Jpn. J. Cancer Res. 81, 1045–1051, October 1990

A Novel Model of Solitary Hepatic Tumor in Rats Using Ascites Hepatoma AH13: Suitability for Chemotherapeutic Studies

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A highly reproducible model of a solitary hepatic tumor in rats using ascites hepatoma AH13 has been developed using a two-step method which was suitable for quantitative chemotherapeutic studies. Diffuse hepatic metastases were induced first by inoculation of three different ascites hepatomas, AH13, AH130 and AH7974 into the portal vein in a dose-dependent fashion. Second, the induced hepatic tumor (3×10^7 cells) was minced into $1\times1\times4$ mm fragments and implanted in the liver of normal rats. In this procedure, the AH13 strain proved best suited for the generation of a solitary hepatic tumor. The growth of the solitary liver tumor using AH13 was highly reproducible. To demonstrate the suitability of this solitary hepatic tumor model for the evaluation of chemotherapy, the tumor-burdened rats were treated with adriamycin (ADR) and mitomycin C (MMC). The reduction in tumor size was proportional to dosage, and the statistical significance of the differences between the treatment group and control group was proportional to dosage. A synergistic effect of ADR and MMC on the tumor also was demonstrated. This model should prove to be a useful tool for the testing of newly developed treatments of hepatic cancer.

Key words: Rat model — Solitary hepatic tumor

The liver is a major site of both primary and metastatic cancer. Hepatocellular carcinoma, which is the most common primary hepatic cancer,[1)] ranks third in incidence among all malignancies in Japan[2)] and its prognosis remains disappointing.[3-5)] Hepatic metastases occur frequently in a variety of human cancers.[6)] However, few animal models exist which are appropriate to evaluate the efficacy of hepatic cancer treatment.

Previous hepatic tumor models in rats generally used long-term, continuous exposure to a carcinogen added to the diet[7-11)] or injection of an established tumor cell line into the portal circulation.[12-20)] However, these preparations require a long time for tumor development and data concerning the "change in tumor size" are unavailable. Therefore, experiments designed to evaluate new approaches to the treatment of hepatic cancer have been hampered by the lack of an appropriate model.

In this paper, we describe a two-step method of generating a solitary hepatic tumor using ascites hepatoma AH13 in rats, and its suitability for chemotherapeutic studies.

MATERIALS AND METHODS

Animals and tumor cell lines Male Donryu rats aged 5 to 6 weeks were obtained from Japan SLC Co., Ltd. (Shizuoka) and used in all experiments.

AH13, AH130 and AH7974 rat ascites hepatomas were generous gifts from Dr. H. Nagayama, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai. Cells were maintained in ascites in normal Donryu rats.

Diffuse hepatic metastases induction by injection of tumor cells into the portal vein of rats Tumor cells used for injection were centrifuged twice in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) at 250*g* for 6 min, and the cells to be injected, 1×10^6 to 3×10^7, were resuspended in 0.3 ml of RPMI medium for each injection. Animals anesthetized with 50 mg/kg of pentobarbital sodium intraperitoneally (ip) were placed in the supine position, and their abdomens were prepared in a sterile fashion. A midline celiotomy incision was performed and the intestines were retracted caudally so that the portal vein was visualized. Tumor cells were injected into the portal vein (pv) through a 27-gauge needle on a 1-ml syringe. Hemostasis was obtained using cotton swabs. The abdomen was closed with Auto Sutures (United States Surgical Corporation, Norwalk, CT).

