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The Establishment of a Rat Colon Cancer Cell Line, ACL-15, with an Analysis of Its Metastatic Properties

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Summary. **A new cultured cell line (ACL-15) was established from a transplantable rat colon cancer strain induced by 1, 2-dimethyl hydrazine (DMH). Histologically, the primary colonic tumor was a poorly differentiated adenocarcinoma; all transplanted tumors showed the same histologic features. The doubling time of the ACL-15 cell line population was 24 h, and the saturation density was 5.6×10^5 cells/cm^2 in culture. The chromosome number and DNA content of the ACL-15 cells were near tetraploid. ACL-15 cells frequently metastasized to the liver and lungs *in vivo*. Thirty percent of those rats inoculated with ACL-15 cells into the subserosa of the cecum and all rats which received intrasplenic inoculations of ACL-15 cells developed hepatic metastases. All rats injected with ACL-15 into subcutis and into the tail vein developed multiple pulmonary metastases. This newly established cell line, ACL-15, can serve for a model of metastatic colonic cancer.**

INTRODUCTION

In patients with colorectal cancer, the presence of hepatic and pulmonary metastases determines their prognoses.[1] Many investigators, therefore, have concentrated their efforts on controlling the tumor to prevent distant metastasis.[2,3] Some murine colon carcinomas have been used as models of human colorectal cancer because of their similar histopathology.[4,5] However, these rarely metastasize to distant organs.[6,7] There are still few experimental models of colon cancer which have the potential to spontaneously metastasize to the liver or lungs.[8-10] For these reasons, the author has established a new cultured cell line, ACL-15, derived from a 1,2-dimethyl hydrazine (DMH)-induced transplantable rat colonic carcinoma. ACL-15 cells frequently form metastatic foci in the liver and lungs of the rats by inoculation. This report describes the biological characteristics and metastatic behavior of this cell line, ACL-15.

MATERIALS AND METHODS

Rats

F344 male rats were obtained from Charles River Japan, Inc. (Atsugi). Eight week-old rats were used for tumor transplantation and 6 week-old rats were used in metastasis assays. The animals were fed with standard rat pellets and water *ad libitum*.

Induction of transplantable colonic carcinoma

Sixty male F344 rats received subcutaneous(s.c.) injections of DMH (20 mg/kg) once a week for 20 weeks. More than 15 weeks after the last injection, each rat was examined for the occurrence of a colonic tumor, using a fiberoptic scope. The rats were then anesthetized by intraperitoneal pentobarbital injection (3 mg/100 gm), the abdomen was opened aseptically, and the tumor-bearing colonic segment was irrigated with normal saline containing kanamycin and piperacillin. Following this, a small part of the tumor was fixed for histological examination and the remaining tumor was minced into 2 mm fragments in RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan) containing 0.02% kanamycin. The recipients were anesthetized with pentobarbital and a small skin incision was made over the right lower abdomen. The minced colonic tumor was then transplanted s.c. into two recipients. In total, 15 different

colonic tumors, all derived similarly, were transplanted; only two of these tumors grew subcutaneously in the recipients. These were transplanted serially and maintained in the subcutis of syngeneic rats. After 15 serial transplantations, one of the two tumor strains was cultured *in vitro*.

Establishment of the cell line

Under sterile conditions, the transplanted tumor was excised and minced without enzymatic processing in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco). The medium containing minced tumor then was filtered through a stainless steel mesh. The filtered cell suspension was centrifugated and the tumor cells were resuspended in the RPMI-1640 medium with 10% FBS. The cell suspension was poured into a 35 mm tissue culture dish and maintained at 37℃ in humidified air with 5% CO_2. Twenty-four days after the primary inoculation, the cells were detached with 0.25% trypsin and subcultured. Cultures were passaged every 6 to 7 days and maintained in our laboratory for 120 passages in the RPMI-1640 medium supplemented with 10% FBS. This cell line was designated ACL-15.

