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A New Rat Colon Cancer Cell Line Metastasizes Spontaneously: Biologic Characteristics and Chemotherapeutic Response

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A new cell line (RCN-9) was established in culture from a transplantable rat colon adenocarcinoma, which was induced in the colon of a male Fischer F344 rat by subcutaneous administration of 1,2-dimethylhydrazine. When RCN-9 cells were injected subcutaneously or into the cecal subserosa of syngeneic rats, carcinomas with progressive growth were obtained and the development of lung (63.6%) and liver (40.0%) metastases, respectively, ensued. Antitumor effects of 5-fluorouracil (5-FU), adriamycin (ADM) and mitomycin C (MMC) against RCN-9 were examined *in vivo* and *in vitro*. 5-FU and ADM had antitumor effects both *in vivo* and *in vitro;* MMC had antitumor effects *in vitro*. These results show that the RCN-9 cell line can be used both as a model to study mechanisms of metastasis from colon carcinoma and as a model in chemotherapeutic studies of metastatic disease from colon carcinoma.

Key words: Experimental colonic carcinoma — Cultured cell line — Spontaneous metastasis — F344 rat

The usefulness of experimental models in the study of carcinoma is well recognized. Nude mice have been used for studies of chemotherapeutic agents against human carcinomas,[1,2] and mice or rats in studies of carcinogenesis.[3,4] However, comparatively few established cell lines of chemically induced colorectal cancer in culture exist,[5–7] and furthermore, few such cell lines metastasize spontaneously.[8–14] We established a colonic cancer cell line in culture from a chemically induced rat colon cancer after 9 passages of subcutaneous transplantation. This cell line (RCN-9) metastasizes spontaneously. This paper describes the biologic characteristics and *in vivo* and *in vitro* anticancer chemotherapeutic responses of RCN-9.

MATERIALS AND METHODS

Animals and primary tumor Sixty male inbred F344/DuCrj rats (Charles River Japan Inc., Atsugi) were given subcutaneous (s.c.) injections of neutralized 1,2-dimethylhydrazine (DMH) 20 mg/kg body weight once weekly for 20 weeks beginning at 6–7 weeks of age. Before injection, the DMH solution was adjusted to pH 6.7 by the addition of ethylenediaminetetraacetic acid (EDTA). After 20 DMH injections, colonofiberscopy was performed to locate colorectal tumors, and a sessile tumor was found in the distal colon of one rat.

Subcutaneous transplantation The rat with the colonic lesion was killed by ether inhalation, and the tumor was excised rapidly and washed in a solution of normal saline with 0.2% kanamycin (KM) and 0.2% piperaciline. Then the primary tumor tissue was bisected, and one half was placed in 10% formalin solution for histologic examination. The other half was minced into fragments in tissue culture medium RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo). The fragments were transplanted using a sterile technique into the subcutis of the inguinal region of a 6-week-old male syngeneic rat. About 60 days after transplantation, a subcutaneous tumor developed sufficiently for retransplantation and was transplanted into the subcutis of another syngeneic rat.

Primary culture and subculture After 9 passages of subcutaneous transplantation, the tumor was excised, minced with scissors and transferred through a wire mesh gauze (42 mesh, Ikemoto, Tokyo) to RPMI-1640 culture medium supplemented with 0.006% KM and 10% fetal bovine serum (FBS) (GIBCO, NY). The cultures were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. After 3 days of initial culture, the tissue debris and spent medium were removed. Culture medium was replaced in part, and all dishes were examined for growth of inoculated cells under a phase-contrast microscope twice a week. After 75 days of initial culture, the cultures were confluent and were subcultured as follows. The culture medium was replaced by 2 ml of 0.25% trypsin EDTA solution and incubated for 15 min at 37°C in an atmosphere with 5% CO_2. Two milliliters of culture medium was added to stop digestion by trypsin. After centrifugation at 1000 rpm for 5 min, the supernatant was decanted, and the cell pellet was resuspended in culture medium at a final concentration of 2×10^5 viable cells/dish. Viable cell count was estimated by using the trypan blue exclusion test. When the

culture dishes were confluent, subculture was repeated, and the established cell line was named RCN-9.

