HELA CELL-TUMOR IN NUDE MICE AND ITS RESPONSE TO ANTITUMOR AGENTS

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HeLa cells were heterotransplanted to nude mice and their response to some of antitumor agents was investigated. HeLa cell-tumor readily grew in nude mice and no regression was observed. Metastases to the lung and other organs were noticed in some of the animals. Histopathological examination revealed that the tumor retained the original characteristics of human epidermoid carcinoma.

Standardization of HeLa cell-tumor in nude mice for the screening and evaluation of antitumor chemotherapeutics was attempted. Marked inhibition of tumor growth was observed with lower doses of Mitomycin-C, 5-fluorouracil, Adriamycin, and Bleomycin. The tumor regression was observed with high doses of Mitomycin-C, Adriamycin, and 5-fluorouracil. However, cyclophosphamide, cytosine arabinoside, and daunorubicin had little effect on the tumor growth. Complete regression was not obtained with any of the test agents and active regrowth took place even with the most effective compound. Considerable variation in the effect on tumor growth was observed among the test compounds, while histopathological findings were much alike; few mitotic figures, vacuolization, and pyknosis were main changes in tumor cells, and large foci of necrosis and hemorrhage were present in the degenerative areas. The regrowth was initiated around the capillaries in the necrotic tumor tissue.

Considerable efforts have been devoted to find a reliable method for selecting the antitumor agents most likely to be clinically effective in particular patients. It is still uncertain whether screening systems of experimental animal tumors, especially transplanted for a long period, could find the most suitable agent for human tumors. For these reasons, human tumor tissues in xenogenic host seemed desirable for examining the sensitivities to potential chemotherapeutic agents. Well-known hosts for heterotransplantation are thymectomized and irradiated animals,3) animals treated with anti-lymphocyte serum (ALS),1) and hamster cheek pouches.8,16) However, most of them are technically difficult and time consuming.

With the introduction of an immunologically incompetent mouse mutant nude, many human tumors were transplanted successfully,10,11,13) and Povlsen et al.12) first described preliminary results with an in vivo model for testing the action of antitumor agents.

On the other hand, HeLa cell culture has long and widely been preferred for the primary screening of antitumor agents in Japan. In fact, a number of antitumor antibiotics have been isolated and developed by means of screening or assay systems employing this...
cell culture. Curtis and Perkins\textsuperscript{4} transplanted HeLa cell culture to mice treated with ALS and to nude mice. However, their results lack detailed data necessary for the antitumor screening. It was, therefore, of interest to know for the first time in vivo response of HeLa cells transplanted in nude mice to various antitumor agents in standardized system. This paper describes the growth and microscopical characteristic of HeLa cells in nude mice, and experimental results on the effect of some antitumor agents on the tumor growth.

**Materials and Methods**

**Animals** The animals used in the present experiments were 6- to 8-week-old BALB/c nude mice. The colony of nude mice (from Charles River Breeding Laboratories, Massachusetts, U.S. A.) was maintained in the barrier-system at the Laboratory of Chiba Cancer Center Research Institute. Cages laid with sawdust and overalls were sterilized by ethylene oxide gas before use. The diet sterilized by \textsuperscript{60}Co-irradiation was purchased from the Oriental Yeast Co., Tokyo. Water was autoclaved and supplemented with 160 mg/liter of streptomycin sulfate (Meiji Seika Co., Tokyo). The male nude mice (\textit{nu/nu}) and female heterozygotes were bred ordinarily.\textsuperscript{7,15}

**Cell Line and Transplantation in Mice** A rapidly growing line of HeLa cells maintained at the National Institute of Health, Japan, and kindly made available by Dr. Nitta in 1971 was used throughout. The line was maintained in Eagle’s minimum essential medium (MEM) containing 10\% fetal calf serum. Mice were inoculated subcutaneously in the lateral abdominal wall with 10\textsuperscript{7} HeLa cells suspended in saline. After subcutaneous inoculation, mice were observed daily, weighed, and tumors measured in two diameters with a slide caliper. Their volume was computed according to the formula \( (A \times B)^{3/2} \), where \( A \) and \( B \) are the lengths of long and short axes, respectively. In the succeeding experiments, a part of the nodule was removed under sterile condition and subdivided into small fragments (8 mm\textsuperscript{3}), which were then serially transplanted subcutaneously by a trocar.

