

Three-dimensional alginate spheroid culture system of murine osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common primary malignant tumor of the bone and often forms pulmonary metastases, which are the most important prognostic factor. For further elucidation of the mechanism underlying the progression and metastasis of human OS, a culture system mimicking the microenvironment of the tumor *in vivo* is needed. We report a novel three-dimensional (3D) alginate spheroid culture system of murine osteosarcoma. Two different metastatic clones, the parental Dunn and its derivative line LM8, which has a higher metastatic potential to the lungs, were encapsulated in alginate beads to develop the 3D culture system. The beads containing murine OS cells were also transplanted into mice to determine their metastatic potential *in vivo*. In this culture system, murine OS cells encapsulated in alginate beads were able to grow in a 3D structure with cells detaching from the alginate environment. The number of detaching cells was higher in the LM8 cell line than the Dunn cell line. In the *in vivo* alginate bead transplantation model, the rate of pulmonary metastasis was higher with LM8 cells compared with that of Dunn cells. The cell characteristics and kinetics in this culture system closely reflect the original malignant potential of the cells *in vivo*.

Introduction

Osteosarcoma (OS) is the most common primary malignancy of bone in adolescents and young adults. Despite an aggressive combination of chemotherapy and surgery, the

pulmonary metastasis that occurs in approximately 40-50% of patients with OS (1) leads to an unfavorable prognosis with limited therapeutic options. To further elucidate the underlying mechanism of the progression and distant metastasis of human OS, it is important to develop a culture system that reflects the clinical manifestation of human OS.

To investigate tumor cell biology it is advantageous to utilize a cell culture system that mimics the microenvironment of a tumor *in vivo* (2). Tumor cells have close cellular contact and a complex extracellular matrix with non-uniform vasculature formation leading to a hypoxic microenvironment with extracellular acidity. Heterogeneous regions of dividing cells, hypoxic cells, and necrosis are mixed within a tumor with a three-dimensional (3D) structure (3). In contrast, conventional monolayer-cultured cells grow in a nutrient and oxygen uniform environment with a two-dimensional homogeneous structure that differs from the microenvironment of the tumor *in vivo* (2). Therefore, to successfully investigate the pathobiology of a tumor, including OS, it is necessary to establish a 3D culture system that closely resembles the tumor tissue architecture and microenvironment *in vivo*.

In this study, two different murine OS cell lines with high metastatic potential to the lung, LM8 (4) and its original Dunn cell line (5), were encapsulated in alginate beads to develop the 3D cell culture system. The purpose of this study was to determine whether murine OS cells are able to grow within an alginate environment and to form a 3D structure. The cellular characteristics and kinetics in this *in vitro* culture system were evaluated to establish whether the cells maintained their malignant phenotype. Furthermore, the beads containing murine OS cells were also transplanted into mice, where tumor growth and pulmonary metastasis were examined to determine the metastatic potential of the OS cells in the alginate beads.

Materials and methods

Cell culture. Two murine OS cell lines, the parental Dunn (5) and the derivative line LM8 with a higher metastatic potential to the lung (4), were used in this study. The LM8 cell line was established from the original Dunn cell line by eight Fidler's repeated *in vivo* selections (6). These two cell lines were kind gifts from Osaka University, Japan. The

