Metastatic Potential of Human Colorectal Carcinomas Implanted into Nude Mice: Prediction of Clinical Outcome in Patients Operated upon for Cure


Abstract

To determine whether the production of experimental hepatic metastases in athymic nude mice by human colorectal carcinomas (HCC) correlated with the clinical outcome in patients, we harvested colorectal carcinomas from 82 patients, dissociated the tumors with collagenase and DNase, and injected them into groups of nude mice, either in the flank to assess experimental tumorigenicity or into the spleen to produce experimental metastases in the liver. Growth in mice was then associated with clinicopathological factors and clinical outcome. Growth of HCC in the flank or the livers of nude mice was associated with the time to recurrence in a Wilcoxon analysis. Analysis of the outcome data in a Cox proportional hazards model suggested that there was an interaction between tumorigenicity and metastatic potential of HCC in nude mice and serum CEA concentration in the patient and stage of disease. A univariate analysis indicated that both tumorigenicity and metastatic potential of HCC in nude mice were significantly associated with the serum CEA concentration of the patient but not with the other variables of stage of disease, mucin production, local tissue invasion, state of differentiation, or sex. A subset of 57 patients was operated upon for cure and followed prospectively for up to 61 months. Tumorigenicity and, to a lesser extent, experimental metastatic potential were associated with disease recurrence in 23 of these patients. Seventy-eight percent of the subset of patients who were operated upon for cure developed liver metastasis as one site of their progressive disease. Thus, the ability of HCC cells isolated from surgical specimens to grow in athymic nude mice correlates with the development of advanced disease in patients.

Introduction

Neoplastic cells must enter the circulation, arrest in distant vascular beds, extravasate, and proliferate within the extracellular matrix of an organ to produce metastasis (1, 2). We have developed a model of metastasis for HCC in athymic nude mice where HCC cell suspensions are implanted into the spleen and produce tumor colonies in the liver (3, 4). To do so, HCC cells traverse the spleen, enter the portal circulation, implant in the liver, and invade the parenchyma to establish colonies (3-5). The experimental metastatic potential of HCC in this model is determined largely by the organ environment in which the HCC is placed: orthotopic implantation in either the site of origin of the primary tumor (colon) or the site of metastasis (liver) enhances the development of metastases (6); whereas heterotopic implantation into such sites as the subcutis or muscularis prevents the production of metastases. Type IV collagenolytic activity is directly proportional to the experimental metastatic potential of a HCC line (6). In addition, Bresalier et al. (7) have found that local invasiveness is associated with the experimental metastatic potential of sublines of LS174T, an established HCC line, after i.s. injection. Thus, experimental metastasis in nude mice can serve as a model of clinical metastasis in patients with colorectal carcinoma.

The ultimate test of a model of metastasis is its ability to predict the potential of a cancer of produce metastases in individual patients. Thus, we sought to determine whether the capacity of recently isolated HCC to produce experimental hepatic metastases in nude mice (subsequent to i.s. injection) correlated with the development of metastases in patients operated upon for cure. Our aim was to test the validity of this animal model for investigating how HCC metastasize. Our previous studies used a small number of HCC to evaluate the biology of metastasis. Now we wanted to determine if the model could be generalized to a larger number of HCC. HCC were dissociated into single cell suspensions and then injected i.m. or i.s. to assess experimental tumorigenicity or metastatic potential. We then sought associations between tumor growth in nude mice and clinical and pathological variables in a group of 82 patients and clinical outcome both for the whole group of patients and in a subset who were operated upon for cure.

Materials and Methods

Animals. Six- to 8-week-old BALB/c nude mice were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility. The mice were age and sex matched for each experiment and were housed in laminar flow cabinets under specific-pathogen-free conditions. Neoplasms were implanted into groups of mice without regard for matching the gender of recipient mice with the donor patient.

