HISTOLOGICAL AND HISTOCHEMICAL FINDINGS OF TRANSPLANTABLE OSTEOSARCOMA OF MICE*

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Light microscopic and histochemical investigations were performed on a total of 329 CBA $\times$ C57BL/6 F₁ hybrid mice which were subcutaneously transplanted with Gardner osteosarcoma 519 between the 80th to the 95th transplant generations. The transplantability was 92.4%, and the survival time was 63 ± 23 days. Metastases were seen only in the lung in 45 (21.0%) of the 214 cases which survived over 40 days. The grafted tumors were palpable at about 10 days and became the size of the head of the little finger at about 50 days after inoculation. When the sizes of the tumors were small they mainly consisted of spindle cells with many mitotic figures, and strong activities of both alkaline and acid phosphatase and PAS reaction-positive granules were present. Multinucleated giant cells, reticular stroma and osteoid or bone tissues appeared with tumor growth, but the numbers of mitotic figures and the activity of alkaline phosphatase were reduced. Thus, the author classified this sarcoma in four histological types; the spindle cell type, the giant cell type, the reticular stroma type and the osteoid-bone type. The spindle cell type was considered as a proliferating phase and the reticular stroma and osteoid-bone types were as a differentiating phase. The functions of this tumor to form bone or osteoid tissue and to increase the levels of the serum alkaline phosphatase of the host were proved to be retained after 95 transplant generations; therefore, this sarcoma was regarded as a kind of functioning tumor with the above functions.

It is well known that serial transplantations of experimental tumors generally increase their growth rate and decrease their grade of differentiation. Most spontaneous or induced osteosarcomas also lose or decrease their capability of forming bone or osteoid tissue after a variable number of transplant generations (1, 18, 27, 40, 41).

Previous studies by the present author (37) showed that Gardner mouse osteosarcoma 519 maintained in our laboratory for 95 generations retains the capability of increasing the serum alkaline phosphatase activity of the host with tumor growth. The objective of this investigation was to determine whether this sarcoma could retain the capability of forming bone or osteoid tissue. The results of this investigation and the correlation between the morphological findings of this sarcoma and the mechanism of differentiation will be discussed.

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TABLE 1. *Fixatives and staining methods*

Fixative	Staining method	Author (s)
Bouin's solution	Hematoxylin and eosin	
	Van Gieson	
	Azan	
Cold alcohol	Periodic acid-Schiff	
	Alcian blue	
	Metachromasia with Toluidine blue*	
	Neutral red	Sugiyama (42)
Cold acetone	Calcium stain	Yasue (47)
	Alkaline phosphatase stain	Gomori (15)
	Acid phosphatase stain	Burstone (4)
Fresh tissue (without fixation)	Succinic dehydrogenase stain	Nachlas <i>et al.</i> (28)

* With and without bacterial hyaluronidase pretreatment (2)

MATERIALS AND METHODS

Tumors:

The tumor used in this experiment was found by Dr. W. U. Gardner of the Department of Anatomy, Yale University School of Medicine, in the pelvis of a C57BL $\varnothing$ $\times$ CBA δ F $_1$ $\varnothing$ hybrid mouse which had received 175R of X-rays in 1955. Microscopically, the tumor had features of osteosarcoma and was designated 519CC $_1$ O $\varnothing$ osteosarcoma. It was imported into Japan by Ojima (29) in 1961 and the serial transplantations into the subcutaneous space of the backs of C57 $\times$ CBA hybrid mice by means of a trocar were continued through a total of 80 transplant generations prior to this experiment. In our laboratory this sarcoma is called Gardner mouse osteosarcoma 519. As shown by the present author (37), high levels of the serum alkaline phosphatase activity in growing mice gradually declined to adult levels with maturation and virtually ceased at the age of 80 days. Therefore the tumors were transplanted aseptically into C57BL/6 $\times$ CBA F $_1$ hybrid mice over 80 days old to avoid the influence of their insufficient maturation upon the level of the serum alkaline phosphatase activity.

Transplanted mice were housed in 30 $\times$ 16 $\times$ 10-cm aluminum cages in sets of 3 to 6. Solid chow CMF (manufactured by Oriental Yeast Co., Ltd., Tokyo) and water were available *ad libitum*. The temperature in the animal room was kept at 22 $\pm$ 2°C and relative humidity at 60 $\pm$ 5%. A total of 356 mice from the 80th to 95th transplant generations were used in this experiment. The lineal dimensions of the grafted tumors were measured every 5 days with Vernier calipers until tumor-bearing mice died or were sacrificed.

