Consistent Liver Metastases in a Rat Model by Portal Injection of Microencapsulated Cancer Cells

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Abstract

Consistent liver metastases in animal models is generally observed only with certain cancer cell lines. With the aim of improving on existing animal models of liver metastases, we hypothesized that cancer cells encased in 300 μm microcapsules, mimicking micrometastatic foci, might be effective seeds of liver metastases. A total of 3,000 microcapsules, containing 700 to 1,500 viable cells/capsule in logarithmic growth phase of three human pancreatic cancer cell lines (SUIT-2, AsPC-1, and BxPC-3), were transplanted in nude rats by portal injection. The rate of liver metastases was 100% (12 of 12), 100% (6 of 6), and 83% (5 of 6) for SUIT-2, AsPC-1, and BxPC-3 microcapsules, respectively. In contrast, the administration of an identical number of single cancer cells (2.1–4.5 × 106) did not lead to liver metastases. Metastases was strictly limited to the liver, was quite stable, and could be proportionately tailored by varying the number of cancer microcapsules administered. Microscopic observation showed that two-thirds of the cancer microcapsules were lodged in the peripheral small (20–50 μm) portal veins, although one-third of the cancer microcapsules were trapped in the central wide (200–400 μm) portal vein. Capsules began to burst at day 3, with recognizable metastases produced at day 7, resulting in overt metastases production at days 28 to 42. The present cancer microcapsule method may be useful for obtaining liver metastases in animal models, especially for cell lines that will not form liver metastases with conventional single cell injection methods and/or for experiments requiring the consistent formation of liver metastases. (Cancer Res 2006; 66(23): 11131-9)

Introduction

Liver metastases is one of the most common forms of hematogenous spread in patients with various malignancies (1, 2), and the development of effective treatment modalities for liver metastases using conventional animal models is highly desirable. Because the liver is the largest solid organ, a reliable animal model of liver tumors is also valuable for in vivo evaluation of various therapeutic agents. However, representation of liver metastasis in an animal model using conventional approaches has proved difficult.

One commonly used approach for obtaining liver metastases is by injecting cancer cell suspensions into the hepatic portal veins or the spleen (3). However, liver metastases are consistently generated only with certain cell lines of the pancreas, colon, and stomach that have a high metastatic potential to liver (4–8). Moreover, many cell lines do not generate consistent liver metastases when administered as single cell suspensions (7–9). An additional obstacle with this model is that tumor growth occurs in sites other than the liver, such as the injected spleen and/or peritoneum (10). In fact, these undesirable tumor growths make experiments focusing on liver metastases difficult to interpret and largely inhibit the appearance of liver metastases (11, 12). Alternative strategies for obtaining liver metastases with cancer cells of low metastatic potential include orthotopic implantation, by injecting cancer cell suspensions, or by surgical transplantation of tumor fragments (13–15). Obstacles in the latter strategy include the presence of undesired metastases and are moreover difficult to reproduce in terms of the frequency and extent of metastases (16–18).

The formation of hematogenous metastases has been explained by two major theories, i.e., the seed and soil hypothesis by Paget (19) and the anatomic mechanical trapping theory of Ewing (20). The former suggests that metastases occurs only when the metastatic capacity of certain cancer cells (= seed) and environments of target organs (= soil) are compatible. The latter theory proposes that the anatomic location of the primary tumor and target organs, i.e., nonspecific trapping of cancer cells in the microvasculature, plays an important role in the development of metastases. Both mechanisms may also jointly contribute to the development of liver metastases in a clinical setting. The fact that even pancreatic cancer cell lines, which are clinically notorious for frequent presentation of liver metastases, do not consistently present liver metastases in animals (7, 8) forces the consideration that one of the difficulties in developing liver metastases in an animal model may be that the mechanical trapping process is not adequately reproduced.

With the aim of improving on existing animal models of liver metastases, we hypothesized that mechanical trapping could be rendered more efficient by aggregating multiple cancer cells. For this purpose, we employed cell encapsulation technology, previously used for pancreatic islet cell transplantation (21), which enabled us to consistently encase cancer cells into uniform 300 to 700 μm capsules. The preincubation of cancer cell containing cancer microcapsules ex vivo could substitute the initial proliferation step of cells, effectively providing cells in the logarithmic growth phase. When these cancer microcapsules are injected in the portal vein, they may become physically trapped in the peripheral vasculature of the liver before bursting and could thus act as seeds of liver metastases.
Herein, we report a novel utilization of cancer cell microencapsulation that acts as an effective seed of liver metastasis in a rat model. Transplantation of ex vivo precultured 300 μm cancer microcapsules, formed from three different human pancreatic cancer cells, into the liver of nude rats via a portal vein resulted in efficient and stable production of liver metastases.