Tumorigenicity *in vivo* When the cultured cells reached confluence in the dishes, they were detached with a rubber cleaner and the cultures were pipetted gently into single-cell suspension, followed by centrifugal washing and resuspension in culture medium. Syngeneic rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight), and an s.c. injection of 1×10^7 viable tumor cells suspended in 0.2 ml of culture medium was given. Injection of 1×10^7 viable tumor cells suspended in 0.1 ml of culture medium into the cecal subserosa was performed in another group of animals.

Metastatic potential Rats were divided into three groups according to the method of tumor inoculation: s.c. inoculation of tumor, cultured cells and intramural cecal inoculation of cultured cells. All animals were observed and weighed weekly, and were killed 2 months after tumor inoculation. The livers and lungs were examined macroscopically to determine whether metastatic nodules were present.

Light microscopy The primary tumor, the subcutaneous tumor, the cecal tumor and metastatic liver and lung tumors were fixed in 10% formalin solution, stained with hematoxylin-eosin (H and E) and examined by light microscopy.

Electron microscopy Cultured cells and subcutaneous tumors formed by the inoculation of tumor cells were fixed in 2.5% glutaraldehyde solution and 2% osmium tetroxide solution. The specimens were dehydrated in series of alcohol and propylene oxide solutions, and embedded in Epon-812. Ultrathin sections were cut with a ultramicrotome using a diamond knife. Observation and photography were performed using a Hitachi HS-9 electron microscope after staining the sections with uranyl acetate and lead acetate.

Chromosome number Cultured cells in the 41st and the 130th passage were maintained for 2 h in 0.2 μg/ml colcemid solution, treated with 0.075 *M* KCl for 15 min, fixed in 3:1 methanol:glacial acetic acid and dropped onto vapor-moistened slides. The slides were dried and stained with Giemsa, and chromosome counts were performed on 100 well-spread, intact cells in metaphase.

Colony-forming ability Tumor cells in the logarithmic phase of growth in the 44th passage were harvested from the cultures by treatment with trypsin/EDTA solution. Cells were pipetted into tissue culture medium to create a single-cell suspension. After centrifugation and dilution, cultures of 100 viable cells/dish were prepared in 60 mm plastic dishes. Cultures were incubated at 37°C in 5% CO_2 in humidified air. Colonies were counted after 14 days under an inverted phase microscope.

Sensitivity to chemotherapeutic drugs *in vivo* Six-week-old syngeneic rats were anesthetized by intraperitoneal injections of pentobarbital sodium. The rats were given an s.c. injection of 1×10^7 viable tumor cells suspended in 0.2 ml of culture medium, and were examined twice weekly to determine body weight and tumor growth. Tumor size was measured in two dimensions by using calipers. Relative mean tumor weight (RW) was calculated by the Battelle Columbus Laboratories Protocol.[1,2] Treatment commenced when the estimated tumor weight reached 100–300 mg. Rats were divided randomly into 4 groups (8 rats/treatment group, 10 rats/control group) and received one anticancer drug. Adriamycin (ADM, Kyowa Hakko Co., Ltd., Tokyo) was given intravenously (i.v.) in a single dose of 3 mg/kg body weight. Mitomycin C (MMC, Kyowa Hakko Co., Ltd., Tokyo) was given intraperitoneal (i.p.) in a single dose of 2 mg/kg body weight. 5-Fluorouracil (5-FU, Kyowa Hakko Co., Ltd., Tokyo) 50 mg/kg was given i.p. 3 times every 4 days. Control rats received i.p. injections of 0.9% NaCl solution. Antitumor activity was determined after 3 weeks.

