ESTABLISHMENT OF A METASTATIC MODEL OF HUMAN HEPATOCELLULAR CARCINOMA IN NUDE MICE VIA ORTHOTOPIC IMPLANTATION OF HISTOLOGICALLY INTACT TISSUES

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A highly metastatic LCI-D20 model of human hepatocellular carcinoma (HCC) was obtained via orthotopic implantation of a histologically preserved metastatic tumour selected from 1 of 30 surgical specimens, and has been maintained for 18 passages in nude mice. All mice with implanted tumours exhibited 100% transplantability and metastatic ability, as well as various manifestations reminiscent of tumour behaviour in HCC patients. These included: local growth, regional invasion, spontaneous metastasis to liver, lymph nodes and lungs, and peritoneal seeding. Histological characteristics of the LCI-D20 tumour were similar to those of the original tumour. Karyotypic analysis revealed heteroploid cells. Immunohistochemically, expression of AFP and HBsAg was shown. Our nude mouse model with its high metastatic rate and short latency period could be an interesting tool for the study of human HCC.

Invasion and metastasis are the major obstacles to successful cancer treatment. (Aznavorian et al., 1993). Relevant animal models for human cancer are of great importance when seeking new therapies for human cancer (Furukawa et al., 1995). However, human tumours grown subcutaneously (s.c.) in nude mice rarely metastasize, although they closely resemble the original tumours morphologically, biologically and biochemically (Sharkey and Fogh, 1984). Fidler (1990) has indicated that implanting human tumour cells orthotopically into the corresponding organ of nude mice results in much higher metastatic rates. For example, human colon cancer cells dissociated, grown in culture and injected into the cecum of nude mice produce tumours which eventually metastasize to the liver, demonstrating that orthotopic implantation can enhance the metastatic ability of human tumour cells in nude mice. Similar results have also been achieved with the orthotopic implantation of cell lines from human lung cancer, pancreatic cancer, bladder cancer, melanoma, breast cancer, head-and-neck cancer, and stomach cancer (Manzotti et al., 1993).

However, cell suspensions used for orthotopic implantation may not necessarily express the full metastatic potential of the original tumour, whereas the orthotopic implantation of histologically preserved tissue avoids disruption of tumour integrity and retains native cell-to-cell interactions (Hoffman, 1994). With the latter method, models of human cancers in nude mice exhibit a variety of clinical manifestations that occur in human patients (Fu et al., 1991a). Models utilizing orthotopic transplantation of intact tumour tissue with subsequent growth and metastasis include lung (Wang et al., 1992), bladder (Fu et al., 1991b), pancreatic (Fu et al., 1992a), prostate (Fu et al., 1992b), ovary (Fu and Hoffman, 1993) and stomach cancers (Furukawa et al., 1993).

It remains difficult, however, to obtain satisfactory models for human cancers in nude mice, particularly when aiming at spontaneous metastatization as well as a reduction of the latent period, when cell suspensions or histologically intact tissue are used for orthotopic implantation (Furukawa et al., 1993; Sharkey and Fogh, 1984; Fidler, 1990; Manzotti et al., 1993; Hoffman, 1994; Fu et al., 1991a,b, 1992a,b; Wang et al., 1992).

Our approach included the selection of highly invasive and metastatic human HCC samples from 30 different surgical specimens, using orthotopic transplantation of histologically intact tissue, followed by selection and isolation of metastatic cells from the samples in nude mice. The metastatic cells were inoculated in nude mice to establish tumour lines. Our metastatic model derived from the orthotopic implantation of metastatic tumour-line cells greatly increased the rate of metastasis in nude mice and displayed a variety of the manifestations seen in human patients. We have thus successfully obtained a model for human HCC (named LCI-D20, Liver Cancer Institute, passage time-20 days) which is described in detail below.

MATERIAL AND METHODS

Mice

BALB/cA male nude mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China) at 4 to 6 weeks of age were used in this study.

