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Establishment and Characterization of a Rat Yolk Sac Tumor Cell Line, NMT-1, Producing α-Fetoprotein, with Potential for Lymphatic Metastasis

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A new cell line producing α-fetoprotein, designated NMT-1, was established from rat yolk sac tumor with the potential for lymphatic metastasis in inbred Wistar rats. In this paper, we investigated the characteristics of NMT-1 cells. The cell line grew in a monolayer and had a polygonal epithelioid appearance in phase-contrast microscopy. The doubling time of NMT-1 cells during exponential growth was approximately 16 h in RPMI-1640 with 10% fetal calf serum. The D_0 value for radiation sensitivity was 97 ± 3 cGy. The extrapolation number, n, for NMT-1 was 1.08 ± 0.15. In the log phase, the G_0/G_1, S and G_2/M fractions were 23.2%, 62.1% and 14.3%, respectively. Tumor take was observed in all of the rats inoculated with NMT-1 cells. In the case of flank tumor, the mean survival time was 104 days after inoculation with 10^6 tumor cells. Inguinal, axillar, paraaortic, mesenteric and mediastinal lymphnode metastases were observed in all rats inoculated with NMT-1 cells. Rats which survived for a long time developed metastases in a lymphatic vessels of the liver, lung and/or kidney microscopically. The biological behavior and the histopathological features of the tumor induced by inoculation with NMT-1 cells were the same as those of the original tumor induced by fetectomy.

Key words: Rat yolk sac tumor cell line — AFP — Lymphatic metastasis potential

Human yolk sac tumor is well known as a tumor that has specific histopathological characteristics and an α-fetoprotein (AFP)-producing potential.[1,2] These tumors are considered to be induced in the process of dedifferentiation of primordial germ cells, but the precise pathogenesis has not been established.[3–5]

On the other hand, the development of yolk sac tumor from a displaced yolk sac after fetectomy is well documented in rats, humster and mice.[6–8] Yolk sac tumor cell lines have also been established in man, rat and hamster.[9–12] We recently reported the establishment of a transplantable slow-growing yolk sac tumor with the potential for lymphatic metastasis in inbred Wistar rats by almost the same method as reported previously.[13] From this tumor, we have established a new yolk sac tumor cell line with the potential for lymphatic metastasis, producing AFP, and we designated it NMT-1. In this paper, we deal with the characteristics of NMT-1 cells.

MATERIALS AND METHODS

Tumor origin Pregnant inbred strain rats (Wistar Mishima, WM/Ms) from which the fetuses were removed on the 12th day of gestation, developed a slow growing yolk sac tumor with distant metastatic potential derived from the fetal membranes left outside the uterus, as described.[13] This tumor was transplantable to closed-colony strain rats.

Morphological studies Part of the original tumor was fixed in 10% buffered formalin and embedded in paraffin. Histological specimens were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), digestive PAS and Alcian Blue. Cultured cells, seeded in a petri dish, were observed by phase-contrast microscopy.

Tissue culture The original tumor was excised under aseptic conditions. A portion was removed for histological examination, and the remainder was minced with forceps and scissors. Cells were cultivated in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (both from GIBCO, Grand Island, NY) in a 25 cm^2 culture flask at 37°C with 5% CO_2 in air. An epithelial cell colony grew among the fibroblasts in about 3 months and gradually became dominant. The cells were removed with a cell scraper and transferred to another 25 cm^2 culture flask. The cultured cells have been maintained for 30 months and more than 50 passages.

Growth kinetics The ciltured cells were trypsinized and 2×10^5 cells were seeded in each 25 cm^2 culture flask. Cells were harvested every day with trypsin-EDTA treatment and the number of cells was counted with a hemocytometer. The doubling time was determined from the number of cells at 1, 2 and 3 days after seeding.

Colony formation To determine the plating efficiency of passaged cells in a single-cell suspension, the following procedure was adopted. Various numbers of cells were

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plated into 60 mm petri dishes each containing a total of 5 ml of RPMI-1640 medium with 10% FCS. Cells were incubated at 37°C for 13 days in an atmosphere of 5% CO_2-95% air. At the end of this time, the dishes were rinsed in saline, and the colonies were fixed and stained in a crystal violet solution in metanol. Colonies containing 50 or more cells were counted visually.

Radiation sensitivity For these experiments, appropriate numbers of logarithmically growing cells were plated in 60 mm petri dishes by the method mentioned above. Three petri dishes containing cells were used each treatment condition. Radiation was started 16 h after seeding. After treatment, dishes were incubated for 13 days, and the colonies were fixed and counted. Surviving fractions were calculated as the fractional cell survival for a given treatment relative to the mean of that for the control petri dishes. Experiments were usually repeated 3 or 4 times, with a minimum of two repeats.

