Establishment of Transplantable Murine Osteosarcoma Cell Line with Endochondral Ossification

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Abstract. *Osteosarcoma is defined as a malignant mesenchymal tumor in which tumor cells produce bone matrix. Osteosarcoma cell line (Nishi-Hirosaki osteosarcoma, NHOS) from a spontaneous soft tissue tumor in an athymic mouse was established. The cultured NHOS cells formed a monolayer consisting of spindle to polygonal cells without extracellular matrix formation or mineralization. In contrast, the transplanted NHOS tumor in immunodeficient mice consisted of short spindle and pleomorphic cells, associated with networks of calcified bone and osteoid matrices, as well as small foci of chondroid matrix. Pulmonary metastasis was detected in 22 (42.3%) out of the 52 tumor-bearing mice when NHOS cells were transplanted into the murine flanks. Pulmonary metastasis was detected in all mice (6/6) at post-injection days 21-49 when the cells were injected into the murine tail veins. The NHOS transplantable osteosarcoma cell line with ossifying ability would be useful to clarify the mechanisms of aggressive metastatic potential, as well as for studying the ossification process involved in cell-to-matrix interactions.*

Osteosarcoma is defined as a malignant mesenchymal tumor in which the tumor cells produce bone matrix. It is the most common primary malignant tumor of bone (approximately 20% of primary bone cancers), exclusive of myeloma and malignant lymphoma. Osteosarcoma is one of the most aggressive tumors spreading through the bloodstream and approximately 10% to 20% of patients show pulmonary metastasis at the time of diagnosis. In these patients who die of the neoplasm, 90% have metastasis to the lungs, bones, brain and elsewhere. Recent advances in treatment have improved the prognosis of osteosarcoma. Long-term survival rates are now 60% to 70%, compared to previous rates of 25% (1). However, the inhibition of osteosarcoma metastasis, as well as the suppression of tumor growth, is still one of the most urgent issues in orthopedic oncology.

Recent advances in molecular biological techniques have provided insight into carcinogenesis, neoplastic growth and differentiation in cell culture systems. However, *in vivo* molecular biological analyses have been restricted due to the limited amounts of tumor samples available for study, especially in primary neoplasms. An *in vivo* transplantable tumor system provides sufficient amounts of tumor tissue which retains the original biological characteristics of the tumor. Transplantable osteosarcoma cell lines with ossifying ability would be useful to clarify the mechanisms of aggressive metastatic potential, as well as for studying the ossification process involved in cell-to-matrix interactions (2). There are several human and rat osteosarcoma cell lines with bone formation (3-6), but they are only transplantable into athymic mice. Only a few murine cell lines have been reported (7-11). Therefore, too few transplantable osteosarcoma cell lines with bone formation and frequent metastasis have been established.

An osteosarcoma cell line (Nishi-Hirosaki Osteosarcoma, NHOS) has recently been established from a spontaneous soft tissue tumor in an athymic mouse in our laboratory. In this study, we investigated whether this cell line has ossifying ability when transplanted *in vivo*, and characterized the tumor growth and metastatic potential of the cell line.

Materials and Methods

Primary mouse tumor. The NHOS murine osteosarcoma tumor line was established from a soft tissue tumor which occurred spontaneously in a female BALB/c mouse, purchased from CLEA Japan, Inc. (Tokyo, Japan) and bred under specific pathogen-free conditions at the Institute for Animal Experiments, Hirosaki University School of Medicine, Japan. The NHOS tumors were serially passaged through transplantation into BALB/c mice using injection needles. All animal experiments in this paper followed the Guidelines for Animal Experimentation, Hirosaki University, Japan.

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Key Words: Murine osteosarcoma, cell line, transplantation, osteoblastic differentiation, endochondral ossification.