Morphology

The primary DMH-induced tumors, transplanted tumors and subcutaneous tumors formed by the injection of ACL-15 cells were fixed in 10% formalin and stained with hematoxylin and eosin. For electron microscopy, cultured cells were fixed in 2.5% glutaraldehyde and 2% osmium tetroxide. The fixed cells were dehydrated in a series of ethanol and propylene oxide solutions, and embedded in Epon-812. Ultrathin sections were stained with uranyl acetate and lead acetate.

Cell growth

Suspensions of 2×10^5 viable cells (at their 54th passage) were inoculated into 60 mm plastic culture dishes (Corning) and received a change of medium every 2 days. Cells were counted every 2 days from Day 1 to Day 15 after inoculation in duplicate. The doubling time of the cell population and cell saturation density were calculated from the growth curve.

Chromosome analysis

Cells in their logarithmic growth phase were treated with 0.1 μg/ml colcemid for 2 h. These cells were lysed hypotonically with 0.075 M KCl for 15 min and fixed in a 3:1 methanol: acetic acid solution. After Giemsa staining, the chromosomes of 50 unbanded metaphases were counted.

DNA content by flow cytometry

At their 87th passage, cells in the logarithmic growth phase were fixed in 70% cold ethanol and treated with ribonuclease (1 mg/ml) (Sigma). Then, the cells were stained with propidium iodide (50 μg/ml) (Sigma) and the DNA content of the nuclei was analyzed by flow cytometry (Facscan, Becton-Dickinson, USA). Normal rat peripheral leucocytes were used as an external standard.

Modeling spontaneous hepatic metastases

Ten rats were anesthetized with pentobarbital intraperitoneally and laparotomies were made through a small incision over the lower abdomen. The cecum was exteriorized and suspensions of 5×10^6 cells/0.1 ml in RPMI-1640 serum free medium were inoculated into the subserosa (s.s.). Sixty five days after this inoculation, the rats were sacrificed and autopsied.

Experimental induction of hepatic metastases

Seven rats received intrasplenic (i.s.) injections as described by Kozlowski et al.[11] Briefly, a left lateral abdominal incision was made and the spleen exteriorized. After this, 2×10^6 cells/ml were injected slowly into the spleen. Twenty one days after injection, the rats were sacrificed and the metastatic nodules on the hepatic surface were counted macroscopically.

Modeling spontaneous pulmonary metastases

Seven rats were given s.c. injections of ACL-15 (1×10^7 cells/ml). Seventy days after this inoculation, the rats were sacrificed and autopsied. Tumors were then weighted and the pulmonary surface was examined for metastatic nodules.

Experimental induction of pulmonary metastases

A suspension of 2×10^6 cells/ml was injected into the tail vein (t.v.) of 5 rats. Twenty-one days after this injection, the rats were sacrificed and the number of metastatic nodules on the pulmonary surface were counted.

RESULTS

Morphology

Microscopically, primary DMH-induced colon tumors were poorly differentiated adenocarcinomas containing some signet-ring cells. The transplantable and subcutaneous tumors caused by cell injection also showed histologic features similar to the primary tumor (Fig. 1). In electron microscopy, ACL-15 cells showed large indented nuclei, poorly developed organelles and a few microvilli. The nucleus-cytoplasm ratio was high (Fig. 2).

In vitro growth characteristics

Cells inoculated into a culture dish adhered immediately to the bottom and grew monolayered (Fig. 3). Cells multiplied logarithmically for ten days before entering a stationary growth phase (Fig. 4). The population doubling time was 24 h and the saturation density in the culture dish was 5.6×10^5 cells/cm^2.

Chromosome analysis

The number of chromosomes ranged from 84 to 91, the mode chromosome number being 89. This value showed as near tetraploid compared with the normal chromosome number, 42, of the F344 rat. Forty-two percent of the cells contained the mode number of chromosomes (Fig. 5).

DNA quantification by flow cytometry

Flow cytometry of the DNA content distribution of the ACL-15 cells revealed that the ACL-15 cell line was composed of cell subpopulations containing at least a tetraploid quantity of DNA. The DNA index was 2.1 (Fig. 6).

