Metastatic Model of Human Colon Cancer Constructed using Orthotopic Implantation in Nude Mice

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Summary: Nude mice have been used to grow subcutis (s.c.) growing human colorectal tumors, but these tumors rarely metastasize. This is a problem for studies into the biological behavior of metastatic subpopulations of human colorectal cancers. We have followed the evolution of the parental line and of a variant of human colon carcinoma KM12 cells, that were both tumorigenic, following implantation into the s.c. or cecal wall of nude mice. The tumors growing s.c. did not produce visceral metastases, whereas the cecal tumors metastasized to the regional mesenteric lymph nodes and to the liver. However, the incidence of liver metastases was different between the parental cell line KM12C cells and the in vivo selected cell line KM12SM cells after orthotopic inoculation. The morphological findings of KM12 cells proliferating in a monolayered sheet revealed that these two cell lines consisted of various cell populations. These results suggest that in the orthotopic colon cancer models, liver metastasis is defined by difference in subpopulations of metastatic phenotypes to the liver with early dominance of its growth in the implanted organ. As a result, our new model using orthotopic implantation of KM12SM cells, which produce a 50% incidence of liver metastasis, can help to provide a technique to study the biological behavior of metastatic subpopulations of human colon cancers.

Key words metastatic animal model, human colon carcinoma, orthotopic implantation, liver metastasis, lung metastasis, mesenteric lymph node metastasis

INTRODUCTION

Colon cancer is one of most prevalent cancers, and its absolute as well as relative occurrence has been increasing recently. Liver metastasis is major problem in colon cancer, because very few treatment strategies are effective. Development of methods for predicting metachronous liver metastasis after curative resection and for effective treatment of liver metastasis is very important to improve prognosis after resection. Therefore, relevant animal models for liver metastasis from colon cancer would be useful for research into the metastatic biology and in the search for new therapeutics.

With regard to animal models, human tumor xenografts grown s.c. in athymic nude mice closely resemble the original tumors morphologically, biologically and biochemically [1,2]. However, these tumors do not metastasized [3,4]. In contrast, it has been shown that implanting human tumor cells orthotopically into the corresponding organ of nude mice resulted in an adequate rate of metastasis, indicating enhanced metastatic capability of human tumor cells in nude mice by orthotopic implantation.

In this study, we compared the tumorigenicity and the metastatic rates for orthotopic and ectopic implantation between the parental cell line KM12C colon carcinoma cells and the variant cell line KM12SM cells selected from KM12C cells in vivo. We conclude that our orthotopically implanted colon cancer model which developed and produced patient-like metastases could be useful for further cancer research.
MATERIALS AND METHODS

Mice

Male KSN-nu/nu mice were obtained from SLC Japan, Inc., Tokyo, Japan. The mice were 5 weeks old and weighed 20 to 22 g. They were maintained in a laminar flow cabinet under specific-pathogen-free conditions.

Cell culture

The human colon carcinoma KM12C and KM12SM cells were kindly provided by Dr. M. Nakajima, Novartis Pharma., K.K. The KM12C parental cell line was isolated from a primary colon carcinoma classified as Dukes' stage B. The KM12SM cell line was isolated from a rare liver metastasis produced by parental KM12C cells growing in the cecal wall of a nude mouse [5]. All tumor cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

Orthotopic or ectopic implantation of tumor cells

A total of $2 \times 10^6$ viable tumor cells in 0.02 ml of Ca$^{2+}$- and Mg$^{2+}$-free Hanks' balanced salt solution (HBSS) were injected into the subcutis (ectopically) or subserosa of the carefully exposed cecum (orthotopically) under general anesthesia with ether.

Evaluation of tumorigenicity and metastatic potential

Mice were sacrificed 6 to 8 wk after the tumor cells injection. The tumor on s.c. or cecal wall was removed and examined histologically. Also the liver, lungs, lymph nodes in the peritoneal cavity, and all other organs suspected of disease were removed, fixed in 10% formalin, and processed for histological examination using whole specimens after careful macroscopic examination.

Measurement of serum human carcinoembryonic antigen (CEA) levels

Two ml of the blood was obtained immediately by heart puncture at autopsy, and the serum CEA was measured using enzyme immunoassay (EIA) method.

Statistical analysis

Student's t test and Fisher's exact test were used for statistical analysis.

RESULTS

Light microscopic findings of the tumor cells in a monolayered culture

Figure 1 shows the microscopic features of KM12SM cells on a plastic dish proliferating in a monolayered sheet with pavement-like arrangement. They tended to pile up in some regions. KM12C and KM12SM cells presented various shapes with large or small, spindle or round, cytoplasm, and with large or small oval nuclei with prominent nucleoli.

Tumorigenicity and production of metastasis

After s.c. implantation, both human colon carcinoma cell lines were tumorigenic, and no discernible

| Tumorigenicity and metastatic ability after orthotopic or ectopic implantation of KM12 colon carcinoma cells in nude mice (S.C. injection) |
|---|---|---|
| Tumor cell line | Tumorigenicity | Visceral metastasis |
| KM12C | 5 / 5 | 0 / 5 |
| KM12SM | 5 / 5 | 0 / 5 |

<table>
<thead>
<tr>
<th>Regional mesenteric lymph node metastasis</th>
<th>Liver metastasis</th>
<th>Lung metastasis</th>
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<tr>
<td>KM12C</td>
<td>10 / 10</td>
<td>2 / 10</td>
</tr>
<tr>
<td>KM12SM</td>
<td>41 / 41</td>
<td>10 / 41</td>
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Values are No. of positive mice / No. of inoculated mice
† p=0.035 as compared with incidence in KM12C cells
‡ Metastases were observed microscopically
Fig. 1. Light microscopic findings of KM12SM cells proliferating in a monolayered culture. KM12SM cells consisted of cells of various shapes and sizes. The cells were large or small, and had a spindle or round shape. The nuclei were oval in shape and large or small, and had prominent nucleoli. The cells proliferated in a monolayered sheet, and tended to pile up in some regions ($\times 200$).

