[原著] Establishment of a Cell Line Derived from Human Osteosarcoma

琉大保医誌 5 (3) : 187～ 195, 1982.

Establishment of a Cell Line Derived from Human Osteosarcoma

Katsuaki Matsui , Tokichi Yumoto*, Junko Nashiro,*
Kimio Hashimoto* , Kiyoo Furuse*, Kanehito Morimoto,**
Masahiro Akeho** and Iwao Maeyama**

1st Department of Pathology, University of the Ryukyus, School of Medicine
(Director : Prof. Etsuo Ito)

* 1st Department of Pathology, Tottori University School of Medicine
(Director : Prof. Tokichi Yumoto)

** Department of Orthopedics, Tottori University School of Medicine
(Director : Prof. Iwao Maeyama)

Summary

Establishment of the cell line (HOS-O) of osteosarcoma was successfully accomplished. The materials for this study were obtained from surgically removed tumor mass metastasized to the lung. The patient was a 10-year-old girl, suffering from an original tumor in the right distal femur.

The tissue culture has been started in September 1977, and the cell line has been maintained in a good condition for about five years. The shape of cultured cells is elongated-spindle and its chromosome number is 46. By histochemical examination, the cells revealed weakly positive at alkaline phosphatase, and moderately positive at acid phosphatase, and highly positive at non-specific esterase and 5′-nucleotidase. Each of twice transplantations of those cells, to the back of nude mice, was successful. Histological features of the trasplanted tumors were resembled to that of the original sarcoma, showing a remarkable formation of osteoid tissue.

Introduction

There are scarce reports about establishment of cell lines of human osteosarcomas up to date[1-7]. There are a lot of differences in its characteristics among each case of osteosarcoma. Therefore, the osteosarcoma has to be studied by means of not only histology, but also biological properties with many cases. Especially in the experimental in vitro study about their therapy, a lot of tumor cells should be supplied at a time. Establishment of maintained cultured cell line which can be transplanted to nude mice will fill the above requirement and our cultured cell line of human osteosarcoma (HOS-O) fills those conditions. It is believed that they would offer useful materials to study the osteosarcoma from now.

Materials and Methods

The patient was a 10-year-old girl who was amputated the right thigh for an osteosarcoma in January 1977. After about 8 months from operation, a metastasis to the lung was found and the pulmonary lesion was excised on 15, September. The tumor tissue to be cultured was obtained from

the metastatic pulmonary lesion.

The removed tumor mass was cut into small pieces by scissors after sufficient washing with phosphate buffer solution without Ca^{++} and Mg^{++} (hereinafter PBS(−)) containing 500 unit／ml of penicillin (Meiji Seika, Tokyo). The small pieces of tumor tissue were washed again in PBS(−). The pieces of tumor tissue were trypsinized with PBS(−) containing 0.1% of trypsin (Wako Junyaku, Osaka), in an incubator for one hour at 37°C. After the incubation, the same quantity of culture medium containing blood serum was added to the above solution and mixed sufficiently by pipetting. The pieces of tumor tissue and separated tumor cells were collected by means of centrifugation and washed twice in PBS(−). The mixture of tissue fragments and separated cells was suspended in the medium and divided into a proper quantity and seeded into plastic dishes of 60×15㎜ (NUNC). They were incubated at 37°C, in the 5% CO_2 incubator (TOKIWA,CO-1). The medium was changed with approximate intervals of 2 or 4 days, when it became yellowish in color. When the cells became confluent, they were harvested by trypsinization and were implanted continuously in other dishes after adjustment of cell density. At the counting of the cell generation number, one generation was added at the procedure of every trypsinization.

The medium was Eagle's MEM (Nissui ① with Kanamycin 60㎎／1, Nissui Seiyaku, Tokyo) supplemented with 10% of fetal calf serum (GIBCO), 0.292 g/1 L-glutamine (Kishida Kagaku, Osaka), and 4×10^5 unit/1 penicillin. The pH of medium was adjusted between 7.2 and 7.6 with 10% $NaHCO_3$ solution.

Medium has been changed at every 2-4 days intervals and the first passage of the cells was carried out at the 18th day after the start. Regular passages of the cells were carried out repeatedly at the intervals of 6-13 days.