Hepatic metastatic properties

In five rats, inoculated tumors grew in the cecal wall, and three of these five rats developed hepatic metastases. All rats which received i.s. injections of ACL-15 developed hepatic metastases (Fig. 7), and the median number of metastatic nodules was 63. Metastatic tumors were found in the mesenterial lymph nodes and splenic hilumglands (Table 1).

Pulmonary metastatic properties

Injections s.c. of 1×10^7 cells produced subcutaneous tumors in all rats. Seven days after such inoculation, the tumors became palpable, but thereafter their size increased slowly. When the rats were sacrificed, the median tumor weight was 10.3 gm (range 2.7-22.2 gm). Pulmonary metastases occurred in all rats (Fig. 8) and the median number of metastatic nodules was 55 a rat. No metastatic lesions was found in any other organ except the axillary lymph nodes. In all rats which received an injection into the tail vein, pulmonary metastases were observed and the median number of metastatic nodules was 11 a rat (Table 2).

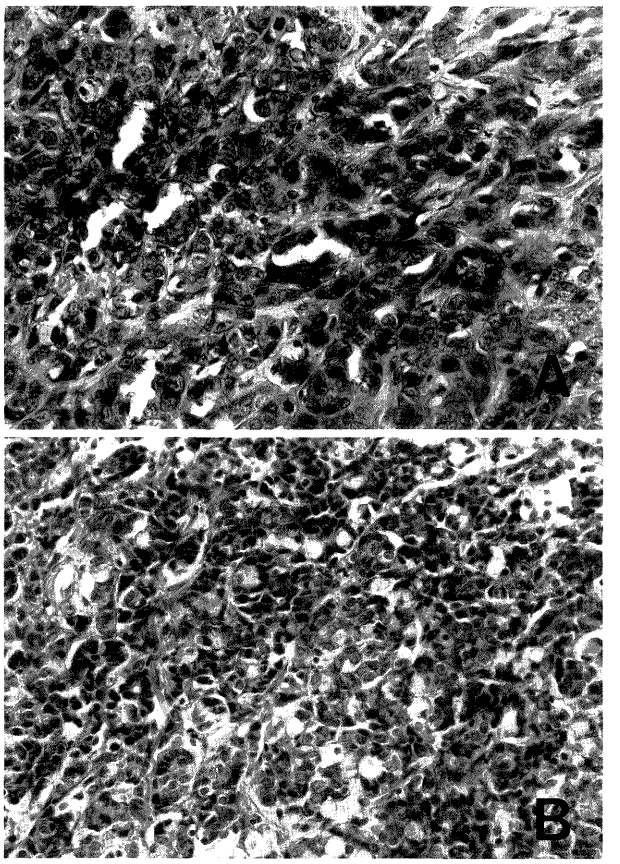

Fig. 1. The histologic appearance of a serially transplantable tumor (A) and a subcutaneous tumor grown by cell injection (B). Both tumors are classified as poorly differentiated adenocarcinomas. (HE, ×80)

DISCUSSION

Murine colonic carcinomas induced by DMH and other carcinogens are used extensively as human models in the study of carcinogenesis and histopathology.[4,12] Many transplantable tumors derived from

