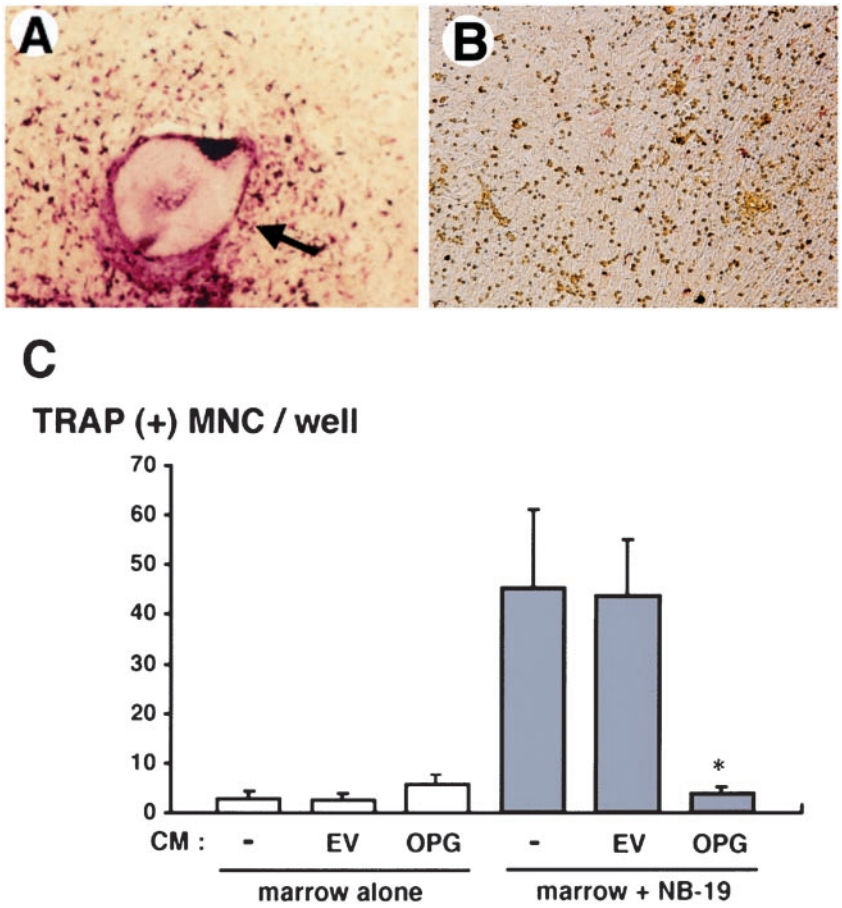

Fig. 4. TRAP(+)MNC formation observed in the coculture of murine bone marrow cells and NB-19 human neuroblastoma cells and the effect of recombinant OPG. Murine bone marrow cells (1×10^6 cells/well) were plated with (*A*) or without (*B*) NB-19 cells (1×10^2 cells/well) onto 48-well culture plates and cultured for 6 days in the absence of any osteoclastogenic stimulator. When the culture was terminated, the cells were stained for TRAP activity. The *arrow* indicates the TRAP(+)MNC formed in the coculture of marrow cells and NB-19 cells. Original magnification was ×100 in both *A* and *B*. In *C*, murine bone marrow cells (1×10^6/well) were plated onto 48-well culture plates with or without NB-19 cells (1×10^2 cells/well) and cultured for 6 days in the presence or absence of the conditioned media (*CM*) harvested from the COS-7 cells transfected with the OPG expression vector or the empty vector (*EV*). After culture for 6 days, the numbers of TRAP-positive cells with two or more nuclei were determined. Data are expressed as the means ± SE of triplicate cultures. *, significantly different from the value in the culture of marrow cells and NB-19 cells treated with the conditioned media from empty vector transfectants ($P < 0.05$).