Pathological preparations:

Autopsies were performed when mice died or were sacrificed under chloroform anesthesia at various times. Blood was collected by heart puncture, centrifuged in

capillary tubes (diameter=6 mm), and the serum was used for alkaline phosphatase activity determination (37). Tumor tissues were fixed in Bouin's solution, cold alcohol and cold acetone for various histochemical and histological studies. Sections fixed in Bouin's solution were stained with hematoxylin and eosin, azan, and van Gieson stains. Sections fixed in cold alcohol were stained with periodic acid-Schiff (PAS) reaction, alcian blue stain, toluidin blue stain with and without bacterial hyaluronidase pretreatment (2), neutral red stain (42), and calcium stain with Yasue's method (47). Sections fixed in cold acetone were used for alkaline phosphatase activity, using Gomori's method (15) and for acid phosphatase by Burstone (4). The cryostat sections were treated to demonstrate succinic dehydrogenase activity according to Nachlas *et al.* (28). Fixatives and staining techniques are summarized in Table 1.

RESULTS

General properties of tumors:

The transplantability was 92.4% (329/356) (Table 2), and the survival time was 63 ± 23 days (Table 3). The grafted tumors were palpable at about 10 days. In 30 to 50 days, they grew to the size of the head of the little finger. The mean growth curve of the tumors was formulated by the logarithmic-product of their linear dimensions (mm) (Chart 1). In general, the tumors were elastic, hard and covered by a thin fibrous capsule. On section, the cut surface was pinkish gray in areas of the periphery. Hemorrhagic and necrotic areas were scattered in the center of the tumor. A few transplants were palpable in about 2 to 3 months after transplantation, but no difference was observed in the tumor-bearing period when compared with those palpated at about 10 days. Of the 214 mice which survived over 40 days after transplantation, 45 (21.0%) had lung metastases. Pulmonary metastases showed a miliary-sized greyish white nodule or nodules. Metastases to organs other than the lung were not noted. Invasion into the peritoneal cavity was often observed, but no ascites tumors appeared.

Histopathologic features:

The morphological features of this tumor altered with its growth; therefore it was classified in four types by its microscopic and histochemical characteristics. Needless to say, the tumor did not consist of only one type but of various types.

1. Spindle cell type

The tumor was very cellular and mostly composed of spindle-shaped cells which had fusiform or ovoid nuclei with abundant coarse chromatin and distinct nucleoli. Many mitotic figures were present, but intercellular substance was scanty (Fig. 1). The tumor cells showed a strong activity of alkaline phosphatase in the cell membrane (Fig. 2). Acid phosphatase activity was also strongly positive in the tumor cells (Fig. 3). PAS reaction revealed granules, and a weak reaction of succinic dehydrogenase were also seen in the tumor cells. Deposits of calcium between tumor cells were sparse (Fig. 4). A few foci of necrosis and of giant cells were found.

2. Giant cell type

This type was characterized by the accompaniment of many multinucleated giant cells, which had several nuclei similar to those of mononuclear cells (Fig. 5).

TABLE 2. *Transplantability*

Transplant Generation	Males		Females		Total	
	Frequency	%	Frequency	%	Frequency	%
80~85	73/76	96.1	65/76	85.5	138/152	90.8
86~90	44/46	95.7	56/62	90.3	100/108	92.6
91~95	39/41	95.1	52/55	94.5	91/96	94.8
80~95	156/163	95.7	173/193	89.6	329/356	92.4

TABLE 3. *Survival time (days)*

Transplant Generation	Males	Females	Total
80~85	51± 9	56±19	53±13
86~90	62±21	62±31	61±26
91~95	69±22	71±20	71±22
80~95	61±20	66±24	63±23

CHART 1. Tumor growth curve. The mean growth curve of tumors was formulated by the logarithmic-product of their lineal dimensions. (mm)

The giant cells showed no alkaline phosphatase activity in contrast to mononuclear cells (Fig. 6). However, intense acid phosphatase activity was observed in these giant cells, whereas it was negative in the necrotic ones (Fig. 7). PAS reaction-positive granules were not present in the giant cells, but succinic dehydrogenase activity was strongly positive in them (Fig. 8). Mononuclear tumor cells around the giant cells were round or polyhedral in shape with abundant cytoplasm, and the nuclei showed few mitotic activity. Numerous vascular channels and faint fibers were present between these cells. A small amount of stroma homogeneously stained by eosin was observed in the intercellular space. Twig-like deposits of calcium were present in small amounts in this stroma (Fig. 9).