Patients. The 82 patients in this study were admitted to the surgical service of The University of Texas M. D. Anderson Cancer Center at Houston and underwent resections of primary or metastatic colorectal adenocarcinomas. Informed consent was granted for this study in accordance with institutional and federal guidelines. Patients were operated upon by one investigator (J. M. J.) and selected sequentially beginning in 1984. Each patient was followed for at least 6 months. Pathological staging was performed according to the method of Astler and Coller (8). There were 64 primary lesions: 1 Dukes' A, a lesion that is limited to mucosa alone; 6 Dukes' B1, carcinomas that penetrated into the muscularis propria without lymph node metastasis; 25 Dukes' B2, carcinomas that penetrated through the bowel wall but without lymph node metastasis; 18 Dukes' C2, carcinomas that penetrated the bowel wall and the regional lymph nodes; and 14 Dukes' D, carcinomas that had metastasized to distant sites. There were also 18 metastases: 11 were from liver; 4 from lymph nodes; 2 from peritoneal implants; and 1 from lung. Seven patients had a primary removed along with a metastasis and 5 patients had more than 1 metastasis removed. When multiple neoplasms were removed from a single patient, the most aggressive neoplasm was used in the analysis. The mean age of the patients was 55.2 years with a range of 31 to 77 years. Median follow-up was 20 months (range, 6–61 months). Forty-four patients were men. Five patients were Hispanic, 9 patients were black, 6 Oriental, and the rest white. Fifty-seven of these patients underwent operations for cure: all of the patients with Dukes' A, B, and C stages of disease as well as 7 patients with Dukes' D lesions (3 liver metastases, 4 peritoneal metastases, and 12 lymph node metastases)
3 retroperitoneal lymph node metastases, and 1 peritoneal implant). Twenty-three of the patients operated upon for cure developed clinical metastases. Median follow-up of the entire group of 82 patients was 12 months, while the median follow-up of patients operated upon for cure was 18 months.

Preparation of Cell Suspensions. Specimens removed at surgery were immediately examined by a surgical pathologist. Within 10 min, tumor samples were removed and placed into cold minimal essential medium (Gibco, Grand Island, NY) containing 300 units/ml penicillin and 300 μg/ml streptomycin. Immediate tissue dissociation was performed as described previously (9) using collagenase type I and DNase type I (Sigma Chemical Company, St. Louis, MO). The viability of the dissociated cells was always >70% as determined by trypan blue dye exclusion and the cells were either injected into nude mice or cryopreserved. Cryopreserved cells were recovered as needed by rapid thawing.

Tumorigenicity. We assessed tumorigenicity after implanting 3 × 10^6 dissociated viable human colorectal carcinoma cells through a 27-gauge needle into the middle of the quadriceps femoris of 3 to 11 nude mice. Implantation sites were inspected twice a week. When nodules were approximately 1 cm in diameter, they were resected under aseptic conditions and divided into aliquots. One aliquot was dissociated and reimplanted into additional nude mice, and the other aliquot was submitted for histopathological study. Tumorigenic nodules were successfully transplanted and appeared histologically similar to the original tumor. Whether the tumor cells produced a tumor in nude mice was determined 6 months after implantation.

Metastatic Potential. Experimental metastatic potential was assessed in the manner described by Giavazzi et al. (4). Briefly, 3 × 10^6 viable tumor cells were injected into the spleens of 3 to 5 nude mice. Experimental hepatic metastasis was considered to have occurred if at least one macroscopic colony was found in the liver of any of the recipients 90 days after inoculations. Metastatic potential was assessed within 4 transplant generations of isolation from the patient. Nine HCC that were tumorigenic in nude mice were not assessed for metastatic potential in nude mice: three tumors because nude mice were not available at the time of testing; four because intrasplenic injection of tumor cells killed the mice within 24 h; and two because the HCC were contaminated with microorganisms (these last two were not in patients operated upon for cure; the other HCC were from those patients whose neoplasms were resected during potentially curative operations).

