Characterization of a Xenograft Model of Human Ovarian Carcinoma Which Produces Ascites and Intraabdominal Carcinomatosis in Mice

Thomas C. Hamilton, Robert C. Young, Karen G. Louie, Brent C. Behrens, Wilma M. McKoy, Karen R. Grotzinger, and Robert F. Ozols

Medicine Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205

ABSTRACT

We have used in vivo and in vitro procedures to select a subpopulation of cells from the human ovarian carcinoma cell line, NIH:OVCAR-3, with the capacity to grow i.p. in female nude athymic mice. After i.p. injection of these cells, animals develop metastatic spread similar to that of clinical ovarian cancer. Disease progression is characterized by the development of massive ascites, extensive invasive i.p. tumors, and pulmonary metastases. The malignant ascites cells are transplantable, manifest cytoplasmic androgen and estrogen receptors, and express the ovarian cancer associated antigen CA125 (116,000 units/ml of ascites supernatant). The cells also have the same chromosome markers which were present in the original cell line, NIH:OVCAR-3. Survival following i.p. passage of ascites is dependent on tumor cell inoculum ranging from a median survival of 39 days with 40 million cells to 84 days for 11.5 million transplanted cells. The characteristics of this unique in vivo model make it well suited for the evaluation of new drugs and novel experimental therapies in ovarian cancer. In addition, this in vivo model, together with ovarian cancer cell lines, may prove particularly useful for the study of pharmacological ways to specifically increase the cytotoxicity of anticancer agents in tumor cells while not increasing toxicity in normal tissues. The presence of hormone receptors should facilitate the experimental evaluation of hormonal therapy in ovarian cancer.

INTRODUCTION

A variety of experimental models of ovarian cancer have been examined to identify an optimum system for the study of the biology of the human disease and to evaluate the potential role of new experimental therapeutic modalities. Both in vivo and in vitro systems have been studied, and these include ovarian cancer cell lines (3, 5, 8, 9, 12, 14, 19, 23, 29), s.c. human ovarian tumor xenografts in immunodeficient or immunodeprived hosts (4, 16, 26), growth of xenografts at immunoprivileged sites (22), direct cloning of human ovarian cancer cells in soft agar, i.e., the human tumor stem cell assay (10, 20, 24), and a transplantable murine teratocarcinoma (18). None of these models exhibits all the following features of an ideal model system: (a) histopathology and embryony consistent with human ovarian cancer; (b) a reproducible pattern of metastases which parallels human disease (30); (c) drug sensitivity profiles similar to that of human ovarian cancer; (d) immunological and biological properties consistent with human ovarian cancer; and (e) suitable markers for following response to treatment. In addition, an experimentally useful model system must also provide adequate numbers of malignant cells for detailed biochemical and biological analyses.

We have used in vivo and in vitro selection procedures to obtain a subpopulation of cells from the well-characterized human ovarian carcinoma cell line, NIH:OVCAR-3 (12) [NIH:OVCAR-3 refers to NIH ovarian carcinoma cell line 3; NIH:OVCAR-3<sup>adri</sup>] refers to a variant of OVCAR-3 selected for growth in nude mice (nu) and substrate-independent growth in agarose (Ag+), which grows i.p. in female nude athymic mice. This new in vitro-in vivo model system fulfills many of the requirements for a suitable experimental system of ovarian cancer as set forth above. The tumor is transplantable, and progression closely parallels the human disease with animals developing intraabdominal carcinomatosis and ascites which leads to death from respiratory compromise and bowel obstruction. Because NIH:OVCAR-3 was derived from a patient refractory to combination chemotherapy with Adriamycin, cisplatin, and cyclophosphamide and the cell line retained resistance to these drugs in vitro (12), this model is an appropriate system in which to study drug resistance and its pharmacological manipulation. In addition, NIH:OVCAR-3 contains steroid hormone receptors making the model potentially useful for the evaluation of hormonal regulation of malignant cell growth and function and the potential significance of hormonal therapy in ovarian cancer.

MATERIALS AND METHODS

Chemicals and Reagents

[2,4,6,7-<sup>3H</sup>]Estradiol (90 to 115 Ci/mmol) and [17α-methyl-<sup>3H</sup>]R1881 (70 to 87 Ci/mmol; 17α-hydroxy-17α-methylstra-4,9,11-trien-3-one) were purchased from New England Nuclear, Boston, MA. Hydroxyflutamide (SCH 16423) was a gift from Schering Corp., Bloomfield, N.J. Unlabeled 5α-dihydrotestosterone, diethylstibestrol, 9α-fluoro-11β,21-dihydroxy-18α,17α-isopropylidenedioxy-1,4-pregnadiene-3,20-dione (trimcinolone acetonide), and agaron (type VII) were acquired from Sigma Chemical Co., St. Louis, MO. Lymphocyte separation medium (Ficoll) was from Bionetics Laboratory Products, Kensington, MD.