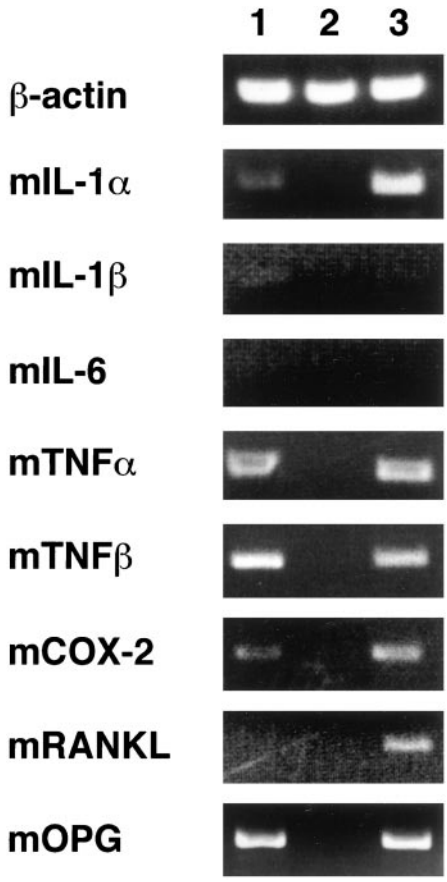

Fig. 5. RT-PCR analysis for the cytokines involved in OC-like cell formation in the coculture of NB-19 cells and marrow cells. Total RNA was extracted from the cultures of murine bone marrow cells alone (*Lane 1*), NB-19 cells alone (*Lane 2*), or the coculture of NB-19 and marrow cells (*Lane 3*) as described in "Materials and Methods." Two μg of each RNA sample were reverse transcribed after the DNase treatment, and then PCR was carried out using the specific primer sets to amplify the cDNAs for the indicated molecules. PCR using the primer set for β-actin confirmed the equal efficiency of first-strand cDNA synthesis between the RNA samples.

with NB-19 cells, all of them had a mass at the inoculation site, and 7 of them exhibited osteolytic lesions detectable by X-ray. The total number of these regions was 14 (5 lesions in right tibiae, 6 lesions in left tibiae, and 3 lesions in left femurs), and the diameter of these lesions was 1.71 $\pm$ 0.67 mm (mean $\pm$ SD). In the histological analysis, although the decrease in trabecular volume was obvious in NB-19-bearing mice, massive metastasis was not apparent (Fig. 2). To confirm the presence of NB-19 cells in the bone marrow of the host animals, we performed genomic PCR for Alu sequences using the genomic DNA extracted from the tumor-bearing mice. Specific signals were detected when DNA from the bone marrow of NB-19-bearing mice was used as template, but not when DNA obtained from the bone marrow of nude mice bearing other cell lines was used (Fig. 3). In the RT-PCR assay for tyrosine hydroxylase as well, specific signals were detected only when the RNA from the NB-19-bearing mice was used as template (Fig. 3), confirming the presence of human neuroblastoma cells in the bone marrow of NB-19-bearing mice.

The sensitivities of these PCR-based assays were tested, and it was found that 100 neuroblastoma cells in one million murine cells were detectable by these assays (data not shown).

TRAP(+)MNC Formation in the Coculture of Murine Bone Marrow Cells and NB-19 Human Neuroblastoma Cells. Murine bone marrow cells were cultured for 6 days in the presence or absence of NB-19 cells, and the number of TRAP(+)MNCs was determined. In the absence of NB-19 cells, the murine bone marrow cells did not form TRAP(+)MNCs without the addition of OC-inducing reagents such as 1,25-dihydroxyvitamin D (Fig. 4*B*). In the presence of NB-19 cells, a number of TRAP(+)MNCs were formed even in the absence of an osteoclastogenesis stimulator (Fig. 4*A*).

RT-PCR Analysis for the Factors Involved in OC-like Cell Formation Induced by NB-19 Cells. To identify the soluble factor(s) involved in the TRAP(+)MNC formation in this system, RT-PCR analysis was performed, and we detected that murine IL-1α expression was stimulated in the coculture of bone marrow cells and NB-19 cells compared with the marrow cells cultured in the absence of NB-19 cells (Fig. 5). These PCR products were revealed to be identical to murine IL-1α cDNA by sequencing analysis (data not shown). No signals were detected when the primer set for human IL-1α was used (data not shown). Murine TNF-α and TNF-β were detected in the culture of murine bone marrow cells alone and in the coculture of murine bone marrow cells and NB-19 cells at similar levels but were not detected in the culture of NB-19 alone (Fig. 5).

Because IL-1α has been reported to increase PG production, we also examined the expression of COX-2 in this coculture system. In the RT-PCR analysis, the expression of COX-2 appeared to be increased in the coculture of NB-19 cells and bone marrow cells compared with the culture of bone marrow cells alone (Fig. 5). The PCR product amplified from the cDNA of the coculture was subjected to cloning into pT7-Blue vector (Novagen), followed by sequencing. All of the eight clones examined were identical to the cDNA for murine COX-2 (data not shown).