Surgical specimens of human HCC

Fresh surgical specimens were obtained from resected lesions of 30 patients with HCC who underwent surgery at the Liver Cancer Institute. The surgical specimens were rinsed in Hanks’ BSS and transported to the laboratory as soon as possible. After necrotic tissue and non-cancerous tissue of the specimens had been removed, the remaining cancerous tissues were divided into small pieces about 2 mm in diameter.

Implantation procedure

Tumour pieces were transplanted to the liver of the nude mouse as histologically preserved tissue, modified according to the method reported for colon cancer (Fu et al., 1991a). Briefly, a left upper abdominal pararectal incision was made under anaesthesia; the left lobe of the liver was exposed and a part of the liver surface mechanically injured with scissors. Then, a tumour piece was fixed within the liver tissue, the liver was returned to the peritoneal cavity and the abdominal wall finally closed. Mice were kept in laminar-flow cabinets under specific-pathogen-free conditions.

Evaluation of growth and metastases

Mice were killed if they developed signs of distress. At autopsy, the liver, lymph nodes, lungs and other organs were dissected and processed for routine gross and microscopic examination. Metastases were considered to have occurred if at least one microscopic metastatic lesion was found in any organ of the recipients.

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Selection of metastatic cells and establishment of highly metastatic model

When a lung metastasis in nude mice was found, the lungs were removed and cut into 1-mm³ pieces, then the lung pieces were implanted s.c. with a trocar into the flanks of nude mice. Lung cubes from each of the donor animals were implanted into 2 mice. Over a given period, a progressively growing s.c. tumour grew to 1 cm³ and the mouse was killed; the tumour was then resected, cut into 2-mm³ pieces and implanted into the livers of nude mice. The procedure was repeated twice, as shown in Figure 1. If a tumour line was obtained after 2 successive cycles of selection, it was routinely maintained by orthotopic passage (liver to liver) in nude mice to produce a passaged tumour line. Our LCI-D20 model derived from such a passaged tumour line.

Chromosome analysis

The human origin of the tumour grown in the nude mouse was confirmed by chromosome analysis. Tumour tissues were finely minced and incubated for 2 hr at 37°C in RPMI-1640 medium (GBCO, Grand Island, NY) with 10% FCS (GBCO, Grand Island, NY) and 8 mg/ml of collagenase II (Sigma, St. Louis, MO). The tissues were then washed repeatedly in a dish with fresh medium and filtered through a 100-μm mesh screen. The cell suspensions were incubated for 48 hr at 37°C in RPMI-1640 supplemented with FCS and harvested. The G-banding technique was used for karyotype analysis (Ochi et al., 1984).

Immunohistochemical studies

Expression of 2 markers, AFP and HBxAg, was studied by using rabbit anti-human alphafoetoprotein antibody (Dako, Glostrup, Denmark) and mouse anti-human HBxAg monoclonal antibody (prepared by our Liver Cancer Institute) (Liet al., 1994), respectively. Tumour tissues obtained from the mice were fixed in formalin and embedded in paraffin. Sections of 5 μm were assayed for AFP and HBxAg expression by immunoperoxidase staining by the ABC method (ABC Kits, Vector, Burlingame, CA). Briefly, tissue sections were deparaffinized and blocked with endogenous peroxidase. The sections were incubated with 10% normal serum for 20 min, incubated with Abs AFP and HBxAg at 4°C overnight, incubated for a further 30 min with biotinylated antibody and then for 40 min with ABC reagent at room temperature. The slides were then rinsed in TBS/0.04% dexamethasone (Sigma); 0.001% H₂O₂ was added for 7 min and followed by counterstaining with haematoxylin (3 min).

RESULTS

Establishment of the metastatic LCI-D20 model

Out of 30 specimens consisting of 13 metastatic lesions and 17 primary tumours, 14 gave rise to locally growing tumours in the mice. Take rates in the 2 groups were 69% (9/13) and 29% (5/17) respectively (p < 0.05). Over a period ranging from 6 to 24 weeks, 7 of 14 specimens gave rise to spontaneous metastasis in nude mice; the metastatic sites included liver (4/7), lymph nodes (4/7), lungs (1/7) and peritoneal seeding (3/7).