Materials and Methods

Cell Lines

Three human pancreatic cancer cell lines (SUIT-2, AsPC-1, and BxPC-3) were used. SUIT-2 cells were generously provided by Dr. Ishimura (Miyazaki Medical College, Miyazaki, Japan; ref. 22). AsPC-1 and BxPC-3 were obtained from the American Type Culture Collection (Bethesda, MD). SUIT-2 was maintained in DMEM (Sigma-Aldrich, Taufkirchen, Germany) containing 5% fetal bovine serum (Sigma-Aldrich). AsPC-1 and BxPC-3 cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum.

Cancer Cell Encapsulation to Form Artificial Cancer Cell Aggregates

Cancer microcapsules were engineered by conventional coaxial airflow methods (23). The size of the cancer microcapsules was targeted to ~300 μm because capsule sizes >100 μm were assumed to be beneficial for physical trapping in the peripheral portal vein in the liver, whereas those <300 μm are technically difficult to produce. SUIT-2 cancer cell pellets were suspended in a 1.5% solution of potassium alginate (Kimica Corp., Tokyo, Japan) and the density was adjusted to ~1 × 10^7 cells/mL. AsPC-1 and BxPC-3 cancer cell pellets were encapsulated in the same manner, except that Matrigel (BD Biosciences, Bedford, MA) in a 25% (vol/vol) was added and the cell density was increased to ~2 × 10^7 cells/mL, because the proliferation of these cell lines was slow in pure alginate alone. The cell-alginate mixture was extruded through a 51-gauge needle at 5.0 mL/min and sheared by airflow, resulting in the formation of droplets having a diameter of 300 μm. The alginate droplets were allowed to directly fall into a cationic solution of 1% CaCl₂, promoting gel formation. The calcium alginate beads were chemically cross-linked with 0.05% (wt/vol) poly-L-lysine in 0.9% NaCl for 4 minutes. Finally, the remaining alginate core was dissolved with 1.6% sodium citrate for 6 minutes.

In vitro Culture of Cancer Microcapsules: Capsule Burst, Histology and Cell Proliferation

Cancer microcapsules were incubated in vitro for several days at 37°C in 5% CO₂ to ensure that they were fully viable at the time of administration to rats. The time when >10% of cancer microcapsules burst was defined as the bursting day for each cell line. Two or 3 days before the bursting day was assumed to be the optimal time for portal injection. The histology of cancer microcapsules at the optimal day for portal injection was observed by embedding cancer microcapsules in optimum cutting temperature compound (Diagnostics Division, Miles, Inc., Elkhart, IN) and frozen sections were stained with H&E. To analyze the cell number included in each capsule at the optimal day for portal injection, microcapsules were sampled, enzymatically digested, and cells were counted using a hemocytometer.

Injection of Cancer Microcapsules in Nude Rats

Nude rats (male F344/NcI-crl-mu rats), 6 weeks of age with a weight of 100 to 125 g (Clea Japan, Tokyo, Japan), were employed. The rats were anesthetized by i.p. injection of pentobarbital, a midline incision was made, and the portal vein was exteriorized and linearized, thus enabling the insertion of a heparinized 20-gauge catheter (Terumo, Tokyo, Japan). A catheter was inserted at the very distal part of the mesenteric vein, near the cecum, and advanced 4 cm towards the liver and the tip of catheter was placed at the major trunk of the portal vein, with the point 5 to 8 mm near the liver hilum. The cancer microcapsules suspended in 1 mL saline were injected manually at ~0.1 mL/s and flushed with 0.5 mL of saline. The site where the catheter was inserted was ligated for hemostasis with 5-0 nylon sutures. Ligation of this point never caused intestinal necrosis because collateral vessel networks are well formed in the rat.

All animal experiments were done with the approval of the Animal Research Committee of the University of Tsukuba. Animals were maintained in a barrier facility on HEPA-filtered racks and fed with autoclaved laboratory rodent chow.

