Development and Characterization of a Model of Liver Metastasis Using Human Colon Cancer HCT-116 Cells

Kazuhiko ISHIZU,a,b Naohide SUNOSE,c Kanami YAMAZAKI,a Takashi TSURUO,c Sotaro SADAHIRO,b Hiroyasu MAKUUCHI,b and Takao YAMORI*a,c

As a Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research; 3–10–6 Ariake, Koto-ku, Tokyo 135–8550, Japan; b Department of Surgery, Tokai University School of Medicine; Bohseidai, Isehara, Kanagawa 259–1193, Japan; and c Division of Experimental Chemotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research; 3–10–6 Ariake, Koto-ku, Tokyo 135–8550, Japan.

Received June 16, 2007; accepted June 28, 2007; published online July 3, 2007

In order to develop a model of liver metastasis of human gastrointestinal cancer cells, we examined the potential of 10 human colon and stomach cancer cell lines (HT-29, WiDr, HCT-116, HCT-15, HCC-2998, MKN7, MKN28, MKN45, MKN74 and St-4) to form liver metastases in nude mice. Among the cell lines, HCT-116 cells consistently formed gross liver metastases when injected into the spleens of nude mice. In contrast, other human colon and stomach cancer cells produced little or no liver metastasis. In order to analyze the high metastatic potential of HCT-116 cells, the adhesion potential was compared between HCT-116 cells and the other colon cancer cell lines. HCT-116 cells showed more efficient adhesion to fibronectin (FN) than other cells. Furthermore, FN enhanced haptotaxis of HCT-116 cells, but not of other colon cancer cells. The high adhesion potential to FN and enhanced haptotaxis may contribute, at least in part, to the high metastatic potential of HCT-116. To assess the value of this newly developed model of liver metastasis, we compared the ability of four anticancer drugs (fluorouracil, doxifluridine, paclitaxel and irinotecan) to inhibit the formation of liver metastases. Paclitaxel and irinotecan showed strong inhibition of liver metastasis but fluorouracil and doxifluridine showed only slight inhibition. Therefore, this model of metastasis may be useful for screening anti-liver metastatic reagents. These results indicate that the HCT-116 liver-metastasis model should be useful for analyzing the molecular mechanism of liver metastasis and for evaluating new anti-liver metastatic drugs.

Key words HCT-116; human colon cancer; intra-splenic injection; liver metastasis; nude mouse

The control of cancer metastasis is one of the most important strategies for cancer therapy from the current viewpoint of considerable limitations of surgical cancer resection. During the process of metastasis, metastasizing tumor cells interact with various host cells (platelets, lymphocytes and endothelial cells), extracellular matrix and basement membranes, leading to the development of metastases. Experimental models are essential for the analysis of the mechanisms of cancer metastasis and a number of animal models have been developed. Although these have been extremely useful for the elucidation of the mechanism of cancer metastasis, the number of metastatic models based on human cancer cell lines is limited.

From a clinical point of view, the liver is the most common target organ for metastasis of cancers of the digestive system, especially hematogenous metastasis of colon cancer, and the prognosis for cases with liver metastasis is extremely poor. Millikan et al. reported that, considering the metastasis of colon cancer, up to 25% of patients have synchronous hepatic metastasis and 50% develop metachronous metastasis. Welch and Donaldson reported that the liver is the most frequent target organ of hematogenous metastasis from colon cancer and 40–70% of the deaths from colon cancer in-
Hanks Balanced Salt Solution (HBSS) and washed three times with HBSS, and suspended in HBSS at a final concentration of $5 \times 10^7$ cells/ml. The mice were anesthetized with an intra-peritoneal injection of pentobarbital (Nembutal, Dainippon Sumitomo Pharma Co., Ltd., Osaka), which was regulated to 75 mg/kg. Then the mice were incised about 10 mm on the left subcostal, the spleen was confirmed under the peritoneum, the peritoneum was opened for about 8 mm and the spleen was exposed over the peritoneum. The cell suspension of $5 \times 10^5/100 \mu l$ of human colon cancer cells or $3 \times 10^5/100 \mu l$ of human gastric cancer cells was injected into the spleen using a 27G needle, and then the spleen was returned to the abdominal cavity, the peritoneum was sutured with one stitch and the wound was closed with a clip. Thirty days after inoculation with tumor cells, the mice were killed and the liver weight was recorded for evaluation of tumor metastasis. A micro specimen of the liver was produced and liver metastasis was confirmed pathologically.