To determine the growth curve, the cells were counted in every two days at the beginning, and in everyday when became near to confluent, after the explantation of 10^4 cells in 30 × 15㎜ plastic dishes. As to the determination of plating efficiency, the number of colonies was counted, after twenty days from the explantation of 10^2 or 10^3 of cultured cells in 30 × 15㎜ plastic dishes. In order to obtain the results, the cell numbers of 3 dishes for growth curve, and those of 10 dishes for plating efficiency were averaged. For the determination of chromosome number, 8 gamma of Oncovin (Shionogi Seiyaku, Osaka) was added to the plastic dishes (60 × 15㎜) in which the cultured cells increased in proper density. After four hours incubation, the cells were collected by trypsinization. The cells were treated by 1% of citric acid at 37°C for 8 minutes after washing and were fixed with Carnoy's fixing fluid. The cells were stained with Giemsa solution and the chromosome number was examined.

For electron microscopic observation, a pellet of cultured cells collected after trypsinization was fixed with 2.5% of glutaraldehyde for one hour. After cutting into 1㎜ cubes and postfixation with 1% of O_sO_4 for 3 hours, the specimens were dehydrated through graded ethanol series and embedded in epoxy resin. Ultrathin sections were abtained with a MT-1 ultramicrotome (Ivan Sorvall), and stained with lead citrate and uranyl acetate. They were examined with a HU-11 electron microscope (HITACHI). As to the specimens for SEM, the plastic dish with cultured cells was fixed directly with 2.5% of glutaraldehyde and O_sO_4.

In order to make a transplantation to nude mice, 10^7 of cultured cells were inoculated into the back of female nude mouse of ten weeks old subcutaneously.

Results

The start of cell culture of the osteosarcoma cell line (HOS-O) was on 15 September,1977.

Chromosome number of cultured HOS-O cell line is distributed between 44 and 48, occupying 76.1 %, and the number of 46 is most numerous occupying 30.9% of all (Table 1). Growth curve of the 21st generation of HOS-O is shown in Figure 1. The number of explanted cells decreased at first, two days after the explantation, from 10^4 to 0.6×10^4 cells. But the cell number recovered up to 1.6×10^4 after four days and increased rapidly till 9 days when it reached plateau with 1.3×10^6 cells. Doubling time of the cells is pretty short of 12.0 to 16.8 hours. As to plating efficiency of the cells, explantation of 10^2 cells in one dish formed 36.6 colonies, and 10^3 cells fromed 201.4 colonies in twenty days on the average.

Table 1. Chromosome number of HOS-O (9)

Chromosome number	Count number	%	
38	2	2.2	
39	1	1.1	
40	3	3.3	
41	1	1.1	
42	5	5.4	
43	6	6.5	
44	15	16.3	76.1%
45	12	13.0	
46	28	30.4	
47	7	7.6	
48	8	8.7	
49	2	2.2	
55	1	1.1	
82	1	1.1	

HOS-O(9) : 9th generation of cultured cells

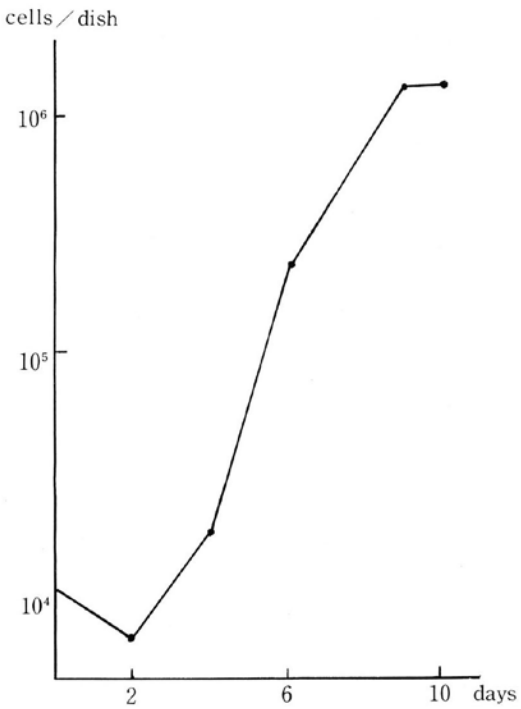


Fig. 1. Growth curve of 21st generation of HOS-O.