Liver Metastases Production by Portal Vein Injection of Cancer Microcapsules or Single Cell Suspension

To produce liver metastases in nude rats by injection of cancer microcapsules via the portal vein, 3,000 cancer microcapsules for each rat were administered: 12, 6, and 6 nude rats were employed for SUIT-2, AsPC-1, and BxPC-3 microcapsules, respectively. The same number of single cells included in 3,000 cancer microcapsules (2.1 × 10⁶ for SUIT-2 and 4.5 × 10⁶ for AsPC-1 and BxPC-3) were also injected via the portal vein. In order to inject a homogeneous single cell suspension, excluding cell aggregates or clumps, cell solutions were passed through a mesh strainer with a 40 μm pore size prior to administration. Six nude rats were employed for single cell injection of SUIT-2, AsPC-1, and BxPC-3, respectively. We assumed that the appropriate metastatic extent for evaluating the procedure would be ~10% to 20%. All rats were sacrificed at different times depending on the cell lines (SUIT-2 at 4 weeks, AsPC-1 at 6 weeks, and BxPC-3 at 5 weeks) with the intent of obtaining metastases with a suitable extent.

Evaluation of Sacrificed Nude Rats, Injected Cancer Microcapsules, or Single Cancer Cells

Incidence of liver metastases. To assess the potential for liver metastases, the incidence of liver metastases was evaluated macroscopically. The rate of liver metastases was defined as the number of rats positive for liver metastases divided by the number of experiments. Formalin-fixed, paraffin-embedded sections were subjected to microscopic examination.

Undesired metastases to sites other than the liver. To evaluate whether metastases occurred only in the liver, other organs and areas, i.e., peritoneal cavity, injection site, and lungs were carefully examined macroscopically. Any suspicious lesion was removed and subjected to histologic analysis.

Numerical evaluation of the extent of metastatic liver nodules: volumetric examination. In order to quantify the objective extent of liver metastases, a numeric calculation was employed. The metastatic extent was defined by the following formula: metastatic extent (%) = (metastatic volume / volume of entire liver) × 100. Formalin-fixed livers were divided into four lobes (left, middle, right, and caudate) and each lobe was then cut to a thickness of 2 mm. Next, the area of metastatic nodules in all serial sections was measured using image-processing software WinROOF (Mitani Corporation, Fukushima, Japan). The metastatic volume and total volume of the liver were calculated by integration.

Variation of the Extent of Liver Metastases by Injecting Different Numbers of Cancer Microcapsules

To determine whether the extent of liver metastases varied according to the number of cancer microcapsules injected, various numbers of SUIT-2 microcapsules were injected. Five, 12, 7, and 8 nude rats were injected with 6,000, 3,000, 1,000, and 333 microcapsules, respectively, and both the incidence of liver metastases and the extent of tumor volume affected were calculated.

In vivo Sequential Observation of Cancer Microcapsule–Derived Liver Metastases

In order to assess the development of liver metastases from cancer microcapsules, livers were extracted from nude rats at days 3, 7, or 28 after portal injection of 3,000 SUIT-2 microcapsules. Livers were cut into serial 2 mm sections and stained with H&E to determine (a) distribution of cancer microcapsule, (b) status of microcapsules, i.e., whether capsules were ruptured or unruptured, and (c) distribution and size of liver metastases.

Pathophysiology of Liver Metastases: Cancer Microcapsules in Rats and Single Cell Injection in Mouse

The pathophysiology of liver metastases in nude rats generated by cancer microcapsules and those derived with conventional methods, i.e., injection
of single cells into the spleen in nude mice were assessed. The following variables were evaluated: (a) macroscopic location of metastatic nodules, (b) microscopic histopathology of tumors, (c) desmoplastic reaction, and (d) neovascularization. Cancer microcapsule–derived liver metastases were assessed on day 28 in nude rats injected with 3,000 SUIT-2 microcapsules. Because single cell injection to the spleen of nude rats never generated liver metastases in our hands, nude mice were employed. Liver metastases derived from single cells were assessed 28 days after splenic injection of $2.1 \times 10^6$ cells/50 µL of SUIT-2 cells in nude mice. The macroscopic location and histopathology were analyzed using representative H&E stained slides. The extent of the desmoplastic reaction was compared by evaluation of collagen fibers visualized by Masson trichrome staining. Neovascularization was evaluated by the microvessel count (MVC) method as reported by Weidner et al., with minor modifications (24). Representative sections were stained immunohistochemically with anti–von Willebrand factor antibody (polyclonal rabbit anti-human factor VIII–related antigen; Dako Corporation, Santa Barbara, CA). The number of von Willebrand factor–positive vessels were counted and the average counts of five selected hotspots, i.e., the highest neovascularization areas in high power ($\times 200$) fields, were recorded as the MVC for each case (25).