Selection for I.p. Growth

The initiation of NIH:OVCAR-3 from the malignant ascites of a patient with common epithelial ovarian cancer and the characteristics of the cell line have been described in detail previously (12). Subsequently, this cell line has been subjected sequentially to selection procedures which favored acquisition of a subpopulation of cells with the capacity for i.p. growth in heterologous hosts. NIH:OVCAR-3 was first selected for in vitro growth by s.c. injection of the cell line (10<sup>6</sup> cells) into the subcapsular areas of nude athymic mice (BALB/c genetic background). After in vivo passage, the s.c. tumors were selected for anchorage-independent growth. This was accomplished by mechanical dissociation of tumors (10) when they were 1 cm in diameter (at approximately 6 weeks) and...
In Vivo Passage of Tumor Cells

All animals given injections of the tumor cell preparation described above developed abdominal distention due to ascites. The peritoneal cavity was irrigated with sodium chloride solution (0.9%, w/v), and the washings were combined with the ascites. The cell suspension thus obtained was centrifuged (300 x g at 4° for 10 min), and the cell pellet was resuspended in sodium chloride solution (0.9%, w/v), after which aliquots were counted, as nuclei, on an electronic cell counter (Model ZBi; Coulter Electronics, Hialeah, FL). Between 10⁶ and 10⁷ cells were injected i.p. into subsequent hosts. Ascites cells were tested periodically by the mouse antibody production test and by this criterion were deemed to be negative for the 13 mouse-associated viruses assayed by this procedure.³

Assessment of Steroid Hormone Receptor Status

Buffer. The buffer used in the following procedures consisted of Tris-HCl (10 mm), pH 7.4, containing EDTA (1 mm), diethothreitol (1 mm), phenylmethylsulfonyl fluoride (1 mm), and sodium molybdate (10 mm).

Protein Estimation. Cytosol protein was measured by the modified method (13) of Lowry et al. (17) with bovine serum albumin as standard.

Preparation of Cytosol. Ascites harvested from animals (as above) was maintained on ice, and cells were obtained by centrifugation (as above). Cells were suspended in 3 volumes of buffer and homogenized with a motor-driven glass-Teflon homogenizer. The homogenate was centrifuged at 100,000 x g for 1 hr at 3° to obtain the cell cytosol (12).

Sedimentation Analysis. Cytosol labeled with [3H]estradiol or [3H]estradiol; R1881 in the absence or presence of potential competitors (3 hr at 4°) and then treated with charcoal to remove free steroid was analyzed on continuous gradients of Ficoll (28). The tumor cell fraction was suspended in sodium chloride solution (0.9%, w/v), and 10⁶ cells were injected i.p. into 3 female 8-week-old nude athymic mice.

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Measurement of Radioactivity. Scintillation fluid (10 ml; Aquassure; New England Nuclear) was added to vials and mixed. Samples were counted at an efficiency of approximately 40%.

Survival Studies

The effect of cell inoculum on duration of survival was determined by i.p. passage of either 11.5 million or 40 million malignant ascites cells to groups of 9 female nude mice.

Detection Of CA125 Antigen

The presence of an antigen, CA125, which is associated with common epithelial tumors of the human ovary (1), was kindly determined by Dr. J. Whang-Peng (Medicine Branch, National Cancer Institute), personal communication.

Intraperitoneal Model of Human Ovarian Cancer

Cytogenetics. Cytogenetic analysis on short-term cultures of NIH:OVCAR-3-derived malignant ascites was kindly performed by Dr. J. Whang-Peng as described previously (12).