RANKL expression was up-regulated in the coculture of NB-19 and marrow cells compared with the culture of marrow cells alone, whereas the expression level of OPG was unchanged (Fig. 5). Sequencing analysis revealed that these PCR products were identical to murine sequences (data not shown).

Biological Activities of Recombinant RANKL and OPG. The biological activity of recombinant RANKL and OPG was examined in murine bone marrow culture. In the presence of recombinant human M-CSF (10 ng/ml; Genzyme/Techne, Minneapolis, MN), the conditioned media from the RANKL-transfected COS-7 cells stimulated TRAP(+)MNC formation in a dose-dependent manner (Fig. 6, *A* and *C*). Crude conditioned media harvested from the COS-7 cells transfected with the OPG expression vector or empty vector pFLAG-CMV-1 were added to the murine bone marrow culture in the presence of 10 ng/ml M-CSF and the conditioned media from the RANKL-transfected cells (20% concentration of the culture media). The conditioned media from OPG-transfected cells inhibited RANKL-induced TRAP(+)MNC formation in a dose-dependent manner, whereas the conditioned media from empty vector-transfected cells did not (Fig. 6, *B* and *D*). These data suggested that recombinant RANKL and recombinant OPG prepared in the study were biologically active.

Recombinant OPG Inhibited TRAP(+)MNC Formation in the Coculture of Murine Bone Marrow Cells and NB-19 Neuroblastoma Cells. Because RT-PCR analysis suggested the involvement of RANKL in TRAP(+)MNC formation in the coculture of marrow cells and NB-19 cells, we next examined whether the recombinant OPG could inhibit the TRAP(+)MNC formation in this coculture system. Crude conditioned media harvested from the COS-7 cells transfected with the OPG expression vector or the empty vector were added to the coculture of NB-19 cells and marrow cells, and the cells were cultured for 6 days in the absence of any osteoclastogenic stimulator such as 1,25-dihydroxyvitamin D. The conditioned media from the OPG-transfected cells inhibited TRAP(+)MNC formation in the coculture of NB-19 cells and marrow cells, whereas the conditioned media from the empty vector-transfected cells did not (Fig. 4*C*).