Assessment of the Efficacy of Anticancer Drugs Using the Present Liver Metastases Rat Model

To investigate whether the rat liver metastases model was useful in evaluating the effect of anticancer drugs, 15 nude rats portaly injected with 3,000 SUIT-2 microcapsules were randomly subdivided into three groups (five animals per group) on day 7. The first group was treated with gemcitabine (Gemzar, Eli Lilly, Indianapolis, IN) administrated via the dorsal vein at 80 mg/kg twice a week for 3 weeks (26). The second group was treated with irinotecan (Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan) at a dose of 60 mg/kg twice a week for 3 weeks (10). The third control group received 0.5 mL saline solution twice a week for 3 weeks. All rats were sacrificed on day 28 and the extent of metastases was determined as described above.

Statistical Analyses

Differences in the metastatic rate between cancer microcapsules and single cell suspensions were analyzed using Fisher’s exact test. Variations in the extent of liver metastases by injecting different numbers of cancer microcapsules were compared by one-way ANOVA. A $P < 0.05$ was considered statistically significant. Statistical calculations were done with the StatView software package (version 5.0, Abacus Concepts, Inc., Berkeley, CA).

Results

**In vitro Culture of Cancer Microcapsules: Capsule Burst, Histology, and Cell Proliferation**

The size of cancer microcapsules engineered in the present study was quite uniform (Fig. 1A). The average diameter of 100 randomly sampled microcapsules of SUIT-2, AsPC-1, and BxPC-3 microcapsules was 305 ± 39, 298 ± 21, and 362 ± 35 µm, respectively. Sequential in vitro observation of microcapsules showed that the cancer cells in microcapsules proliferated well and formed spheroids at days 5 to 7. Histologic observation of cancer cells infected with SUIT-2, AsPC-1, and BxPC-3 cells at day 0, immediately after production. B, after 5 to 7 days of in vitro incubation, cancer microcapsules were filled with proliferated cells forming spheroids (arrows). Histologic observation of frozen section stained with H&E of cancer microcapsules at the optimal day of portal injection showed three-dimensional proliferation of cancer cells. C, cancer microcapsules began to burst at days 7 to 10. Cancer cells, extruded from ruptured capsules continued to proliferate (white arrowheads). Bars, 100 µm.
microcapsules showed that all the cancer microcapsules proliferated in a three-dimensional manner (Fig. 1B). Further in vitro culture resulted in bursting of the outer layer of cancer microcapsules, and cancer cells were finally deviated outward and continued to grow in flasks (Fig. 1C). Although the speed of cell proliferation in cancer microcapsules differed between the three cell lines, all three cancer microcapsules burst nonetheless. The bursting times of SUIT-2, AsPC-1, and BxPC-3 microcapsules were on days 7, 7, and 10, respectively. From these results, the optimal day for portal injection of microcapsules from SUIT-2, AsPC-1, and BxPC-3 was assumed to be on days 5, 5, and 7, respectively. The average number of cells that were encased in one cancer microcapsule, at the optimal day for portal injection, was 718 cells for SUIT-2, 1,530 for AsPC-1, and 1,640 for BxPC-3.

Assessment of Liver Metastases Production in Nude Rats, Injected Cancer Microcapsules, or Single Cancer Cells

The metastatic potential to the liver of these three cell lines, by either single cell injection into the spleen and/or orthotopic implantation, has been reported to be different. SUIT-2 provides constant overt liver metastases (7). AsPC-1 is known to generate liver metastases, however, the extent of metastases is variable (7, 27). BxPC-3 does not cause liver metastases as reported by several authors (7, 17). Injection of three cancer microcapsules formed from different cell lines resulted in the successful production of overt liver metastases in all cases (Fig. 2).

Rate of liver metastases. The rate of liver metastases in rats injected with SUIT-2 microcapsules, AsPC-1 microcapsules, and BxPC-3 microcapsules was 100% (12 of 12), 100% (6 of 6), and 83% (5 of 6), respectively. In contrast, no liver metastases were formed by the injection of nonencapsulated single cancer cells (Table 1). Macroscopic observation and hepatic cross-sections showed that metastatic nodules tended to precipitate at peripheral hepatic margins (Fig. 2).

Metastases at organs other than the liver. No metastatic lesions other than in the liver were seen, and peritoneal, injection site, or pulmonary dissemination was not observed. However, occasional skin incisional wound metastases was seen in two rats that were injected with 3,000 SUIT-2 microcapsules (Table 1).

