Establishment and Characterization of the Growth and Pulmonary Metastasis of a Highly Lung Metastasizing Cell Line from Canine Osteosarcoma in Nude Mice

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ABSTRACT. Highly lung metastasizing model of canine osteosarcoma in nude mice was established from five subcutaneous implantation cycles of lung tumor deposits. The selection of cells with increased metastatic properties from the parent POS canine osteosarcoma cell line recovered medium sized and polygonal Highly Metastasizing POS cells (HMPOS). The doubling time of HMPOS and POS in culture averaged 30 ± 1.2 hr and 32 ± 1.3 hr respectively, and their cell growth patterns in vitro were comparable to their in vivo growth patterns. HMPOS cells produced more tumor deposits (>20 nodules, >1-mm in diameter) of various sizes with replacement of lung tissues at 12 weeks after implantation. POS cells produced fewer and smaller lung deposits (<10 nodules, 1-mm in diameter). Tumor size and number of metastatic tumor deposits showed a regular association. HMPOS cells developed an osteoblastic type of cellular differentiation subcutaneously and in the lungs. HMPOS micrometastasis along the alveolar walls and blood vessels at 4 weeks averaged 6–7 small tumor locus. Each micrometastatic locus contained an average of 5–7 tumor cells, and developed a pleomorphic osteoblastic type of cellular differentiation. An average of 4 macrometastatic nodules could be seen at 6 weeks, composed of an average of 23 tumor cells, 10 nodules at 8 weeks, 12 nodules at 10 weeks and 20 nodules at 12 weeks. These model provides an opportunity for the evaluation of new treatments against canine lung metastatic osteosarcoma in a nude mice model.—KEY WORDS: canine osteosarcoma, cell line, implantation, lung metastasis, nude mouse.


The most devastating aspect of cancer is the emergence of metastasis in organs distant from the primary tumor. Metastasis may have already been established when a definite diagnosis for the cancer is made. It is the uncontrolled growth of metastasis resistant to therapy that kills most patients despite significant advances in surgical techniques and chemotherapy.

Osteosarcoma is one of the most malignant tumors of both man and animals. It has a high percentage of lung metastasis in its early stages [18]. Canine osteosarcoma closely resembles human osteosarcoma in its histological appearance, biological behavior and pathogenesis, such that it becomes an excellent model for the application of tumor in vivo biological and preclinical studies [11]. There is however, very few reports characterizing its lung metastatic behaviour.

Experimental tumor metastasis models promote advances in the evaluation of potential new treatment strategies based on local tumor growth and metastasis formation, development of alternative treatment modalities, the application of simple estimation of anti-metastatic effects of anti-cancer drugs, and the testing of new agents and new therapeutic regimens [3, 4, 21, 23]. Likewise, the mechanism and the distribution of metastases can improve understanding of the metastatic process, elucidate the cellular and the molecular basis of the metastatic phenotype, and the production of information with practical implications for the supression of secondary tumor growth [10, 13, 24, 25].

We have previously established and characterized a new cell line derived from a spontaneous canine osteosarcoma in a dog and was called POS [7]. The original osteosarcoma developed on the left femur in a 1.5-year-old male mongrel dog, with the femoral neck considerably destroyed by the tumor. Cell suspensions were made from the surgically excised and minced tissues and were expanded in culture for four months with 20 passages routinely cultured in RPMI-1640. Six clonal cells were also established, characterized and classified from POS using a limiting dilution technique [16].

This study presents the establishment and characterization of the growth and lung metastasis of canine osteosarcoma in a nude mice model produced by the selection of tumor cell populations with increased lung metastatic properties from the previously established, characterized and cloned POS canine osteosarcoma cell line.

MATERIALS AND METHODS

Animals and irradiation: Five week old female BALB/cAJcl nude mice (Clea Lab., Tokyo, Japan), housed under specific pathogen free conditions, and fed with sterile feed and water continuously were used for the study. They were irradiated at 63.5 cGy for 6 min and 18 sec before subcutaneous transplantation of tumor cells.