Implantation of minced fragments of the induced hepatic metastasis into the liver of new hosts Five days after the initial injection of tumor cells pv at a dose of 3×10^7, the animals were again anesthetized with pentobarbital sodium ip, and a celiotomy incision was remade. The induced hepatic tumor was resected and placed in a sterile Petri dish and minced finely into $1\times1\times4$ mm

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fragments with surgical scissors for each implantation. Hepatic tumor induction was recognized easily by the appearance of an enlarged liver. New hosts were placed in a supine position, and a 3-cm celiotomy incision was made aseptically just below the xyphoid. The liver was exposed and the fragment prepared as described above was implanted into a pocket in the left lobe of the liver created by blunt dissection with a trocar. During implantation, the left side of the abdominal incision was covered with a 2×2-cm sterile gauze and the right side was pulled up with forceps to avoid contact between the fragments and the abdominal wall. Hemostasis of the liver capsule was obtained by using cotton swabs, and the abdomen was closed with Auto Sutures. At the end of each study, the livers were removed and the lengths of the major and minor axes of the tumor on a section taken through the center of the tumor were measured with calipers to determine the growth of the implanted tumor. The size of the tumor was expressed as the product of the length of the major and minor axes.

Histological examination Forty rats received implants, and the samples including liver, heart, lung and kidney, were removed 14 days after implantation and fixed in 10% phosphate-buffered formalin. Sections stained with hematoxylin and eosin were prepared for histological examination.

Chemotherapy model The chemotherapy protocol, using seven animals per treatment and seven animals per control, was as follows.

Day 0: Normal rats received implants of AH13 liver tumor fragments in the liver and were randomized to treatment and control groups..

Day 7: Chemotherapy consisting of adriamycin (ADR, Kyowa Hakko Kogyo Co., Ltd., Tokyo) and mitomycin C (MMC, Kyowa Hakko Kogyo Co.) was initiated. The detailed schedules of the treatments are shown in Tables III and IV. Drug dosage was chosen based upon the reported lethal dose.

Day 14: Animals were killed, and their livers were removed to determine the size of the tumor.

Statistical analysis The significance of differences in tumor size between the treatment group and the control group was determined by using the Wilcoxon rank sum test. No rats were excluded from the statistical evaluation. Two-tailed *P*-values are presented for all experiments.

RESULTS

Dose titration of tumor cells for induction of diffuse hepatic metastasis The first step in the generation of the solitary hepatic tumor model was the generation of diffuse hepatic metastases using three different ascites hepatoma cell lines.

The data summarized in Table I show the relationship between hepatic metastases induction and the number of injected ascites hepatoma cells in the three cell lines. Each tumor presented a different microscopic character[18] and needded to be evaluated by dose titration prior to implantation in the new host. The animals generally were killed two weeks after the injection of tumor cells to determine hepatic metastases induction, though the injection of 3×10^7 tumor cells resulted in a shorter survival time, ranging from 5 to 7 days, for each strain. Each cell line induced diffuse hepatic metastases in a dose-dependent manner.

For the second implantation, hepatic metastases five to seven days after the inoculation of 3×10^7 cells pv were used for each cell line (Fig. 1). At this point, the proliferation of tumor cells were sufficiently diffuse that any heterogeneity between prepared minced fragments could be neglected.

Table I. Hepatic Metastasis Induction by Injection of Ascites Hepatoma Cell Lines into the Portal Vein of Rats

No. of injected cells	No. of rats with hepatic metastasis[a]		
	AH13	AH130	AH7974
1×10^6	0/5	0/5	0/5
3×10^6	2/5	ND[b]	ND
5×10^6	3/5	2/5	2/5
1×10^7	3/5	3/5	2/5
2×10^7	4/5	ND	ND
3×10^7	5/5[c]	5/5[c]	5/5[c]

a) Animals were killed 2 weeks after the injection.
b) Not done.
c) All rats died within 7 days after the injection.

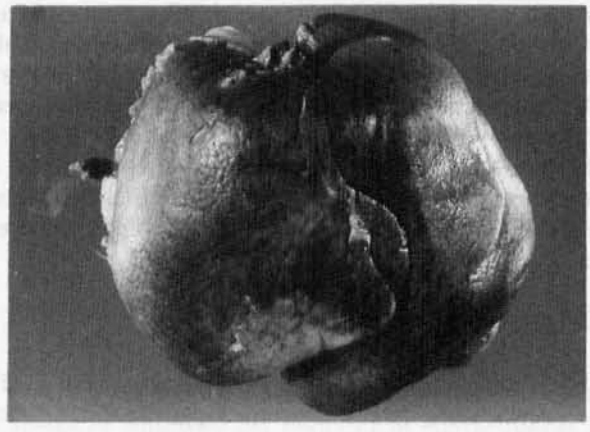

Fig. 1. A photograph of a formalin-fixed diffuse hepatic metastasis 7 days after injection of 3×10^7 of AH13 cells pv. No tumor nodule is apparent macroscopically at this stage.