Histopathological Examination and Evaluation. Specimens used in our studies were classified by routine histopathological examination in the Pathology Department at The University of Texas M. D. Anderson Cancer Center. Xenografts of tumor tissue or fresh tissues from surgical specimens were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 4-μm intervals, and stained with hematoxylin and eosin. Representative tumor and mucosa samples were reviewed by one investigator (K. R. C.) who defined the morphological variables according to standard techniques: state of differentiation, well, moderately, or poorly (10); mucin production, either extracellularly (40% of tumor volume composed of mucin lakes or pools) or intracellularly (signet ring cells contained intracellular mucin droplets and comprised at least 5% of tumor) (11); local invasion of lymphatics, blood vessels, or nerves at the periphery of a primary or mass of metastatic tumor.

CEA Measurement. The serum CEA level was determined by a commercially available enzyme immunoassay kit (Abbott Laboratories, Chicago, IL) and was considered elevated if ≥5 ng/ml.

Statistics. Associations of tumorigenicity and metastatic potential of HCC in nude mice analysis with time to recurrence (the DFI) were first tested with a Wilcoxon analysis in the procedure LIFETEST in SAS (12). Proportional hazards analyses were then performed using a Cox model of the clinical outcome of the sets of patients whose HCC had been assessed for tumorigenicity and metastatic potential in nude mice. The stepwise proportional hazards procedure PHGLM was used in SAS (13) with the following dummy variables for the covariates: stage of disease, Dukes' A-C2 (0) versus Dukes' D (1); mucin present (0) versus mucin absent (1); local invasion present (0) versus local invasion absent (1); male sex (0) versus female sex (1); serum CEA level less than 5 ng/ml (0) versus serum CEA level ≥5 ng/ml (1). Tumorigenicity and metastatic potential were considered in separate analyses. Growth either in the flank or as liver colonies was 1 compared to 0 for failure to grow at either site. Potential interactions between tumorigenicity and metastatic potential and the covariates were sought in 2 × 2 contingency tables using the two-tailed Fisher's exact test in the procedure FREQ in SAS (14). Significance was set at the 5% level.

RESULTS

Clinical Outcome. A Kaplan-Meier analysis of the time interval between surgery and recurrence of cancer, the DFI, was performed for all patients whose HCC had been tested for tumorigenicity or metastatic potential. The curves were compared by a Wilcoxon analysis. Tumorigenicity and metastatic potential were both significantly associated with DFI (Fig. 1). The median DFIs for the entire group of HCC that were tumorigenic or produced experimental metastases were 12 and 9 months, respectively, compared to HCC that were not tumorigenic (median, 34 months) or metastatic in nude mice (median, 29 months). Tumorigenicity, but not metastatic potential, was significantly associated with DFI in the subset of patients who underwent potentially curative operations (Fig. 2). Although the DFI for the HCC operated for cure that produced experimental metastases were not significantly different from the HCC in the potentially cured that were not metastatic in nude mice, there was a suggestion that a subset of these experimentally metastatic tumors were more aggressive in the patients because the 25th percentile for the metastatic group was shorter than that for the nonmetastatic group (12 months for the former compared to 28 months for the experimentally nonmetastatic HCC (Fig. 2B)). We then assessed whether tumorigenicity and metastatic potential in nude mice remain significant after accounting for the effects of other covariates.

Proportional Hazards Analysis. A stepwise proportional hazards analysis using the Cox model was performed for the whole group of HCC and then that subset of HCC that was operated upon for cure. When all covariates and tumorigenicity are included in the Cox model of all the HCC, stage of disease is significant. Both forward and backward stepwise analyses yield stage of disease as the only significant covariate (Table 1). However, when only stage of disease is in the model, the proportional hazards assumption is not met (Table 1). When the covariates and experimental metastatic potential are present simultaneously, stage of disease is significant. Forward and backward stepwise analyses yield both stage of disease and invasion without rejecting the proportional hazards assumption (Table 1).

In the subset of patients operated upon for cure, nothing is significant when the covariates and tumorigenicity are present simultaneously. However, forward and backward stepwise analyses yield stage of disease as a significant covariate with acceptance of the proportional hazards assumption (Table 1). Similarly, when the covariates and experimental metastatic potential are present simultaneously, there are no significant covariates. However, stepwise analysis yields stage of disease, again with acceptance of the proportional hazards assumption (Table 1). These results do not clearly distinguish whether tumorigenicity or experimental metastatic potential are significant variables by themselves or because of their association with some other variable. Thus, tumorigenicity and metastatic potential in nude mice may interact with other covariates, e.g., stage of disease and serum CEA level, to influence clinical outcome.