Morphology of the cultured cells reveals round nuclei and elongated-spindle or polygonal cytoplasms. The cytoplasm is stained slightly acidophilic with H. E. staining (Fig. 2). Those cells have a tendency to form spiral shaped colonies. The cells adhered to the plastic dish show a little difficulties in releasing with trypsin treatment.

The results of histochemical studies of cultured HOS-O cell line are listed in Table 2. Alkaline phosphatase is weakly positive (Fig. 3), and acid phosphatase is moderately positive (Fig. 4) while addition of tartaric acid makes the reaction negatively. Non-specific esterase (Fig. 5) and 5′-nucleotidase are strongly positive. Both naphthol AS acetate and naphthol AS-D acetate are positive. β-Glucuronidase and ATP-ase are pseudo-positive. Oil-red O and Sudan III are negative.

Table 2. Histochemical findings of the cultured cells (HOS-O) and the transplanted tumor of HOS-O cells

Histochemical agents	HOS-O(7)	HOS-O(23)	T_1	T_2
Alkaline phosphatase	+	+	+++	+++
Acid phosphatase	++	++	+	++
Acid phosphatase + tartaric acid	−	−	+	+
β-Glucuronidase	−	+	−	+
Non-specific esterase	+++	+++	++	+
5′-Nucleotidase	+++	+++	++	+++
ATP-ase	−	+	+	+
Naphthol AS acetate	+	+	+	+
Naphthol AS-D acetate	+	+	+++	+++
Oil-red O	−	−	−	−
Sudan III	−	−	−	−

HOS-O(7): 7th generation of cultured cells.

HOS-O(23): 23rd generation of cultured cells.

T_1 : Tumor of nude mouse occurred by inoculation of HOS-O cells.

T_2 : Tumor of nude mouse occurred by transplantation of T_1 tumor.

Electron microscopically, the cell shows irregularly shaped nucleus with deep clefts and clear nucleolus. Nuclear chromatin is finely dispersed. The cytoplasm has villous projections on one side. Well developed Golgi's apparatus, abundant rough surfaced endoplasmic reticulum and filaments are observed (Fig. 6,7).

Figure 8 shows a transplanted tumor in the back of nude mouse which was inoculated 10^7 cells of HOS-O, of the 44th generation. The tumor is index finger tip sized and hard in consistency. It is sharply demarcated showing yellowish-white in color on the cut surface. Transplantation of the cells to nude mouse was performed twice and all of them was successful. The tumors are further transplantable to the other nude mouse but did not show any metastasis to the other organ. Survival time of the tumor bearing nude mice made by inoculation of the cultured cells is average 66.5 days, and that of the secondarily transplanted tumor from the former tumor is elongated to 93 days.

Histology of the tumor developed by the inoculation of HOS-O cells reveals rather scarce bone formation comparing with original tumor (Fig. 9), while osteoid and cartilage formation are relatively abundant. The stroma of this tumor reveals remarkably increased collagen fibers (Fig. 10). The aggregated spindle cells which varied in size are noted as well as original tumor. The secondarily transplanted tumor of the other nude mice also reveals a remarkble formation of osteoid tissue and increased collagen.

Table 2 shows the results of histochemical studies on the transplanted tumor of the nude mice. Alkaline phosphatase is strongly positive in the most of tumor cells (Fig. 11), and acid phosphatase is positive mainly in round larger tumor cells (Fig. 12). Non-specific esterase, 5′-nucleotidase and naphthol AS-D acetate are intensely positive and all of the others are positive except Sudan III and Oil-red O.

Electron microscopy of the tumor occurred in the nude mouse by transplantation reveals an

irregular nucleus having deep clefts and clear nucleolus. There are numerous villous cytoplasmic projections, and the cytoplasm contains abundant rough surfaced endoplasmic reticulum, mitochondria, filaments and lipid droplets. Stroma of the tumor is filled with a lot of collagen fibers (Fig. 13).