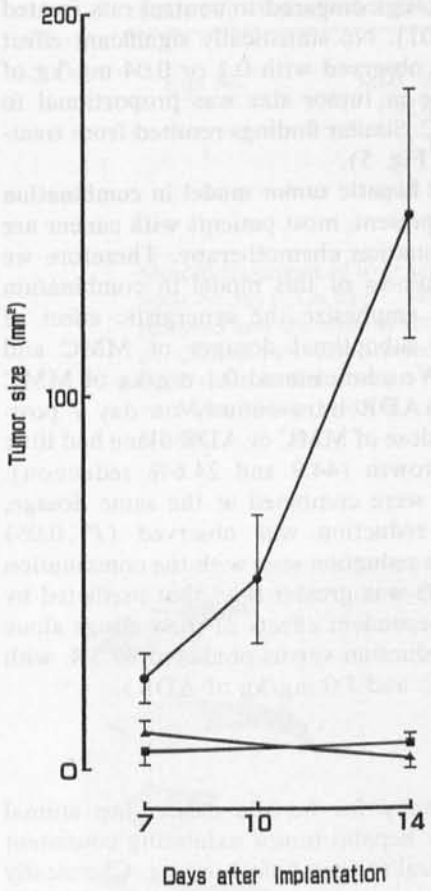

Fig. 2. Growth profile of minced, implanted liver tumor of AH13 (●), AH130 (▲), AH7974 (■) in the liver of rats from 7 to 14 days after implantation. Points, mean of tumor sizes of twelve rats; bars, SE.

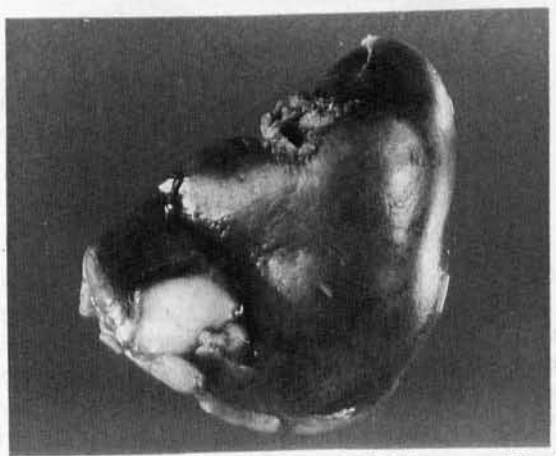

Fig. 3. Photograph of an implanted AH13 tumor. The liver was removed two weeks after implantation and fixed in 10% phosphate-buffered formalin.

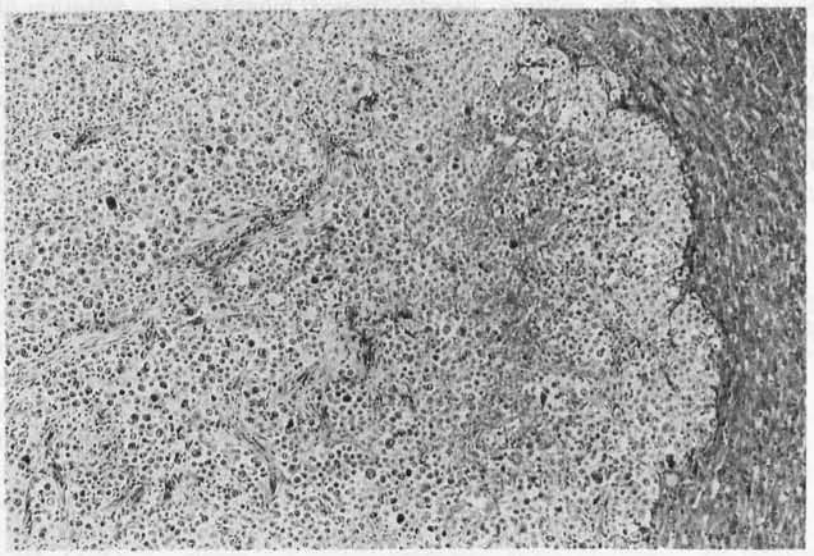

Fig. 4. Light photomicrograph of a portion of the margin of an implanted AH13 tumor. The tumor is clearly distinguishable from normal liver. Hematoxylin and eosin, ×200.