Irradiation The X ray machine (Hitachi MBR-1505R) was operated at 140 kVp, 4.5 mA with a 0.5 mm Al filter. The dose rate at the irradiation distance (30 cm) was 98 cGy/min. The dose rate was quantified by thermoluminescent dosimetry. Cells were irradiated at room temperature.

Flow cytometry Cells were prepared as a single cell suspension in medium at 5×10^5 to 10^6 cells per ml, then the nuclei were stained by adding ethidium bromide (50 μg/ml) solution containing 1% Triton X-100. DNA determinations were carried out with a FACScan (Becton-Dickinson, Mountain View, CA). Experiments were repeated 3 to 4 times.

Immunofluorescence staining of AFP Expression of AFP was detected by the direct immunofluorescence method. Cells cultured on chamber slides (Lab-Tek Nunc, Inc., Naperville, IL) were washed twice with phosphate-buffered saline (PBS) and fixed for 5 min with acetone at room temperature. The fixed cells were rinsed in PBS and incubated overnight with FITC-conjugated sheep anti-rat AFP immunoglobulin (Nordic Immunological Laboratories) at 4°C. Stained cultures were examined under an Olympus BHS-RF-B microscope.

Transplantation to rats NMT-1 cells (2×10^6) at the 30th passage in a volume of 0.2 ml were inoculated intramuscularly at the base of the gastrocnemius muscle of the right leg of 4-week-old inbred Wistar rats (Wistar Mishima, WM/Ms). The volume of tumors was determined by the measurement of one leg diameter. Autopsy was performed and histopathological examination was carried out when the rat died of the tumor.

RESULTS

Morphology The histopathological features of the original tumor were almost the same as those reported previously. The tumor was composed of epithelial cells with a thick, homogenous, eosinophilic, intracellular substance that is PAS-positive (Fig. 1) and digestive PAS-positive. Tumor cells, however, were not stained by Alcian Blue. Electronmicroscopic examination revealed that the substance surrounding the tumor cells was laminate and containd abundant endoplasmic reticulum. This material closely resembled the Reichert membrane of the parietal yolk sac in the normal placenta (Fig. 2). The established cell line, NMT-1, had a polygonal epitheliod appearance in phase-contrast microscopy. The cytoplasm of the

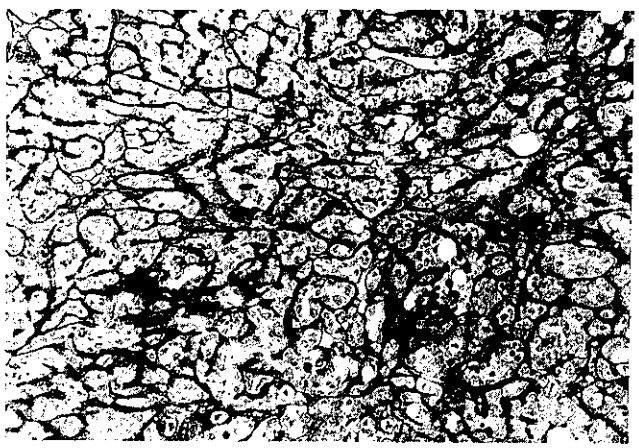

Fig. 1. Histopathological features of original tumor. The tumor was composed of epithelial cells with a thick homogenous intercellular substance that is PAS- and digestive PAS-positive. (PAS staining ×270)

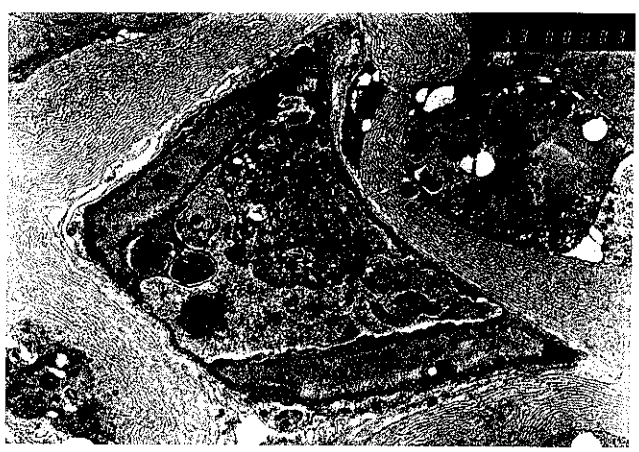

Fig. 2. Electronmicroscopic features of original tumor. The substance surrounding the tumor cells was laminated and contained abundant endoplasmic reticulum. This material closely resembles the Reichert membrane of the parietal yolk sac in the normal placenta. (×3,700)