Discussion

It is said that the establishment of cultured cell lines of the human soft tissue tumor and bone sarcoma is very difficult even with progressed culture technique at the present time. As to the cause of difficulty in the cell culture, some preoperative therapies, for example administration of anticancer drugs to general or local through intra-arterial injection and local irradiation, were supposed as playing a role to decrease the activity of tumor cell. According to the report of Sethi et al.[8], they succeeded only in 22 cases out of 49 cases (45%) in establishment of the cultured cell line from human soft tissue tumor and bone tumors. We succeeded the establishment of 12 cases of cultured cell line out of 25 cases of soft tissue tumor and bone sarcoma including this HOS-O case (48%).

Sethi et al.[8] had processed only surgical material obtained immediately after removal from the patient, without refrigeration. For primary cultures, they used RPMI 1640 medium which includes nonessential amino acids as well as other supplementary factors that may be important to support early cell proliferation. They mentioned that it is important for primary culture especially for first growth to maintain the pH under physiological extent and to avoid the medium change and trypsinization in early time. We cultured the mixture of tumor cells and finely cut tissue fragments which remained at the process of trypsinization to separate the tumor cells. In this method, the alteration of pH in the dishes had occurred in an early time, then the number of medium change increased. The medium might be replaced partially with care, at most 4/5 in maximum, so as not to move the tissue fragments. We believe that this method has a benefit as to be expected a proliferation of cells from the tissue fragments and to obtain good results.

Established cultured cell lines of human osteosarcoma have been reported as follows : SAOS-2 by Fogh and Trempe[1], SI I and SI II of Giovanella et al[2], TE-85 by McAllister et al[3], 393T by Pontén[6], 2T, 2OS, $T_2$56, $T_2$278 of Pontén and Saksela[5], 755T_2, 768T, 788T, 791T and 803T by Smith et al[7] and three cell lines by Ohboshi et al[4].

This time, we established a cell line (HOS-O) from osteosarcoma occurred in the 10-year-old girl, and it has passed 4 years and 10 months since the start of cultivation. HOS-O cell line shows a difficulty in releasing the cells from the wall of dishes after one hour trypsin treatment. This nature agrees to the facts explained by Varani et al[9], that highly malignant cells were much more resistant to protease-mediated release from plastic flasks than were the low grade malignant cells. HOS-O cells grew rapidly and 10^4 cells in 30 × 15 mm dishes reached confluent after 8 to 9 days and their average doubling time was 12.0～16.8 hours.

The chromosome number of HOS-O was distributed between 44 and 48, and the most numerous number was 46. According to the results of Smith et al[7], average chromosome's mode of 5 strains of cultured human osteosarcoma cell was 46. These results seemed to correspond to the speculation of DiPaola and Papescu[10] suggesting that tumorigenicity needs not to be related to an imbalance of chromosome number or structure.

Smith et al[7] produced a subcutaneous tumor by inoculation of cultured cells of 5 strains in immunosuppressed mice in high rate soon after inoculation, but most of them were regressed. McAllister et al[3] produced tumors with TE-85F cells with a small frequency. In order to determine the tumorigenicity of HOS-O, transplantations of the cells of the 44th generation were performed to nude mice twice. In both experiments, tumors were produced in all nude mice, and they were transplantable to other nude mice.

Histological examination of the tumors induced in mice showed less bone formation than that of the original tumor, but the formation of abundant osteoid and cartilage-like materials and the increase of collagen fibers were noted. Histochemically, the majority of cultured cells was positive to acid phosphatase except a number of cells which were positive to alkaline phosphatase. Conversely, the majority of histologically examined transplanted tumor cells was positive to alkaline phosphatase except small number of bigger tumor cells which were positive acid phosphatase. In general, the osteoblast is positive to alkaline phosphatase in contrast to the osteoclast is positive to acid phosphatase[11]. The results of transplanted tumor are in understanding but the results of cultured cells were unconvincing. As to the production of alkaline phosphatase in experimental murine osteosarcoma, an effective dose was composed at 37°C in 30 minutes in vitro from 10^5 cells[12]. Consequently, the produced alkaline phosphatase might be supposedly released in the culture medium. In the case of in vivo, the activity of alkaline phosphatase would decrease when the transplanted tumor became bigger with appearance of osteoid and bone tissue[13].