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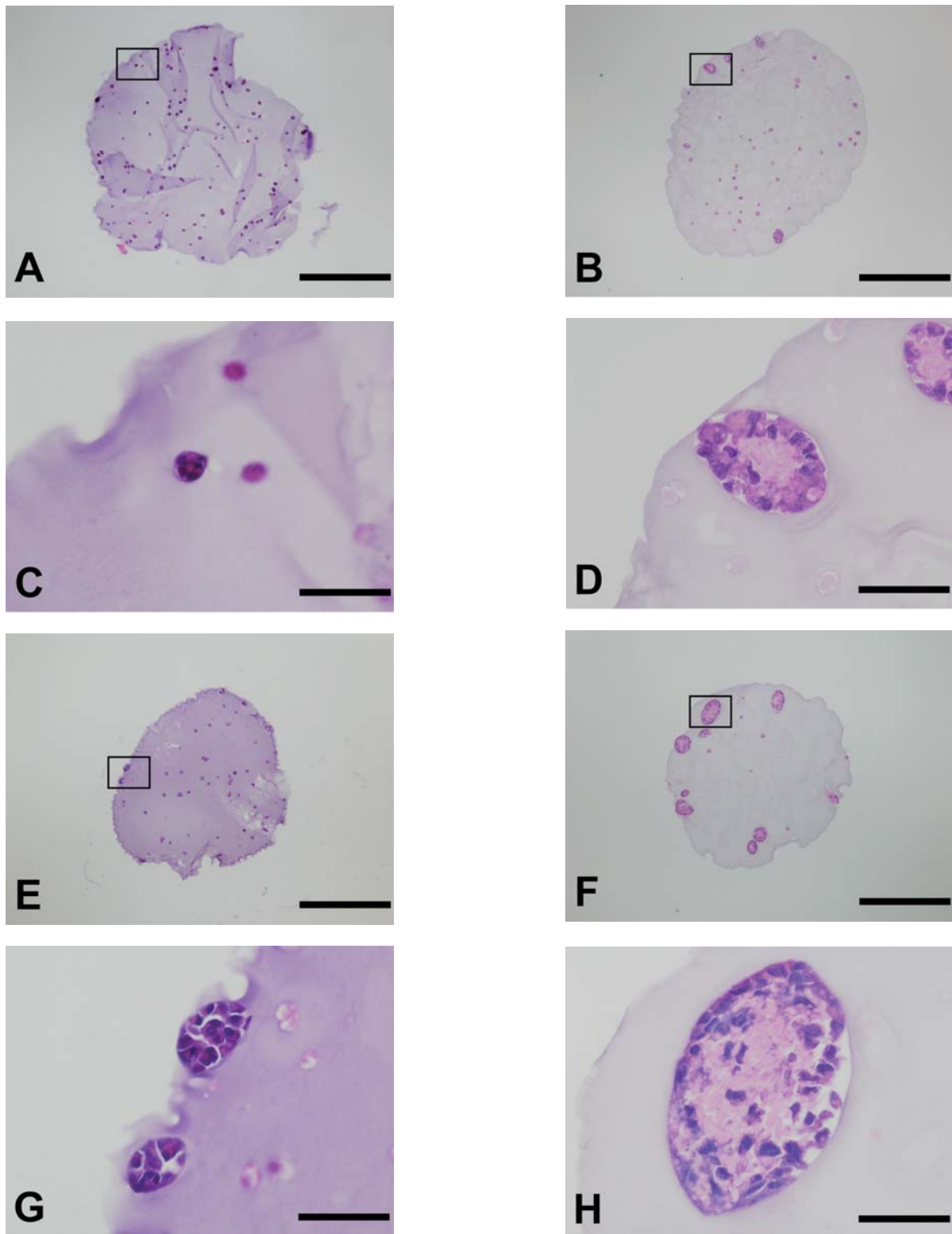


Figure 1. Histology of alginate beads containing the osteosarcoma (OS) metastatic clones, the parental Dunn cell line. Dunn (A, B) and LM8 (E, F) cells encapsulated in alginate beads were cultured for 14 days (A, E) and 28 days (B, F). High magnification images (C, D, G, H) correspond to the image of the area marked by a square (A, B, E, F), respectively. Scale bars: 500 μm (A, B, E, F), 50 μm (C, D, G, H).

Dunn and LM8 cells were seeded at a density of 2.0×10^5 cells in 175-cm² culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; CELlect[®], MP Biomedicals Inc., Eschwege, Germany) and 50 $\mu\text{g}/\text{ml}$ gentamicin (Gibco). At >90% confluency (on day two), the cells were released using 0.05% trypsin digestion. After several

washes in DMEM media, the isolated cells were encapsulated in 1.2% low-viscosity sterile pharmaceutical grade alginate [Keltone LV-(HM), a gift from ISP Alginate Inc., San Diego, CA, USA] in 0.15 M sodium chloride (NaCl) at 4 million cells/ml as previously described (7,8). Briefly, the cell suspension in alginate solution was slowly expressed through a 21-gauge needle and dropped into a 102 mM CaCl_2 solution. The beads



SPANDIDOS PUBLICATIONS were to polymerize in this solution for 10 min before successive washes with 50 ml of 0.15 M NaCl. The beads were then cultured in DMEM supplemented with 10% FBS and gentamicin (50 $\mu\text{g}/\text{ml}$). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Each bead contained $\sim 4.0 \times 10^4$ cells. The media were changed daily.

