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

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INTRODUCTION

Neuroblastoma originates in cells of the neural crest 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 NB-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 osteolytic cancer cells, 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 D₃ 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 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.

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 NB-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 osteolytic cancer cells, 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 D₃ 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 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.

Received 1/31/00; accepted 12/13/00.

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1 Supported in part by grants from Japan Space Forum (to T. M. and K. O.) and Ministry of Education, Japan (to T. M.).

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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.
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 osteoporosis 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 D₃, parathyroid hormone, PGE₂, 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 4T1 cells. Metastatic breast tumors develop into a mass, and the data are described as the mean ± SE.

### Tumor Cell Injection into Nude Mice

#### 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 NB-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% CO₂ atmosphere.

#### Primers used in the RT-PCR analysis

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<th>Primers</th>
<th>Sequences (5′→3′)</th>
<th>Product size (bp)</th>
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<td></td>
<td>Antisense CTCCCTAAAGGCAAGATTC</td>
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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′-TGCCTCTTTCAGAAAGCGG-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 × 10⁵ cells/well with various numbers of NB-19 cells (0–1 × 10⁴ 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 × 10⁷ cells/well) and NB-19 cells (1 × 10⁵ 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-
extracted 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 373 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′-GGAAGCTTGAAACCCTTCCTCCAAAG-3′) and msRANKL-R (antisense; 5′-CCAGATCTTTCAAGGGTGACATCTATTCC-3′). The amplified cDNA products were sequenced and 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 (LipfectAMINE; 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 secreted proteins were confirmed by Western blotting using the anti-FLAG M2 monoclonal antibody (Sigma; data not shown).

**Statistical Analysis.** The results 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...
found that 100 neuroblastoma cells in one million murine cells were neuroblastoma cells in the bone marrow of NB-19-bearing mice. Mice was used as template (Fig. 3), confirming the presence of human signals were detected only when the RNA from the NB-19-bearing 3). In the RT-PCR assay for tyrosine hydroxylase as well, specific the bone marrow of nude mice bearing other cell lines was used (Fig. 4). To the RT-PCR analysis, the expression of IL-1α 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).

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).

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 tibia, 6 lesions in left tibia, and 3 lesions in left femurs), and the diameter of these lesions was 1.71 ± 0.67 mm (mean ± 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. 4B). In the presence of NB-19 cells, a number of TRAP(+)MNCs were formed even in the absence of an osteoclastogenesis stimulator (Fig. 4A).

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/Technne, 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. 4C).
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 radiography, 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 neuroblasta 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 PGE2 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 suggest 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 PGE2 synthesis. PGE2 is known to be involved in osteoclastogenesis, and 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 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 αβ1 promoted the bone metastasis of Chinese hamster ovary cells, suggesting that the interaction between integrin αβ1 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 αβ1 were also reported to stimulate OC-like cell formation in vitro (41). However, NB-19 cells did not express integrin αβ1 (data not shown). Some surface molecule other than integrin αβ1 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.

ACKNOWLEDGMENTS

We thank Tomoko Hayashi for secretarial assistance.

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Cancer Res 2001;61:1637-1644.

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