Isolation and Identification of Cell Sublines with Different Metastatic Capacities from Human Osteosarcoma MG-63

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Abstrcat **Objective** To supply a good experimental model of studying osteosarcoma metastatic mechanism by establishment of human osteosarcoma MG-63 cell sublines with different metastatic capacities. **Methods** Four cell sublines derived from human osteosarcoma MG-63 cell line were isolated preliminarily and established by clone technique *in vitro* and transplantation in nude mice.We compared, analysed and identificated their biologic characters by using cell electrophoresis, cell proliferation, agarose clony-formation , cell migration assay in vitro, subcutaneous and orthotopic implantation in nude mice. **Results** The cell electrophoretic and proliferatic rate, agarose clony-formation ability , migration ability in vitro and spontaneous metastatic ability to lung in nude mice of A2, A3 were obviously higher than that of A6, A20, The *t*-test showed the difference between A2, A3 and A6, A20 was statistically significant ($P<0.01$). **Conclusion** The establishment of human osteosarcoma MG-63 cell sublines with different metastatic capacities can supply a better experimental model for the further studies of metastatic mechanism of osteosarcoma.

Key words MG-63 cell line; Osteosarcoma; Metastasis; Cell sublines

Osteosarcoma is the most common primary malignant bone tumor, which is characterized by a high propensity for metastasis (i.e, the lungs, liver and bone). In spite of successful control of the primary tumor , its mortality due to metastatic spread is beyond 30% of patients within 5 years[1]. Though recently there are a lot of the studies about osteosarcoma metastatic mechanism [2,3], the establishment of better metastatic experimental model of osteosarcoma is needed. In order to the destination we isolated preliminarily and established four cell sublines derived from human osteosarcoma MG-63 cell line with different metastatic capacities by clone technique in vitro and transplantation in nude mice. In the same time, we analysed and identificated their biologic characters.

MATERIALS AND METHODS

Reagents MEM was purchased from Gibico BRL, USA. FBS was obtained from Hyclone Co.Ltd. Trypsin was purchased from Sigma Corporation and Agarose LMP was obtained from Promega Co.Ltd.

Mice BALB/C-nu/nu nude mice were purchased from the Experimental Center of Shanghai Drug Institute of Academy of China. These animals were between 4~5 w of age, 18~22g in weight and male and female nude mice were respectively constitute a half of the total nude mice when they were used in all experiments. Animals were maintained in standard ambient conditions with free access to food and water. After purchased, they were allowed to adapt to their environment for 1w before initiating the experiments.

Cell Line Human osteosarcoma MG-63 cell line (ATCC Number:CRL-1427)was purchased from the cell collection center of Wuhan University who obtained the cells originally from American Type Culture Collection (Manassas, VA).

Cell culture MG-63 cell line was grown in MEM supplemented with 10% heat-inactivated(30min,55 °C) fetal bovine serum, penicillin (50U/ml) and streptomycin (50μg/ml). All cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. The culture medium was exchanged every 3 to 4 days. When approximately 80% of them were confluent, cells were trypsinized with 0.1% trypsin in Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). The cells were subcultured with a 1:3 to 1:4 split for several passages. Throughout the experiment, the cells which had been used were in logarithmicphase of growth.

Isolation of cell sublines MG-63 cells were collected until about 80% confluent. Four cell sublines was isolated from the cultured cell line with the limiting dilution method and use of 96-well culture plates.

Electrophores migration rates of cell sublines Electrophores migration rate of the cells (1.0×10^6/ml) of 20 cell sublines were measured (40V, 37℃) with the cell electrophores instrument (LIANG-100)which was purchased from the instrument factory of the medical college of Shanghai. The samples of every cell subline were 10 totally and every sample had 10 cells. The electrophores migration rates were averaged and were analysed statistically with the SPSS software.

Proliferation curves of cell sublines The cells of cell sublines were collected and planted into a 24-well plate (1.0×10^4/ml for each well). Each sample was repeated 21 parallel wells and counted for the number of cells (3 wells) everyday and cell numbers were averaged.

Colony formation assay in soft agarose 1 portion of 5% Agarose LMP and 9 portion of culture medium were mixed and then solidified in a 24-well plates (1 ml for every well). Cells (125) were mixed well with 9.4ml culture medium with 20% fetal bovine serum and 0.6ml 5% Agarose LMP. The mixture was overlaid on the bottom gel (0.8ml for each well), allowed to set at room temperature and then grown at 37℃ in a humidified atmosphere containing 5% CO_2 for 21 days. Colony formation was monitored under microscope, and 50 cells or more in a cluster were defined as a colony. Six parallel wells were set up for each cell sublin, and mean value was used in comparison.