***In vitro* colony assay** ADM, MMC and 5-FU were prepared in sterile water and diluted in RPMI-1640 solution for incubation. Tumor cells in the log-phase were harvested from the cultures by treatment with trypsin/EDTA solution. Cells were pipetted into tissue culture medium to make a single-cell suspension and incubated with or without drug for 1 h at 37°C in culture medium. The cell suspension was centrifuged, washed twice and prepared for culture. Cells to be tested were suspended in 0.3% agar in enriched RPMI-1640 medium supplemented with 10% FBS to yield a final concentration of $1–3\times10^3$ viable cells/ml. One milliliter of this mixture was pipetted into each of three 35 mm plastic dishes (Corning) containing 1 ml of 0.5% agar in enriched RPMI-1640 solution. Cultures were incubated at 37°C in 5% CO_2 in humidified air. (Cells were not incubated with 5-FU before suspension in soft agar culture medium; 5-FU was diluted appropriately and added directly to the soft agar culture medium.) Three levels of drug concentrations were chosen as 1/100, 1/10, and 1/1 of the peak plasma concentration in human pharmacokinetic studies.[15] Final concentrations (in μg/ml) were 0.01, 0.1, 1.0 for MMC, 0.004, 0.04, 0.4 for ADM and 0.1, 1.0, 10.0 for 5-FU. After 2 weeks of incubation, the number of colonies on control and drug-treated plates was determined by counting under an inverted stage microscope.

RESULTS

Primary tumor and subcutaneous transplantation The colonic tumor transplanted from the distal colon of one rat after DMH injection transplanted to inbred rats ceased to be palpable about one week after transplan-

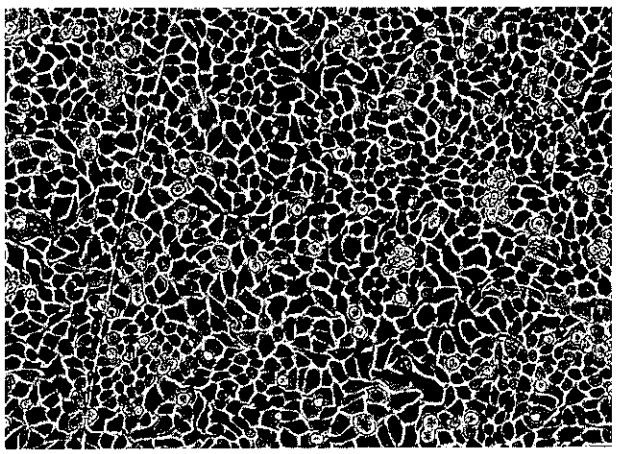

Fig. 1. Phase contrast photomicrograph of the RCN-9 colon cancer cell line grown on a cell culture dish.

tation. The tumor, however, became palpable again after about 4 weeks and subsequently grew gradually and progressively, eventually resulting in host cachexia. This tumor was excised 104 days after transplantation. After tumor fragments were retransplanted into the syngeneic rat, the tumor showed stable growth and became transplantable 1.5 to 2 months after transplantation.

Primary culture Tumor cells transferred to the culture medium formed an insular colony, but were admixed with fibroblasts.

Subculture In replacing the culture medium, a mark was put on the colony of tumor cells from the reverse side of the dish and only fibroblasts were detached with a rubber cleaner. Thus, the proportion of tumor cells increased gradually with the sequential procedures. Subculture was performed when the piling up of the colony of tumor cells became conspicuous and a cell suspension appeared. Fibroblasts no longer were detectable in the 3rd passage. In the subcultured cell line, polygonal cells grew in a single layer pavement (Fig. 1), and the dish became confluent about seven days after subculture. At the time of writing, after 24 months of culture, the cells are in the 140th passage.

The cell doubling time and the cell confluence density calculated from the growth curve in the 8th and 28th passages were 32 h and $5.6\times10^5/cm^2$, respectively.

Biologic characteristics The tumor survival rates in subcutaneous transplantation and cecal subserosa transplantation were 91.9% (34/37) and 35.7% (5/14), respectively. The rate of lung metastasis following subcutaneous transplantation and the rate of liver metastasis in cases of cecal subserosa transplantation were 63.6% (7/11) and 40% (2/5), respectively (Fig. 2 and Table I).