Tumorigenicity and Metastatic Potential in Nude Mice. Interactions between tumorigenicity and metastatic potential and
Fig. 1. Kaplan-Meier estimates of DFI and tumorigenicity (A) or metastatic potential (B) of HCC implanted in nude mice. Tumorigenicity was tested by implanting HCC from 82 patients i.m. in the flanks of nude mice. Those patients whose tumor did not grow in the subcutis of nude mice (Tum−) had a significantly longer interval between surgery and recurrence of disease than did patients whose HCC was tumorigenic in nude mice (Tum+) by a Wilcoxon analysis of the curves. Metastatic potential was tested by injecting viable HCC cells i.s. in groups of nude mice that were examined within 3 months for the production of liver colonies. Patients whose HCC formed experimental metastases (Met+) had a significantly shorter time to recurrence than those patients whose HCC were not metastatic in nude mice (Met−).

Fig. 2. Kaplan-Meier estimates of DFI and tumorigenicity (A) and experimental metastatic potential (B) of HCC from patients who were operated upon for cure and disease-free interval. Tumorigenicity and metastatic potential were analyzed in 57 and 50 patients, respectively, as described for Fig. 1. Patients whose HCC were either tumorigenic (Tum+) or metastatic (Met+) in nude mice had a significantly shorter time to recurrence than did patients whose HCC were Tum− or Met−.

Table 1 Proportional hazards (PH) analysis of human colorectal carcinomas grown in nude mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>N</th>
<th>% Tumorigenic</th>
<th>P*</th>
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<td>CEA</td>
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<td>0.017</td>
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<tr>
<td></td>
<td>&gt;5.0 ng/ml</td>
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<td>81</td>
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<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
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<td>76</td>
<td>0.063</td>
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<tr>
<td>Mucin</td>
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<tr>
<td></td>
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<td>77</td>
<td>0.189</td>
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<td>Invasion</td>
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<td></td>
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<td></td>
<td>3</td>
<td>12</td>
<td>67</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* Those clinicopathological factors (variables) that were included in the proportional hazards model for all neoplasms were analyzed for interaction with tumorigenicity in the cross-tabulations program of SAS. Variables are defined in "Materials and Methods." * Two-tailed Fisher's exact test.

Heterogeneity of the Growth Potential of Human Colorectal Carcinoma. Inter- and intratumoral heterogeneity are important characteristics of experimental neoplasms (15). This was investigated in 11 patients who had had more than one HCC resected. Three patients (27%) had one lesion that was not tumorigenic while other lesions from the same patient grew in the flank. Similarly, 30% of those patients who had more than one lesion tested for metastatic potential in nude mice had one lesion that did not form liver colonies when one or more other lesions from the same patient did. Thus, at least two-thirds of HCC were homogeneous in their growth potential in nude mice.

Metastatic Potential as a Model for Site of Clinical Recurrence or Progression. Eighteen of the 23 patients (78%) operated upon for cure whose disease recurred developed metastases in the other variables were then sought in 2 x 2 contingency tables. When all the HCC were analyzed, both tumorigenicity and metastatic potential were significantly associated with the serum CEA level and not any other clinicopathological variables, including stage of disease (Tables 2 and 3, respectively). Similar results were obtained in those HCC that were resected for cure (data not shown). Thus, growth of HCC in nude mice is associated with the serum CEA level in the patient from whom the tumor was removed, but not with the stage of disease and various other clinicopathological characteristics.
their livers as one component of their advanced disease. Thirty-five % developed lung metastases, 17% developed retroperitoneal nodal metastases, 9% developed pelvic metastases, and 9% developed brain metastases. Thus, assessing the ability of HCC cells to grow in the livers of athymic nude mice models the various steps of metastasis in the liver, the organ most commonly involved with metastasis by HCC.