Cell migration assay Cell migration assays were performed as described [3] using modified Boyden chambers with transwell membrane filter inserts in a 24-well tissue culture plate (Corning Costar Corp., Cambridge, MA). The transwell filters were 6.5 mm in diameter, 8μM pore size, 10μM thick polycarbonate membrane. In brief, only the underside surface of the polycarbonate membrane on the upper chamber was coated with 10μg/ml fibronectin in PBS for 2h. After the chamber was rinsed with PBS, it was placed into the lower chamber filled with 400μl of MEM containing 1% fetal calf serum. The cells (5.0×10^4) suspended in the MEM containing 0.1% BSA at 5.0×10^4 cells/ml were applied to the upper chamber and allowed to migrate to the underside of the upper chamber for 12h at 37℃ with 5% CO_2. After the nonmigrated cells on the upper membrane surface were removed with a cotton swab, cells that migrated to the underside of the upper chamber were fixed in 4% formaldehyde and stained in 1% crystal violet. The number of the stained cells were counted under a microscope in five different high-power fields in duplicate wells, in at least four independent experiments. Data are expressed as mean±S.D. of four independent experiments.

Xentransplantation in nude mice The nude mice were injected s.c. subcutaneously into the right back leg of nude mice with 5.0×10^6 cells of each cell subline. The nude mice were randomly divided into each group, with 5 mice in each group. The mice were maintained in a sterile animal facility and monitored for tumor growth. Tumor size was measured at the three weeks. Four weeks later, the mice were killed and the tumor was cut up into 1 mm^3 pieces.

Orthotopic transplantation in nude mice Two to three pieces (1 mm^3) were implanted into the right tibia of nude mice. The nude mice were randomly divided and each group had 15 mice. The each cell subline was as a group. After eight weeks, the mice were killed and the tumor tissues such as tibia and lung were dissected and checked by naked eye for histological observation. Frozen or paraffin section of the lung tissues were made, stained with HE observed for micrometastasis under microscope.

Statistical analysis Date were expressed as mean± S. D. Statistical significance was determined by Student´s *t*- tests. Difference with a $P< 0.05$ was considered to be statistically significant.

RESULTS

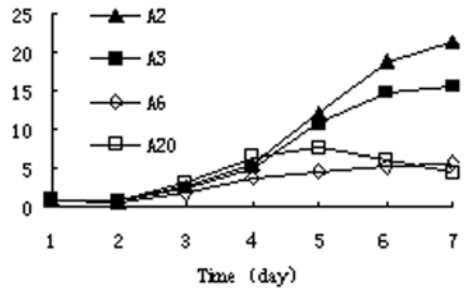

Fig.1 Growth curves of MG－63 cell sublines with different metastatic capacities .

Table 1. The formation rate of metastatic tumor to lung of each cell subline.

Group	Mouse	Metastasis to lung	Formation rate (%)
A2	15	15	100
A3	15	13	86.67
A6	15	3	20
A20	15	1	6.67

Isolation of Cell Sublines Twenty cell sublines were obtained by the limited dilution method and were named respectively as MG－63－A1～ A20.

Electrophores Migration Rates of Cell Sublines By being measured the electrophores migration rates of 20 Cell Sublines, The experiment obtained two cell sublines that had the highest rate and two cell sublines that had the lowest rate . They were A2,A3 and A6,A20. Their electrophores migration rates were respectively 1.02 ±0.09μm,1.08 ±0.12μm,0.69 ±0.11μm, 0.64 ± 0.13μm. The t－ test showed that no significant difference between A2 and A3, and between A6 and A20 (P>0.05). but the difference was significant in statistics as compared A2, or A3 with A6, or A20 respectively (P <0.01) .

Proliferation Curves of Cell Sublines To determine the growth ability of each cell subline, the cell growth of A2, A3, A6 and A20 was observed and their growth curves were drawn (Fig.1). It shows that the growth ability of A2,A3 was obviously higher than that of A6, A20.The platform phase of A6, A20 was earlier than that of A2,A3 .The cell number of A2,A3 was much more than that of A6,A20.

Colony Formation Assay in Soft Aga－rose The colony of the four cell sublines was 21.0 ± 2.3, 19.3 ± 1.8, 9.5 ± 2.9, 12.5 ±3.5 respectively. The t－ test showed that no significant difference between A2 and A3, and between A6 and A20 (P>0.0 5). But A2, A3 demonstrated significant difference as compared with A6, A20 (P<0.01). The colony diameters of A2, A3 were bigger than that of A6,A20.

Cell Migration Assay The cell migration index of A2, A3, A6 and A20 was 186 ± 16.7, 178 ± 11.5, 79 ± 13.8, 84 ±12.6 respectively. The t－ test showed that no significant difference between A2 and A3, between A6 and A20 (P>0.05). A2, A3 demonstrated significant difference as compared respectively with A6, A20 (P <0.01).