0250-7005/2008 $2.00+.40

Table I. *Metastasis of the transplanted NHOS osteosarcoma in mice.*

	Metastatic organs		
	Lung	Liver	Spleen
Post-transplantation			
Day 30	15.8% (3/19)	5.3% (1/19)	0% (0/19)
Day 45	50.0% (9/18)	0% (0/18)	0% (0/18)
Day 60	72.7% (8/11)	18.2% (2/11)	9.1% (1/11)
Day 90	75.0% (3/4)	0% (0/3)	0% (0/3)

Table II. *Tumor spread of the NHOS osteosarcoma through the bloodstream (after tumor cell injection into the tail).*

	Metastatic organs		
	Lung	Liver	Spleen
Post-injection (tail vein)			
Day 7	0% (0/2)	0% (0/2)	0% (0/2)
Day 14	0% (0/2)	0% (0/2)	0% (0/2)
Day 21	100% (2/2)	0% (0/2)	0% (0/2)
Days 28, 35, 42 and 49	100% (4/4)	0% (0/4)	0% (0/4)

The tumor-bearing mice were radiologically examined using a soft X-ray apparatus (Type EMB, Softex, Ebina, Janan) under deep anesthesia. They were then sacrificed and the tumors were taken from the mice. Tumor tissue specimens were rapidly fixed with 10% buffered formalin for 24 to 48 hours and routinely embedded in paraffin. Tumor tissues were histologically examined on sections 4 μm-thick stained with hematoxylin and eosin (H&E), as well as special stainings such as Ráliš tetrachrome, alcian blue stain at pH 2.5 and von Kossa's method.

Cell culture. For establishment of the cell line, the NHOS tumor tissues were chopped with a sharp blade. The small tissue fragments were washed with Ca^{++}- and Mg^{++}-free buffer and incubated in trypsin-EDTA solution at 37˚C. Single cells were collected and washed with the buffer. The final cell pellet was resuspended in culture medium (RPMI-1640, Nissui Pharmaceutical, Tokyo, Japan; 10% heat-inactivated fetal bovine serum, JRH Biosciences, Lenexa, KS, USA) and seeded to culture dishes. The cell line was maintained over one year of continuous *in vitro* culturing and stored in liquid nitrogen. The cells used in the present study were those at three passages after thawing. The cultures were incubated at 37˚C in a humidified atmosphere of 5% CO_2: 95% air. A growth medium consisting of RPMI-1640 (Nissui Pharmaceutical) was supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences), 100 units/ml of Penicillin G Potassium (Banyu Pharmaceutical, Tokyo, Japan), 100 μg/ml of Streptomycin Sulfate (Meiji Seika, Tokyo), and 2.5 μg/ml of Amphotericin B (Life Technologies, Grand Island, NY, USA). Contamination with mycoplasma was checked by the polymerase chain reaction (PCR) method using a PCR Mycoplasma Detection Set (Takara Shuzo, Otsu, Japan) according to the manufacturer's instructions. Growth experiments were conducted by plating 2.0×10^5 cells after trypsinization in 35-mm Petri dishes with a base area of 9 cm^2, using 2 ml of growth medium. The medium was changed every other day during the experiment. Three dishes were trypsinized each day and the viable cells were counted by the trypan blue exclusion method using a hemocytometer. Doubling time (DT) was evaluated by the formula:

$$DT = (t1 - t0) \times \log 2 / (\log N1 - \log N0)$$

where N1 and N0 were the cell numbers at t1 and t0, respectively, during the logarithmic growth phase (12).

Transplantation to mice and examination for metastasis. Total 62 BALB/c mice, 6- to 10-week-old female, were used for the experiments, *i.e.* 52 mice for tumor transplantation (Table I) and 10 for tumor spread through the bloodstream (Table II). Suspended 5.0×10^4 cells of the established NHOS cell line were inoculated into the subcutaneous flank regions of the BALB/c mice. At post-transplantation day 30, 45, 60 and 75, the tumor-bearing mice were individually sacrificed under deep anesthesia, and the transplanted tumors and metastases were analyzed (Table I). Tumor tissue specimens and mice organs fixed with 10% buffered formalin were excised routinely embedded in paraffin, and histologically examined on 4 μm-thick sections stained with H&E and the other stainings described above.

In addition, tumor spread through the bloodstream was analyzed. Suspended 2.0×10^4 NHOS cells were injected into the tail veins of the BALB/c mice using needles. At post-injection day 7, 14, 21, 28, 35, 42 and 49, the mice were individually sacrificed under deep anesthesia, and metastases were histopathologically analyzed in the systemic organs of mice (Table II).