Histological analysis. On days 14 and 28 of culture, the beads of each cell line were pretreated with 100 mmol/l BaCl₂, which causes irreversible polymerization of the alginate molecules, for 15 min and then fixed in 2.5% glutaraldehyde solution containing 10% cetyl pyridinium chloride and 2% sucrose in phosphate-buffered saline (PBS) (9). The beads were then embedded in paraffin and 5- μm sections were cut. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Cell proliferation. At culture days 2, 4, 6, 8, 10, 12 and 14, the beads were solubilized with dissolving buffer containing 0.15 M NaCl, 30 mM ethylenediamine tetraacetic acid (EDTA) and 55 mM sodium citrate at 4°C for 20 min. The cells were recovered by centrifugation at 100 x g for 5 min. The DNA content of the nine beads at each time-point of culture was determined using the bisbenzimidazole fluorescent Hoechst 33258 dye assay (Polysciences, Warrington, PA, USA) assay as previously described (10).

Cell detachment assay. One bead containing either the Dunn or LM8 cell line was cultured in 100 μl of DMEM with 10% FBS (n=10) in a 96-well plate (Cellstar, Greiner Bio-One, Tokyo, Japan) for 24 h, following which the bead was then transferred to a new well. The number of cells that detached from the alginate bead and adhered to the bottom of the culture plate during the next 24-h culture period was determined. The adhered cells were fixed in 4% paraformaldehyde, stained with H&E and manually counted under a light microscope. This assay was repeated daily until day seven of culture.

In vivo alginate beads transplantation model. Seventeen C3H mice (male, 5-weeks-old) were used for the *in vivo* alginate bead transplantation model. C3H mice were obtained from Japan Oriental Yeast Co., Ltd. (Tokyo, Japan). All mice were housed under specific pathogen-free conditions with a 12-h light and dark cycle. The housing care rules and experimental protocol were approved by the Animal Care and Use Committee of Mie University. The animals were anesthetized with ketamine (80 mg/kg) and xylazine (7 mg/kg). Five alginate beads containing Dunn or LM8 cells ($\sim 2.0 \times 10^5$ cells) were transplanted subcutaneously under the dorsal skin of mice (Dunn, n=8; LM8, n=7). For the control group (n=2), five beads without tumor cells were transplanted as described above. Body weight and tumor volume in mm³ [(minor axis)² x (major axis)/2] were measured every other day. Measurement of tumor volume was started from day eight after transplantation when the solid nodular lesion was first palpable on the dorsal skin of mice. After sacrifice on day 28, the tumors and lungs were removed, fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 5- μm sections. The sections were stained with H&E, and evaluated by light

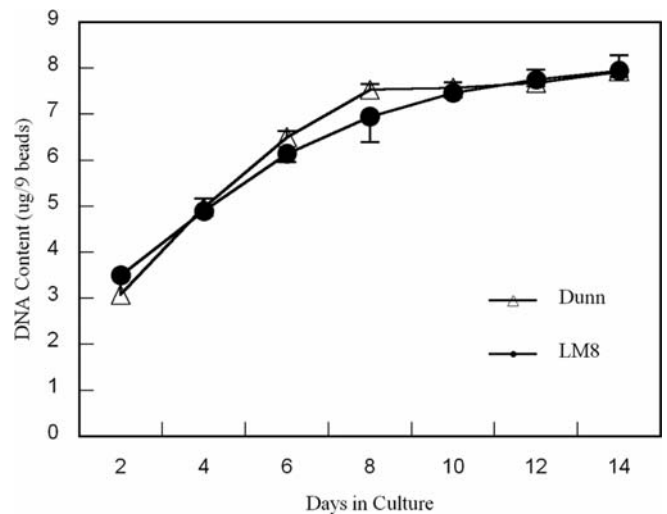


Figure 2. The DNA content of alginate beads containing the osteosarcoma (OS) metastatic clones, the parental Dunn cell line or its derivative, the LM8 cell line. The DNA content of the alginate beads increased over time for both Dunn and LM8 cells until day 14 of culture; there were no significant differences throughout the experimental period.

microscopy. On the maximal area of thin sections of lungs, the number of tumor nodules (metastasis) was counted microscopically (x200).

Statistical analysis. Statistical significance was determined using the unpaired Student's t-test (Stat-View software, Abacus Concepts, Berkeley, CA, USA). P-values <0.05 were considered significant. All data are expressed as the mean \pm standard deviation (SD).