It is uncertain whether the special in vitro characteristics will correlate to the type and grade of malignancy[7], so that the differentiation of normal cells from malignant tumor cells is difficult[14]. To distinguish malignant cultured cells, today we use the techniques of morphological examination, karyotyping, determination of enzymatic activity, and growth test of tumor cells on the soft agar or the nude mice. But the results of these examinations are remarkably reflected by cell density and the passaged generations of the cell lines[14].

In the studies of human malignant tumor, especially in the etiology, immunology, chemotherapy and so on, nevertheless the investigations using cultured cells are important. Even at present, final diagnosis of osteosarcoma depends on the histological diagnosis by light microscope. In general, the histological features of the osteosarcoma are very variable, and making diagnosis become difficult when peculiar findings of bone formation are obscure[16]. For above reason, the studies of biological characteristics of cultured malignant cells are required to make sure final diagnosis.

Cultured cell line NZB A~F which was formerly established by us from murine osteosarcoma naturally occurred in New Zealand Black mouse was used in the studies of bone sarcoma[17-20]. Similarly, we believe that our HOS-O cells become useful in the future as materials for studies concerning the peculiar nature of osteosarcoma like a bone formation, procollagen[14], fibronectin[21], and alkaline phosphatase.

(This study was presented at the 69th Annual Meeting of the Japanese Pathological Society, Sapporo,1980)

References

1) Fogh J. and Trempe G.: New human tumour cell lines, Chapter 5. In; J.Fogh (ed.) Human tumour cells

in vitro, pp. 115-159, Plenum Press, New York and London, 1975.

2) Giovanella B.D., Stehlin I.S. and Williams Jr. L. J.: Heterotransplantation of human malignant tumours in "nude" thymusless mice. II. Malignant tumours induced by injection of cell cultures derived from human solid tumour. J. Nat. Cancer Inst. *59,* 921-930, 1974.

3) McAllister R.M., Gardner M.B., Green A.E., Bradt C., Nichols W.W. and Landing B.H.: Cultivation in vitro of cells derived from human osteosarcoma. Cancer *27,* 397-402, 1971.

4) Munehiro C., Nomura S., Manabe S., Tomita K., Yamada Y., Nomura T., Nakamura T. and Chiba H.: Transplantation of the human malignant bone and soft part tumors to nude mice. Orthop. Surg. *32,* 1-10, 1981. (in Japanese)

5) Pontén J. and Saksela E.: Two established in vitro cell line from human mesenchymal tumours. Int. J. Cancer *2,* 434-447, 1967.

6) Pontén J.: Neoplastic human glia cells in culture. In ; J. Fogh (ed.), Human tumour cells in vitro. Chapter 7, pp. 175-206, Plenum Press, New York and London, 1975.

7) Smith H.S., Owen R.B., Hiller A.J, Nelson-Rees W.A. and Johnston J.O.: The biology of human cells in tissue culture. Characterization of cells derived from osteogenic sarcomas. Int. J. Cancer *17,* 219-234, 1976.

8) Sethi J., Hirshaut S., Hadju I. and Clements L.G.: Growing human sarcomas in culture. Cancer *40,* 744-755, 1977.

9) Varani J., Lovett E.J., Elgebaly S., Lundy J. and Ward P.A. : In vitro and in vivo adherence of tumor cell variants correlated with tumor formation. Am. J. Pathol., *101,* 345-352, 1980.

10) DiPaola J.A. and Popescu N.C.: Relationship of chromosome changes to neoplastic cell transformation. Am. J. Pathol. *85,* 709-738, 1976.

11) Remagen W.: The bone cell system; from and function. Beitr. Path. Bd., *150,* 1-10, 1973.

12) Chanta V.K., Hiramoto R.N., Weiss A.B. and Caudill L.: Monitoring of murine osteosarcoma by serial alkaline phosphatase determinations. J. Nat. Cancer Inst. *57,* 837-839, 1976.

13) Shimokawa K.: Histological and histochemical findings of transplantable osteosarcoma of mice. Acta Histochem. Cytochem. *7,* 112-125, 1974.