Growth profile of implanted hepatic tumor in the new hosts Animals receiving implants of minced fragments of hepatic metastases induced by the injection of AH13, AH-130 or AH7974 cells pv were killed on day 7, 10, or 14 postimplantation. The livers were removed, and the size of each tumor was measured (Fig. 2). AH13 implants demonstrated favorable growth, while most AH130 and AH7974 implants failed to show measurable growth. The mean tumor sizes using implants of AH13, AH130 and AH7974 14 days after implantation were 148.2 $mm^2 \pm$ 33.3 SE, 2.8 $mm^2 \pm 2.1$ SE and 6.2 $mm^2 \pm 1.9$ SE, respectively. These data indicated that AH13 strain was best suited for the development of a solitary hepatic tumor model. No operative death occurred in 304 consecutive implantations in this study.

Figure 3 shows a representative solitary hepatic nodule of AH13 14 days after implantation. The tumor was distinguishable clearly from normal liver tissue (Fig. 4). The lung, heart and kidneys of the animals receiving AH13 implants also were examined grossly and microscopically to determine whether or not tumor was present. No evidence of tumor was observed in any other organ in 40 rats two weeks after implantation and other intrahepatic metastases were not observed.

Reproducibility of growth of implanted AH13 hepatic tumor The growth of the solitary liver tumor using AH13 was highly reproducible by this technique. The tumor was measured in eight consecutive experiments on day 14 postimplantation to determine the stability of growth (Table II).

Suitability of the solitary hepatic tumor model for chemotherapeutic studies A dose-dependent response (size of the tumor) was demonstrated (Table III). Treatment with 0.4 mg/kg of MMC iv daily for 4 days resulted in a significant reduction in tumor size (98.8% reduction, $P<0.005$). A lower dose was less effective, but a significantly smaller tumor was found in rats treated with single injection of 0.2 mg/kg compared to control rats treated with saline ($P<0.01$). No statistically significant effect on tumor size was observed with 0.1 or 0.04 mg/kg of MMC. The change in tumor size was proportional to the dosage of MMC. Similar findings resulted from treatments with ADR (Fig. 5).

Table II. Reproducibility of Solitary Hepatic Tumor Size in Rats

Expt No.[a]	Tumor size[b] (mm^2) mean ± SE
1	64.8 ± 24.2
2	111.8 ± 33.0
3	192.4 ± 41.8
4	78.9 ± 29.6
5	72.0 ± 17.8
6	125.3 ± 33.9
7	115.7 ± 27.5
8	143.1 ± 39.1

The liver tumor induced by the injection of AH13 cells pv was cut into 1×1×4 mm fragments and implanted in the liver of new hosts on day 0. On day 14, the livers were removed and the sizes of the tumors were measured.
a) There were 8 animals per group.
b) The tumor size was expressed as the product of the lengths of major and minor axes of the section through the center of the tumor.

Use of the solitary hepatic tumor model in combination chemotherapy At present, most patients with cancer are treated with combination chemotherapy. Therefore we evaluated the usefulness of this model in combination chemotherapy. To emphasize the synergistic effect of anticancer agents, suboptimal dosages of MMC and ADR were used. We administered 0.1 mg/kg of MMC and 1.0 mg/kg of ADR intravenously on day 7 post-implantation. This dose of MMC or ADR alone had little effect on tumor growth (44.9 and 24.6% reduction). When these drugs were combined at the same dosage, significant tumor reduction was observed ($P<0.05$) (Table IV), and the reduction seen with the combination of anticancer agents was greater than that predicted by addition of the independent effects of these drugs alone (82.5 to 89.7 % reduction versus predicted 69.5% with 0.1 mg/kg of MMC and 1.0 mg/kg of ADR).