**Chemicals** Mitomycin-C, 5-fluorouracil, and Adriamycin were obtained from Kyowa Hakko Co., Tokyo, and cyclophosphamide, cytosine arabinoside, Bleomycin, and daunorubicin from Shionogi & Co., Osaka, Nihon Shinyaku Co., Kyoto, Nippon Kayaku Co., Tokyo, and Meiji Seika Co., Tokyo, respectively. Maximum tolerated doses of the chemicals as determined by preliminary experiments were given intravenously or intraperitoneally. They were dissolved in saline as occasion demands, the required dose being given in a volume of 0.1 ml/10 g body weight. The administration of drugs was started when the nodule became about 5 to 6 mm in diameter, approximately 3 weeks after transplantation.

**Morphological Preparation** Complete autopsies were made on all the animals sacrificed after the inoculation of HeLa cells. Blocks were taken from inoculated tumors and each of visceral organs. They were fixed in 10\% buffered Formalin, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin, and by the periodic acid-Schiff reaction. Selected blocks from inoculated tumors in control animals were also studied by electron microscopy. Tissues were fixed for 12 hr in 2.5\% glutaraldehyde in Eagle’s MEM (pH 7.4) at room temperature, post-fixed in 1\% OsO\textsubscript{4} in MEM for 2 hr, then dehydrated, and embedded in Epon 812. Thin sections were cut with a Porter MT-1 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with Hitachi HU-12 electron microscope.

**Results**

**Development of Tumors** Several days after subcutaneous injection of 10\textsuperscript{7} HeLa cells, a small nodule appeared at the site of inoculation and continued to grow. Spread of the tumor to the lung (Photo 3) was found in one mouse sacrificed 78 days after inoculation. Incidence of tumor growth in serially transplanted mice was about 100\%. Other routes of inoculation were also tried. Intravenous inoculation of 10\textsuperscript{6} HeLa cells induced several nodules in the lung after 68 days. Peritoneal dissemination was seen without any ascites, 41 days after intraperitoneal inoculation. Intravenous or intraperitoneal inoculation might thus present metastatic model for lung or peritoneal cavity, but it is difficult to evaluate the effect of a chemotherapeutic. Consequently, in the present experiments, subcutaneous inoculation was employed. There was a good and relatively constant growth of tumors and their volume reached 200 mm\textsuperscript{3} at 15 to 25 days after implantation, depending on the inoculum size. In general, their growth curves showed...
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![Growth curves of HeLa cell-tumors in nude mice](image)

Fig. 1. Growth curves of HeLa cell-tumors in nude mice

a logarithmic increase but they grew more rapidly until they became several hundred mm³ in size (Fig. 1). In company with their growth, a dominant confluent necrosis was observed throughout them, but no spontaneous regression was observed unless tumors were injured. There were also little variance in body weight throughout tumor growth.

Histologically, the tumors were made up of large polygonal parenchymal cells with large irregular nuclei and pale eosinophilic cytoplasm. Arrangement of the tumor cells varied in form even within the same tumor. Pavement-like, trabecular, and duct-like structures were often seen. No squamous pearls were observed in the tumor masses. Each nucleus had one to two prominent nucleoli (Photo 1). Mitotic figures including abnormal polypolar ones were numerous (Photo 2). Intercellular bridges could not be observed in the tumor cells. In the case which showed metastasis into the lung, sheets of tumor cells invaded into the vein in the muscular layer of subcutis adjacent to the tumor masses (Photos 3 and 4). Invasion of vein in this manner probably supplied emboli for metastatic lesions. Electron microscopically, well-differentiated granular endoplasmic reticulum, rosette-like aggregated free ribosomes, and bundles of microfibrils in the cytoplasm were prominent. Microvilli at the luminal surface were also noted. Occasionally, typical junction complexes were present between neighboring cells. In some cells bundle of tonofilament-like substances was seen at the supranuclear regions (Photo 5).