Fig. 2. Macroscopic view of metastases from orthotopically implanted colon cancer. Liver metastasis (arrow 1), lymph node metastasis (arrow 2) and intraluminal growth of implanted cecal tumor without serosal invasion (arrow 3) were observed in a nude mouse inoculated with KM12SM cells into the cecal wall. Dilated intestine due to obstruction by the tumor was also noted.

Fig. 3. Microscopic view of locally growing and of metastatic tumors. The tumor implanted orthotopically in the cecal wall was a poorly differentiated adenocarcinoma with gland formation in part (a; H.E. $\times 40$), which showed severe lymphatic permeation (b; H.E. $\times 100$). Liver metastatic foci were poorly differentiated adenocarcinoma as the cecal tumor, and were associated with central necrosis (c; H.E. $\times 100$).
TABLE 2.
Relationship between serum CEA levels and liver metastasis in mice with orthotopic inoculation of KM12SM cells

<table>
<thead>
<tr>
<th>Serum CEA (ng/ml)</th>
<th>Liver metastasis (-)</th>
<th>Liver metastasis (+)</th>
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<tr>
<td></td>
<td>3.3 ± 1.8</td>
<td>8.2 ± 3.7</td>
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Values are mean ± SD, p=0.0014 (student’s t-test)

differences were observed in the size of the primary tumors. Despite the high incidence of tumorigenicity, no visceral metastasis was produced (Table 1). In contrast, when the cells were injected in the cecal wall, both cell lines were tumorigenic (Table 1). After injection in the cecal wall, regional mesenteric lymph node metastases occurred in 24% of mice inoculated with KM12SM cells (Fig. 2) and in 20% of mice inoculated with KM12C cells (Table 1). Mice inoculated with KM12SM cells produced liver metastases in 20 of 41 (Fig. 2), whereas mice inoculated with KM12C cells had a low incidence (1 of 10) of liver metastases, which were solitary or a few in number. Microscopic lung metastases occurred in 4 of 41 mice inoculated with KM12SM cells, and in no mice inoculated with KM12C cells (Table 1). The histological findings from the locally growing tumor after orthotopic implantation of the KM12SM cells revealed poorly differentiated adenocarcinoma with glandular formation in part (Fig. 3a), and severe lymphatic permeation (Fig. 3b). As in the primary cecal tumor, the metastatic tumors in the liver and lungs were poorly differentiated and were associated with central necrosis (Fig. 3c). These data established the validity of the model for subsequent studies.

Relationship between serum human CEA levels and liver metastasis

The mean value of the serum human CEA in 10 mice with liver metastasis was 8.2±3.7 ng/ml. This was significantly higher than that (3.3±1.8 ng/ml) in another 10 mice with no liver metastasis (Table 2).

DISCUSSION

Cancer metastasis is a multistep process involving tumor growth at the primary site, venous invasion (intravasation) and lymphatic permeation at the primary site, cell aggregation during circulation, adhesion to vascular endothelial cells at the metastatic organs, extravasation, tumor angiogenesis, and tumor growth in the metastatic organs. Tumor implantation s.c. or tumor injection into the portal or tail vein has been a standard methodology for many years for establishing animal models for human cancer research [4,6,7]. Although such models have helped us to understand the nature of human cancer and to test the efficacy of treatment, major problems still remain unresolved. Although the tumor can sometimes grow when injected s.c., the tumor is encapsulated and usually fails to metastasize either regionally or distantly. Metastatic models after injection of tumor cells into the portal or tail vein have been devoid of most of the essential steps for metastasis such as tumor growth, venous invasion and lymphatic permeation at the primary site. These animal models could not allow study of the biology of human cancer metastasis.

In our experiment, the parental cell line KM12C and the in vivo selected cell line KM12SM were both tumorigenic when injected s.c., but they produced neither regional nor distant metastasis. In contrast, orthotopic intracecal inoculation of KM12 cells resulted in the production of mesenteric lymph node metastasis, and intracecal inoculation of KM12SM cells also produced a remarkable 50% incidence of liver metastasis.

From the morphological findings of KM12 cells proliferating in a monolayered sheet, these two cell lines have been thought to consist of several multi-clonal subpopulations including highly metastatic phenotypes. The KM12SM cell line selected from the KM12C cell line in nude mice may mean that highly metastatic phenotypes were selected, resulting in a high incidence of liver metastasis after orthotopic inoculation. It is also possible that liver metastasis may depend to some unknown extent on the early predominant growth of highly hematogenous metastatic subpopulations of KM12SM cells after orthotopic implantation, as Karbel [8] has suggested.

It has previously been postulated that KM12SM cells could demonstrate a higher metastatic potential than KM12C parental cells due to different production of matrix metalloproteinases (MMPs)-2 and -9, and of heparanase, which are affected by the organ environment [9]. In addition, there is a close correlation between the incidence of liver metastasis and the levels of serum CEA, suggesting that a model using KM12SM cells implanted into the cecal wall orthotopically could behave in a "patient-like" manner. Using these ideas, we could develop a liver metastatic model - involving a 50% incidence of
metastasis in the liver - that can now allow us to study the biological behavior of metastatic subpopulations in colon cancer. We hope to increase our understanding of the mechanisms of metastasis through similar further investigations.

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REFERENCES