3. Reticular stroma type

Abundant intercellular stroma was characteristic in this type. The tumor cells became bizarre and small, and the nuclei pyknotic (Fig. 10). The numbers of giant cells and mitotic figures decreased in comparison with the giant cell type, whereas necrotic foci increased. Activities of acid and alkaline phosphatase (Fig. 11) became weaker than those in the previous two types. An increasing intercellular reticular stroma was demonstrated to become pale blue, blue and red with alcian blue, azan and van Gieson stains, respectively. Thick, twig-like deposits of calcium were present (Fig. 12). PAS reactive and metachromatic materials (pH 2.5) were not present in this stroma. Although the umbilical cord used as a control for metachromasia was not stained, but the cartilage plates of bronchus as another control were positive under the same conditions. At pH 4.1 and pH 7.0 the intercellular stroma as well as the umbilical cord showed positive metachromasia. The metachromatic material was digested at pH 5.0 with bacterial hyaluronidase being similar to the umbilical cord, whereas the cartilage plates of bronchus were resistant. The stroma was stained brown using Sugiyama's neutral red method (42).

It is memorable that pulmonary metastases had a tendency to consist of this reticular stroma type, even though they were of miliary size (Fig. 13).

4. Osteoid-bone type

The tumor cells were smaller and the bizarre nuclei became more pyknotic than those in the reticular stroma type. The number of tumor cells was also reduced. While osteoid or bone formation was found in increased intercellular stroma, cartilage has never been identified (Fig. 14). Histochemical findings of intercellular stroma of the osteoid-bone type were similar to those of the reticular stroma type. However, deposits of calcium were thicker than those in the reticular stroma type, and activities of both alkaline and acid phosphatase of the tumor cells also became more faint. Giant cells and mitotic figures were hardly seen.

Size of tumor and histological type:

The grafted tumors were about 3×3 mm in size at approximately 10 days after

FIG. 1. Spindle cell type illustrating the spindle-shaped cells with many mitoses. (H & E) $\times 200$.

FIG. 2. Alkaline phosphatase activity of the spindle cell type. Strong activity is observed in the cell membrane of the spindle-shaped cells. (Alkaline phosphatase stain) $\times 200$.

FIG. 3. Acid phosphatase activity of the spindle cell type. The activity is strong in the spindle-shaped cells. (Acid phosphatase stain) $\times 200$.

FIG. 4. Calcium stain of the spindle cell type. The deposition is slightly seen in the intercellular space. (Yasue's method (47) for calcium stain) $\times 100$.

TABLE 4. *The relationship of tumor size to the frequency of histological type*

Size of Tumor (mm)	Histological Type			
	Spindle Cell Type	Giant Cell Type	Reticular Stroma Type	Osteoid-bone Type
~3×3	‡‡	—	—	—
~10×10	‡‡	‡‡	÷	—
~20×20	‡‡	‡‡‡	‡‡	÷
20×20~	‡‡	‡‡	‡‡	+

The plus signs indicate increases of various degrees

TABLE 5. *Histological type, giant cell index and mitotic index*

Histological Type	Giant Cell Index*	Mitotic Index*
Spindle Cell Type	0.7	7.3
Giant Cell Type	7.3	3.1
Reticular Stroma Type	1.1	1.1
Osteoid-bone Type	0.4	0.7

* Mean of numbers in 10 separate high-power fields ($\times 400$)

transplantation, and they mostly consisted of the spindle cell type. At about 20 days the tumors measured approximately 10×10 mm in size, most of which were of the spindle cell type and part were of the giant cell type. They reached approximately 20×20 mm in size at about 50 days and had hemorrhagic and necrotic areas in the center of the tumors. Adjacent to those areas, the reticular stroma and giant cell types were present. The peripheral areas of the tumors mainly consisted of the spindle cell type. When the tumors measured over 20×20 mm in size, osteoid or bone formation was observed around the central hemorrhagic and necrotic areas. In Table 4 the changes of histological types with the tumor growth are summarized.

Indices of giant cell and mitosis:

The numbers of giant cells and mitotic figures in each histological type were counted in 10 separate high-power fields ($\times 400$). Then their mean numbers were

FIG. 5. Giant cell type illustrating the giant cells with several nuclei similar to those of the mononuclear sarcoma cells. The sarcoma cells around the giant cells are round or polyhedral in shape with abundant cytoplasm. (H & E) $\times 200$.

FIG. 6. No alkaline phosphatase activity of the giant cells in contrast to the mononuclear sarcoma cells. (Alkaline phosphatase stain) $\times 200$.

