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Research Paper

The SK-N-AS human neuroblastoma cell line develops osteolytic bone metastases with increased angiogenesis and COX-2 expression

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ABSTRACT

Neuroblastoma (NB), which arises from embryonic neural crest cells, is the most common extra-cranial solid tumor of childhood. Approximately half of NB patients manifest bone metastasis accompanied with bone pain, fractures and bone marrow failure, leading to disturbed quality of life and poor survival. To study the mechanism of bone metastasis of NB, we established an animal model in which intracardiac inoculation of the SK-N-AS human NB cells in nude mice developed osteolytic bone metastases with increased osteoclastogenesis. SK-N-AS cells induced the expression of receptor activator of NF-κB ligand and osteoclastogenesis in mouse bone marrow cells in the co-culture. SK-N-AS cells expressed COX-2 mRNA and produced substantial amounts of prostaglandin E_2 (PGE_2). In contrast, the SK-N-DZ and SK-N-FI human NB cells failed to develop bone metastases, induce osteoclastogenesis, express COX-2 mRNA and produce PGE_2. Immunohistochemical examination of SK-N-AS bone metastasis and subcutaneous tumor showed strong expression of COX-2. The selective COX-2 inhibitor NS-398 inhibited PGE_2 production and suppressed bone metastases with reduced osteoclastogenesis. NS-398 also inhibited subcutaneous SK-N-AS tumor development with decreased angiogenesis and vascular endothelial growth factor-A expression. Of interest, metastasis to the adrenal gland, a preferential site for NB development, was also diminished by NS-398. Our results suggest that COX2/PGE_2 axis plays a critical role in the pathophysiology of osteolytic bone metastases and tumor development of the SK-NS-AS human NB. Inhibition of angiogenesis by suppressing COX-2/PGE_2 may be an effective therapeutic approach for children with NB.

1. Introduction

Neuroblastoma (NB), which arises from embryonal neural crest cells, is the most common extra-cranial solid tumor of childhood and accounts for more than 15% of all child cancer death [1,2]. Patients with NB frequently manifest disseminated disease at first visit and prognosis of these patients is poor with 3-year survival rate less than 40% [1,2]. It has been well-known that bone is one of the most common target sites of NB spread. More than 60% of advanced NB patients manifest bone metastases that are radiologically osteolytic or a mixture of osteolytic and osteoblastic type [3,4]. Children with bone metastases suffer from complications including intolerable bone pain, pathological fractures and bone marrow failure. Currently-available anti-NB therapies are not satisfactorily effective at treating bone metastases and these complications. Accordingly, these children show miserable clinical courses and poor survival rate [5,6]. Therefore, control of bone metastases is an important goal in the management of infants with NB [7] and effective therapeutic interventions designed based on the understanding of the pathophysiology of bone metastasis of NB have been awaited. However, the mechanism underlying the preferential metastasis of NB to bone remains poorly understood.

Recent studies suggest that the interactions between cancer cells and bone microenvironment are critical to the pathophysiology of bone metastasis [8,9]. Bone provides a fertile soil for metastatic cancer cells by releasing growth factors such as insulin-like growth factors (IGFs) [10] and transforming growth factor β (TGFβ) [11] as a consequence of osteoclastic bone resorption during bone remodeling. Cancer cells stimulated by these bone-derived growth factors consequently produce increased levels of osteoclast-stimulating factors such as parathyroid hormone-related protein (PTH-rP) [11] and prostaglandin E_2 (PGE_2) [12], which in turn further promotes osteoclastic bone resorption, establishing a vicious cycle between bone-resorbing

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osteoclasts and metastatic cancer cells [8,9]. Contribution of similar vicious cycle between NB and bone microenvironment [13] mediated by receptor activator of NF-κB ligand (RANKL) [14,15] and IGF-1 [16] to the development and progression of NB bone metastasis has been also suggested.

In the present study, we developed an animal model of NB bone metastasis to advance our understanding of the mechanism of bone metastasis. We showed that an inoculation of the SK-N-AS human NB cells into the left heart ventricle of nude mice caused osteolytic bone metastases with increased osteoclastogenesis. SK-N-AS cells expressed COX-2 mRNA and produced substantial amounts of PGE_2. The selective COX-2 inhibitor NS-398 inhibited PGE_2 production and osteolytic bone metastases with reduced osteoclastic bone resorption and subcutaneous SK-N-AS tumor enlargement with reduced angiogenesis. These results suggest that COX-2/PGE_2 system plays a critical role in the pathophysiology of osteolytic bone metastases and tumor progression of NB. Inhibition of COX-2/PGE_2 is a potential therapeutic intervention for bone metastases in children with NB.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human osteoprotegerin (OPG) was purchased from BioSource International (Camarillo, CA). 1,25-dihydroxyvitamin D_3 ($1,25D_3$) was from BIOMOL International (Plymouth Meeting, PA). Dimethylsulfoxide (DMSO), PGE_2, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO). NS-398, a selective COX-2 inhibitor, was purchased from Cayman Chemical Co. (Ann Arbor, MI). For *in vitro* use, indomethacin and NS-398 were reconstituted in DMSO and diluted with culture media. The final concentration of DMSO in the culture media was less than 0.1%.