Results

Histological analysis of alginate beads. On day 14 of culture, Dunn cells were evenly distributed within alginate beads and several small nodular formations were mainly found at the periphery of the beads (Fig. 1A, C). The Dunn cell line formed tumor spheroids at the periphery of the beads on day 28 of culture (Fig. 1B, D). Tumor spheroid formations by the LM8 cell line were identified at the periphery of the beads on day 14 of culture (Fig. 1E, G). The LM8 cell tumor spheroids were enlarged within the alginate beads on day 28 of culture (Fig. 1F, H), while few cells were identified at the center of the bead (Fig. 1F). Both Dunn and LM8 cells grew within the alginate beads and formed tumor spheroids at the periphery of the beads. The tumor spheroids of the LM8 cell line were larger than those of the Dunn cell line.

DNA content. The DNA content of the alginate beads increased over time for both Dunn and LM8 cells until day 14 of culture; there were no significant differences between the cell lines at any time-point (Fig. 2).

Cell detachment assay. The cells that had detached from the alginate environment were attached to the bottom of the culture plate in both the Dunn and LM8 cell lines (Fig. 3B, C). On day one of culture, no cells had detached from the alginate beads for both the Dunn and LM8 cell lines. However,

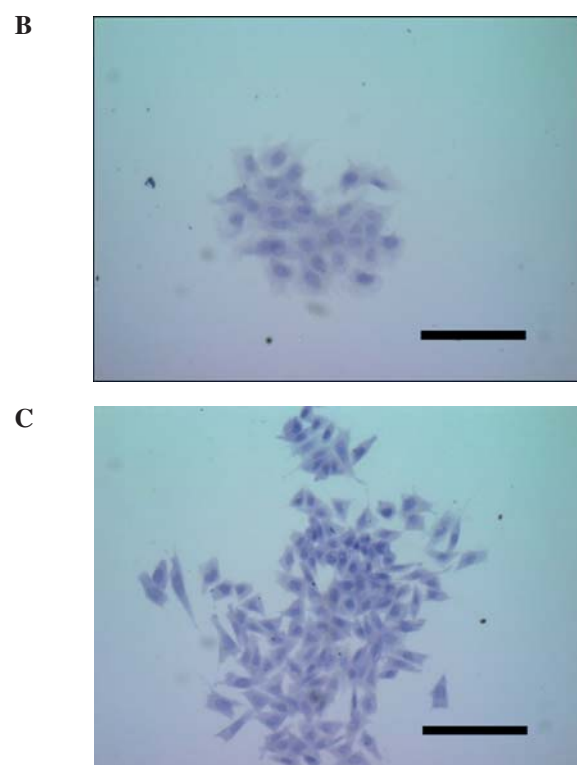
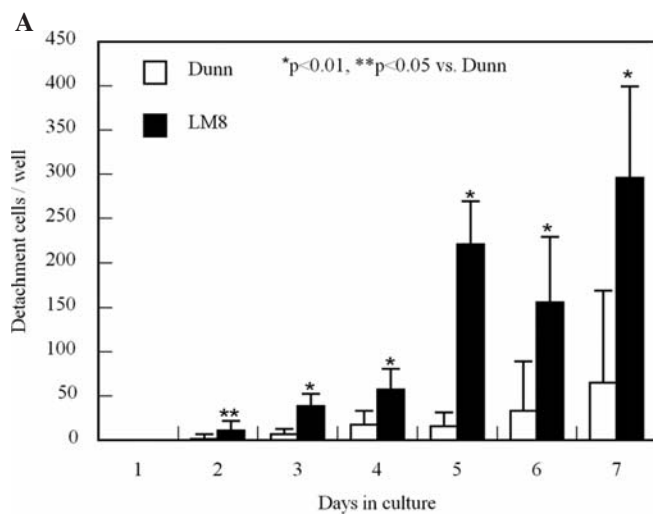


Figure 3. Cell detachment assay for the osteosarcoma (OS) metastatic clones, the parental Dunn cell line and its derivative, the LM8 cell line. The number of detached cells for the LM8 cell line was significantly higher than that for the Dunn cell line on days two to seven of culture (A). Representative images of Dunn (B) and LM8 (C) cells that detached from the alginate bead and adhered to the bottom of the culture plate on day six of culture. Scale bars: 200 μ m (B, C).

from day two of culture, the number of cells increased over time for both Dunn and LM8 cell lines. The number of detached cells for the LM8 cell line was significantly higher than that for the Dunn cell line on day two ($p < 0.05$) and days three to seven ($p < 0.01$) of culture (Fig. 3A).