All animals were used and cared for according to the approved guidelines in the handling of experimental animals by the Graduate School of Veterinary Medicine, Hokkaido University.

Establishment of highly lung metastasizing POS cell line:
POS canine osteosarcoma parent cell line [7], at the 14th passage, were selected for populations of cells with high lung metastatic capacity in vivo. POS cells in RPMI-1640 were prepared at $5 \times 10^6$ in 1.0 ml of physiological buffered saline (PBS, pH 7.4) and injected subcutaneously into the back of each 5-week-old irradiated BALB/cAJcl nude mice. Developed subcutaneous tumors were grown until 12 weeks. Cells were selected in vivo by harvesting metastatic tumor deposits in the lungs and immediately reimplanting subcutaneously into new nude mice, completing the selection process of 5 cycles for 15 months. After the selection process, developed subcutaneous tumors were dissected and enzymatically digested with trypsin to yield tumor cell suspensions with enhanced pulmonary metastatic capacity. These selected cells were expanded in culture, and named Highly Metastasizing POS (HMPOS) cells.

**Cell line suspensions and tumor cell inoculations:** HMPOS single cell suspensions, with greater than 90% viability (trypan blue staining), at $5 \times 10^6$/ml in PBS, were subcutaneously injected at the back of each nude mice 3 days after irradiation. Nude mice were divided into 5 groups: 4, 6, 8, 10 and 12 weeks, according to the time of necropsy, with 5 nude mice in each group. POS cells as a control were also used as above and nude mice were necropsied at 12 weeks.

**Morphology and growth in cultured cells:** HMPOS and POS cells were seeded into 8-well chamber slides (Falcon Co., Tokyo, Japan) at $1 \times 10^5$ cells per well in 300 ml RPMI-1640 supplemented with 10% fetal calf serum (FCS), and grown to near confluency at 37°C, in 95% air and 5% CO$_2$. The cells were then washed with PBS and stained with giemsa solution. The cells were morphologically compared by light microscope under 200X and 400X magnifications. HMPOS and POS cells were cultured in 12 well plates (Falcon Co., Tokyo, Japan) at $2 \times 10^5$ cells/cm$^2$ well in 2.0 ml RPMI-1640 with 10% FCS. The cell number were determined for 8 consecutive days by trypan blue staining. The growth curves and their corresponding doubling times were compared. All counts were done in triplicates.

**HMPOS and POS lung metastasis colonization potential:** Tumor cell suspensions of HMPOS cell line and POS at $5 \times 10^6$/ml single cell suspensions were each implanted subcutaneously at the back of each preirradiated nude mice, with the developed subcutaneous tumor grown for 12 weeks. The incidence, pattern, number and size of neoplastic lung macrometastases formed were compared.

**Tumor growth characterization:** Subcutaneous tumor size (TS) $= L \times W$, where $L$ = length of tumor and $W$= width of tumor, and Tumor Weight (TW=gm) after careful dissection at necropsy time, were characterized for the HMPOS and POS cells.

**HMPOS subcutaneous tumor and lung histopathology:** Gross and histopathological description of the developed subcutaneous tumors and the lung tissues were done under stereomicroscope and hematoxylin and eosin (HE) stained sections respectively. Identification of the type of cellular differentiation of the subcutaneous tumors in each group were described.

**HMPOS lung macrometastasis and histopathology:** Spontaneous metastases into the lung were described according to occurrence, location and pattern of colonization, gross pathology, number of tumor deposits, and histopathology of tumor deposits and lung.

**HMPOS lung micrometastasis:** Micrometastases were characterized according to occurrence, location, number of locus formed, number of tumor cells in each locus, and their type cellular differentiation. Continuous parallel histopathological sections were made for each lung sample to screen for micrometastasis.