The histology of the present transplanted HeLa cell-tumor resembles that of the original description of the carcinoma of the patient "He., La.". The histological diagnosis of this cervical carcinoma cited by Leighton⁹ was as follows: "The microscopic picture in all the metastases was that of an anaplastic epidermoid carcinoma. Intercellular bridges could be made out in some tumorous areas, but no keratin pearls were found." Although neither squamous pearls nor intracellular bridges could be found, presence of the bun-
Fig. 2. Effect of cyclophosphamide on HeLa cell-tumor (100 mg/kg/day, i.p.)

Fig. 3. Effect of cytosine arabinoside on HeLa cell-tumor (150 mg/kg/day, i.v., □; 200 mg/kg/day, i.v., ○, △)

diles of tonofilaments in the tumor cell cytoplasm suggests that the present transplanted HeLa cell might originate from the squamous cell carcinoma.

Effect of Antitumor Agents Fig. 2 illustrates the effect of cyclophosphamide on HeLa cell-tumor in nude mice. A dose of 100 mg/kg/day for 5 days had no therapeutic effect despite a considerable weight loss. Cytosine arabinoside also had no effect on tumor growth (Fig. 3). In the case of the mouse treated with 150 mg/kg/day of cytosine arabinoside, one tumor invaded into the peritoneal cavity and produced 4 small nodules on the mesenterium. One of these nodules was attached and infiltrated into the liver. Low dose of 5-fluorouracil, 15 mg/kg/day, exhibited no effect and caused no weight loss of animals. As shown in Fig. 4, a dose of 30 mg/kg/day of 5-fluorouracil for 4 successive days had a growth inhibitory effect when administered peritoneally. Yet, the
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Fig. 5. Effect of Mitomycin-C on HeLa cell-tumor (2 mg/kg/day, i.v.)

decrease in body weight due to its toxicity was significant. On the other hand, this drug in a dose of 60 mg/kg/day for 2 days had a considerable anticancer activity. Mitomycin-C at a dose of 1 mg/kg/day for 5 successive days had no effect although a slight weight loss of the animals was observed. When its 2 mg/kg/day was administered only the growth inhibition was observed (Fig. 5). With 4 mg/kg/day of this antibiotic for 3 days, the tumors regressed within 10 days but progressive growth was resumed soon after the regression. Marked weight loss was observed with two mice and the other one was dead 12 days after discontinuation of the treatment. The effect of Adriamycin was most remarkable as shown in Fig. 6. When 4 mg/kg/day of this antibiotic was daily administered for 5 days almost complete regression of the tumor was attained. Similar but less marked effect was observed with 3 and 4 injections of the same dose. After transitionary regression, however, rapid regrowth of the tumors took place in all the mice. With 2 mg/kg/day of Adriamycin, a slight growth inhibition of tumor and body weight loss were observed. On the other hand, the growth inhibition was not observed with daunorubicin at the same doses (Fig. 7). The growth inhibition and marked toxicity continued for a long time when 50 mg/kg/day of Bleomycin was administered for 5 days (Fig. 8).

The HeLa cell-tumor after treatment with various antitumor agents showed similar histopathological characteristics. Photos 6 and 7 show the effect of cyclophosphamide and Adriamycin after 3 days of the treatment. The principal changes in the tumor cells were vacuolation and the presence of pyknotic nuclei. There were few mitotic figures in the tumor tissue. Large foci of necrosis and hemorrhage were present in the degenerative areas. Usually there were no signs of inflammatory processes such as cell infiltration, exudation,
Fig. 6. Effect of Adriamycin on HeLa cell-tumor (4 mg/kg/day, i.v.)

Fig. 7. Effect of daunorubicin on HeLa cell-tumor (4 mg/kg/day, i.v.)

Fig. 8. Effect of Bleomycin on HeLa cell-tumor (50 mg/kg/day, i.v.)
and mesenchymal reactions around and/or in the tumor tissues.

At the beginning of the regression of a HeLa cell-tumor only a few surviving tumor cells were found in the necrotic areas. Dilated blood capillaries were always present at the center of the cluster of surviving tumor cells. Numerous mitotic figures were seen around the blood capillaries. In the ensuing weeks the tumor cells proliferated centrifugally from these capillaries. The small clusters of tumor cells gradually became confluent with each other. Photos 8 and 9 show the regrowth of tumor cells 20 days after cyclophosphamide and Adriamycin treatments.