DISCUSSION

To improve therapy for hepatic cancer, an animal model of a solitary hepatic tumor exhibiting consistent growth would be ideal for preclinical testing. Chemically induced hepatic tumors in rats are often used as models

Table III. Modulation of Growth of Implanted AH13 Tumor by iv Administration of Anticancer Agents

Drug	Dose (mg/kg)	Day of administration	Tumor size (mm^2) Treatment group	Tumor size (mm^2) Control group[a]	% Reduction	P[b]
MMC	0.4	7, 8, 9, 10	1.9 (0.6)[c]	149.6 (28.5)[c]	98.8	<0.005
	0.4	7, 8	3.6 (2.0)	125.4 (27.8)	97.1	<0.005
	0.4	7	7.7 (7.0)	170.2 (40.6)	95.5	<0.01
	0.2	7	27.4 (9.6)	116.5 (26.4)	76.5	<0.01
	0.1	7	48.2 (14.0)	87.4 (32.4)	44.9	NS[d]
	0.04	7	111.7 (33.5)	150.2 (46.8)	25.6	NS
ADR	2.0	7, 8, 9, 10	13.8 (4.2)	181.6 (29.6)	92.4	<0.005
	1.0	7, 8	11.3 (6.6)	40.1 (12.4)	71.8	<0.05
	1.0	7	59.3 (32.5)	78.6 (29.4)	24.6	NS
	0.5	7	74.1 (22.9)	49.7 (16.9)	−49.1	NS

On day 0, fourteen rats were implanted with small fragments of the liver tumor induced by injection of AH13 cells pv and were randomly assigned to two groups. On day 7, the following treatments were started; on day 14, the livers were removed and the sizes of the tumors were measured. All rats used in a single experiment received implants on the same day using fragments of the liver tumor of the same rat.
a) Control group was treated with saline iv.
b) *P*-value as described in "Materials and Methods." No rats were excluded from statistical evaluation.
c) Mean of tumor size in a group of seven rats. The figures in the parentheses show the standard errors.
d) Not significant.

Table IV. Synergistic Effect of MMC and ADR on Implanted AH13 Tumor

Expt No.	Tumor size (mm^2)		% Reduction	$P^{b)}$
	MMC 0.1 mg/kg + ADR 1.0 mg/kg	Control[a)]		
1	31.1 (11.0)[c)]	177.4 (30.6)[c)]	82.5	<0.05
2	24.1 (4.1)	233.8 (39.2)	89.7	<0.05
3	13.6 (4.3)	84.9 (21.1)	84.0	<0.05

Minced fragments of liver tumor induced by injection of AH13 cells pv were implanted in the liver of new hosts on day 0. On day 7, MMC 0.1 mg/kg and ADR 1.0 mg/kg were given iv; on day 14, the livers were removed and the sizes of the tumors were measured.

a) Control rats were treated with saline iv.

b) *P*-value as described in "Materials and Methods." No rats were excluded from statistical analysis.

c) Mean of tumor size in a group of seven or eight rats. The figures in the parentheses show the standard errors.

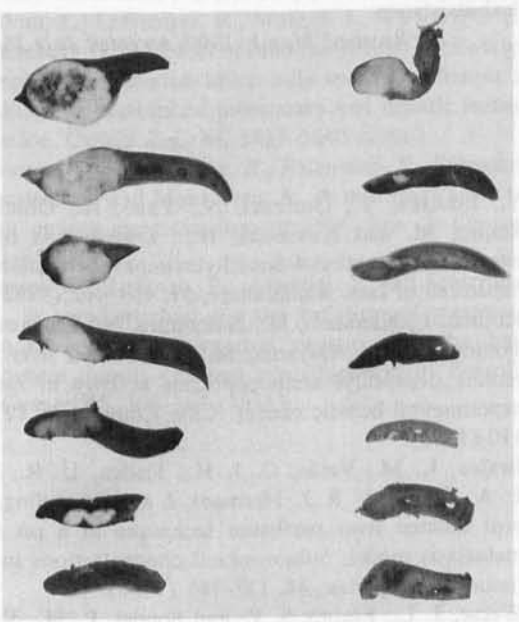

Fig. 5. Representative liver sections from rats given saline (left) or adriamycin 2 mg/kg daily for 4 days starting on day 7 postimplantation (right).