**Metastasis to other organs:** Spontaneous metastasis into the liver, spleen, kidney, stomach and intestines were investigated grossly and histopathologically.

**Statistical analysis:** For the comparison of the changes between HMPOS and POS in the cell number, tumor size, tumor weight and the number of macrometastatic nodules, analysis of variance, followed by Scheffe’s multiple comparison as post hoc test was used. Data were expressed as the mean $\pm$ S.E. A $P$ value less than 0.05 was considered statistically significant.

**RESULTS**

**Morphology and growth in the cultured cells:** HMPOS cells were generally medium sized cells and showed a polygonal morphology (Fig. 1-1). POS cells appeared in mixed sizes from small to large cells and different shapes from spherical, fibroblastic or polygonal cells (Fig.1-3). HMPOS and POS cells grown in culture for 8 days averaged $30 \pm 1.2$ hr and $32 \pm 1.3$ hr doubling time respectively, and the cell number of HMPOS at 4 days were significantly increased as compared to POS (Fig. 2-1).

**Subcutaneous growth of HMPOS and POS:** Tumor size in HMPOS was progressively increased from $17.7 \pm 0.6$mm at 4 weeks, to $22.0 \pm 3$ mm at 6 weeks, $26.2 \pm 0.3$ mm at 8 weeks, $27.0 \pm 0.3$ mm at 10 weeks and $28.0 \pm 0.5$ mm at 12 weeks, being significant at 6, 8, and 10 weeks as compared to POS (Fig. 2-2). Tumor weight in HMPOS progressively increased similarly with increase in the size, being significant at 8 weeks as compared to POS (Fig. 2-3).

**HMPOS and POS subcutaneous tumor characterization:** Grossly large round moderate soft to hard neoplasm were developed subcutaneously with HMPOS cells, with occasional rupture in the center from 6 weeks. Soft white tissue was grossly observed upon cutting. Histologically, the subcutaneous tumors were arranged in solid sheets located in the dermis consisting of large pleomorphic cells, with varied sizes of vesicular nuclei from round to ovoid, with distinct to prominent nucleoli. An osteoblastic type of cellular differentiation was developed in all nude mice. POS subcutaneous tumors mainly consisted of osteoblastic type of osteosarcoma showing characteristic osteoid trabeculae.

**HMPOS and POS lung colonization potential:** After 12 weeks from subcutaneous implantation, HMPOS cells
produced more tumor deposits (>20 nodules, >1-mm in diameter) of various sizes with considerable replacement of lung tissues (Fig. 1–2, Fig. 2–4), while POS cells produced fewer and smaller lung metastatic deposits (<10 nodules, 1-mm in diameter) and occasional larger ones (Fig. 1–4, Fig. 2–4).

**HMPOS lung macrometastasis:** Macrometastasis was absent at 4 weeks, but were observed from 6 weeks. An average of 4 visible pulmonary neoplastic nodules could be seen at 6 weeks, with each nodule composed of an average of 23 tumor cells, 10 nodules at 8 weeks, 12 nodules at 10 weeks and 20 nodules at 12 weeks. Initially, the macrometastases were small to medium sized, white glossy, soft to hard nodules attached into the lung parenchymas. Some neoplastic nodules exist singly when they are big, especially from 10 weeks, and usually small and sometimes occur in clusters from 6 weeks. No particular lung lobule was preferred and the distribution pattern was scattered. Progressive replacement of the lung tissues with lung tumor deposits were observed at 12th weeks.

**HMPOS lung micrometastasis histopathology:** The lungs showed mild congestion, moderate thickening of the alveolar walls, hyperplasia of bronchioles, mild multifocal hemorrhages, and occasional mild emphysema. The neoplastic nodules showed mild mitotic figures, sometimes surrounded by plasma cells, accompanied by local extensive thickening of the alveolar walls (hyperplasia of type II pneumocytes), and multifocal necrosis. The nodules were mainly composed of small to large pleomorphic osteoblastic cells occurring in clumps along the alveolar walls and near the basement membranes of blood vessels. An increase in the number of tumor nodules, and the number of its tumor cells in the lungs were observed as tumor size increased on every necropsy schedule (Fig. 3).