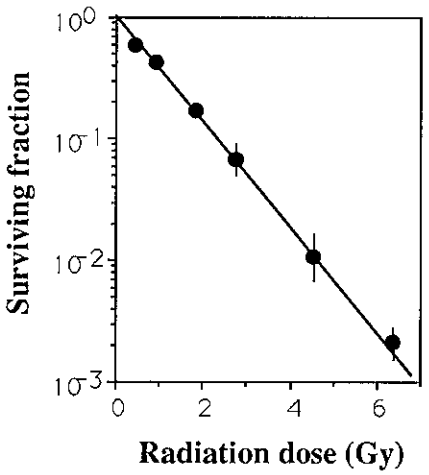

Fig. 3. Survival curve of irradiated NMT-1 cells *in vitro*.

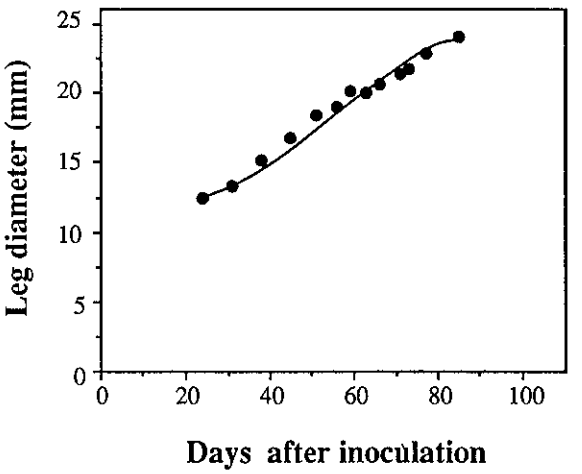

Fig. 5. Growth curve for inbred Wistar rats into which NMT-1 cells at the 30th passage were inoculated.

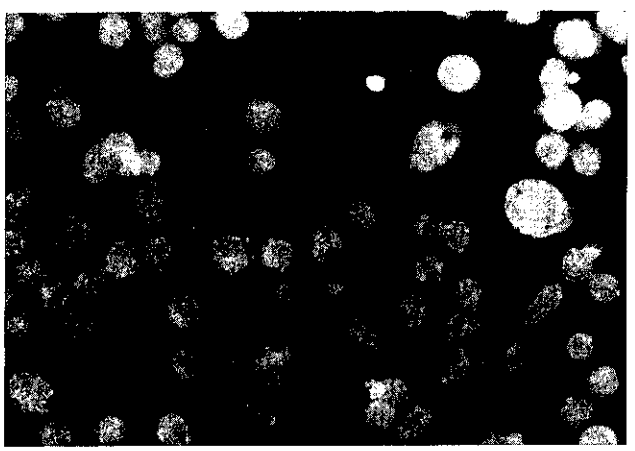

Fig. 4. Immunofluorescence staining of AFP. The NMT-1 cells react positively for AFP by direct immunofluorescence staining. (×540)

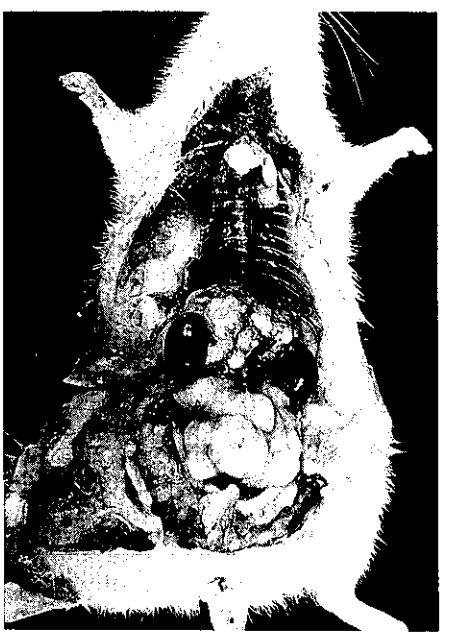

Fig. 6. Macroscopic appearance at autopsy of rat inoculated with NMT-1 cells in the right thigh. Metastatic lesions are observed in the inguinal, axillar, paraaortic, mesenteric and mediastinal lymph nodes.

NMT-1 cell was rich in glycogen granules, in accordance with the PAS reaction.

Growth kinetics The doubling time for NMT-1 cells during exponential growth was approximately 16 h in RPMI 1640 with 10% FCS. This growth curve was almost the same in each passage between the 10th and 30th passages.