**Cell Adhesion Assay** Log-phase cultures of human colon cancer cells were washed twice with PBS, harvested with PBS with 0.05% trypsin-0.02% EDTA and then resuspended in 0.02% bovine serum albumin (BSA)-RPMI to form a single-cell suspension. The tumor cell suspensions ($5 \times 10^5/100 \mu l$/well) were added to microculture wells precoated with 50 $\mu l$ of 100 $\mu g$/ml human FN (COSMO BIO Co., Ltd., Tokyo), laminin of rat tail (COSMO BIO Co., Ltd., Tokyo) or mouse collagen type1 FN (COSMO BIO Co., Ltd., Tokyo), and incubated at 37°C for 60 min. Non-adherent cells were washed away, and adherent cells were stained for 10 min with 0.04% crystal violet in 20% methanol. After washing twice with PBS, the residual stained cells were lysed with 100 $\mu l$ of 10% DMSO and the absorbance of the lysates was measured at 590 nm.

**Haptotaxis Assay** The haptotaxis of tumor cells was assayed using a Chemotaxicell chamber (Kurabo Co., Ltd.) according to the experimental method of Kubota et al. Filters with a pore size of 8.0 $\mu m$ were precoated with 3 $\mu g$ FN in a volume of 50 $\mu l$ on their lower surfaces and dried at room temperature in a cleanbench. Log-phase cultures of tumor cells were harvested with trypsin, washed with serum-free RPMI with 0.1% BSA and resuspended in serum-free RPMI with 0.1% BSA to a final concentration of $1 \times 10^7$ cells/ml. Serum-free RPMI with 0.1% BSA (600 $\mu l$/well) was added to the lower compartment, and cell suspensions (100 $\mu l$/ chamber) were added to the upper compartment and incubated for 60 min at 37°C in a 5% CO2 atmosphere. The filters were fixed and stained with Diff-Quick (Green Cross Co., Ltd.). The cells on the upper surfaces of the filters were removed by wiping with a cotton swab. The cells that had infiltrated through the FN to the lower surfaces of the filters were counted microscopically on ten predetermined filters at $\times 200$ magnification; each assay was performed in triplicate.

**Assessment of Efficacy of Chemotherapeutic Reagents** HCT-116 cells ($5 \times 10^5/100 \mu l$) were injected into the spleens of nude mice and anti-cancer drugs were administered from the next day. Fluorouracil (KYOWA HAKKO Co., Ltd., Tokyo) was administered orally for 4 terms (1 term is 5 d) at a dose of 15 mg/kg. Doxifluoridine (Nippon Roche, Kamakura) was administered orally for 4 terms at a dose of 125 mg/kg. Paclitaxel (Bristol Myers K.K., Tokyo) was administered intravenously for 1 term at a dose of 28 mg/kg, and irinotecan (Yakult Honsha Co., Ltd., Tokyo) was administered intravenously for 1, 5 and 9 d at a dose of 100 mg/kg after tumor cell inoculation. Liver metastasis was evaluated by the above-mentioned methods 30 d after inoculation.