### DISCUSSION

The ability of HCC cells harvested from surgical specimens to proliferate in nude mice subsequent to i.m. (tumorigenicity) or i.s. (experimental metastasis) implantation correlated with development of metastases in the patients, although clinical outcome was more strongly associated with tumorigenicity in a subset of patients operated upon for cure. These observations support the data of others who either demonstrated (16–18) or suggested (19, 20) a correlation between tumorigenicity of human neoplasms implanted in nude mice and the clinical outcome of patients with sarcomas and various epithelial neoplasms. The association between recurrence of cancer and tumorigenicity was weak, possibly because the microenvironment of the subcutis of the nude mouse is not similar to the microenvironment in which these neoplasms originate and metastasize in the patient. Morikawa et al. (6) have shown that orthotopic implantation of HCC into the submucosa of the murine colon enhances expression of the metastatic phenotype. Thus, implantation of HCC cells in an appropriate organ site for metastasis may improve their ability to grow and thereby improve the correlation with clinical outcome. Our data suggest that the implantation of HCC into the flanks of nude mice provides data equivalent to that of i.s. inoculation.

The tumorigenicity and experimental metastatic potential of HCC implanted in athymic nude mice measure an aspect of the malignant phenotype that may not be assessed by standard morphological criteria. Neither tumorigenicity nor experimental metastatic potential were associated with variables that correlated with poor prognosis in large groups of patients: mucin production (11); poor differentiation (10, 21); and invasion of vessels, lymphatics, or nerves (22–25). Further, only local invasion of these variables was associated with clinical outcome in our sample and then only in those HCC assessed for experimental metastatic potential. Possibly, our sample was too small to detect an effect of these variables on either clinical outcome or growth potential in nude mice. If so, these prognostic variables do not have as strong an association with clinical outcome as do stage of disease, elevated donor serum CEA level, and tumor growth in nude mice, which were associated with outcome in our study. While morphological variables are associated with clinical outcome in other studies, their impact on neoplastic behavior may be weak and observable only in larger samples of patients.

The current data extend our previous observation that tumor growth in athymic nude mice is associated with the serum concentration of CEA in the patient (9) by indicating that experimental metastatic potential is also associated with serum CEA level. Thus, the presence of an elevated concentration of CEA in the sera of patients with colorectal carcinoma portends a poor prognosis, even in patients without objective evidence of clinical metastasis (26, 27), because neoplasms from these patients are biologically more aggressive than neoplasms that do not induce CEA elevations. Although CEA was first identified in 1965 by Gold and Freedman (28), its function remains to be elucidated. Since CEA is a member of the immunoglobulin supergene family (29), it may participate in the attachment of neoplastic cells to liver cells.

Although the development of metastases in the majority of patients was associated with the growth of their tumor cells in nude mice, some HCC that did not grow in nude mice produced recurrences in patients. These HCC clearly represent failures of the model. The approximately 30% of multiple carcinomas with discordant growth characteristics may represent a similar experimental failure or a manifestation of inter- and intratumoral heterogeneity. While variations in the toxicity of enzymes used to dissociate the HCC and amount of tumor necrosis may inhibit the growth of a xenograft, it is also possible that the HCC cell suspension may not have contained enough cycling cells to produce a neoplasm in the nude mouse. Some HCC have low growth fractions (30) and a random sample may include only cells in G0. DNA content measurements will be performed on paraffin-embedded sections of the original material to assess this point (31).

While the implantation of colorectal carcinomas cells into athymic nude mice may improve the determination of prognosis for individual patients, our intent was not to develop a predictive bioassay. Instead, our objective was to validate a model in nude mice that may help explain HCC metastasis. Our previous data were derived from a few HCC lines, most of which were established in tissue culture. In the current study, a large number of freshly isolated HCC were analyzed and found to behave similarly to the established cell lines. Thus, our data indicate that tumorigenicity and experimental liver metastasis are valid models for the processes that produce clinical HCC metastases.

### REFERENCES

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