In vivo alginate beads transplantation study. All mice receiving transplants of beads containing either Dunn or LM8 cells formed subcutaneous elastic hard tumor nodules, which were identifiable at day eight after transplantation (Fig. 4C).

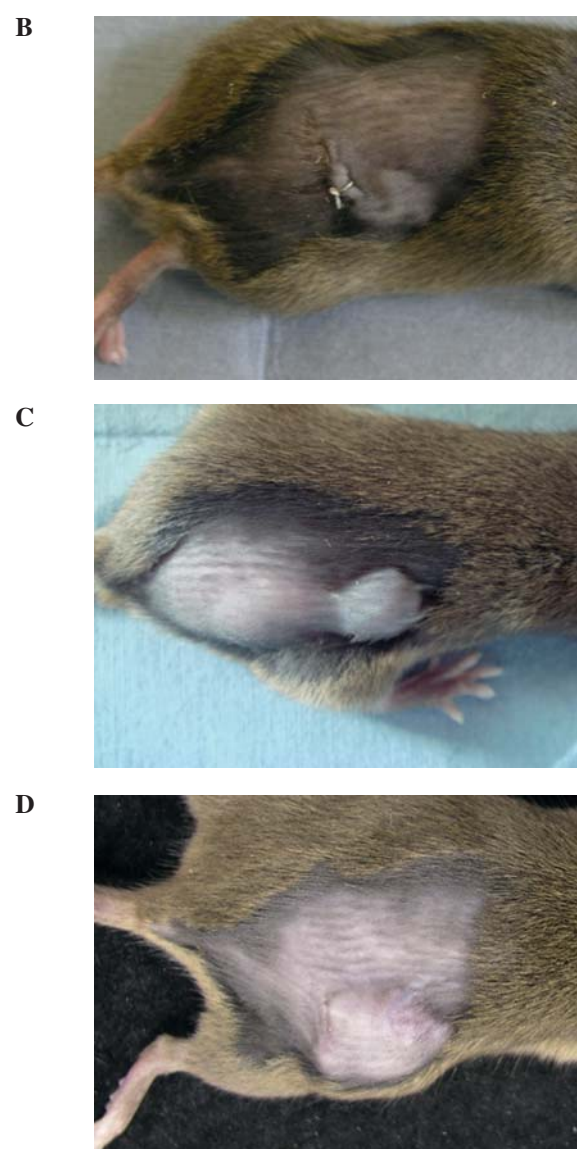
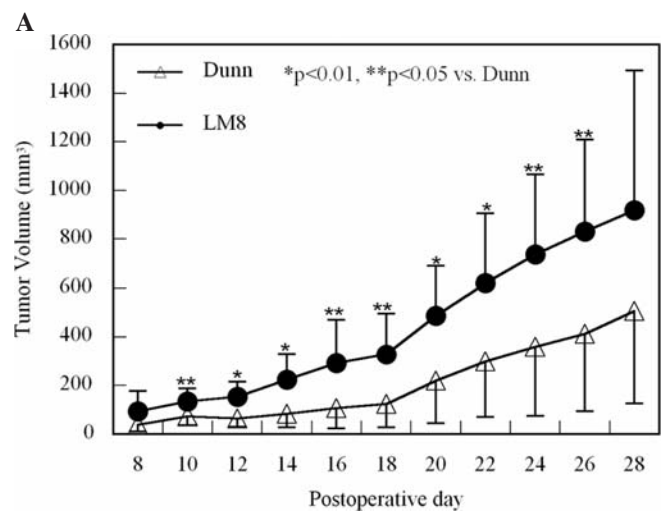


Figure 4. Tumor volume and macroscopic images of local tumor formation. Five alginate beads containing the osteosarcoma metastatic clones, the parental Dunn cell line or its derivative, the LM8 cell line, were transplanted under the dorsal skin of C3H mice. The tumor volume of the LM8 group was significantly larger than that of the Dunn group on day 10 to day 26 (A). Representative macroscopic pictures of mice after transplantation of five alginate beads containing LM8 cell line are shown. Immediately after transplantation (B), eight days after transplantation (C), 28 days after transplantation (D).

