ABSTRACT

In vivo studies aimed at therapy of spontaneous human tumor metastases have been hampered by the lack of practical experimental models. The LOX amelanotic melanoma model described here represents a transplantation model which rapidly and reproducibly results in spontaneous pulmonary metastasis following s.c. inoculation into athymic mice. Pulmonary lesions can be detected using a simple bioassay procedure which is useful for estimation of metastatic cell killing. Using this model we demonstrate that systemic therapy with cyclophosphamide or dacarbazine can produce metastatic cell killing consistent with complete eradication of established pulmonary metastases. This model may also prove useful for future experimental therapeutic studies aimed at prevention of metastases by manipulating tumor staging interval and treatment schedule.

INTRODUCTION

Human neoplasms frequently have already spread to secondary sites at the time of clinical diagnosis (1). While modern surgical and radiotherapeutic techniques may be very effective in controlling primary tumors, treatment directed toward cure of patients with advanced disease requires eradication of metastases (2). Identification of new agents with enhanced ability to eradicate or prevent metastases could potentially be important in the development of improved treatment regimens for many tumor types. Preclinical models used for identification of such agents should, ideally, mimic the patterns of metastasis seen clinically and represent all of the steps in the metastatic process.

While the introduction of genetically immunodeficient mice for in vivo transplantation of human tumors has provided new opportunities for detailed investigation of the metastatic phenotype (3), preclinical research on treatment of spontaneous human tumor metastasis has been constrained by the lack of practical experimental models. The general experience in xenografting human tumors to genetically immunodeficient mice has been that tumors grow locally and rarely metastasize spontaneously (3-6). The great majority of human tumor xenografts derived therefrom have been shown to be of human origin on the basis of isozyme studies as well as cytogenetic analysis. The cells of this subline have an aneuploid human karyotype with a modal chromosome number of 74/75 and several marker chromosomes. The cell stock derived from LOX-L has been shown to be of human origin by marker chromosome analysis. The establishment of LOX xenograft as well as in vitro studies will be described in detail (30-32).

In this report we describe a spontaneous metastasis model based on a subline of LOX derived from experimental metastases and present results illustrating the usefulness of this model for therapeutic studies aimed at treatment of established metastases.

MATERIALS AND METHODS

Establishment of LOX Xenograft. The LOX xenograft was originally established from an amelanotic lymph node metastasis of a 58-year-old male patient. The establishment of this xenograft as well as in vitro lines derived therefrom has been described in detail (30). Growth of the LOX tumor in serial s.c. passage in athymic nude mice is characterized by rapid formation and progressive growth of tumors which when examined histologically are found to be composed of large, undifferentiated tumor cells. This appearance corresponds closely to the histological appearance of the metastatic lesion from which the xenograft was derived. No evidence of metastatic growth was observed in tumors maintained in serial s.c. passage. An in vitro cell line designated LOX-L was derived from lung tumor colonies formed following i.v. injection of cells from the 16th generation of s.c. passage. These cells were transferred from Oslo, Norway, to the National Cancer Institute-Frederick Cancer Research and Development Center, expanded in mass culture, cryopreserved at several passage levels, and characterized to provide a standardized starting material for further experimental studies.

Characterization of in Vitro LOX Line. The cryopreserved in vitro cell stock derived from LOX-L has been shown to be of human origin on the basis of isozyme studies as well as cytogenetic analysis. The cells of this subline have an aneuploid human karyotype with a modal chromosome number of 74/75 and several marker chromosomes. The cells have been shown to be free of contamination with Mycoplasma and were negative in a mouse antibody production test for pathogenic murine viruses. A positive test result for reverse transcriptase was observed, and this can be attributed to the presence of a murine xenotropic virus.

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2 To whom requests for reprints should be addressed, at Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute-Frederick Cancer Research and Development Center, Building 1652, Room 121, Frederick, MD 21702-1201.
Experimental Animals. Athymic nude mice were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center or from various suppliers under contract with the Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Animals were provided with food and water ad libitum and maintained on a 12-h light/dark cycle.