Results

Morphology of primary murine tumor. The NHOS primary tumor was a soft tissue tumor which occurred spontaneously in a female BALB/c mouse. Histologically, the tumor

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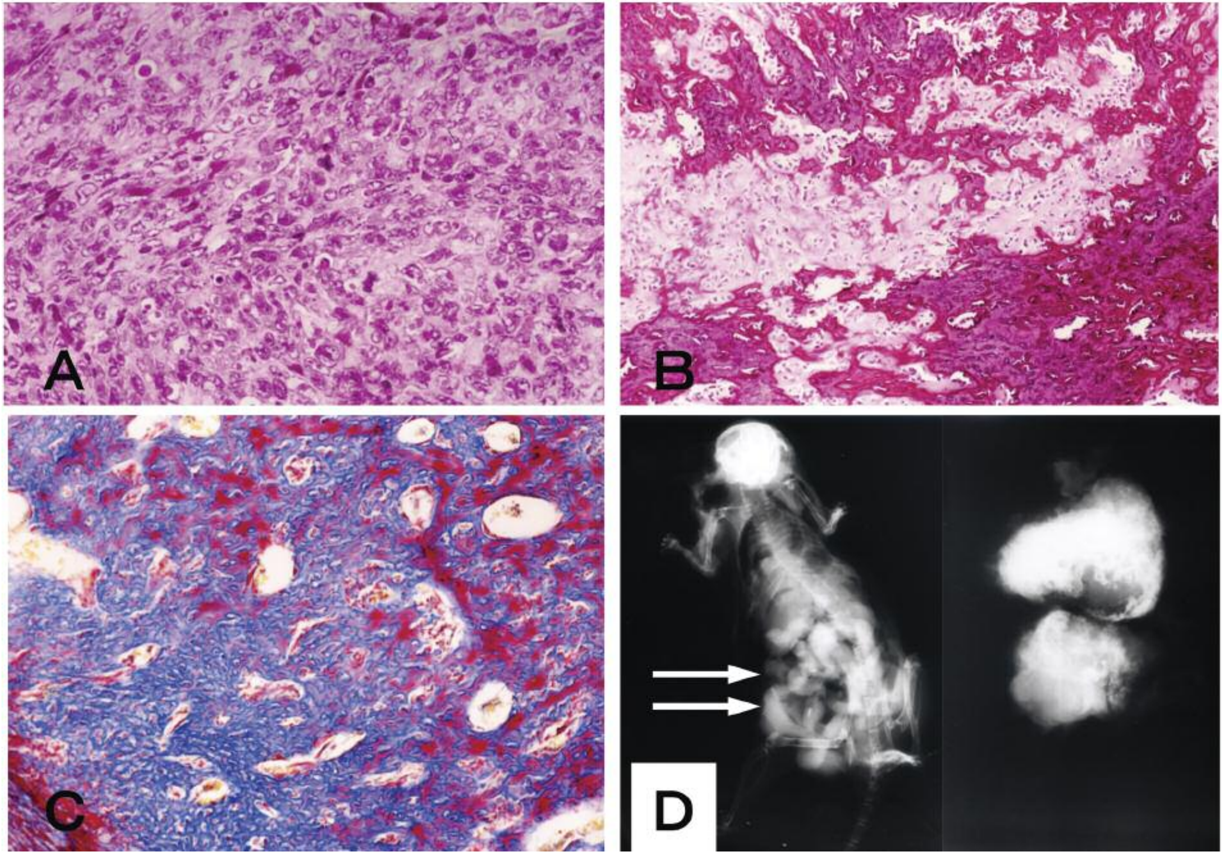
Figure 1. *Histological features of the NHOS osteosarcoma cell line. The primary tumor consisted of short spindle and pleomorphic cells, focally exhibiting a fascicular pattern (A, hematoxylin & eosin). The tumor formed networks of calcified bone and osteoid matrices (B, hematoxylin & eosin). Section with Ráliš tetrachrome staining showed calcified bone tissues in dark red, and osteoid matrices in dark blue (C). Soft X-ray photography showed significant calcified parts in the primary tumor, but no apparent destruction of the murine bones (D, arrows).*