Numerical evaluation of the metastatic liver nodules: volumetric analyses. The metastatic extent to the liver produced by the injection of SUIT-2, AsPC-1, and BxPC-3 microcapsules was 14.6 (15.9/107.1 cm³) ± 7.0%, 9.7 (11.4/113.2 cm³) ± 5.8%, and 15.0 (19.1/111.4 cm³) ± 12.5%, respectively. The macroscopic extent of liver metastases is known to be larger than the calculated tumor volume. Our previous study showed that the tumor volume of clinically massive liver metastases remains ~10% to 30% when calculated by computed volumetry (28).

Control of the Extent of Liver Metastases by Injection of Varying Numbers of Cancer Microcapsules

The rate of liver metastases in rats injected with 6,000, 3,000, 1,000, and 333 microcapsules was 100% (5 of 5), 100% (12 of 12), 86% (6 of 7), and 50% (4 of 8), respectively. The extent of metastases in rats injected with 6,000, 1,000, and 333 microcapsules was 29.5 (46.0/146.2 cm³) ± 13.1%, 1.9 (1.8/96.5 cm³) ± 1.9%, 0.2 (0.2/88.4 cm³) ± 0.3%, respectively (Fig. 3).

In vivo Sequential Observation of Liver Metastases Derived from Cancer Microcapsules

Distribution of cancer microcapsules at 3 days after portal injection in liver showed that two-thirds were lodged in the small peripheral (20–50 μm) portal veins, although one-third were trapped in the central wide (200–500 μm) portal vein (Fig. 4A). It should be noted that 300 μm of microcapsules reached peripheral regions more than initially expected because the diameter of Glisson’s sheath or the portal vein neighboring cancer microcapsules lodged were ~20 to 50 μm in diameter (Fig. 4A). A total of 175 cancer microcapsules were observed in 10 representative slices on day 3, 35% (62 of 175) of which were ruptured. The proportion of ruptured microcapsules increased to 70% (88 of 128) at day 7 and 100% at day 28. Although intact cancer microcapsules should also be involved in the formation of metastatic foci, all cancer microcapsules at day 28 were assumed to be ruptured because almost all were buried in tumor nodules. Sequential

Figure 2. Overt liver metastases in nude rats by injection of three different human pancreatic cancer microcapsules via the portal vein. Representative gross appearance of rat livers at 28 days after injection of SUIT-2 microcapsules (A), 42 days after injection of AsPC-1 microcapsules (B), and 35 days after injection of BxPC-3 microcapsules (C). The hepatic metastatic extent produced by injection of SUIT-2, AsPC-1, and BxPC-3 microcapsules was 14.6 (15.9/107.1 cm³) ± 7.0%, 9.7 (11.4/113.2 cm³) ± 5.8%, and 15.0 (19.1/111.4 cm³) ± 12.5%, respectively. Note that metastatic nodules, especially of AsPC-1 microcapsules, tended to precipitate at the peripheral regions of the liver.

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analysis of metastases revealed that cancer cells gradually extruded from the outer layer of the microcapsules at day 3, which could not be recognized macroscopically (Fig. 4a2–3). At day 7, metastatic foci developed to 0.5 to 2 mm, accounting for 6% of the sectional area (Fig. 4b1). Tumor growth was equally achieved with cancer microcapsules in both peripheral (Fig. 4b2) and central regions (Fig. 4b3). At day 28, overt liver metastases occupied 53% of the sectional area (Fig. 4c1) and were diffusely distributed from the peripheral to proximal regions (Fig. 4c2–3).

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<th>Table 1. Liver metastasis take rate in rats by different injection form of cancer cells: cancer microcapsule versus single cells</th>
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*Three thousand microcapsules with a 300 µm diameter were injected.
†A single cell suspension of 2.1 to 4.5 × 10⁶ cancer cells were injected.

**Pathophysiology of Liver Metastases: Cancer Microcapsules in Rats and Single Cell Injection in Mouse**
Using both methods, metastatic foci were mainly distributed in the peripheral one-third of the hepatic hilum, i.e., marginal area of the liver (Fig. 5a1 and b1). Microscopic observation of liver metastases from cancer microcapsules showed glandular formation around remnant microcapsules (Fig. 5a2). Single cell–derived liver metastases showed medullary proliferation of cancer cells.