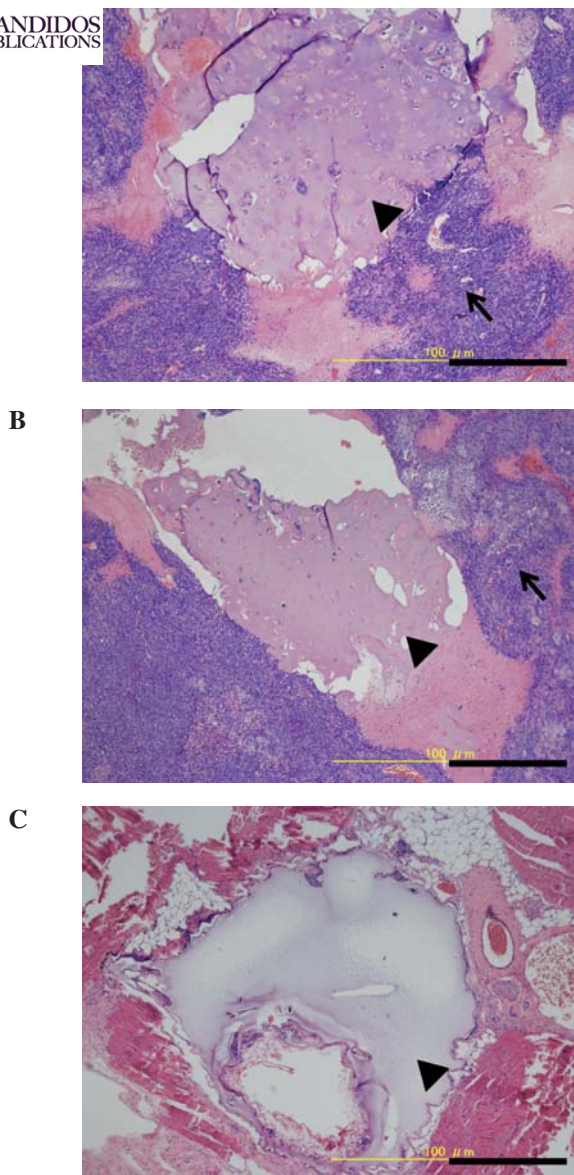


Figure 5. Local tumor histology. Five alginate beads containing the osteosarcoma metastatic clones, the parental Dunn cell line or its derivative, the LM8 cell line were transplanted under the dorsal skin of C3H mice. Tumor nodules (arrow) formed around the alginate beads (allow head) by both Dunn (A) and LM8 (B) cells. In the control (no cell) alginate transplantation group (C), alginate beads were identified within the muscle layer. Scale bar: 50 μ m.

The tumor nodules progressively increased in volume until sacrifice on day 28 (Fig. 4A). The body weight of mice in all experimental groups increased over time [no cell beads: 21.1 ± 0.6 g (day 0), 25.0 ± 0.1 g (day 28); Dunn: 21.4 ± 1.0 g (day 0), 24.4 ± 1.5 g (day 28); LM8: 19.6 ± 1.2 g (day 0), 23.0 ± 2.4 g (day 28)]. There were no significant differences in body weight among the three groups throughout the experimental period.

The tumor volume for both Dunn and LM8 cell lines increased over time. The tumor volume of the LM8 group was significantly larger than that of the Dunn group on days 10, 16, 18, 24 and 26 ($p < 0.05$) and on days 12, 14, 20 and 22 ($p < 0.01$) (Fig. 4A).

Histologically, tumor nodules formed around the alginate beads for both the Dunn and LM8 cells (Fig. 5A, B). Alginate