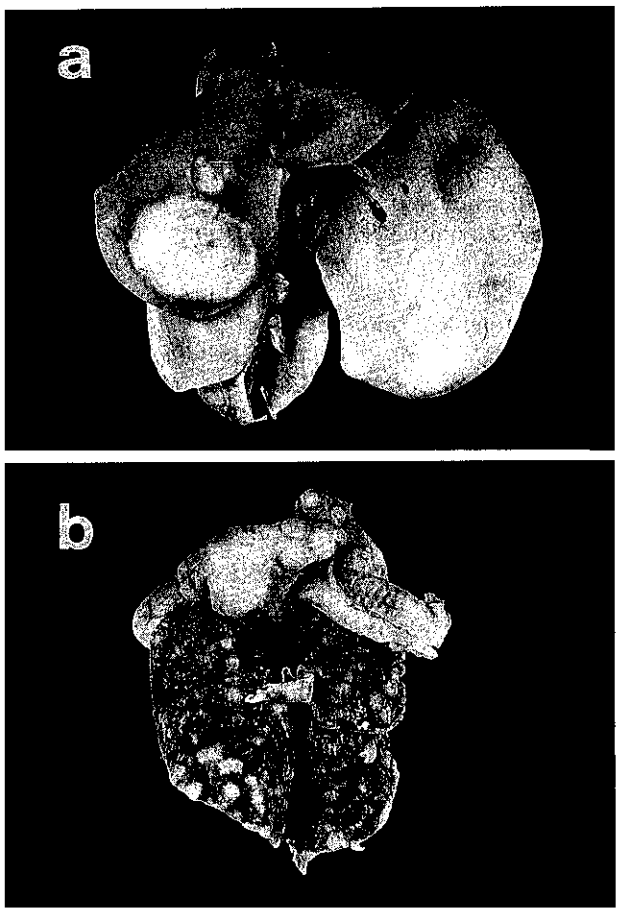

Fig. 2. Experimental model of colon cancer metastasis from the RCN-9 cell line. (a) Liver metastasis from a cecal tumor. (b) Lung metastasis from a subcutaneous tumor.

Table I. The Tumor Survival Rate and Rate of Metastasis of RCN-9 Colon Cancer Cell Line

Site of transplantation	Tumor survival rate	Metastasis rate
Subcutis	91.9% (34/37)	63.6% (7/11)[a]
Cecal subserosa	35.7% (5/14)	40.0% (2/5)[b]

a) Lung metastasis.
b) Liver metastasis.

Histopathologically the primary tumor and subcutaneously transplanted tumor were poorly differentiated adenocarcinomas with some signet ring cells. Metastatic liver and lung tumor cells were similar to the primary tumor (Fig. 3). Electron microscopically, both tumor