**RESULTS**

**Metastatic Potential of Human Colon and Stomach Cancer Cell Lines** The metastatic abilities of human colon and gastric cancer cell lines were evaluated by macroscopic observation and by the weight of the livers 30 d after intra-splenic injection (Fig. 1). Among the cancer lines, HCT-116 cells developed the greatest liver metastases. The weights of the livers were HT-29: 1.46±0.09 g, WiDr: 1.59±0.18 g, HCT-116: 3.35±1.14 g, HCT-15: 1.48±0.25 g, HCC-2998: 1.48±0.25 g, MKN7: 1.73±0.22 g, MKN28: 1.56±0.54 g, MKN45: 1.33±0.28 g, MKN74: 1.74±0.23 g, and St-4: 1.55±0.13 g. These results clearly demonstrated that the liver metastatic potential of HCT-116 was by far the highest (Fig. 2). The metastatic areas in the livers of the mice injected with HCT-116 cells were similar to the clinical histology that is typically observed in the metastasis of colon cancer, with a clear boundary between the tumor and the normal liver tissue (Fig. 3).

**Adhesion Potential of Human Colon Cancer Cell Lines** To analyze the cellular properties of HCT-116 that are related to its metastatic potential, we examined the adhesive capabilities of HCT-116 cells to substrates in vitro. When we carried out the in vitro experiments of liver metastasis described above, we treated the cancer cells with trypsin to make single-cell suspensions before intra-splenic injection. Therefore, we treated the cells with trypsin in the adhesion assay, too, to bring the in vitro conditions close to the in vivo conditions. Highly metastatic HCT-116 cells adhered to FN-coated sub-

![Fig. 1. Liver Metastasis after Intra-splenic Injection of Colon Cancer Cells or Stomach Cancer Cells](image-url)
strates more efficiently than did other cancer cells (Fig. 4). The adhesiveness to other matrices (laminin and collagen type 1) did not correlate with the metastatic potential.

**Haptotaxis of Human Colon Cancer Cell Lines**

We examined the haptotaxis of HCT-116 cells using a Chemo-taxicell chamber assay to investigate the high metastatic potential of HCT-116 cells in vivo. HCT-116 cells migrated to the lower surface of the filter coated with FN, while the other cells failed to migrate to the lower surface (Fig. 5).

**Evaluation of the Effect of Anti-cancer Drugs Using the HCT-116 Liver Metastasis Model**

Control mice, which were injected with HCT-116 cells into the spleen, developed marked liver metastasis. Treatment of the mice with paclitaxel or irinotecan dramatically reduced liver metastasis (control: 2.39 ± 0.66 g, paclitaxel: 1.40 ± 0.17 g, and irinotecan: 1.39 ± 0.15 g) (Fig. 6a). However, the mice treated with fluorouracil or doxifluridine did not demonstrate significant prevention of liver metastasis (Fig. 6b).

**DISCUSSION**

When human colon cancer cells are injected into the spleens of nude mice, highly malignant cells develop metastatic colonies in the livers. In recent years, there have been some reports of liver-metastatic models using human colon cancer cells. However, there are only a few colon cancer cell lines that can cause significant liver metastasis. To develop a new model of liver-metastasis, we screened several cancer cell lines. Among the gastro-intestinal cancer lines tested, HCT-116 showed the greatest potential for liver metastasis. Morikawa et al. established a highly liver-metastatic human colon cancer KM-12 line after selection in vivo. In this study, however, HCT-116 was found to have a great and sufficient metastatic potential without further manipulation and therefore we did not carry out selection in vivo.

Hoffman and co-workers reported orthotopic implantation as a more faithful model of clinically observed liver metastasis. However, orthotopic implantation results in a di-
verse degree of metastasis among the animals and involves a long experimental duration of several months. In contrast, the model established here by intrasplenic injection of HCT-116 cells has rather high reproducibility and takes only a short time to reach the experimental endpoint.