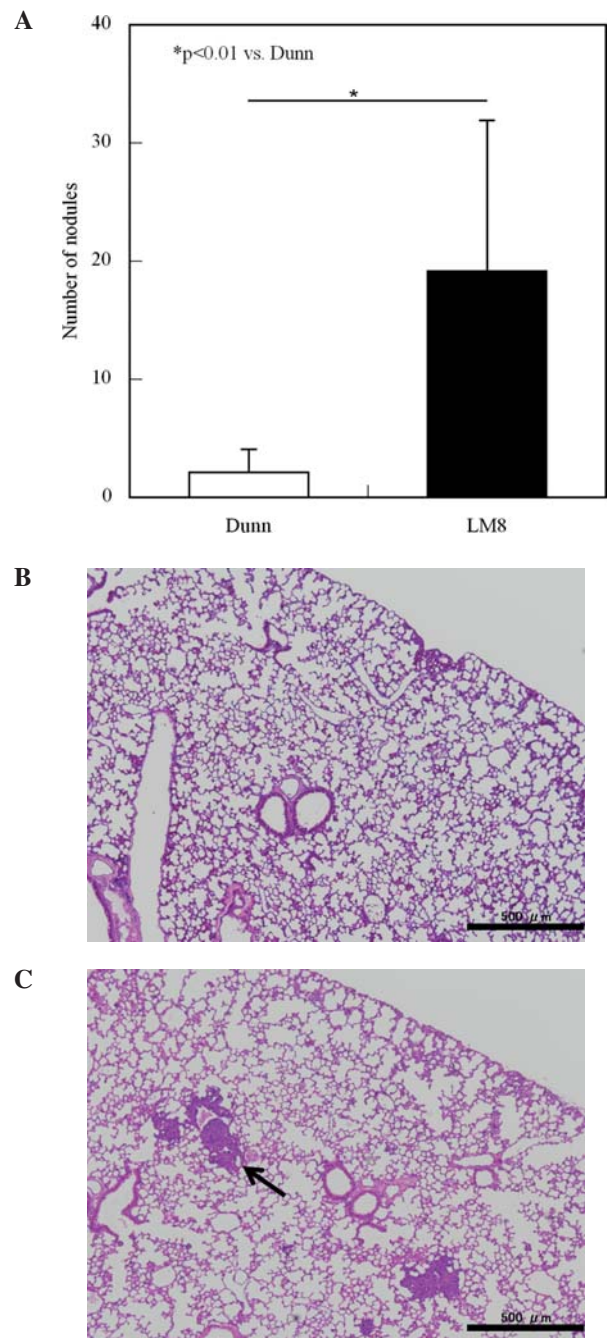


Figure 6. Histological analysis of pulmonary metastases. Five alginate beads containing the osteosarcoma metastatic clones, the parental Dunn cell line or its derivative, the LM8 cell line, were transplanted under the dorsal skin of C3H mice. The number of pulmonary metastatic nodules formed by LM8 cells was significantly higher than that of Dunn cells (A). Representative histological images of lung by Dunn (B) and LM8 (C) groups are shown. Arrow indicates metastatic nodules. Scale bars: 500 μ m (B, C).

beads remained within the tumor nodule (Fig. 5A, B). In the control (no cell) alginate bead transplantation group, the alginate beads were identified within the subcutaneous muscle layer. No inflammatory cell infiltration into the alginate beads was seen (Fig. 5C).

Pulmonary metastases spontaneously formed at high rates 28 days after transplantation by both the Dunn and LM8 cell lines [Dunn: 6/8 (75%); LM8: 7/7 (100%)]. The number of pulmonary metastatic nodules formed by LM8 cells was significantly higher than that for Dunn cells ($p < 0.01$) (Fig. 6A).

Discussion

We have established a new 3D alginate spheroid culture system for murine OS using two different metastatic clones (the parental Dunn and its derivative line LM8 with a higher metastatic potential to the lung). In this culture system, murine OS cells encapsulated in alginate beads were able to grow in a 3D structure with cells detaching from the alginate environment. The number of detached cells was higher in the LM8 cell line than in the Dunn cell line. In the *in vivo* alginate bead transplantation model, the tumor volume of the LM8 cell line was larger and the rate of pulmonary metastasis was higher compared with those of the Dunn cell line; this suggests that the cell kinetics in this culture system reflect the original malignant potential of the cells *in vivo*.

It is becoming increasingly recognized that the tissue microenvironment, including complex cell-cell and cell-matrix interactions, is crucial to the regulation of metabolism and function of the constituent cells. The field of tissue engineering has developed 3D culture systems to create a more realistic environment that better reflects the *in vivo* microenvironment. During the past decade a number of 3D culture systems of *in vitro* tumor models have been developed to screen and analyze the effect of chemotherapeutics or gene therapy (reviewed in ref. 2). To support the extracellular matrix in 3D culture, scaffold-based culture systems have been fabricated and shown to recreate the complex heterogeneous 3D structure of tumors *in vitro* (11-15). These 3D culture technologies have been utilized for the study of OS (13,15-19).