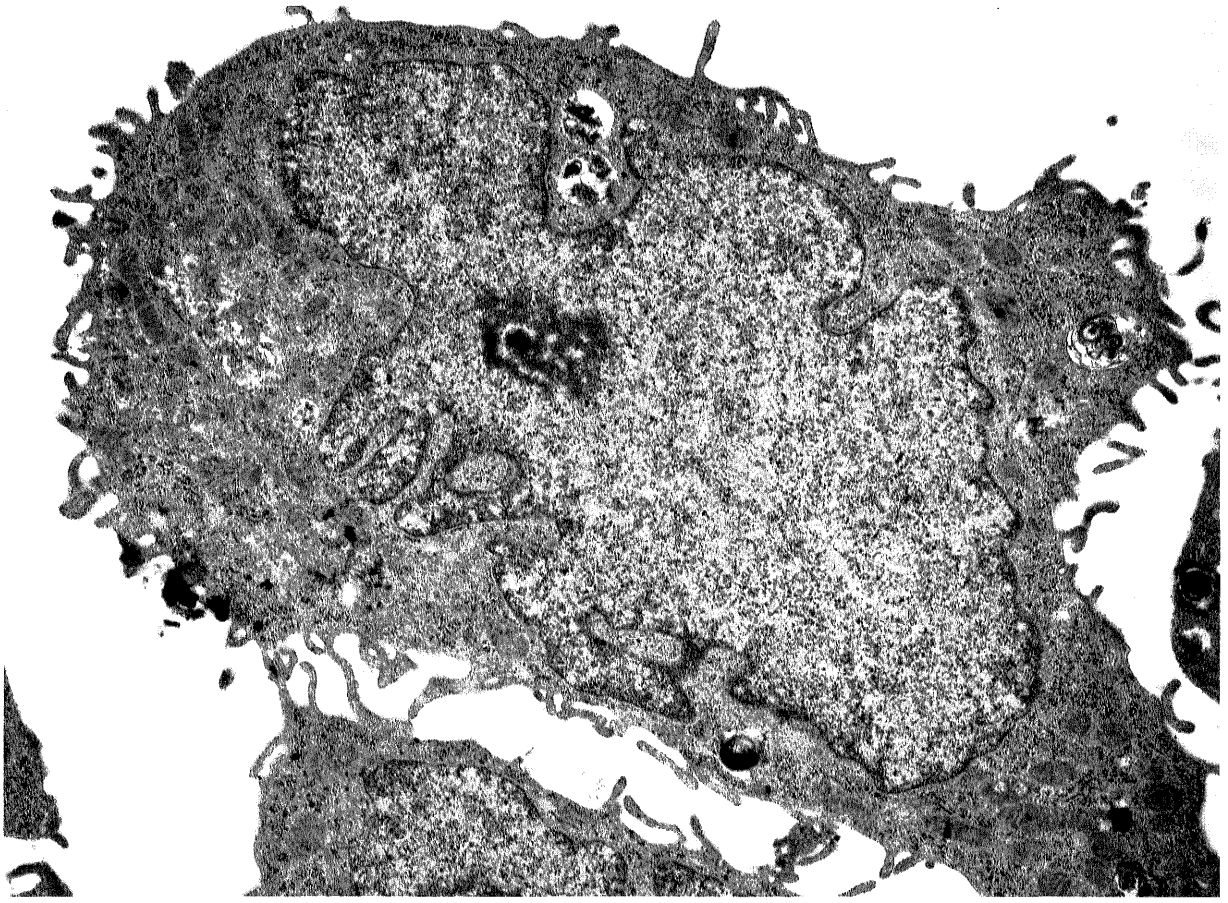

Fig. 2. Ultrastructure of ACL-15 cells at the 54th passage. The cells have a large nucleus and poorly developed intracellular organelles and microvilli. (×3000)

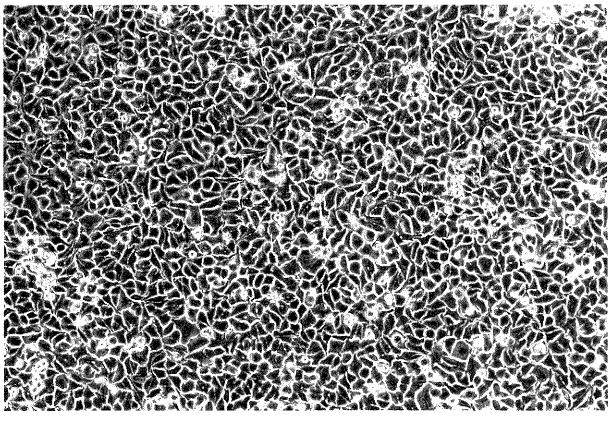

Fig. 3. Phase contrast photomicrograph of ACL-15 cells grown in a culture dish. (×100)

these colonic carcinomas are also used for evaluation of the effect of anticancer agents[13,14] or biological response modifiers.[15,16] Several reports have described the establishment of colonic carcinoma cell lines in rodents,[17-20] and a few investigations have reported these cell lines to have metastatic potential.[21-24] However, most metastasis models are formed by cell injection directly into the blood stream, i.e., into the tail vein, portal vein or spleen. On the other hand, Bresalier et al.[10,25] reported a spontaneous metastasis model using the 51B cell line. They formed the first tumors by cell injection into the cecal wall and found that hepatic metastasis occurred in 20% of the recipients.