Xenotransplantation in Nude Mice The four kinds of cells (A2,A3,A6 and A20) had been respectively inoculated subcutaneously into the right back leg of nude mice. The tumor growth was monitored at every other day. The time of new tumor formation of A2 and A3 in the locus of injection was 6~8 days, but that of A6 and A20 was 11～13 days. After 3 weeks, the tumor size (length×width) was measured. A2, A3, A6 and A20 was 1.12×0.56cm, 0.98×0.32cm, 0.69×0.52cm, 0.61×0.47cm respectively. After 4 weeks , all mice were killed and no metastatic focus was founded in the nude mice of the four cell sublines. The tumorigenesis rate of each cell subline was 100%.

Orthotopic Transplantation in Nude Mice After 8 weeks, all mice were killed. The tibia and lung were dissected and observed with naked eye. The tumors

forming in the locus of orthotopic transplantation of all mice were obvious. The lungs of some mice had the formation of tumor metastatic focus with different size (1~3 mm).The results of tumor metastatic focus formation are shown in Table 1. The $t-$ test showed that it was not significant difference between A2 and A3, and between A6 and A20 ($P> 0.05$), but the difference was significant in statistics as compared A6, or A20 with A2,A3 respectively ($P<0.01$) . Microscopic observation of HE paraffin sections revealed that the parenchymatous structure was in the tumor metastatic focus of lung .The focus located in the parenchyma of lung. The distribution of tumor cells with different sizes was crowded together and disorderly. The heterotype of tumor cell was founded and there were a lot of the division phases of cell nucleus. A little of tumor giant cell were founded and shown the change of undifferentiated carcinoma tissue. Sometimes it was shown that tumor cells infiltrated peripherad. The partial normal structure of lung was destroyed. The cell appearance of metastatic tumor was unanimous basically with MG−63 cell line.

DISCUSSION

Since Fidler [4]put forward the concept of tumor metastatic heterogeneity in 1977, people have gradually known that the heterogeneity exist in the compo − sition of primary tumor. In a tumor, many kinds of cell sublines can exist simultaneously with different biologic character and metastatic behaviour. The invasive and metastatic ability of these cell sublines is different in the process of tumor growth. So, if the cell sublines were isolated and identificated by the method of screening *in vitro* and transplantation *in vivo,* their differences in biologic characters could be compared and analysed. It would be of important significance in theory and practice to study the mechanism of tumor metastasis, and look for the new tumor metastatic genes and guide clinical therapy of tumor.

In theory, tumor cell sublines could be screened from the cell population of primary tumor with heterogeneity. But it is very difficult in fact that the lower metastasis or no− metastasis cell sublines were screened from the tumor cell population with higher metastatic character. Because the growth ascendancy may make the cell subline with high malignancy degree to take gradually the major part of the whole tumor cell population during the multiplication *in vivo* or tumor growth. At present , studies have proved the above mentioned opinion[5,6].

MG−63 cell line is a human osteosarcoma cell line with high metastatic capacity. In this study , 20 cell sublines of MG−63 cell line were primarily screened *in vitro* by the limiting dilution method. Studies indicated that the metastatic potentiality of tumor cell was positive correlation with the electrophores migration rate of tumor cell [7]. Hence, 20 cell sublines were screened by the method of the cell electrophoresis and the two cell sublines (A2 and A3) with highest rate and the two cell sublines (A6 and A20) with lowest rate were obtained. The result suggested that the metastatic potentiality of A2 and A3 were higher than that of A6 and A20. In the same time, by the cell growth curve and the colony formation assay in soft agarose, the proliferation situation *in vitro* was observed. The result shown that the proliferation ability *in vitro* of A2 and A3 was obviously higher than that of A6 and A20 . In the cell migration assay, the result indicated the cell invasive ability *in vitro* of A2 and A3 was higher than that of A6 and A 20 .

For further proving the relation of tumor cell invasive ability *in vitro* with tumor cell metastatic ability *in vivo,* the difference in metastatic character of each cell subline was observed by the transplantation in nude mice. Nude mice are the animal with immunodeficiency and can eliminate the influence of host immune surveillance to tumor cell [8]. So, the transplantation in nude mice can make tumor cell to adapt to the microenviroment in host body. The studies proved the orthotopic transplantation *in vivo* of tumor tissue could express fully the tumor biologic behaviour, natural character and screeded well cell subline with different metastatic ability [9]. The result of this study shown the tumor cell invasive ability *in vitro* were positive correlation with the tumor cell metastatic ability to lung *in vivo.*

All above mentioned results indicated what the

method of cell culture *in vitro* and transplantation *in vivo* was used to isolate the cell subline of MG−63 cell line with different metastatic characters could be used and efficient. Though the study had screened preliminarily four cell sublines, we will make further study by the method of continuous passage transplantation in nude mice, look for the purer cell subline of MG−63 with full metastasis and no− metastasis. It will supply a better experimental model for the further studies of metastatic mechanism of osteosarcoma.

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