Polyclonal rabbit anti-vascular endothelial growth factor-A (VEGF-A) and polyclonal rabbit anti-proliferation cell nuclear antigen (PCNA) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-COX-2 and polyclonal rabbit anti-factor VIII were from Zymed Laboratories, Inc. (South San Francisco, CA).

2.2. Human neuroblastoma cells

Human NB cell lines, SK-N-DZ, SK-N-AS, SK-N-FI, were obtained from American Type Culture Collection (Rockville, MD). All cells were maintained in Dulbecco's modified Eagle minimal essential medium (DMEM, Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Life Technologies, Inc.) at 37 °C under 5% CO_2 in air.

2.3. Tumorigenicity of human NB cell lines

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science center at San Antonio and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were kept in our specific pathogen-free animal facilities. SK-N-DZ, SK-N-AS or SK-N-FI human NB cells (2.5×10^6) were suspended in 0.1 ml of phosphate buffered saline (PBS) and inoculated subcutaneously into the right flank of 4-week-old BALB/c-nu/nu (nude) mice (National Cancer Institute, Frederick, MD) (Day 0). Tumor volume was determined weekly based on the calculation suggested by the National Cancer Institute formula: Volume (cm^3)=(L cm × I^2 cm)/2, in which L is the largest diameter, and I is the smallest diameter of the tumor. Body weight (BW) of mice bearing with or without NB tumor was measured weekly until sacrifice. Subcutaneous (sc) tumors were excised and their wet weight was determined. A portion of the tumor tissue was fixed in 10% formalin for subsequent histological examinations and remaining tissues were snap-frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.4. Bone metastases of human NB cell lines

Bone metastasis of SK-N-DZ, SK-N-AS or SK-N-FI human NB cells was studied using a well-characterized animal model we described previously [17]. Briefly, NB cells (5×10^5) were suspended in 0.1 ml of PBS and injected into the left heart ventricle of 4-week-old nude mice with 27-gauge needle under anesthesia with pentobarbital (0.05 mg/g body weight) (Day 0). Development of bone metastases was monitored weekly by X-ray. At 5 weeks, mice were euthanized and blood was collected for Ca^{2+} measurement and all bones and soft tissues were harvested and fixed in formalin for subsequent histological analyses.

2.5. Radiological monitoring of bone metastases

Animals were anesthetized deeply, then x-rayed in a prone position against the film (22 × 27 cm^2 X-Omat AR, Eastman Kodak Co., Rochester, NY) and exposed with X-rays at 35 KVP for 6 s using a Faxitron radiographic inspection unit (43855A; Faxitron X-ray Corporation, Buffalo Grove, IL) as described [17]. The radiographs were scanned and the area of osteolytic lesions was measured using NIH Image 1.62 and the results were expressed in square millimeters. The number of bone metastases was also counted in bilateral femurs and tibiae.

2.6. Ca^{2+} measurement

Ca^{2+} concentrations were measured in whole blood using a Ciba Corning 634 ISE Ca^{2+}/pH analyzer (Corning Medical and Scientific, Medfield, MA) and adjusted using the internal algorithm of the instrument to pH 7.4 as described previously [18].

2.7. Administration of a selective COX-2 inhibitor NS-398

Three days after the tumor cell inoculation, mice were divided into two groups, one group of mice received daily intraperitoneal (ip) injections of 100 μl vehicle (50% v/v DMSO) and another group of mice received NS-398 (15 mg/kg body weight) [12] until the end of the experiments.

2.8. Histology

Soft tissues and bones were removed from mice at the time of sacrifice, fixed in 10% buffered formalin for 2 days. Bones were further decalcified in 14% EDTA solution for 2 weeks at room temperature with gentle stirring. Subsequently, they were embedded in paraffin and stained with hematoxylin and eosin (H&E). Tartrate-resistant acid phosphatase (TRAP) staining was also performed according to the procedure described [12].

2.9. Immunohistochemistry

Paraffin sections (5 μm) were deparaffinized in xylene and rehydrated through graded alcohol [100%, 95%, 80% ethanol/double distilled H_2O (v/v)], and then rehydrated in PBS (pH 7.4). Sections were microwaved in 10 mM trisodium citrate (pH 6.0) for 4 min for antigen retrieval and then endogenous peroxidase activity was blocked by incubation in 3% H_2O_2/methanol for 30 min at room temperature. After a 30-min treatment with 10% normal goat serum diluted in PBS, sections were incubated