The author established a cultured cell line, ACL-15, from a transplantable tumor of DMH-induced poorly differentiated adenocarcinoma of the rat colon, and found it to have spontaneous metastatic potential. After s.s. inoculation, ACL-15 cells were found to be

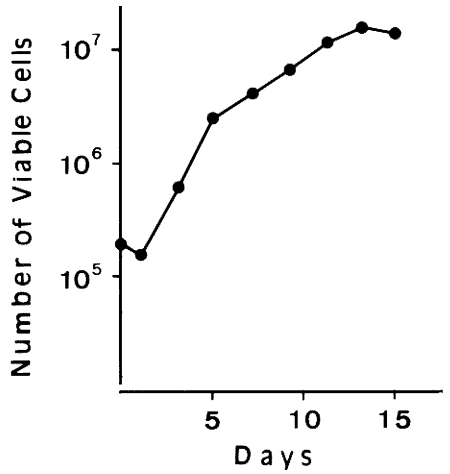

Fig. 4. Growth curve of ACL-15 cell line at the 54 passage in a 60 mm plastic culture dish. The population doubling time was 24 h.

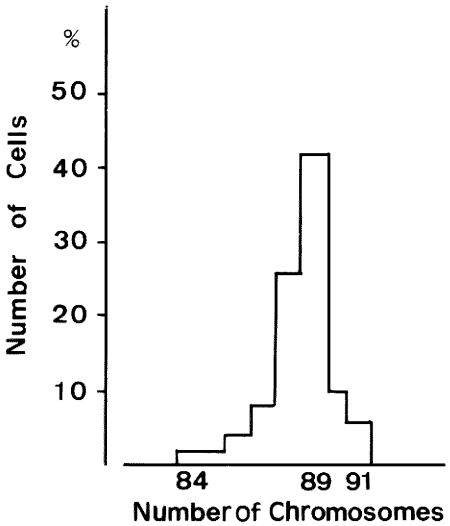

Fig. 5. Distribution of the chromosome number of ACL-15 cells.

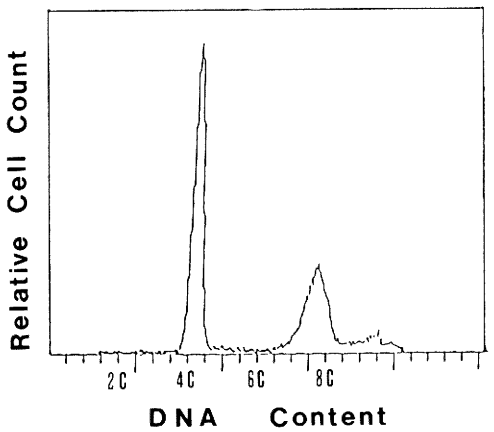

Fig. 6. Histogram of ACL-15 cell DNA content at the 87th passage.

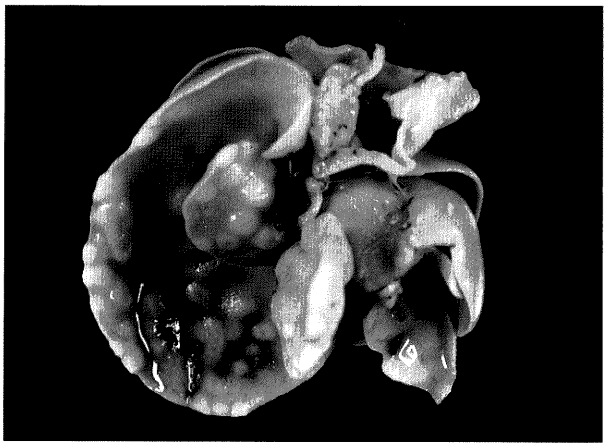

Fig. 7. Multiple metastatic nodules in the liver induced by intrasplenic injection of ACL-15 cells.