Bioassay for Pulmonary Metastases. Detection/quantitation of pulmonary tumor burden was based on a simple bioassay in which lung tissue was removed from mice previously inoculated with LOX cells, and the tissue was transplanted s.c. into naive animals. These mice were observed for tumor development and caliper measurements were taken beginning 10–12 days after transplantation. Tumor outgrowth at the transplantation site served to indicate the presence of LOX tumor in the lungs of experimental animals. Existence of metastatic lesions was confirmed during histological examination of representative lung fragments. In chemotherapy trials, therapeutic effects were assessed on the basis of growth delay of tumor outgrowth in T-C\(^2\) bioassay mice to a predetermined tumor weight (1.0 g). Tumor weight was calculated from caliper measurements of two perpendicular dimensions of the tumor using the formula:

\[
\text{tumor weight (mg)} = \frac{\text{length} \times \text{width}^2}{2}
\]

where length and width are expressed in mm. The T-C value is indicative of the number of surviving cells at the time of transplantation for bioassay. This value is used along with the tumor volume-doubling time to calculate cell killing by the method of Lloyd (33). The volume-doubling time was estimated from tumor growth in the control group of the bioassay. Drugs were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

RESULTS

Growth and Morphology of s.c. Tumors. Tumors formed following s.c. inoculation of LOX cells are comprised of large, round cells with irregular nuclei lacking evidence of pigment formation or other differentiation. A notable feature of these tumors is the relative lack of encapsulation and the tendency for invasive growth at the periphery of the tumors.

LOX s.c. xenografts are characterized by rapid growth whether inoculated from in vitro cell stock or passaged serially as tumor fragments. Typical curves for growth from s.c. tumor implants of 1 × 10\(^6\) cells are shown in Fig. 1.

Growth in the Peritoneal Cavity. Following inoculation into the peritoneal cavity, LOX cells give rise to a lethal ascites. Formation of the ascites is accompanied by abdominal distention and a generalized cachexia and results in death of animals in approximately 20 days with inocula of 1 × 10\(^6\) cells. Necropsy of mice bearing advanced disease has indicated extensive invasion of visceral organs, notably the pancreas and liver, as well as formation of pulmonary metastases. To define the time course for formation of pulmonary metastases, a group of mice was inoculated with 1 × 10\(^6\) LOX cells harvested from ascites-bearing animals, and individual mice were sacrificed at intervals. Lung tissues from these mice were histologically examined and transplanted to bioassay animals. As indicated in Table 1, pulmonary metastases could be detected as early as 1 day following s.c. inoculation of LOX cells.

Spontaneous Metastasis following s.c. Implantation. LOX cells rapidly metastasized to the lungs following s.c. inoculation. Histological examination of tissues removed from animals held

\[3\] The abbreviations used are: T-C, test minus control; CPA, cyclophosphamide; DTIC, dacarbazine.

Table 1 Time course for metastasis of LOX cells

<table>
<thead>
<tr>
<th>Days after implantation</th>
<th>i.p. cells</th>
<th>s.c. cells</th>
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* Animals were implanted with LOX cells or tumor fragments, as indicated, and then killed at intervals for bioassay of lung tissue. Animals were observed for evidence of tumor formation for up to 3 months.

Growth and Morphology of s.c. Tumors. Tumors formed following s.c. inoculation of LOX cells are comprised of large, round cells with irregular nuclei lacking evidence of pigment formation or other differentiation. A notable feature of these tumors is the relative lack of encapsulation and the tendency for invasive growth at the periphery of the tumors.

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Spontaneous Metastasis following s.c. Implantation. LOX cells rapidly metastasized to the lungs following s.c. inoculation. Histological examination of tissues removed from animals held up to 30 days after inoculation has also revealed evidence of metastases in other tissues (primarily in lymph nodes and spleen). Pulmonary lesions are found as multiple, discrete foci of growth with cellular morphology similar to s.c. tumors. Macroscopically, these lesions are associated with areas of hemorrhage on the surfaces of the lung.