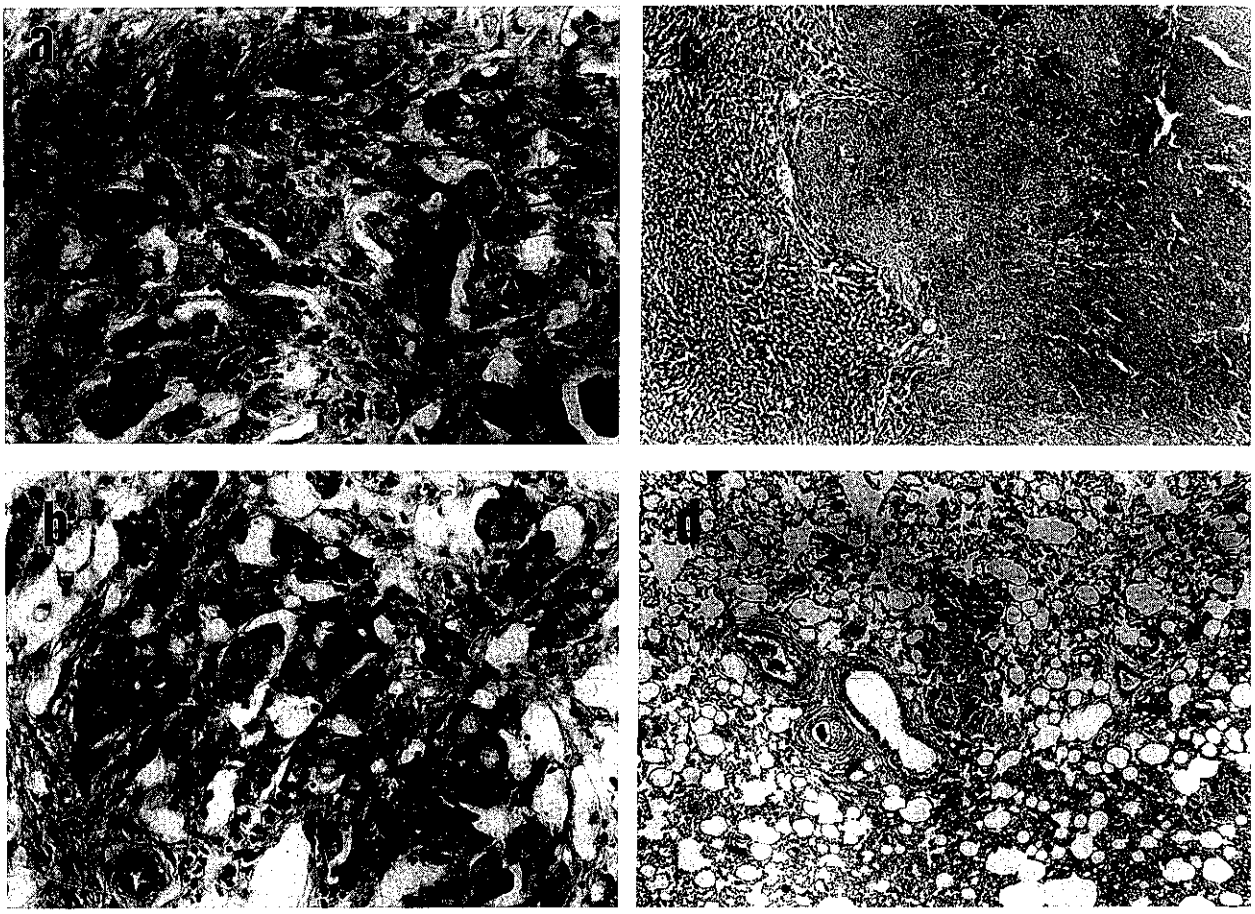

Fig. 3. Histopathology of the tumors associated with the RCN-9 colon cancer cell line. Photomicrographs of the primary tumor (a) (H and E ×80), the subcutaneously transplanted tumor (b) (H and E ×80), liver metastases (c) (H and E ×40) and lung metastases (d) (H and E ×16). Metastatic tumor cells were similar to the primary tumor cells.

and cultured cells were composed of small cells with a high nucleus-cell ratio and having few intracellular organelles (Fig. 4). Among 100 cultured cells in the 41st and 130th passage, the values of the mode of chromosome number were 42 (range, 34–79) and 43 (range, 34–88), respectively. The colony-forming ability of cultured cells in the 44th passage was 69.8%.

Sensitivity to chemotherapeutic drugs *in vivo* and *in vitro*

The T_{RW}/C_{RW} ratios of ADM, MMC and 5-FU after 3 weeks of chemotherapy were 35.0%, 83.8% and 30.2%, respectively. The tumor was sensitive to ADM and 5-FU (Fig. 5). Sensitivity, defined as at least 50% inhibition of colony formation in a culture plate containing at least 30 colonies, was demonstrated for MMC at 1/10 the maximum blood concentration in humans, for ADM at the maximum blood concentration in humans and for 5-FU at 1/100 the maximum blood concentration. The concentrations of drug required for 50% inhibition of cell growth (IC_{50}) of MMC, ADM and 5-FU were 0.035 μg/ml, 0.064 μg/ml and 0.058 μg/ml, respectively (Fig. 6).

DISCUSSION

Many reports of experimental models of rat or mouse colon carcinoma induced by DMH and other carcinogens have appeared since the reports of Druckrey[3] and Thurherr *et al.*[4] These carcinomas have been reported to be similar morphologically to human colon carcinomas in many respects. On this basis, these carcinomas have been accepted as experimental models of human colon carcinoma.[16–18] However, the models are concerned mostly with carcinogenicity and few experiments on metastasis have been performed.[19, 20] The main reason for this is the

Fig. 4. Electron photomicrograph of the cultured RCN-9 cells. ×7000.