**HMPOS lung micrometastasis:** Lung micrometastasis seen along the alveolar walls and blood vessels at 4 weeks averaged 6–7 small tumor locus. Each tumor locus contained an average of 5–7 tumor cells. All micrometastatic tumor cells developed a pleomorphic osteoblastic type of cellular differentiation (Fig. 3-1).

**Metastasis to other organs:** Spontaneous metastasis into the liver, spleen, kidney, stomach and intestines were not found grossly and histopathologically for all groups of nude mice.

**DISCUSSION**

Selection of tumor cell populations with increased metastatic properties can be achieved by isolating metastases and establishing new variants of a tumor. This procedure had been previously done in the selection of highly metastatic cells to the lungs in murine osteosarcoma cell
line (LM8) [1], tumor cell dissemination studies from metastasized organ transplantations in murine mammary carcinoma [7], in vivo selection of highly metastatic human colon carcinoma cells by nude mice implantation [13, 15], isolation, growth and metastasis of several human renal cell carcinomas in nude mice [16] and the selection of murine cells with enhanced metastatic potential [21]. In this study, an initial selection process was similarly done using the POS canine osteosarcoma cells. Metastatic cells were first selected in vivo by harvesting metastatic tumor deposits produced in the lungs, and ectopically implanting small intact tumor deposits subcutaneously into the back of nude mice and allowed to spontaneously metastasize into the lungs. This selection process was repeatedly done in 5 continuous cycles, 12 weeks for each cycle, with the selected cells finally recovered and expanded in culture. This derived line from the parent line POS was named HMPOS.

After the in vivo selection process, the isolated HMPOS cells was compared to the POS cells. Morphologically, HMPOS cells appeared as medium sized cells and polygonally shaped. In contrast, the original parent POS cells were composed of spherical, polygonal, and fibroblastic shaped cells appearing in various sizes as shown in the results and described previously [8]. HMPOS cells also showed a slightly higher doubling time than POS cells when grown in culture. HMPOS cells grown subcutaneously in the back of nude mice showed a significant increase in tumor size and weight at 6–10 weeks and 8 weeks respectively as compared to POS tumors. HMPOS cells also formed osteoblastic type of cellular differentiation when grown subcutaneously, while POS tumors mainly consisted of osteoblastic type of osteosarcoma, with characteristic trabeculae and partial amounts of chondroblastic type, fibroblastic type and undifferentiated type of tissues. The...
Fig. 3. Histopathology of lungs at 4 weeks showing micrometastases formation (1), and the lungs with macrometastases formation at 6 weeks (2), 8 weeks (3), 10 weeks (4) and 12 weeks (5). (HE stain, × 100).
lung colonization potential of HMPOS cells *in vivo* has shown enhanced metastatic capacity and considerable replacement of lung tissues when compared to the parent POS cells after 12 weeks.

With the type of cellular differentiation formed by HMPOS cells subcutaneously and in the lungs occurring as osteoblastic and osteoblastic pleomorphic in nature respectively, this may suggest that drugs to which osteoblastic tissue forming osteosarcoma cells are sensitive to, might be of significant value in reducing the neoplasia’s proliferation and spread in the lungs. We have previously demonstrated that vitamin D3 and retinoids can induce apoptosis in the parent POS cells *in vitro* [2]. We have also demonstrated that these differentiation-inducing vitamins could induce morphological differentiation, inhibit the growth and reduce the proliferative ability of POS and its fibroblastic (POS 14A), chondroblastic (POS 53B), undifferentiated (POS 53C) and osteoblastic (POS 53D) clonal cell lines *in vitro* [3] and implied that loss of control of differentiation in osteosarcoma can be reversed. Therefore, this growth and lung metastasis model presents potential application for testing the differentiating and anti-metastatic efficacy of these vitamins *in vivo*.