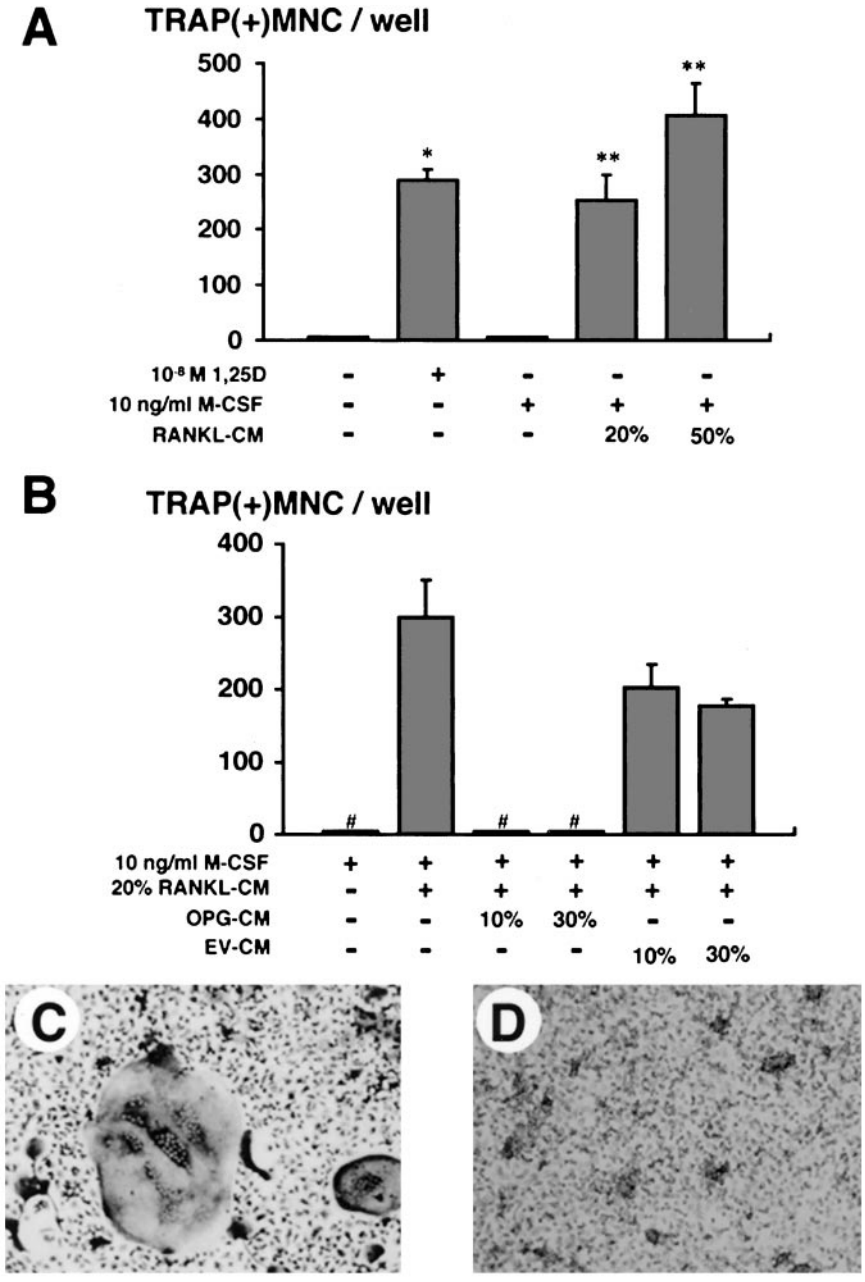

Fig. 6. Effect of recombinant RANKL and recombinant OPG on TRAP(+)MNC formation in murine bone marrow culture. Expression vector encoding either RANKL or OPG or pFLAG-CMV-1 as empty vector was introduced into COS-7 cells, and the effect of the conditioned media of these transfectants on TRAP(+)MNC formation was investigated as described in "Materials and Methods." In *A*, marrow cells were cultured in the presence or absence of human recombinant M-CSF (10 ng/ml) and the conditioned media of RANKL transfectants. We used 10^{-8} M 1,25-dihydroxyvitamin D as a positive control. In *B*, marrow cells were cultured in the presence of M-CSF (10 ng/ml) with or without conditioned media from COS-7 cells with RANKL, OPG, or empty vector. Features depicted are as follows: *1,25D*, 1,25-dihydroxyvitamin D; *RANKL-CM*, conditioned media from RANKL-transfected COS-7 cells; *OPG-CM*, conditioned media from OPG-transfected COS-7 cells; *EV-CM*, conditioned media from empty vector-transfected COS-7 cells. *Percentages* indicate the concentrations of conditioned media in the culture media of marrow cells. Data are expressed as the means ± SE of triplicate cultures. * and **, significantly different from the value in the untreated culture and the value in the culture treated with M-CSF alone, respectively ($P < 0.01$). #, significantly different from the value in the culture treated with M-CSF and RANKL-CM alone ($P < 0.001$). *C*, TRAP(+)MNC formed in the murine bone marrow cells cultured in the presence of M-CSF and conditioned media harvested from RANKL-transfected COS-7 cells. *D*, murine bone marrow cells cultured in the presence of M-CSF, RANKL-transfectant conditioned media, and OPG-transfectant conditioned media. No TRAP(+)MNCs were observed.

DISCUSSION

In the present study, we screened four cell lines of human neuroblastoma for tumorigenicity and bone metastatic capacity in immunocompromised nude mice, and we detected micrometastasis of NB-19 cells, which had originally been obtained from the bone marrow of a patient with stage IV neuroblastoma (34). After s.c. injection into nude mice, NB-19 cells reproducibly developed osteolytic lesions in the long bones detectable by radiographs. Because bone metastasis in neuroblastoma patients is also usually osteolytic, the radiological manifestation in NB-19-bearing mice is clinically relevant.