The time course for metastasis from the s.c. inoculation site to the lungs was defined in an experiment similar to that described above for i.p. inoculation. A group of animals was inoculated with 1 × 10\(^6\) LOX cells harvested from mice bearing ascites, and individual animals were sacrificed and evaluated for pulmonary metastases at various intervals beginning 6 days after inoculation of cells. As indicated in Table 1, pulmonary
metastases were detected as early as 8 days after s.c. inoculation of LOX cells. To evaluate the time course for metastasis from solid LOX tumors, fragments of lung tissue containing metastatic tumor were implanted into a group of mice and samples were obtained as above. The results of this experiment indicate a similar time course to that obtained with ascites material (Table 1).

Experimental Therapeutic Studies. An experimental protocol was developed to allow measurement of treatment effects on established pulmonary metastases. Athymic mice were inoculated with LOX cells s.c. and then held to allow establishment of metastatic lesions. As illustrated in the results of the metastatic time-course experiments described in Table 1, well-established pulmonary lesions, detectable on bioassay, could be expected in animals held for 2 weeks or more after s.c. implantation of LOX cells. Since the rapid growth of s.c. tumors limits holding of individual tumor-bearing mice to approximately 30 days, the “staging” interval has a practical range of about 14–30 days. Drug treatment was initiated after staging and continued on a schedule appropriate to the individual agent. Pulmonary tumor burden following treatment was evaluated by bioassay of tissue removed 24 h following the last treatment, as described above. Twenty-four h was selected to provide a standardized protocol consistent with measurement of maximal therapeutic effect.

CPA was selected as a prototype agent for development of this protocol on the basis of in vitro studies which indicated a high degree of sensitivity of this cell line to alkylating agents and on previous in vivo studies using an i.p. survival model in which LOX cells were found to be quite sensitive to this agent.

As indicated in Fig. 2A, single-dose treatment with CPA was very effective in reducing pulmonary tumor burden. Dose-related metastatic cell killing was observed with the highest doses (200 or 300 mg/kg), resulting in complete eradication of pulmonary metastases, indicated by a lack of tumor outgrowth from the lungs of these animals. In a similar experiment (Fig. 2B), DTIC was also shown to be highly effective in treating established LOX metastases. In this latter experiment the staging interval was reduced to 16 days, compared to 29 days in the CPA experiment, to provide a more modest therapeutic challenge. Treatment with DTIC at the highest dose resulted in >4.8 log₁₀ units of cell killing and complete eradication of pulmonary metastases.

DISCUSSION

LOX xenografts derived from the subline described here are characterized by rapid and aggressive growth in athymic mice. As we reported previously, s.c. tumors grow in an invasive fashion which is associated with formation of distant metastases (29). Inoculation of LOX cells into the peritoneal cavity results in formation of a lethal ascites which can be the basis for therapeutic studies using survival of animals as the end point (28). Spontaneous pulmonary metastases develop rapidly following inoculation by either the i.p. or s.c. routes. Use of s.c. inoculation provides an opportunity to concurrently measure the effect of treatment on the “primary” lesion and metastatic lesions. Since the purpose of the chemotherapeutic studies reported here was to evaluate the potential of treatment to eradicate established pulmonary metastases, we did not measure
s.c. primary tumors. However, measurement of s.c. tumors could be of importance in defining activity of agents thought to act by inhibiting metastasis. Reduction of metastatic burden in the absence of an effect on the primary tumor would provide support for an antimetastatic effect.