By the second week post-implantation of HMPOS cells, developed tumor sizes averaged from 5 to 6 mm in diameter. Tumors which grow beyond the size of 2 mm in diameter can already synthesize and secrete angiogenic factors that paves the way for intravasation, shedding of tumor cells, and the formation of seeding colonies [21]. This might then suggest that HMPOS cell micrometastases may have already been established as early as a week after implantation, and this however must be confirmed.

It is also known that tumor cells with high metastatic potential tend to aggregate with each other forming homotypic clumps [6]. Similarly, homotypic clumps were also observed with HMPOS in the lungs. It is possible that these cells may have extravasated into the lung parenchyma by destruction of the surrounding vessels or penetration of endothelial basement membranes and thin walled capillaries, since most tumor cell clumps observed developed near intact or destroyed blood vessels.

The patterns of micrometastasis formation seen at 4 weeks showed extravascular type of tumor cell growth occurring in foci composed of 6–8 cells. A sequential observation of HMPOS cell micrometastasis formation in the lungs needs to be further established to specifically detect lung dissemination patterns at the single cell level. This sequential observations of micrometastasis formation have been previously demonstrated by gene tagging rat prostatic adenocarcinoma cells and Lewis lung carcinoma cells through transfection of bacterial lac Z gene with the sequential events in micrometastasis formation, including entry into the blood circulation and arrest, extravasation and initial growth in the lungs clearly demonstrated [8, 9]. Experimental pulmonary micrometastasis models could also be done by the incorporation of viral encoded tumors specific antigens [25].

In this model, the intravascular type of micrometastasis formation was not seen at 4 weeks. This may have occurred very early since clumps of cells, and not single cells, had already been formed in the lung parenchyma. The extravascular micrometastasis seen in the lungs at 4 weeks may suggest that HMPOS cells have already elaborated degrading enzymes which had disrupted blood vessel basement membranes in the lungs and allowed for its extravasation. Similarly, spontaneous lung metastasis model of human osteosarcoma was developed through orthotopic transplantation of histologically intact tumor xenografts into the tibia of nude mice [4]. In this study however, highly lung metastatic cell line was first established and cells subcutaneously injected to characterize its growth and establish a spontaneous lung metastasis model. HMPOS cells also formed “metastasis of metastasis” [5, 20] at 4 weeks, with extravasation of tumor cells to adjacent lung tissues. This suggests that multiplication, growth of vascularised stroma into new tumor mass, and final tumor expansion of these cells via the hematogenous route were already complete at 4 weeks.

An unstrict correlation between tumor size and the number of microscopic and macroscopic metastatic tumor deposits, and the number of tumor cell aggregates inside each neoplastic nodule in the lungs was also observed. Similar correlations with mean tumor diameters and lung metastasis formation were likewise seen in breast carcinoma cell lines when implanted in nude mice [18] and average tumor size and the number of lung micrometastasis formation in prostatic adenocarcinoma cells [10]. However, a regular association between the growth rate and the metastatic capability of HMPOS cells was hard to establish from the present data.

Progressive development into macroscopic metastasis occurred by 6 weeks. Tumor colonization potential of macrometastasis was graded similarly according to the scale described previously [20]. HMPOS cells have no macroscopic colonization activity at 4 weeks; low colonization potential, with a few to small deposits, at 6–8 weeks; moderate colonization potential, with deposits of various sizes, at 10 weeks; and high colonization potential, with numerous deposits of various sizes, and some degree of lung tissue replacement at 12 weeks.

In conclusion, the following characterization of the growth and lung metastases of canine osteosarcoma cells provides an opportunity for the evaluation of new anticancer drugs and treatment strategies against canine lung metastatic osteosarcoma in a nude mice model.

REFERENCES


HIGHLY LUNG METASTASIZING CANINE OSTEOSARCOMA