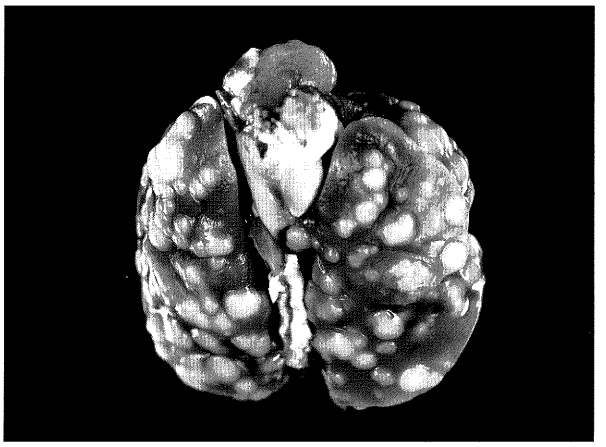

Fig. 8. Multiple lung tumors metastasized from a subcutaneous tumor formed by injection of ACL-15 cells.

tumorigenic in the cecal wall in 50% of the recipients, with hepatic metastases occurring in 60% of the recipients that developed cecal tumors. During inoculation into the s.s., when the cell suspension leaked out from the injection site, the recipient rats died in a short period because of peritoneal spread. If the cell injection is made more carefully, the tumorigenicity and metastatic rate will surely increase. When ACL-15 cells were inoculated into the s.c., they showed remarkable spontaneous metastatic ability to the lungs.

The formation of metastases is a complex multistep process. It involves the detachment of cells from the primary tumor, their penetration into the circulation, binding to the vascular wall, extravasation into the surrounding tissues and proliferation of the cells in the metastatic foci. Since cell injection subserosally or subcutaneously can reproduce all of this proc-

Table 1. Metastasis of ACL-15 to the liver

Injection site	Dose	Incidence of hepatic metastasis	Median no. of hepatic nodules (range)	Autopsy day[a]	Other metastatic legions
s.s.[b]	5×10^6	3/10	6(1-11)	65	mesenterial lymph node
i.s.[c]	2×10^6	7/7	63(34-100)	21	Lymph node of splenic hilus

[a] All rats were sacrificed at this time.
[b] Subserosal space of the cecum.
[c] Intrasplenic.

Table 2. Metastasis of ACL-15 to the lung

Injection site	Dose	Incidence of pulmonary metastasis	Median no. of pulmonary nodules (range)	Autopsy day[a]	Other metastatic legions
s.c.[b]	1×10^7	7/7	93(25-200)	70	Axillar
t.v.[c]	2×10^6	5/5	11(4-19)	21	None

[a] All rats were sacrificed at this time.
[b] Subcutis of anterolateral thorax.
[c] Tail vein.

ess, metastasis induced by an injection of ACL-15 cells is an ideal model for spontaneous metastasis.

By chromosome number and DNA content, ACL-15 cells were mostly near tetraploid. Morikawa et al.[26] selected cell strains with high metastatic potential to the liver from parent human colonic carcinoma cell lines in athymic nude mice. They found that the chromosome mode of highly metastatic strains was near tetraploid in contrast with near diploid of the parent cell line. Also, Inoue et al.[27] established a rat colonic carcinoma cell line (RCN-9) with diploid chromosome mode, from which the author derived a cell line highly metastatic to the liver (RCN-H-4). These RCN-H-4 cells were also near tetraploid by both chromosomal analysis and DNA quantification. These results suggest that DNA-aneuploidy, particularly tetraploidy, correlates with metastatic potential in rodent models.

Many investigators model artificial or spontaneous metastasis of colon carcinoma using athymic nude mice.[28,29] Because these mice are immunodeficient, these metastatic models are not suitable for studying immunological reaction against cancer cells and the effect of biological response modifiers.[24] Therefore, ACL-15, a newly established rat colonic cancer cell line with high metastatic potential, allows for permits the development of better models for the prevention and therapy of colonic cancer metastases in immunologically intact animals.

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