We characterized the high metastatic potential of HCT-116 cells in vitro. Many biological events are involved in metastasis: 1) separation from the primary lesion and invasion to the surrounding tissue, 2) infiltration into vessels, 3) movement in vessels, 4) adhesion to the target organ, 5) infiltration into the extracellular matrix, and 6) proliferation. Steps 4) and 5) are important in the intra-splenic injection model. Therefore, we performed adhesion and haptotaxis assays on the highly metastatic HCT-116 cells in vitro. The extracellular matrix plays an important role in the growth and differentiation of cells and in the process of infiltration into the basement membranes of cancer or inflammatory cells. The adhesion ability of HCT-116 cells to FN, laminin and collagen type 1, major components of the extracellular matrix, was examined in the present study. Ohnishi et al. reported that murine Colon26-L5 cells with a high metastatic potential showed significantly stronger adhesion to FN, laminin and Matrigel than murine Colon26-P cells with a low metastatic potential. Morimoto and Irimura reported that the liver-metastatic mouse colon carcinoma cell line colon38 showed higher motility migration to FN and activated host fibroblasts. The HCT-116 cells did not show a specific reaction with laminin and collagen type 1. However, the adhesion ability to FN was elevated significantly compared with the other cells.

Subsequently, we examined the haptotaxis, which is one of most major steps in the process of metastasis. Ohnishi et al. reported that murine Colon26 L5 cells with a high metastatic potential showed significantly stronger haptotaxis than murine Colon26-P cells with a low metastatic potential. Similarly, the haptotaxis exhibited by HCT-116 cells was significantly more substantial than other cell lines. Extracellular matrix of the liver is rich in FN. Thus, the high metastatic potential of HCT-116 cells in the in vivo experiments may be due partly to its high potential in adhesion to FN and in haptotaxis to FN. In this context, it seems reasonable that HT-15 cells, with high ability in adhesion to FN but with low ability in haptotaxis to FN, did not show high metastatic ability. Recently, one of other mechanisms has been suggested by Bouvet et al. They developed HCT-116 cell lines that expressed green fluorescent protein and/or red fluorescent protein for imaging the interaction of colon cancer cells and splenocytes in the formation of liver metastases, and suggested a novel tumor-host interaction. Further studies are necessary to elucidate the high metastatic potential of HCT-116 cells more precisely.

We also examined the HCT-116 liver metastasis model for its suitability for the selection of anti-metastatic reagents. Four clinically available drugs (fluorouracil, doxifluridine, paclitaxel and irinotecan) were chosen as models of test compounds and examined their anti-metastatic ability. Either drug is known to inhibit cancer cell growth by its own mechanism. Paclitaxel does it by inhibiting tubulin, which is essential for mitosis. Irinotecan does it by inhibiting topoisomerase I which is essential for DNA replication and transcription. Fluorouracil and doxifluridine, anti-metabolites, do it by inhibiting DNA and RNA synthesis. Therefore, they were expected equally to inhibit the later stage of metastasis, i.e. secondary growth of cancer cells in the liver, rather than early stages such as adhesion and invasion of cancer cells in the sinusoids of liver. Indeed, the results showed that paclitaxel and irinotecan prevented liver metastasis of HCT-116 cells significantly, but fluorouracil and doxifluridine did not. Theses results were probably because of the dosage selection. We used maximum tolerated doses for paclitaxel and irinotecan, but suboptimal doses for fluorouracil and doxifluridine. However, we cannot exclude the possibility that the strong anti-metastatic activities of paclitaxel and irinotecan may be due to their unknown modes of action. Thus, using this model, anti-metastatic activities of paclitaxel and irinotecan could be evaluated. This model seems to be valuable for screening certain types of anti-metastatic drugs.

In conclusion, we developed a new model of liver metastasis using the human colon cancer HCT-116 cell line in nude mice.
mice. This could be useful for the analysis of the molecular mechanism of liver metastasis and for screening anti-metastatic drugs.

Acknowledgments This work was partly supported by grants-in-aid of the Priority Area “Cancer” from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to T. Yamori (18015049) and grants-in-aid for Scientific Research (B) from Japan Society for the Promotion of Science to T. Yamori (17390032).

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