Figure 3. Intentional control of the extent of liver metastases by injecting different numbers of cancer microcapsules. Livers from nude rats 28 days after administration of 6,000 (A), 3,000 (B), 1,000 (C), and 333 (D) SUIT-2 microcapsules. E, the extent of liver metastases was numerically calculated (29.5% for 6,000 microcapsules, 14.6% for 3,000 microcapsules, 1.9% for 1,000 microcapsules, and 0.2% for 333 microcapsules), statistically demonstrating that the extent of liver metastases could be intentionally controlled by injecting different numbers of cancer microcapsules (*, P < 0.0001; **, P = 0.0031; ***, P = 0.0009; ****, P = 0.049).
with a cellularity of >90% and no glandular formation (Fig. 5b2). The desmoplastic reaction, proliferation of interstitial collagens, visualized by Masson trichrome staining was more evident in liver metastases originating from cancer microcapsules (Fig. 5a3 and b3). The desmoplastic reaction was heterogeneously distributed in the liver metastases from cancer microcapsules. Dense collagen proliferations predominantly distributed the surrounding areas of cancer microcapsule remnants, presumably due to a foreign body reaction to the extracellular components of the microcapsules. Regarding neovascularity, the MVC of cancer microcapsules and single cell–derived metastases were 30.4 ± 7.0, and 62.8 ± 14.4, respectively.

Application of Liver Metastases Model for Evaluation of Anticancer Drug Efficacy

The extent of metastases in rats treated with gemcitabine, irinotecan, or saline was 0.52 (0.44/822 cm²) ± 0.72%, 0.12 (0.1/80.6 cm²) ± 0.16%, and 23.7 (26.6/106.4 cm²) ± 11.3%, respectively. Objective evaluation for anticancer drug efficacy was possible because undesired metastases to organs other than liver was not observed.

Discussion

The present cancer microcapsule method led to the successful and efficient production of liver metastasis in rats. The mechanism

Figure 4. Sequential in vivo observation of liver metastases generated from cancer microcapsules. Representative pictures of rat liver, at day 3 (A), day 7 (B), and day 28 (C) after portal injection of 3,000 SUIT-2 microcapsules. a1, distribution of cancer microcapsules were marked on loupes observation (C; ruptured; ×, unruptured). Two-thirds of the microcapsules were lodged in peripheral small (20–50 μm) portal veins, although one-third were trapped in the central wide (200–400 μm) portal vein. a2, microscopic observation of the peripheral region on day 3, including a ruptured capsule. Outer layers of microcapsule (white arrowheads) and extruded cancer cell proliferation (black arrowheads). Fibroblastic proliferation around cancer microcapsules, a hallmark of the host reaction to foreign bodies, was sometimes observed at day 3 (white dotted line). It should be noted that 300 μm microcapsules reached regions much more peripheral than expected, because the diameter of the portal vein in Glisson’s sheath (white arrow) neighboring lodged cancer microcapsules were 20 to 50 μm in diameter. Peripheral bile ducts (black arrow), hepatic arteries (black dotted arrows). a3, microscopic observation of the central region at day 3. Four microcapsules, one marked with an “O” was ruptured and three marked with an “×” were unruptured and trapped in the large portal vein (400 μm), on both sides (white arrows). Extruded cancer cells beginning to proliferate (black dotted line). b1, loupes observation at day 7. Recognizable metastatic foci of 0.5 to 2.0 mm accounting for a sectional area of 6% (surrounded by a dotted line). b2, microscopic observation of peripheral regions showed noteworthy proliferation of cancer cells (black dotted line) around microcapsules (white arrowheads). b3, microscopic observation of the central region showed metastatic foci at the tip of a 120 μm portal vein (two white arrows). c1, loupes observation at day 28. Liver metastatic foci, which occupy a sectional area of 53%, were diffusely observed from the proximal to the peripheral area of the liver. c2, microscopic observation of the peripheral region and (c3) the central region showed that cancer microcapsules and fibrotic proliferation were surpassed by cancer cells and could not be recognized in mature metastatic foci.
for successful production of liver metastases may be explained by
the following considerations. First, cancer cells are forcibly trapped
(mechanically embolized) because the microcapsules were large
enough not to pass through the liver. Secondly, the cancer cells
delivered to the implantation sites were fully viable. Lastly,
embolization of cancer microcapsules to the peripheral portal
vein mediates local ischemia, releasing cytokines and/or growth
factors.