Figure 2. *Histological features of the NHOS osteosarcoma cell line. The cultured cells formed a monolayer consisting of spindle to polygonal cells without extracellular matrix formation and mineralization (A). Multinucleated giant cells were spread among the spindle to polygonal cells (B, cell culture, hematoxylin & eosin). The transplanted NHOS cells in mice showed pulmonary metastasis, which consisted of proliferated spindle/pleomorphic tumor cells with bone and osteiod formation (C, hematoxylin & eosin). Tumor thrombus was confirmed in the lungs of the tail-vain injection model (D, arrows, hematoxylin & eosin).*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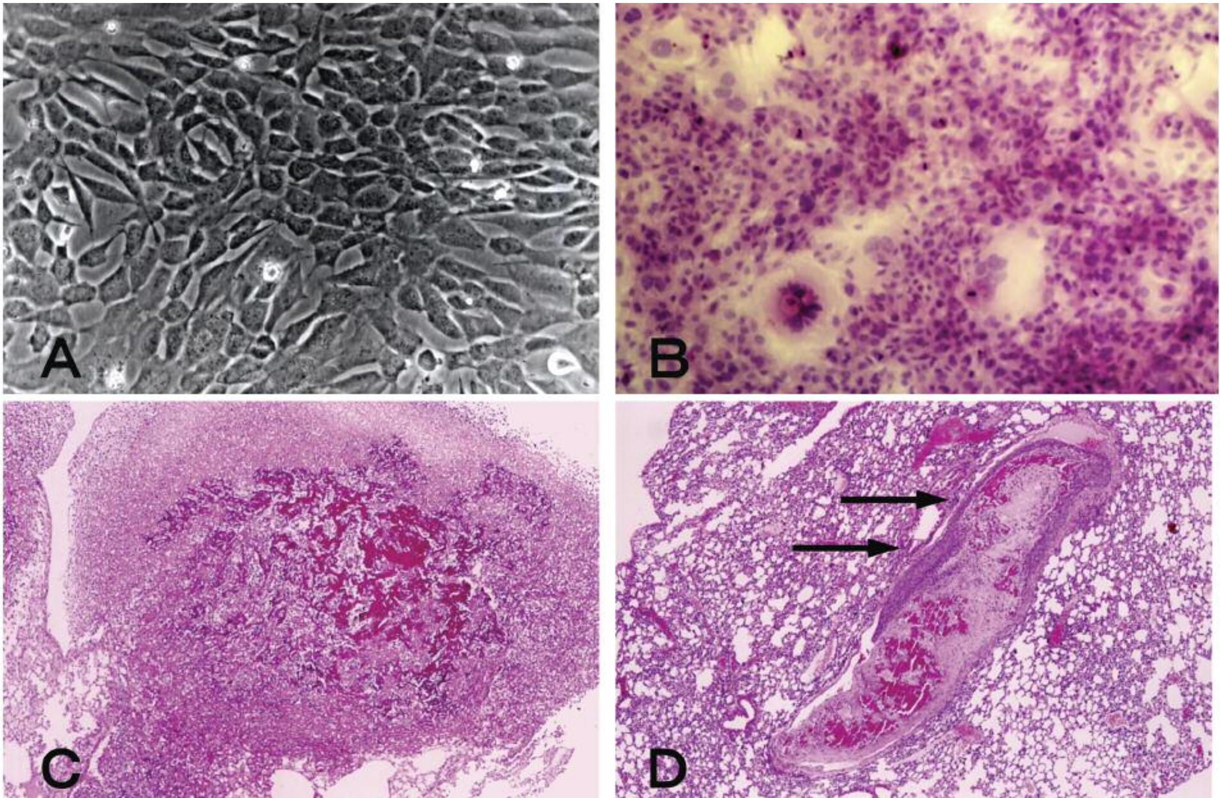
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consisted of short spindle and pleomorphic cells, focally exhibiting a fascicular pattern (Figure 1A). In addition, the tumor formed networks of calcified bone and osteoid matrices (Figures 1B and C), associated with small foci of chondroid matrix which stained positively for alcian blue. The histological features were typical of osteosarcoma, which is defined as a malignant tumor characterized by the direct formation of bone and/or osteoid by proliferating tumor cells. Soft X-ray photography showed significant calcified parts in the primary tumor, but no apparent destruction of the murine bones (Figure 1D). Because it had originated in the soft tissue of a mouse, the tumor represented a murine extraskeletal osteosarcoma.