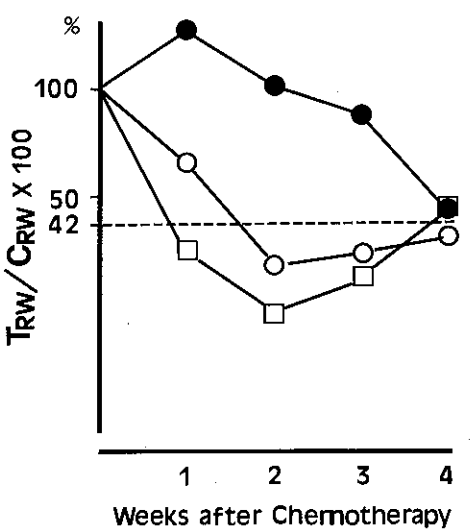

Fig. 5. Sensitivity of RCN-9 colon cancer cell line to three chemotherapeutic agents *in vivo*. Tumor weight (mg) = (W^2 × L)/2; W, width (mm) and L, length (mm). Relative mean tumor weight (RW) = Wi/Wo; Wi, mean tumor weight at any given time; Wo, mean tumor weight at initial treatment. Regression of tumor, RW < 1.0; retardation of tumor growth, $T_{RW}/C_{RW} < 42\%$; inactive, $T_{RW}/C_{RW} > 42\%$; T_{RW}, RW of treatment group; C_{RW}, RW of control group. Mitomycin C (●) 2 mg/kg, adriamycin (○) 3 mg/kg, 5-fluorouracil (□) 50 mg/kg ×3.

low incidence of metastasization of tumors induced by DMH. But it should also be mentioned that the time it takes to form a tumor is considerable and that tumors of other organs, such as the external auditory meatus and small intestine, do develop.[21,22]

In creating an experimental model of metastasis, it is necessary to use subcutaneously transplanted cell lines and cultured cell lines. Cell lines heretofore used in experimental models are mostly nonepithelial tumors, such as B16 melanoma and sarcoma.[23-25] Models using colon carcinoma cell lines are few: colon 26 in mice,[5] 192 NRc,[6] DHD-K12-TR[7] in rats and RPMI 4788 in human colon carcinoma.[26] Moreover, most of these models are induced by the injection of tumor cells into the tail vein, portal vein or spleen. These are referred to as "implantation models." Models of spontaneous metastasis such as DHD-K12-TR,[8] 51B,[9] colon 26,[10] MCA-38,[11,12] KM12SM,[13] LS174T,[14] are very few.

When metastases develop, cancer cells separate from the primary tumor, enter the intercellular space, penetrate the wall of a blood vessel or lymphatic and are fluid-borne to the implantation site.[27] Whether or not a metastasis forms depends on the physical, chemical and immunologic modifications to which the cancer cells are subject at each of these stages.[28-30] A true model of metastasis of human carcinoma cannot be reproduced experimentally since the cancer cells do not separate