of hepatic cancer.[7-10] However, this procedure is very time-consuming, and generally several months are required to induce hepatic tumor. Furthermore, evaluation of therapeutic efficacy in this model is difficult. Subsequently, hepatic metastasis models induced by the inoculation of malignant cell lines into the portal vein,[11-14] spleen,[15-17] cecal wall,[19,20] intravenously[21,22] or subcutaneously[23-25] have been used in rats and mice. In these models, assessment of treatment efficacy is based upon the reduction in the number of hepatic metastases,[13,16,17,21,22] increased survival time,[13] or reduced incidence of hepatic metastasis.[12] However, a model which evaluates treatment based on a change in tumor size would be a more useful tool because this parameter is well understood by both clinicians and investigators and provides more accurate information with regard to the effect of the treatment. Experiments using a solitary hepatic tumor in rats have been performed previously by Hafström and Carlsson.[26] The liver tumor was induced by injection of tumor cells (1.0×10^6 cells in 0.1 ml) directly into the central lobe of the liver of rats. However, the reproducibility of the model is unknown, and peritoneal carcinomatosis due to tumor cell leakage may invalidate the results.

The model we have established was highly reproducible, resulted in no operative death, required little technical expertise and provided a short-term, *in vivo*, animal model.

Three different ascites hepatoma cell lines, AH13, AH130 and AH7974, were used to generate diffuse hepatic tumor as the first step in this model. We chose ascites hepatoma cell lines because they originated in the liver and have been well-characterized in previous studies.[17,27-29] Also, a hepatic tumor model using hepatoma cells would provide a model closely related to hepatocellular carcinoma, which ranks third in incidence among malignancies in Japan,[2] and it might show pharmacokinetic and pharmacodynamic features more characteristic of human hepatocellular carcinoma than the models using colon cancer or sarcoma cell lines.[11-14,16,17,19-22,24-26]

The implantation technique required an average of 4 min per animal. Aside from an incidental abdominal wall tumor in 15% of animals, the model generated a tumor only in the liver. One reason for the high level of reproducibility was that the diffuse hepatic metastases

were used as the implant. Standardization of the initial tumor implants is fundamental to any assay using tumor implants.[30] The use of diffuse hepatic metastases induced by inoculation of tumor cells pv was free from the problem of tumor heterogeneity due to necrotic areas or other factors which might affect results, as in the subrenal capsule assay.[30, 31]

In order for an experimental model to be useful for the prediction of clinical antitumor activity, it should be demonstrable that it is amenable to quantitative studies of variables such as different doses of drugs and treatment regimens.[30] Treatment with MMC and ADR inhibited tumor growth in a dose-dependent manner and the combination of these anticancer agents demonstrated synergy. These data indicate that the model is reliable for quantitative studies.

Generally large numbers of animals are required in therapeutic trials. From this perspective, our model using rats seems to be more suitable when compared to models using larger animals, such as the VX-2 rabbit model, which is often used as a solitary hepatic tumor model.[32-33]

In this model, the tumor was clearly distinguishable from normal liver (Figs. 3 and 4), and the tumor can be enucleated easily. This feature makes it possible to obtain pure tumor tissue and offers the possibility that this model can be used in studies of the pharmacokinetics and pharmacodynamics of anticancer drugs in the liver or in studies of the cell cycle distribution of tumor cells *in vivo*.

A new model in rats of a solitary hepatic tumor which is suitable for use in studies of hepatic cancer therapy has been described. This model may also be useful to investigate which factors influence tumor growth in the liver.

ACKNOWLEDGMENTS

The authors thank Mrs. Reiko Narita for her excellent technical assistance.

(Received May 1, 1990/Accepted July 26, 1990)

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