In addition to the osteolytic lesions revealed in rentgenography, two different PCR-based assays detected micrometastasis of NB-19 cells in the host bone marrow. One of the assays was based on the genomic PCR amplification of Alu sequences specific to the human genome, which had been previously used in the chick embryo metastasis model by Kim *et al.* (30). The other assay, reverse transcription-nested PCR for tyrosine hydroxylase, has been used to detect occult neuroblastoma cells in the bone marrow and peripheral blood of patients on therapy (31). In both assays, the signals were detected in the bone marrow of NB-19-bearing mice but not in those of the control animals, confirming the presence of human neuroblastoma cells in the bone marrow of NB-19-bearing mice. Neither of the assays detected signals when bone marrow obtained from mice bearing one of the other three cell lines (NB-1, NB-16, and NH-6) was used as the source of the template.

A number of reports have demonstrated that osteoclastic bone resorption is important to the development of bone metastasis in some cancer types including breast cancer, lung cancer, and prostate cancer (35). These cancers induce OC formation via secretion of PTHrP, IL-1α, or PGE_2 by themselves (13, 20, 36). Consistent with the role of bone resorption in cancer metastasis, antiresorption therapy such as administration of bisphosphonates or anti-PTHrP neutralizing antibody was reported to be effective in an animal model of breast cancer

in which direct intracardiac injection of MDA-MB-231 cells was performed on nude mice (13, 37).

To study the OC-forming activity induced by the presence of NB-19 cells in the host bone marrow, we performed an *in vitro* osteoclastogenesis assay using a murine bone marrow culture system. In this assay, OC-like TRAP(+)MNCs were formed in the coculture of NB-19 neuroblastoma cells and murine bone marrow cells without the requirement for any osteoclastogenic stimulator, including 1,25-dihydroxyvitamin D and PTHrP. These results suggested that production of some OC-inducing activity is involved in osteolysis in NB-19-bearing mice, which was triggered by the presence of neuroblastoma cells in the host bone marrow.

We further examined which factors were involved in OC-like cell formation induced by the presence of NB-19 cells. We found that the expression of IL-1α was stimulated in the coculture of NB-19 cells and murine bone marrow cells compared with the cultures of NB-19 alone or marrow cells alone. IL-1α has been reported to induce COX-2, which is the key enzyme for PGE_2 synthesis. PGE_2 is known as a potent inducer of OC formation (38), and recent studies have revealed that PGE_2 is one of the inducers for RANKL (22). Consistent with these findings, the expressions of both COX-2 and RANKL as well as IL-1α were up-regulated in the coculture of NB-19 cells and murine bone marrow cells compared with cultures of NB-19 or marrow cells alone. The data suggested that the presence of NB-19 cells stimulated the expression of IL-1α, COX-2, and RANKL in the murine marrow cells.

In the present study, the significant role of RANKL in the osteolysis found in our model was enhanced by the blockage of OC-like cell formation by recombinant OPG in the coculture of NB-19 cells and murine bone marrow cells. These results may lead to a new therapeutic approach to bone metastasis in neuroblastoma such as the administration of recombinant OPG or gene therapy using OPG expression vector. Recombinant RANKL constructed in the study required M-CSF to induce osteoclastogenesis *in vitro*, which was consistent with the previous reports (39).

Cell-cell interaction via adhesion molecules between tumor cells and bone marrow stromal cells also appears to play a role in bone metastasis. The overexpression of integrin $\alpha_4\beta_1$ promoted the bone metastasis of Chinese hamster ovary cells, suggesting that the interaction between integrin $\alpha_4\beta_1$ and vascular cell adhesion molecule 1 expressed on the bone marrow stromal cells is important in bone metastasis (40). The Chinese hamster ovary cells overexpressing integrin $\alpha_4\beta_1$ were also reported to stimulate OC-like cell formation *in vitro* (41). However, NB-19 cells did not express integrin $\alpha_4\beta_1$ (data not shown). Some surface molecule other than integrin $\alpha_4\beta_1$ might be responsible for osteoclastogenesis and metastatic osteolysis in neuroblastoma.

In conclusion, we have developed a unique model of osteolytic bone metastasis where the presence of human neuroblastoma NB-19 cells in bone marrow was proven by two kinds of PCR-based assays 8 weeks after the s.c. injection of tumor cells into nude mice. Tumor-host cell interaction between NB-19 cells and murine bone marrow cells resulted in the increased expressions of IL-1α, COX-2, and RANKL, leading to OC formation. Recombinant OPG was capable of blocking this OC formation, demonstrating the critical role of RANKL in osteolysis induced by neuroblastoma cells.

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