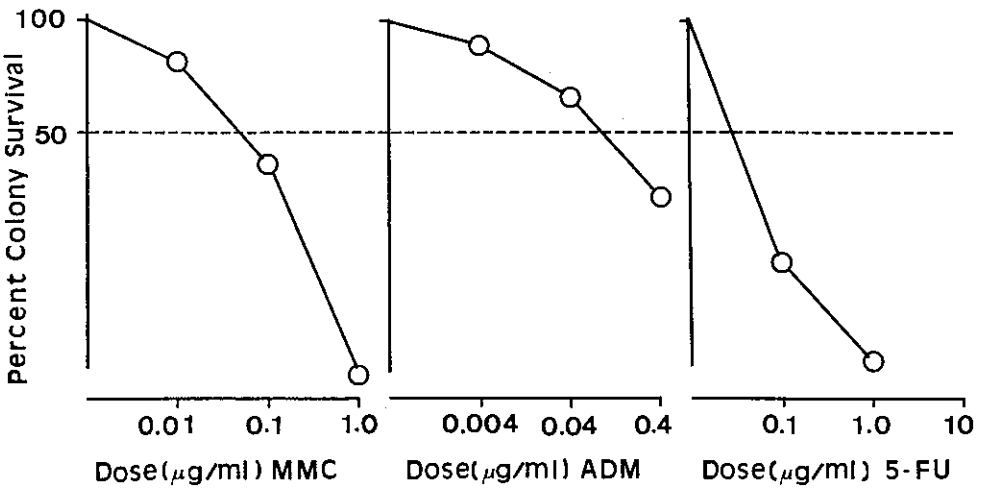

Fig. 6. Sensitivity of RCN-9 colon cancer cell line to three chemotherapeutic agents *in vitro*. Sensitivity was defined as inhibition of at least 50% of RCN-9 colonies on a culture plate containing at least 30 colonies. MMC, mitomycin C; ADM, adriamycin; 5-FU, 5-fluorouracil.

from a primary tumor to enter a vessel naturally. Also some experimental models use cells which do not metastasize spontaneously to the organs in which experimental metastases are created.

Earlier reported models of spontaneous metastasis of colon carcinoma include lung metastasis by subcutaneous transplantation of tumor cells and liver metastasis following cecal subserosa transplantation.[20-26] Models of heterotransplantation using nude mice and models of homotransplantation using mice and rats were also reported. Models using nude mice have the advantage that human cancers can be used. As reported by Hanna and Fidler,[31,32] Wiltrout *et al.*[33] and Skov et al.,[34] these models also have some problems in that anti-asialo GM1 serum or a young nude mouse has to be used to modify the immunologic environment, since the adult nude mouse does not develop metastases easily because NK activity is high and the primary immunologic environment between tumor and host is different. With models using syngeneic mice and rats, the immunologic environment may be considered identical for the host and transplanted tumor. In this respect, such a model is superior to the nude mouse model.

The RCN-9 cultured cell line is associated with an ability of spontaneous metastasis to the lung when transplanted subcutaneously to syngeneic rats and to the liver when transplanted into the cecal subserosa. The incidence of spontaneous metastasis is not so high as compared with that of experimental metastasis models. Therefore the usefulness of RCN-9 for quantitative experiments on metastasis is limited at present. But the authors consider that the incidence of spontaneous metastasis can be increased by developing highly metastatic clones using the method of Fidler.[35]

Since the route of metastasis is similar to that of human carcinoma and since RCN-9 is transplanted to syngeneic rats, this cell line is useful immunologically as an experimental model of clinical lung and liver metastasis.

This model of metastasis, while useful in the elucidation of mechanisms of metastasization, is also extremely valuable in evaluating therapeutic efficacy. In the present study, the antitumor effect of three chemotherapeutic drugs (5-FU, MMC, and ADM) on RCN-9 was evaluated both *in vivo* and *in vitro*. *In vivo* efficacy was evaluated in accordance with the Battelle Columbus Laboratories Protocol.[1,2] Dosage and method of administration were based on the report of Kondo *et al.*[36] The dosages were changed in present experiment, as an LD >50 was noted with the treatment groups of ADM and MMC in the preliminary experiment (the LD >50 of ADM and MMC were 8 mg/kg body weight and 6 mg/kg body weight, respectively). The method for sensitivity testing *in vitro* varies with the investigator. In the present study, the colony-forming ability of RCN-9 was high (69.8%) and was felt to meet the requirements for a colony assay substrate. The method of Hiraki *et al.*,[37] a modification of the method of Hamburger *et al.*,[38,39] was employed. Drug concentrations were established using the maximum blood concentrations in clinical use based on the report of von Hoff *et al.*[15] According to the present study, sensitivity to 5-FU was shown both *in vivo*

and *in vitro* and at the maximum blood concentration *in vitro* with ADM; good results were also obtained *in vivo* with ADM. No sensitivity to MMC was seen *in vivo* despite the sensitivity *in vitro* at one-tenth the maximum blood level.

We believe that RCN-9 can be used as a model for evaluating the efficacy of chemotherapeutic agents against metastatic lesions. Furthermore, surgical procedures can be done more easily with this model than with models using mice. Therefore this model should also be more useful in the investigation of adjuvant chemotherapy combined with surgery.

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