Radiation sensitivity The mean plating efficiency for NMT-1 cells taken from the monolayer culture was 20% with a standard deviation of 4%. Data showing the effects of radiation on NMT-1 cells are shown in Fig. 3. There was no clear shoulder on the radiation dose-response curve. The D_0 value for radiation sensitivity, calculated by taking the reciprocal of the slope of the radiation dose-response curve, was 97 ± 3 cGy. The extrapolation number, n, for NMT-1 was 1.08 ± 0.15.

Flow cytometry The DNA distribution for NMT-1 cells in the log phase was as follows. The G_0/G_1, S and G_2/M fractions were $23.2 \pm 4.9\%$, $62.1 \pm 6.5\%$ and $14.3 \pm 5.6\%$, respectively.

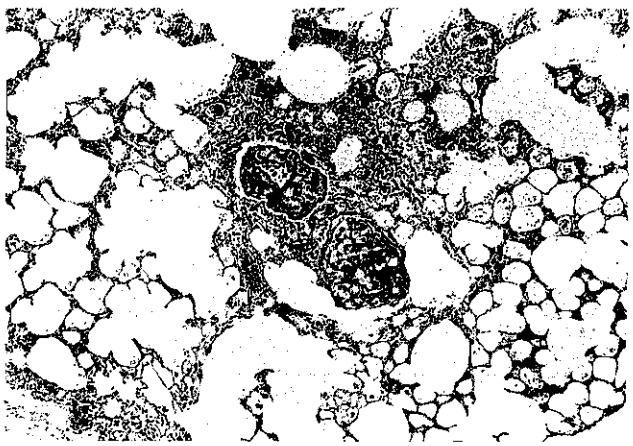

Fig. 7. Microscopic appearance of lung metastasis. The rat develops distant metastatic lesions in the lymphatic vessels of the lung. (HE ×270)

Immunofluorescence staining of AFP A positive reaction for AFP was seen within the cytoplasm of NMT-1 cells (Fig. 4).

Transplantation to rat Tumor take was observed in all of the rats inoculated with NMT-1 cells. The growth curve in 11 inbred Wistar rats into which NMT-1 cells at the 30th passage were inoculated is shown in Fig. 5. For the thigh tumor, the growth of tumors inoculated with cells taken from different passages was similar. The mean survival time was 104 days, with a range of 62 to 129 days. Fig. 6 shows the macroscopic appearance at autopsy of a rat inoculated with NMT-1 cells in the right thigh. Inguinal, axillar, paraaortic, mesenteric and upper mediastinal lymphnode metastases were observed in all rats. There was no difference between the biological behavior of the tumor induced by inoculation with NMT-1 cells and that of the original tumor. Rats which survived for more than 90 days developed metastases to the lymphatic vessels of the lungs, liver and kidneys microscopically (Fig. 7). The histopathological features of the tumor induced by inoculation with NMT-1 cells were also the same as those of the original tumor.

DISCUSSION

There are some reports about the establishment of yolk sac cell lines in man, rat and hamster,[9–15] but these cell lines do not have the potential for metastasis.[4] As far as we know, the NMT-1 cell line is the first transplantable rat yolk sac tumor cell line with the potential for lymphatic metastasis. In particular, this cell line has the potential to form metastatic lesions not only in the lymph nodes but also in lymphatic vessels of the lungs, liver and kidneys in challenged rats which survive for a long time.

The original tumor is considered to have been induced in the fetal membrane, but it is not yet clear what component of the fetal membrane was involved.[4,9] The development of yolk sac tumor from displaced yolk sac after fetectomy was completely inhibited by abdominal irradiation with 200 cGy immediately after fetectomy.[13] Teshima also reported that abdominal irradiation with 150 cGy one day before fetectomy decreased the occurrence of yolk sac tumor.[3] Therefore, there is a strong possibility that yolk sac tumor originates in the radiosensitive primordial germ cells of the fetal membrane. The NMT-1 cell line is very radiosensitive, like lymphocytes, because it does not have a clear shoulder (D_q) and it has a small D_0 value (about 100 cGy) on the radiation dose-response curve. The NMT-1 cell line may inherit these characteristics from the radiosensitive primordial germ cells of the fetal membrane.

These preliminary studies suggest that this model may be a valuable experimental system *in vitro* and *in vivo*, especially for biological studies on cell differentiation, lymphatic metastases and the radiosensitivity and/or drug sensitivity of yolk sac tumor. Furthermore, the NMT-1 cell line will provide a useful model of the production of AFP by cells.

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