Cancer cell implantation in the liver is believed to occur at the
sinusoids, and the principal mechanism is binding between cell
surface adhesion molecules of single cancer cells and receptor
molecules on hepatic endothelial cells (29). We assumed that an
additional factor likely to be equally important is the mechanical
entrapment of cancer cell clumps at the peripheral portal vein.
The diameter of the portal vein of nude rats at the liver hilum is
~1 mm and gradually narrows to 20 to 50 μm before going to the
periphery of the liver. Sequentially, the peripheral portal vein shifts
to the liver sinusoids, i.e., the space between hepatocytes,
the diameter of which is 7 μm. The size of a single cancer cell
is ~8.3 to 47 μm (30), and a previous study has shown that large
cancer cells are advantageous in liver implantation (31). Once
cancer cells form aggregates, the size might increase to ~30 to
50 μm (32). Larger aggregates, compared with cell suspensions,
are known to be more effective in both implantation and survival to
form gross tumor colonies after i.v. injection (33, 34). In order to
improve the ratio of cell aggregates, previous experimental models

Figure 5. Pathophysiologic assessment of
liver metastases generated by cancer
microcapsules in rats versus single cell
injection in mice. Histopathologic and
immunohistochemical findings of SUIT-2
liver metastases, 28 days after
administration of 3,000 cancer
microcapsules in a nude rat (A) and single
cell suspension (2.1 × 10^6 cells) in a nude
mouse (B). a1 and b1, macroscopic image
stained with H&E shows that metastatic
nodules (surrounded by a dotted line)
tend to form at the peripheral margin of the liver in
both methods. a2, microscopic observation
of cancer microcapsule–derived liver
metastases showed glandular formation
(arrows) around remnant microcapsule
(white arrowheads), indicating a histologic
presentation similar to that of primary
pancreatic cancer. b2, microscopic
observation of liver metastases derived
from single cells showed that medullary
proliferation of cancer cells with a cellularity
of >90% with no glandular formation that
is not histologically similar to primary
pancreatic cancer. a3 and b3, proliferation of
interstitial collagens, visualized by Masson
trichrome staining, was more evident in
liver metastases derived from cancer
microcapsules than in those from single cell
suspensions. a4 and b4, the extent of
neovascularity was evaluated by the MVC
method using anti–factor VIII antibodies.
The MVC of cancer microcapsule and single
cell–derived metastases was 30.4 ± 7.0, and
62.8 ± 14.4, respectively. Liver metastases
with cancer microcapsules were less
vascularized with respect to those derived
using a single cell suspension. Bars,
100 μm.
have employed an in vitro rotary cell culture system (35–37). In these models, however, the size and number of cancer cells included in these aggregates vary greatly. Furthermore, these cell aggregates are physically fragile and are easily damaged by transplantation before arriving to potential implantation sites. As a result, the frequency of liver metastases by this method ranges from 20% to 40% even under optimal conditions (36, 38). Moreover, only a highly limited number of cell lines are capable of forming aggregates in this in vitro system, greatly limiting their utility. We used uniform cancer microcapsules with a diameter of 300 μm, which resulted in 100% of the injected cancer microcapsules becoming trapped in the peripheral portal vein, and thus, never pass through the liver sinusoids to the hepatic vein.

A second advantage of cancer microcapsules may be their capacity to deliver viable cancer cells to implantation sites. When suspensions of cancer cells are injected via the portal vein or spleen in animals, the cells are rapidly attacked by the host immune system and also suffer from hemodynamic forces (39). In fact, although the majority of injected cancer cells were found to be arrested in the liver sinusoid several minutes after injection, the vast majority of injected cancer cells were disseminated or no longer viable at 24 hours. Finally, only 1% of injected cancer cells survive in the liver, and further progression to form metastatic foci is even less probable (40, 41). Although observation by intravital video microscopy showed that melanoma cells can survive in the liver (>36% even at day 13; ref. 42), it seems reasonable to assume that, in general, relatively few cells are the seeds of metastases. In our cancer microcapsules, cells were preincubated ex vivo until the logarithmic growth phase and were effectively protected by the outer layer of the microcapsule throughout the processes of initial administration, delivery, embolization, and growth before bursting. Physical protection by the microcapsule may beneficial, although this may not be necessary in all cell types, increasing the ratio of viable cells in liver and therefore aiding in the formation of metastases.

The third mechanism that may explain the success of the present liver metastatic model may be related to liver ischemia, which induces the release of cytokines and/or growth factors, simultaneously possess the potential to stimulate cancer cell growth (43–45). It is quite reasonable to assume that once cancer microcapsules are embolized, more peripheral liver parenchyma will be included in ischemic environments. There is also clinical evidence that hepatic pedicle clamping during liver surgery causes liver ischemia and mediates the release of cytokines such as tumor necrosis factor α, interleukin 1β, and other growth factors, accelerating cancer growth (46–48). Together with these results, we assume that local ischemia of transplanted sites of the liver might contribute, at least in part, to the successful production of overt metastases.