FIG. 7. Intense acid phosphatase activity is seen in the giant cells as well as in the mononuclear sarcoma cells, whereas it is negative in the necrotic ones. (Acid phosphatase stain) $\times 200$.

FIG. 8. Strong reaction of succinic dehydrogenase activity in the giant cells. Weak reaction in the mononuclear sarcoma cells. (Succinic dehydrogenase stain) $\times 200$.

FIG. 9. Calcium deposit of the giant cell type. Twig-like deposits of calcium are present in small amounts in the intercellular space. (Calcium stain) $\times 100$.

presented in Table 5 as giant cell and mitotic indices, respectively. In the mitotic index the spindle cell type showed a maximum and osteoid-bone type a minimum. Therefore it seems possible that the spindle cell type is a proliferative phase. The giant cell index was maximum in the giant cell type and minimum in the osteoid-bone type.

DISCUSSION

In experimental animals, especially in mice, spontaneous bone tumors are extremely rare (7, 11, 25, 27) except for one inbred line derived by Pybus and Miller (32) from the Simpson strain. Several methods to induce bone tumors were undertaken: zinc beryllium (12), radioactive compounds (11, 19, 24, 48), chemical carcinogens (19, 22, 46), and X-ray irradiation (9, 40). Recently a role played by a virus in the oncogenic process of osteosarcoma has been reported (10). The original tumor of Gardner mouse osteosarcoma 519 was found in a C57BL $\times$ CBA F_1 hybrid mouse which had received 175R of X-rays. In general, a very high dose of X-rays is required to induce osteosarcoma; however, Finkel *et al.* (9) reported a few osteosarcomas in mice which had received 250R (500 rads) of X-rays. No spontaneous occurrence of osteosarcoma has been described in either C57BL or CBA strains of mice (7, 27). Accordingly, it is possible that Gardner osteosarcoma might have been induced by X-ray, though the dose was as low as 175R.

As described above, experimental osteosarcomas were apt to lose their bone-producing capability for several transplant generations (1, 18, 27, 41). Alkaline phosphatase activity of osteosarcoma was histochemically observed in the chickens (21), mice (3, 21) and human beings (13, 34), and serial transplantation has a tendency to decrease the levels of the activity (1, 36). However, exceptionally osteogenic sarcoma EM-2 (40) did not lose the capability of bone production, and both Wagner and Ridgway osteogenic sarcomas retained high alkaline phosphatase activity though the bone-forming capability was lost (41). Therefore it seems remarkable that an osteoid or bone-forming property as well as an alkaline phosphatase activity is still retained, even after the 95th transplant generation in Gardner mouse osteosarcoma 519.

In this experiment, the activity of alkaline phosphatase was strong in the spindle cell type, but it was weak in both the reticular stroma and the osteoid-bone types, which seemed to be a more differentiated phase than the former. From these facts it seems possible that the activity of alkaline phosphatase of Gardner osteosarcoma gives evidence of the potentiality to differentiate or produce bone, even when the tumor is composed of the spindle cell type. Previous studies by the present author

FIG. 10. Reticular stroma type illustrating abundant intercellular stroma homogeneously stained pale pink by eosin. The tumor cells are bizarre and small with pyknotic nuclei. (H & E) $\times 200$.

FIG. 11. Alkaline phosphatase activity of the tumor cells in the reticular stroma type is weaker than those in the spindle cell and giant cell types. (Alkaline phosphatase stain) $\times 200$.

FIG. 12. Calcium deposit of the reticular stroma type. Thick, twig-like deposits are present. (Calcium stain) $\times 100$.

FIG. 13. Pulmonary metastases mainly consist of the reticular stroma type. (H & E) $\times 40$.

FIG. 14. Osteoid-bone type. The tumor cells are small, and the nuclei are pyknotic. The numbers of tumor cells are reduced. Osteoid or bone formation is seen. (H & E) $\times 200$.

(37) showed that the activity of serum alkaline phosphatase in the Gardner mouse osteosarcoma-bearing host increased with tumor growth. On the other hand, the osteoid-bone type in the histologically differentiated phase, also increased with tumor growth. From these, serum alkaline phosphatase activity simulated a quantitative marker of the differentiation. When the tumor measured over 20×20 mm in size, the osteoid-bone type appeared; however, the spindle cell type also increased in the areas surrounding the osteoid-bone type. Thus it is reasonable to presume that the source of the increased activity of serum alkaline phosphatase with tumor growth is the spindle cell type. Serum alkaline phosphatase activity could not be regarded as a quantitative marker of differentiation, but rather as a marker of proliferation. Accordingly, Gardner mouse osteosarcoma 519 may be regarded as a kind of functioning tumor with the capability of forming bone or osteoid tissues and of increasing the level of serum alkaline phosphatase activity.