The only case of lung metastasis in nude mice was derived from a 1-cm³ liver metastatic lesion of a 39-year-old man with HCC. Edmonson Grade II, serial alphafoetoprotein (AFP) 96 μg/l; hepatitis virus-B surface antigen (HBsAg) was also detected. During surgery, extensive metastases in all liver lobes and hilus hepatis lymph nodes were found. The patient died of serious metastasis 40 days after operation.

On day 40, 3 mice transplanted with specimens from this patient developed signs of distress. At autopsy, metastases were found in the liver, lymph nodes and lungs; subsequently, according to the experimental process shown in Figure 1, the LCI-D20 model was obtained and exhibited 100% transplantability and spontaneous metastasis to liver, lymph nodes and lungs in nude mice. Up to the present, it has been maintained for 18 passages in nude mice, also exhibiting 100% transplantability and metastasizing capacity. All mice transplanted with the tumour line died of serious metastasis within 6–8 weeks. Detailed data are presented in Table I.

Metastatic characteristics of the LCI-D20 model

The LCI-D20 model exhibited various manifestations reminiscent of tumour behaviour in HCC patients. These included: local growth, regional invasion, spontaneous metastasis to liver, lymph nodes and lungs, and peritoneal seeding. At early stages, the tumour cells disseminated, forming micrometastases in hepatic blood vessels, followed by lymph-node and lung metastases. At later stages, numerous visible metastases were found in the liver and lymph nodes.

Extensive local growth and regional invasion were seen in all mice with transplanted tumours (78/78) and, when the orthotopic tumour grew to 15 mm in diameter, the left upper abdominal wall and the liver parenchyma were invaded. Finally, the gastrointestinal ductus, spleen, pancreas and peritoneum were involved and bloody ascites was often found.

Liver metastasis occurred at the 2nd week, when small visible metastatic colonies were seen around the local tumour. At the 6th week, widespread dissemination of metastases

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<th>Table 1: Transplantability and Metastasizing Ability of the LCI-D20 Model (Sum of the 18 Passages)</th>
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The LCI-D20 tumour line was maintained by orthotopic passage (liver to liver), using 5 to 6 mice for every passage (total of 96 mice, including 18 mice using passage and 78 mice for observing metastasis). After 6 to 8 weeks, when the tumours grew in the liver 2–3 cm in diameter, the mice developed signs of distress and died. At autopsy, metastases were seen in all organs by gross and histological examination (1 showed the histological results of 78 mice).
throughout the lobes of the liver was observed. Histological examination revealed micrometastases in the liver vessels (Fig. 2).

Lymph-node metastases were visible from the 3rd week on and were found in the hilus hepatis, mesenteric, diaphragmatic, mediastinal and iliac lymph nodes. Lung metastasis began at the 3rd week, when a few microscopic masses consisting of tumour cells and blood cells were seen within the pulmonary blood vessels; by the 6th week, the micrometastases had disseminated into the lungs as seen under light microscopy (Fig. 3).

Metastases to other organs were found, upon gross and microscopic examination, in all mice with transplanted tumours.

Histological findings
Histological characteristics of the LCI-D20 tumour were similar to those of the original tumour (Fig. 4).

Chromosome analysis
Chromosome analysis confirmed morphologically the human origin of the LCI-D20 tumour cells and karyotyping showed 90% heteroploid cells (Fig. 5).

Immunohistochemical results
AFP immunoreactivity was localized in the cytoplasm of neoplastic cells (100% of stained cells); no nuclear AFP positivity was found. HBxAg immunoreactivity was also confined to the cytoplasm and the nuclei of neoplastic cells (100% of stained cells). The expression of human AFP and HBxAg was well maintained in the locally growing tumours and in the metastases in all 18 passages.

DISCUSSION
Subcutaneous (s.c.) tumour implantation is a standard method of establishing animal models of human cancer (Fidler, 1986, 1990). Although such models have helped to understand the nature of and therapeutic approach to human cancer, many problems still remained unsolved. One major problem is that the tumour derived from a patient and implanted s.c. into an immunodeficient animal no longer behaves as it did in the patient. Although the tumour can sometimes grow s.c., it is encapsulated and usually fails to metastasize either regionally or at distant sites (Fidler, 1990).