One question that arises is whether the pathophysiology of liver metastases generated by the present cancer microcapsule method is equivalent to that of widely used liver metastases generated by single cell injection. It should be highlighted that liver metastasis using the cancer microcapsule method has only been tested in rats and successful liver metastasis by single cell injection using certain special cells (SUIT-2, in the present report) was observed only in mice; therefore, histopathologic comparisons were made using different species. Regarding the region of liver metastases production, this method is initially expected to provide artificially proximal tumors, located in the proximity of the wide portal vein which has a diameter of 300 μm. In reality, however, the regions of cancer microcapsules embolized were distributed more peripherally than expected, resulting in the precipitation of metastatic nodules in the marginal area of the liver. The fact that 300 μm of cancer microcapsules were found at the 20 to 50 μm peripheral portal vein shows the considerable plasticity of the portal vein. There were some cancer microcapsules that were likely to have been trapped in the central area, where the diameter of the portal veins ranges from 200 to 400 μm. Metastatic formation in the central region, however, was not a phenomenon specific to the cancer microcapsule method because they were also observed following single cell injection. Regarding the region of liver metastases, therefore, cancer microcapsules confer a similar hepatic distribution in rats to that of conventional single cell injection in mice.

Almost invariably, the histopathology of tumors in animal models is quite different from that of primary, clinical cancer specimens. Pancreatic cancer is well-known for its hypovascular nature and extensive desmoplastic reaction. Tumors in single cell–derived liver metastases in mice usually show endocrine tumor–like growth with an expansive growth pattern, without the formation of glands. Given this, it was unexpected that tumors generated by cancer microcapsules in rats formed glands around fibroblasts. Because cancer cells are known to be heavily affected by surrounding fibroblasts and infiltrating hematopoietic cells (49), the presence of a foreign body reaction to cancer microcapsules also seemed to be beneficial in mimicking the histopathology of primary pancreatic cancer. Together with the unique characteristics of the present animal model, such as stable production of liver metastases and the presence of metastases only in liver, this presents immense advantages in evaluating the effect of therapeutics aimed at controlling liver metastases. In fact, the effectiveness of commonly used anticancer chemotherapeutic agents can be evaluated in an objective and quantitative manner.

Although we succeeded in producing consistent liver metastases in rats using cell lines with little or no metastatic potential (AsPC-1, BxPC-3), it is unknown if the cancer microcapsule method will be applicable to all cell lines and will always produce overt liver metastases. The necessary conditions for liver metastases in the present microcapsule system are that cancer cells have two capabilities. The first is a growth potential that is powerful enough to burst the outer layer of the microcapsule, whereas the second is the ability to proliferate in liver parenchyma after being extruded from the ruptured microcapsule. The first condition may be enhanced by the unique application of the present method, i.e., the ability of coencapsulation with different cells or substances. Matrigel, an extracellular matrix that contains several growth factors and cytokines, was used as a "burst-supporting" agent in the present study, although coculture with various growth factors, cytokines, extracellular matrix components, and fibroblasts might also augment cell proliferation and capsule burst. Regarding the second condition, some cancer cells have never been reported to proliferate in liver even after direct intrahepatic injection, indicating that the liver is not an appropriate soil for some cell lines (18, 50). Application of the present microcapsule system to those cell lines might not generate liver metastases, even if they have the capacity to rupture the outer layer of microcapsules.

In conclusion, we succeeded in producing consistent overt liver metastases in nude rats using cancer microcapsules with a diameter of 300 μm, whereas the administration of single cancer cells never produced liver metastases in rats. Although the
advantage of this microcapsule method has been shown here in rats, this method may be applicable for larger animals such as rabbits, dogs, and pigs. In smaller animals such as mice, liver metastasis production was possible with the single cell injection method only if we used certain cancer cell lines. The present cancer microcapsule method may be useful for obtaining liver metastases in mice, especially for cell lines that will not form liver metastases with conventional methods. It should be noted, however, that technical improvements such as the production of smaller (<100 μm) cancer microcapsules and better surgical skill in injecting cancer microcapsules to the narrow portal vein, and especially the hemostasis step after injection, are necessary for applying the present method in mice. Even though the present microcapsule system is artificial, this may nonetheless provide information for understanding the mechanism of clinical liver metastases, highlighting the importance of anatomical-mechanical entrapment. We believe that the present cancer microcapsule method could contribute to the development of new anticancer therapeutics by providing consistent tumor growth in animal models.

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References


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