The lesions induced by Adriamycin and Mitomycin-C in the liver were severe, sometimes showing extensive necrosis, fibrosis, and bizarre giant cell formation in the centrilobular regions. Cyclophosphamide and 5-fluorouracil, on the contrary, caused no pathological changes in any of the visceral organs examined.

**DISCUSSION**

HeLa cells heterotransplanted to nude mice proliferated well with full retention of histological appearance of the initial human carcinoma as revealed by light and electron microscopic observation. As was reported by Povlsen and Rygaad,10,11 most fresh human tumors heterotransplanted to nude mouse develop more or less histological reactions such as infiltration of granulocytes to the graft, and metastases to lymph nodes or organs have never been observed.

When established human cell lines such as amelanotic melanoma6) and HeLa cells were heterotransplanted to nude mice, little tissue reaction is usually observed. They were found to be more invasive and metastasized to lymph nodes or other organs. Standardization of implantation and rapid growth are also readily accomplished with these established cell lines. In contrast to the results by Franks et al.,5) almost 100% takes were attained with our strain of HeLa cells, the variation of tumor growth rate was least, and spontaneous regression was not observed, which warrants the usefulness of this system for in vivo evaluation. The discrepancy might be attributed to the difference of strains.

Among the antitumor agents tested, most significant effect was observed with Adriamycin which has a chemical structure only slightly modified from that of daunorubicin, while daunorubicin itself was much less effective. It is well-established that a considerable discrepancies are usually observed between the effect on HeLa cell culture and on experimental animal tumors. Whether these discrepancies are due to the difference in cell types or to the difference of in vitro and in vivo status of the tumor cells still remains unsettled. Introduction of the nude mouse has enabled one to compare in vitro and in vivo effectiveness of the compounds under study on human tumor directly. The results of the present experiments employing HeLa cells suggest that the status of the tumor cells has a significance for the sensitivity of tumor cells to antitumor agents. As was reported by other authors8,14) employing human tumors heterotransplanted to mice, most antitumor compounds proved to be less effective on HeLa cell-tumor than on transplanted animal tumors regularly used for antitumor screening, in which complete cure is sometimes attained. This discrepancy might be ascribed to the difference in two cell types, human and animal tumor cells. A legitimate conclusion, however, cannot be drawn until more human tumors in nude mice are tested for their sensitivity to those agents.

With some agents as cyclophosphamide, little effect on tumor growth was observed. Nevertheless, considerable histological changes of tumor tissue were observed as those of the agents which caused marked inhibition or regression of the tumor. These results may be taken to indicate that the evaluation of drugs employing human tumors heterotransplanted in nude mice in terms of
chemotherapy will only be possible with concurrent histopathological examination.

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References


Explanation of Plates

Photo 1. A small nodule at the site of HeLa cell inoculation. These cells infiltrated into the subcutis. No squamous pearls are observed in the tumor mass. H-E. ×50.


Photo 3. Lung metastasis of the subcutaneously injected HeLa cells. The tumor cells are grown around the peribronchial spaces. H-E. ×6.

Photo 4. A tumor embolus (arrow) in a small vein of muscular layer beneath the nodule of HeLa-cell tumor (T). H-E. ×200.

Photo 5. An electron micrograph of transplanted HeLa cell-tumor. At the top left, typical microvilli (arrows) are seen. Abundant tonofilament-like substances (T) are seen at the supranuclear region. N: Nucleus. ×25,000.

Photo 6. Three days after treatment with cyclophosphamide. All the tumor cells are vacuolated and their nuclei have become pyknotic. H-E. ×500.

Photo 7. Three days after treatment with Adriamycin. The cytological changes are similar to those seen in Photo 6. H-E. ×500.

Photo 8. Twenty days after treatment with cyclophosphamide. There are surviving and proliferating tumor cells around the dilated blood capillary (C). N: Necrosis. H-E. ×80.

Photo 9. Twenty days after the treatment with Adriamycin. A similar pattern as that in Photo 8 is seen. H-E. ×80.

H-E = Hematoxylin and Eosin stain.