14) Stern R., Wilczek J., Thorpe W.P., Rosenberg S.A. and Cannon G.: Procollagens as markers for the cell of origin of human bone tumors. Cancer Res. *40,* 325-328, 1980.

15) Hirano H. and Urist M.R.: Characteristics of the newly derived human chondrosarcoma cell line OSHU-1; observations on osteoinduction. J. Nat. Cancer Inst. *65,* 299-309, 1980.

16) Amitani K. and Nakata Y.: Characteristics of osteosarcoma cells in culture. Clin. Orthop. *122,* 315-324, 1977.

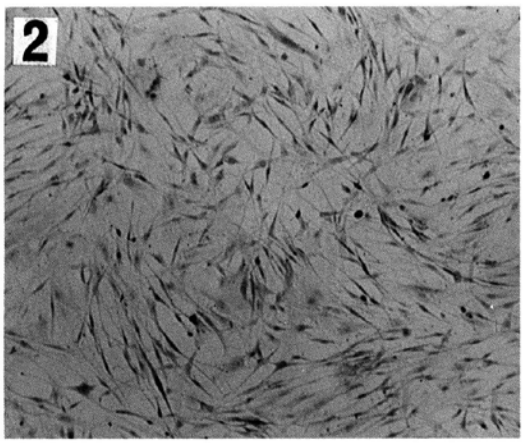
17) Matsui K.: Transplantable osteosarcoma naturally occurring in a NZB mouse. J. Yonago Med. Ass. *26,* 483-511, 1975. (in Japanese)

18) Matsui K., Teitei N., Inada O., Ando K. and Yumoto T.: Long term transplantation of murine ostesarcoma. J. Yonago Med. Ass. *29,* 380-390, 1978. (in Japanese)

19) Matsui K., Hashimoto K. and Yumoto T.: Comparative analysis of bone-forming ability of the long term cultured cell lines, with regard to the number of cell passage and the difference of transplanted regions. Ryukyu Univ. J. Health Sci. Med. *4,* 33-45, 1981. (in Japanese)

20) Yumoto T., Ando K., Yoshida H., Okamoto S. and Matsui K.: Naturally occurring bone tumor in a NZB mouse. Yonago Acta Med. *18,* 145-153, 1974.

21) Murray J.C., Liotta L., Rennard S.I. and Martin G.R.: Adhesion characteristics of murine metastatic and non-metastatic tumor cells in vitro. Cancer Res. *40,* 347-351, 1980.



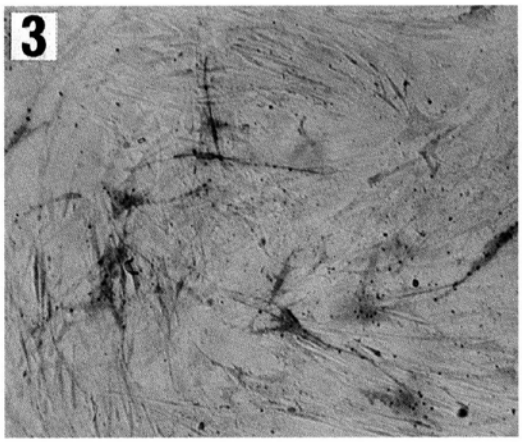
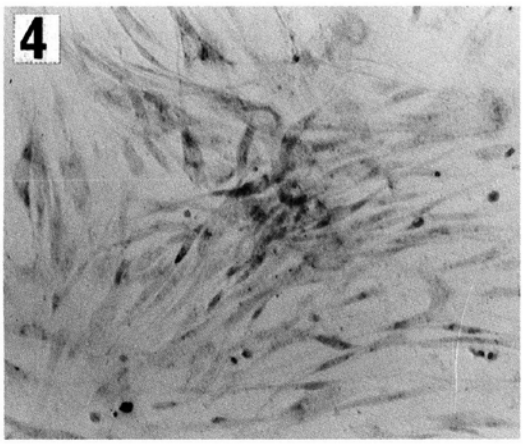


Fig. 2. Cultured cells (HOS-O) are spindled and show a slight spiral arrangement. H.E. ×40.