In the present study, acid phosphatase activity was positive in both the giant and mononuclear sarcoma cells. Gutman *et al.* (16) suggested a possible connection between acid phosphatase and bone resorption, as they found a marked increase of the serum acid phosphatase level in 11 of 15 patients with skeletal metastases of prostate carcinomas which were predominantly osteolytic. De Duve *et al.* (8) described that this enzyme existed in lysosome and had a role in intracellular digestion by tissue fractionation studies. Histochemically, acid phosphatase activity was observed in foreign body giant cells (4, 31), macrophages (6), osteoclasts (4, 6, 20, 44), giant cells in osteosarcoma and giant cell tumors of bone (4, 14, 20, 31, 35, 44). Pepler (31) and Schajowicz (35) described a close resemblance between osteoclasts and giant cells of osteosarcoma and of giant cell tumors of the bone. However, Shibata (36) found low activity of acid phosphatase in both mononuclear and giant cells of Horie's osteogenic sarcoma. The activity of acid phosphatase in mononuclear cells of giant cell tumors of the bone was negative (31, 35). The activity was mostly negative (20) or low to moderate (44) in osteosarcoma cells. As described above, acid phosphatase activity was positive in Gardner mouse osteosarcoma 519 in both the mononuclear and giant cells, therefore this osteosarcoma is somewhat different from other osteosarcomas. In this experiment, the giant cells showed succinic dehydrogenase activity as well as acid phosphatase activity. Positive activity of succinic dehydrogenase was demonstrated in osteoclasts by Burstone (5), and in giant cells of osteosarcoma or giant cell tumor of bone by Schajowicz (35) and Timmer *et al.* (44). Walker (45) suggested that osteoblasts synthesized L-proline and L-hydroxyproline of collagen from pyruvate and osteoclasts participated in the reversed pathway on the basis of histochemical findings of several dehydrogenase, such as succinic dehydrogenase in osteoclasts and osteoblasts. Histochemical behavior of the giant cells of this osteosarcoma was similar to that of osteoclasts reported by many investigators on the activity of alkaline phosphatase (31, 35, 44), acid phosphatase (31, 35, 44) and succinic dehydrogenase (5, 45). Based on the aforementioned observations and the site and time of appearance of the giant cells in the graft, it seems possible that the giant cells functionally might have no relationship to osteolysis, bone absorption and intracellular digestion. Accordingly, there are many problems remaining to be solved in a further study on pathological roles of acid phosphatase.

As to the mode of giant cell-formation, there are two theories; fusion of mono-

nuclear cells (23, 31, 35, 36), and amitotic division of mononuclear cells (35, 36). Studies using the electron microscope and tissue culture may reveal the mechanism of giant cell-formation.

Sobel *et al.* (39) suggested that chondroitin sulfate was important in the mechanisms of mineralization of the stroma. In the present study, histochemical findings by toluidin blue metachromasia with and without bacterial hyaluronidase digestion (2) and neutral red stain (42) did not indicate the presence of chondroitin sulfate but rather hyaluronic acid and/or chondroitin in the stroma of this osteosarcoma. Deposits of calcium in the stroma suggest that formation of the stroma is the first step for calcification or ossification. PAS reaction-positive granules in the sarcoma cells were considered as an important material for ossification (26, 34). In the present work since PAS reaction-positive material was rich in both the spindle cell and the giant cell types but poor in the reticular stroma and osteoid-bone types, it is possible that the PAS reaction-positive material may be important in the production of reticular stroma and osteoid tissue.

The most common site of the metastases in spontaneous bone tumors (1, 17, 33, 38) as well as induced tumors (11, 43) is the lung, and the incidence was about 20% or less. Of the 214 mice which survived over 40 days after transplantation, 45 animals (21.0%) had lung metastases in this experiment. Histologically, pulmonary metastases of this sarcoma mostly consisted of the reticular stroma type and had a strong deposition of calcium and a few osteoid or bone formations. The same histological features of metastases had been reported by Ojima (30) and Pybus (33). Hilberg (18) showed that osteosarcoma of a C3Hb/HeN female mouse which had lost its bone-forming capability in the 6th to 20th serial transplant generations regained its capability by inoculations of the tumor tissue beneath the renal capsule. These findings suggest a possibility that tumors may represent a more differentiated phenotype if the proliferating site of the tumor cells was suitable. Therefore, the next problem for investigation is to analyze the factors which induce this sarcoma to more differentiation.

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