Alginate is a natural polysaccharide extracted from brown algae consisting of linear copolymers of 1,4-linked α -L-guluronic acid and β -D-mannuronic acid. Alginate forms a gel consisting of negatively-charged and cross-linked 3D lattices in the presence of a divalent cation solution, such as calcium chloride (20). Because of its high biocompatibility and the low impact of the gelatin procedure on cell viability, alginate has been used as a scaffold to maintain the phenotype of articular chondrocytes (9,21-23), as well as intervertebral disc cells (7,8,24), during 3D culture and as a dressing to promote wound healing (25,26). Alginate-encapsulated vector-producing cell lines have also been used for cancer gene therapy *in vivo* (27). Chondrocytes encapsulated in an alginate environment are known to reform a heterogeneous matrix similar to that found in native cartilage tissue, including a compartmentalized matrix and matrix volume per cell (22,23). To our knowledge, there is no previous report of OS cells being cultured in an alginate scaffold. Taking the advantages of alginate into consideration, we have developed a 3D alginate scaffold culture system for murine OS.

Our study demonstrated that both Dunn and LM8 cell lines grew in the alginate environment and that the DNA content of alginate beads containing both Dunn and LM8 cells increased equally over time. Histologically, both Dunn and LM8 cells showed a tendency to locate peripherally within the beads over time in culture, forming tumor spheroids that were larger with LM8 cells than with Dunn cells. A similar finding was reported for alginate-encapsulated hybridoma cells (20). During the pre-alginate culture of Dunn and LM8 cells in this culture system, we found that the

cells were also found on the bottom of the culture plates. Therefore, to precisely evaluate cell proliferative activity in this culture system, the cells detached from the alginate beads were also quantitatively evaluated. Our results showed that the number of cells detaching from the alginate environment by the LM8 cell line was significantly higher than that of the Dunn cell line, indicating that LM8 cells have a greater tendency to detach and migrate from the alginate microenvironment. Because the detachment of cells from the primary tumor is the first step in the sequential process of metastasis (28), the cellular detachment observed in this culture system may reflect the initial step of metastasis for the *in vivo* microenvironment.

We next examined whether the Dunn and LM8 cell lines encapsulated in alginate beads are capable of growing and maintaining their original metastatic potential *in vivo*. In a preliminary study, 1, 5 or 10 beads containing LM8 cells were transplanted under the dorsal skin of C3H mice. The survival rate of cells in the transplanted alginate beads was 67% (two of three mice) in the 1 bead transplantation group and 100% (three of three mice) in the 5 and 10 beads transplantation groups. Therefore, in this study, 5 beads containing either Dunn or LM8 cells were transplanted subcutaneously under the dorsal skin of C3H mice. Ten days after transplantation of the beads, histology of the transplantation site showed that LM8 cells had proliferated within the alginate and formed tumor spheroids similar to that observed *in vitro*. Twenty days after transplantation, the beads were buried within the tumor nodule (data not shown). The results of this study showed that both Dunn and LM8 cells encapsulated in alginate beads grow in an *in vivo* environment. In addition, both cell lines readily form lung metastasis, but at a higher rate in the LM8 cell line than in the Dunn cell line; this suggests that the cell kinetics in this culture system reflect the original malignant potential of both the cell types as previously reported (4).

For cancer cells, including OS cells, to form distant metastases, the cells must proliferate locally, detach from the primary site, migrate and invade the microvasculature, survive in the circulation, and adhere and proliferate at a distant organ (28). Major animal models of spontaneous pulmonary metastasis have been developed using subcutaneous and/or orthotopic (e.g., intra-tibial) injections of tumor cells suspensions (4,29-31). There is a possibility that these procedures induce an increase in the local tissue-pressure, which could cause dissemination of the tumor cells into the surrounding tissues or into the circulation. Therefore, the initial steps of spontaneous metastasis, such as local tumor growth, detachment, and invasion, could not be demonstrated. In our animal model, murine OS cells encapsulated in alginate beads proliferated with detachment of the cells from the alginate environment and formed pulmonary metastases, suggesting that this animal model faithfully recapitulates the sequential steps of pulmonary metastasis as observed in the clinical progression of human OS.

In conclusion, we have developed a 3D alginate tumor spheroid culture system for murine OS. The cellular characteristics and kinetics in this culture system closely reflect the metastatic potential of both Dunn and LM8 cell lines; this was confirmed by the *in vivo* alginate beads transplantation



We propose that this newly developed alginate-ated tumor spheroid model could be useful for investigating the pathobiology of OS, as well as for drug screening and the testing of new therapies. Moreover, the alginate bead transplantation model can be used as a spontaneous pulmonary metastasis model that is clinically relevant to human OS.

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