Fig. 3. Some cultured cells stained slight positively by alkaline phosphatase. ×100.



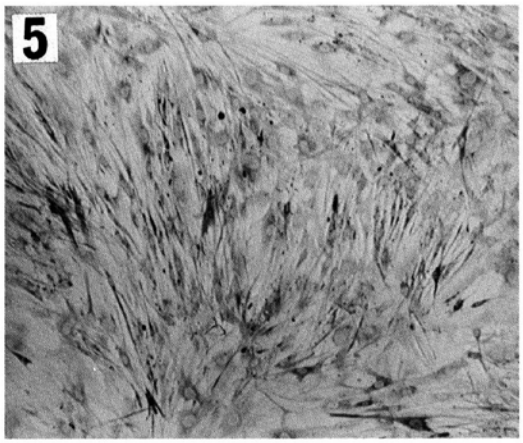
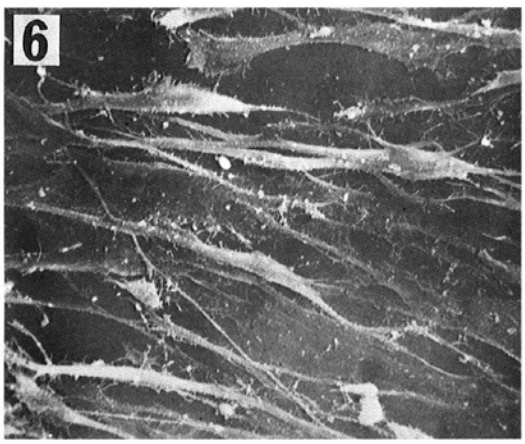


Fig. 4. Most of cultured cells stained positively by acid phosphatase. ×100.

Fig. 5. Most of cultured cells stained positively by non-specific esterase. ×100.



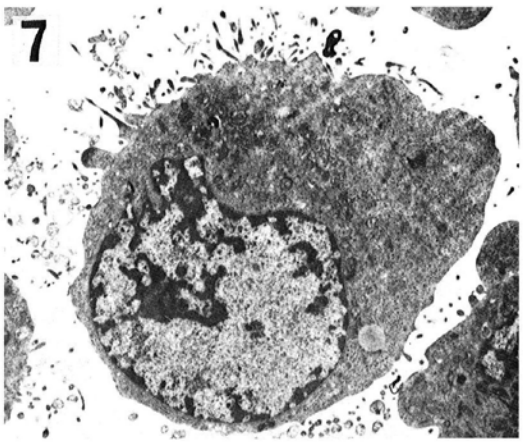
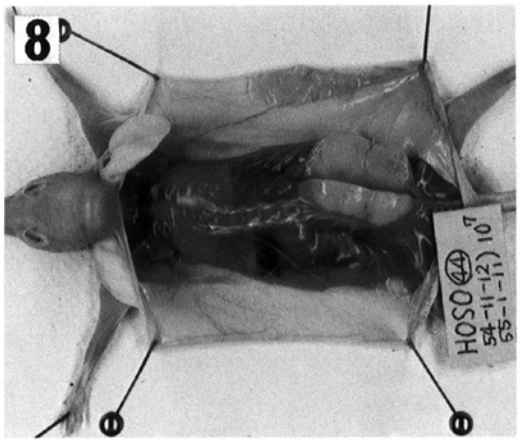


Fig. 6. Cultured cells showing elongated spindled or polygonal cytoplasms with villous cytoplasmic projection. SEM. × 400.

Fig. 7. Cultured cell which shows a clear nucleus and numerous cytoplasmic projections. TEM. ×2600.