Recently, a new strategy of “orthotopic implantation” has been used to develop rodent models of metastatic human cancer (Fidler, 1990; Manzotti et al., 1993). In the first generation of these models, cell lines or disaggregated cells were injected into the organ of the mouse that corresponds to the organ from which the human tumour is derived. This
method allows metastasis to occur, at least in certain cases (Fidler, 1990; Giavazzi et al., 1986; Bresalier et al., 1987; Morikawa et al., 1988a,b). However, the cell lines and disaggregated cells used for orthotopic implantation were obtained by disrupting the original structure of the human tumour tissue, which may lead to a change in the nature and biological behaviour of the tumour and could be the basis of the greatly reduced metastatic rate (Fu et al., 1991b). Hoffman (1992) has developed an orthotopic implantation model utilizing intact tissue such as that obtained directly from surgery. This approach has yielded a high take rate and frequent metastases in colon cancer (Fu et al., 1991a), bladder cancer (Fu et al., 1991b), lung cancer (Wang et al., 1992), pancreatic cancer (Fu et al., 1992a), prostate cancer (Fu et al., 1992b), and stomach cancer (Furukawa et al., 1993). These models of human cancers in nude mice can show various manifestations similar to tumour behaviour in human patients.

However, it remains difficult to obtain satisfactory models for human cancers in nude mice, particularly with regard to spontaneous metastasis and the reduction of the latent period when cell suspensions or histologically intact tissue are used for orthotopic implantation, which precludes effective study of important events in spontaneous metastasis. Therefore, our approach included the selection of highly invasive and metastatic human HCC samples from 30 different surgical specimens by using the orthotopic transplantation of histologically intact tissues, followed by selection and isolation of metastatic cells from the samples in nude mice. The results of this study showed that 14 of 30 surgical specimens yielded growing tumours at the implantation site, while 7 led to spontaneous metastases over a period ranging from 6 to 24 weeks; most of these were located in the liver and regional lymph nodes and were difficult to maintain during the passage process in nude mice. In fact, in only 1 case did we observe spontaneous lung metastasis which was maintained during the subsequent selection and passage processes in nude mice. Spontaneous metastasis of human tumour xenografts in nude mice was extremely rare (Nakanishi et al., 1991). Obviously, the selection of a highly invasive and metastatic sample correlated closely with the patient outcome and was a key factor in the successful establishment of metastatic models for human HCC; subsequent isolation of these metastatic cells from the sample in nude mice was another important factor in obtaining a passaged tumour line with a high metastatic rate and short latency period. This was because cells with a high metastatic capacity can be isolated from their parent tumours (Morikawa et al., 1988a) and the nude mouse can be used to isolate and select metastatic subpopulations of cells from human cancers (Fidler, 1990). Following the establishment of the LCI-D20 model via orthotopic implantation of histologically preserved metastatic tumour tissues, it also displayed a variety of clinical features that occurred in patients with HCC and maintained 100% metastasization in all 18 passages. Furthermore, the LCI-D20 tumour line implanted into the subcutis and peritoneum also exhibited a 70% and a 100% lung metastasis rate respectively (data not shown), which suggested that its high metastatic ability depended mainly on the nature of the tumour cells.

As was shown in the immunohistochemical results, the expression of AFP was well maintained in locally growing tumours as well as in metastases; similar results were seen for HBsAg. These results indicated that this mouse model maintained the native structure of human HCC and some of its original antigenic phenotype, suggesting that this model does, indeed, have features resembling the natural biological behaviour of human HCC.

Aruga et al. (1993) accidentally established a liver metastatic model of human hepatoma in nude mice by subcutaneous transplantation, but its metastasis was only localized to the liver whereas our model exhibited various manifestations resembling tumour behaviour in HCC patients. Thus, the LCI-D20 model with its high metastatic rate and short latent period could be an interesting tool for studying human HCC.

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REFERENCES


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