Characterization of cultured cells. The established NHOS cells seeded onto the dishes began to proliferate without a noticeable lag phase. The cell line formed a monolayer consisting of spindle to polygonal cells without extracellular matrix formation or mineralization (Figure 2A). Multinucleated giant cells were spreaded among the spindle to polygonal cells (Figure 2B). During a logarithmic growth phase, the NHOS cell line showed a doubling time of 13.6 hours.

In vivo metastatic potential. Tumor masses were detected as swelling of the inoculated flanks in all of the mice within five days after transplantation. Pulmonary metastasis was detected in 22 (42.3%) of the 52 tumor-bearing mice, especially the mice at post-transplantion days 60 and 90 (Table I). Hepatic and splenic metastases were less frequently found, in 3 (5.8%) and 1 (1.9%) of the 52 mice, respectively. The other organs, such as kidneys and lymph nodes, showed no metastatic nodules. Histologically, the metastatic nodules consisted of proliferated spindle/ pleomorphic tumor cells with bone and osteiod formation (Figure 2C), which were similar to the primary murine tumor.

Tumor spread of the NHOS osteosarcoma through the bloodstream was analyzed, *i.e.* tumor cell injection into the tail was performed. Pulmonary metastasis was detected in all mice at post-injection days 21-49, while no tumor metastasis was detected at post-injection days 7 and 14 (Table II). Other organs including the liver and spleen showed no metastatic nodules. Histologically, the metastatic nodules, as well as the tumor thrombi in the arteries, were confirmed in the lungs of the tail-vain injection model (Figure 2D).

Discussion

The NHOS osteosarcoma cell line was established from a spontaneous soft tissue tumor in an athymic mouse. In the present study, we demonstrated that the cell line has ossifying ability when transplanted *in vivo*, and further characterized tumor growth and metastatic potential of the cell line. The histological examination revealed that the established tumor had typical features of osteosarcoma, which is defined as a malignant tumor characterized by the direct formation of bone and/or osteoid by proliferating tumor cells (13). Because it had originated in the soft tissue, the tumor represented a murine extraskeletal osteosarcoma (14).

We established both the transplantable tumor and the cultured cell line because the transplantable tumor and the cell line have different advantages for tumor biology. The transplantable tumors in the mice model are useful because they provide sufficient amounts of tumor tissue for analysis of the *in vivo* biological characteristics of neoplasms (15-21). The tumors are shown to retain their original characteristics including morphological phenotypes and biological features (22, 23). On the other hand, *in vitro* characterization, transformation and cloning of the cultured cell lines are easier to compare to those of transplantable tumors in the mice model (24). Established cell lines are useful in analyzing the genetic and cellular characteristics of neoplastic cells.

Osteosarcoma is the most common primary malignant tumor of bone, exclusive of myeloma and malignant lymphoma. However, aggressiveness of the osteosarcoma has not yet been clarified extensively. Moreover, pathological mechanisms of the osteoid and bone formation of the osteosarcoma are poorly understood. Transplantable osteosarcoma cell lines with ossifying ability would be useful for study of the ossification process *in vivo* and to clarify factors derived from such cells and host cells that are involved in cell-to-matrix interactions (2, 25), as well as for analysis of the aggressive behavior and the metastatic potential. Only a few murine cell lines have been reported (7-9), and there are several human and rat osteosarcoma cell lines with bone formation (3-6) which are only transplantable into athymic mice. Therefore, our established cell line is thought to be unique.

In conclusion, the established NHOS cell line has the properties of osteoblastic lineage, differentiating to osteoblasts in the stationary phase *in vitro*, and exhibits an ossifying ability following transplantation. The cells transiently manifest a chondroid phenotype during the ossification processes. Thus, the NHOS cell line is a useful model for the study of not only osteosarcoma, but also the endochondral ossification process, especially with *in vivo* experiments.

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Received August 28, 2007
Revised February 20, 2008
Accepted February 26, 2008