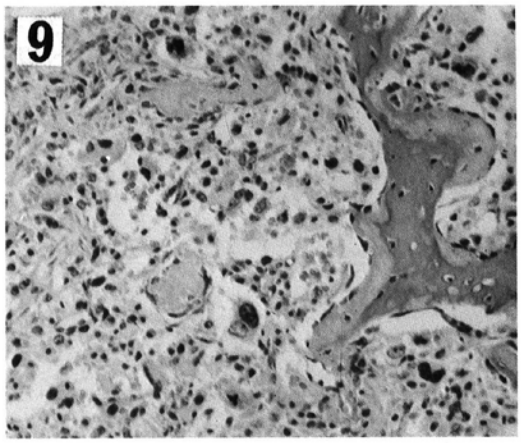
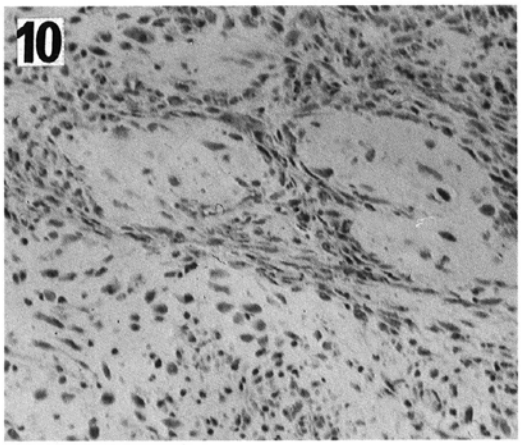


Fig. 8. A tumor occurred in a nude mouse by the inoculation of HOS-O cells.

Fig. 9. Photograph of the original tumor occurred in the right distal femur of the 10-year-old girl. H.E.×100.



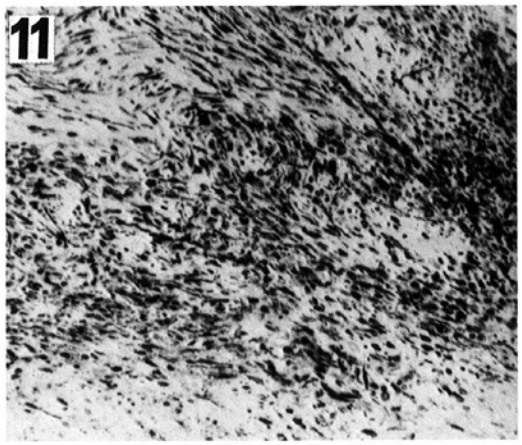
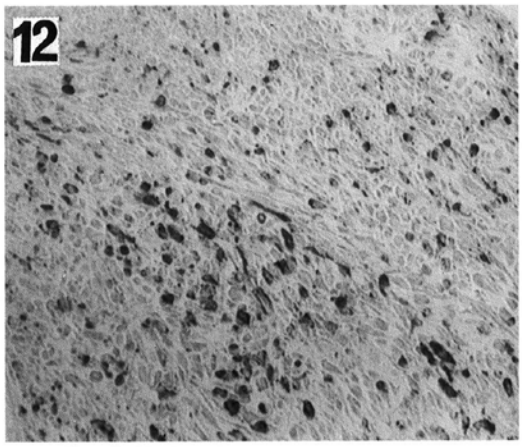


Fig. 10. Photograph of the original tumor occurred by transplantation of HOS-O cells. Middle area shows a remarkable osteoid formation. H.E. × 100.

Fig. 11. Alkaline phosphatase is marked positive to the most of transplanted tumor cells of HOS-O cells. ×100.



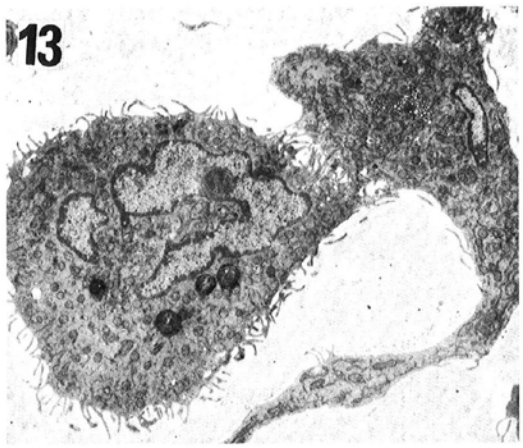


Fig. 12. Acid phosphatase is mainly positive in large rounded tumor cells. X100.

Fig. 13. EM photograph of transplanted tumor cells. The cell has an irregular nucleus, abundant organelle, and numerous cytoplasmic projection. In the stroma, a lot of collagen fibers are seen. TEM. ×2100.