Initiation of metastatic studies using cell suspensions rather than solid tumor fragments eliminates several of the early events in the metastatic process such as invasion of basement membrane, etc. This procedure represents a form of model intermediate between experimental metastases, produced by i.v. injection of tumor cells, and true spontaneous metastasis. For experimental therapeutic studies directed toward treatment of established pulmonary metastasis, we have utilized ascites material, and cell stock expanded in culture (data not shown) for s.c. inoculation, primarily for reasons of convenience. However, as indicated in Table 1, we have also observed rapid formation of true spontaneous pulmonary metastasis from s.c. implanted solid tumors.

Experimental metastases have been produced with high efficiency following i.v. injection of the parental LOX-L cell line (30). However, spontaneous metastases were not observed during serial s.c. transplantation of tumor fragments. The parental line has a near diploid karyotype compared to the highly aneuploid metastatic subline. Thus, it appears that in the course of transferring, expanding, and cryopreserving the cell population we have selected an aggressively metastatic subpopulation from the LOX-L line.

Experimental therapeutic trials utilizing cyclophosphamide and dacarbazine illustrate the potential of this metastatic model for defining treatments effective in eradicating established pulmonary metastases. As illustrated in Fig. 2, single doses of cyclophosphamide of 200 or 300 mg/kg produced high levels of metastatic cell killing and completely eradicated metastases as indicated by the lack of outgrowth of tumors from bioassayed lungs. Likewise, treatment with dacarbazine, 300 mg/kg (two injections), completely eradicated metastases. The high degree of in vivo and in vitro sensitivity of LOX to alkylating agents appears to be due to a DNA repair deficiency. Preliminary studies* have indicated that the LOX line manifests the Mer DNA repair phenotype.

The major complication in conducting and interpreting experiments using the bioassay end point arises when lungs from untreated or vehicle-treated control animals fail to form tumors in the bioassay animals. In the case of the cyclophosphamide experiment (Fig. 2A), this occurred with 2 of 10 of the control animals. This presumably reflects variability in some or all of the multiple steps involved in the bioassay, e.g., maintenance of tissue viability during excision/transplantation, resorption/remodeling of normal lung graft tissue, vascularization of the graft, etc.; variability in experimental animals; and variation in metastatic tumor cell content of the lung grafts. Presumably, the variability in the bioassay procedure also accounts for the findings in Table 1, in which only 2 of 5 mice showed a positive bioassay at 23 days postinoculation (i.p. route). Observation of 100% positive animals at the previous two time points would suggest that this result should have been 100% as well. The other two time-course experiments showed results more in accordance with the expected progressive increase in percentage of positive animals with time (Table 1). All control lungs produced tumors in the DTIC experiment (Fig. 2B) in spite of the fact that the metastatic staging interval was reduced to 16 days and pulmonary tumor burden should have been less than in the CPA experiment. The potential for lack of tumor “takes” in control animals suggests that results from treated animals indicating complete eradication of metastases may occasionally represent spurious results and requires that multiple independent experiments be conducted before making conclusions regarding the effectiveness of treatment.

While other human tumor cell lines have been reported to produce spontaneous metastases in athymic mice and may prove useful for therapeutic studies, the LOX subline reported here is uniquely suited to experimental therapeutic studies because of its rapid growth, relatively reproducible behavior, and compatibility with the bioassay approach to assessment of treatment effects. The high degree of drug sensitivity manifested by LOX for some classes of agents enhances its value for targeted studies of new therapies directed toward eradication of established metastases but is not representative of the typical pattern of drug sensitivity seen in melanoma. We have found this metastatic model to be useful for preclinical evaluation of several new anticancer drug leads, including pyrazine diazohydroxide (34). Simple modifications to the protocols described here, e.g., initiation of treatment immediately following s.c. inoculation of tumor fragments and measurement of the primary tumor, may also allow future investigation of agents designed to inhibit spontaneous metastasis.

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REFERENCES

14. Tan, M. H., and Chu, T. M. Characterization of the tumorigenic and
Practical Spontaneous Metastasis Model for in Vivo Therapeutic Studies Using a Human Melanoma

Robert H. Shoemaker, Donald J. Dykes, Jacqueline Plowman, et al.