RESULTS

General Observations. The i.p. injection of female nude athymic mice with 10 million NIH:OVCAR-3 cells selected for the capacity for substrate-independent and in vivo growth was followed in 40 to 50 days by visible abdominal distention (Fig. 1A). Examination of the peritoneal cavity at this stage revealed diffuse studding of tumor deposits on all peritoneal surfaces, the viscera, and the diaphragm (Fig. 1C), as well as copious ascites (Fig. 1B). The size of the tumors varied from 0.1-mm implants on the diaphragm to 1- to 2-cm masses in the bowel mesentery. Cytological examination of the ascitic fluid (Fig. 1D) revealed groups and sheets of adenocarcinoma cells which in some cases show acinar and papillary-like structures. The volume of ascites usually was 2 to 5 ml per animal, and 10⁶ cells were frequently obtainable by aspiration of the ascites combined with peritoneal lavage. These cells could be grown and passed in vitro. Histological examination of tumor implants revealed invasive malignant cells with a growth pattern which varied from solid tumor to glandular and papillary with numerous mitotic figures (Fig. 1E). Late in the course of the disease, pulmonary metastases frequently (~80%) developed (Fig. 1F).

Animals died from complications of extensive i.p. disease (massive ascites and carcinomatosis) within 1 to 3 weeks after abdominal distention was noted. Malignant cells in the ascitic fluid were readily passaged to subsequent hosts and to date has been serially transplanted 6 times. A dose-response relationship was found to exist between size of the cell inoculum, abdominal distention, and time of death. Injection of 40 million cells i.p. resulted in a mean survival for 9 animals of 38 ± 5 (S.D.) days, while survival increased to 89 days ± 16 (S.D.) days on i.p. inoculation of 11.5 million cells (Table 1). It is feasible to inject 30 to 50 million cells per animal, for experiments requiring a large number of animals, since between 300 million and 1 billion cells can routinely be harvested from a single mouse. An inoculum of this size will produce the degree of reproducibility in median survival required for comparative survival studies with this model. It should be noted, however, that animals inoculated with as few as 1 million cells will ultimately succumb to the disease, although the duration of survival is variable (data not shown).

Cytogenetics. Cytogenetic analysis of malignant ascites subjected to short-term culture revealed the cells to be derived from a human female and to manifest those chromosomal markers described previously for the cell line NIH:OVCAR-3 (12).

³ Animal Health Diagnostic Laboratory, Frederick Cancer Research Facility, National Cancer Institute, personal communication.
CA125 Antigen. The supernatant derived from centrifugation of malignant ascites cells was found to contain 100,000 to 400,000 units of the CA125 antigen per ml, which is expressed by ovarian carcinoma cells from a majority of patients with nonmucinous ovarian cancer (1, 2). The level of CA125 in mice with established tumors was in the range of 12,500 units/ml.5 The level of antigen expressed in the ascites supernatant is 6 times greater than the highest serum level in any ovarian cancer patients thus far studied.6

Steroid Hormone Receptors. Cytosol prepared from ascites cells, incubated with labeled estradiol or labeled R1881 alone or in the presence of potential competitors and analyzed on low-salt sucrose density gradients (Chart 1), was found to contain distinct androgen- and estrogen-binding components with sedimentation coefficients of 7 to 9S and with specificities consistent with the requisite receptors (11, 12). Whole-cell estradiol-binding assays of ascites cells further revealed approximately 5000 estrogen-specific sites (diethylstilbestrol compatible) per cell, assuming equivalent binding in all cells.

**DISCUSSION**

This in vivo model of human ovarian cancer which mimics the human disease in growth pattern and progression was successfully developed through the use of in vivo and in vitro selection procedures to obtain a subpopulation of cells enriched for the combined capacities of substrate-independent and in vivo growth. The use of the approach was successful where previous attempts by us to establish such a model by direct i.p. implantation of human ovarian carcinoma cell lines, fresh ascites, and solid tumor failed.

In an effort to develop more specific therapies for ovarian cancer, we utilized experimental models involving the human tumor stem cell assay (20), a murine teratoma (18), and human ovarian cancer cell lines (3, 9, 12, 19-23) for the preclinical evaluation of potential new therapeutic modalities. Studies in these experimental systems have formed, in part, the rationale for clinical trials of i.p. Adriamycin therapy (21) and enhancement of Adriamycin cytotoxicity by its use in combination with verapamil (19, 23). The ability of these model systems to accurately predict the clinical efficacy of new treatment modalities may be linked by their inability to (a) differentiate between normal tissue toxicity and tumor cytotoxicity and (b) accurately reflect important determinants of in vivo response such as bioavailability and drug metabolism. The model system reported here has the potential to be more predictive for clinical utility of experimental therapies than were previously described models of ovarian cancer. The NIH:OVCAR-3 human ovarian cancer i.p. xenograft model parallels the human disease by producing ascites, intraabdominal carcinomatosis, pulmonary metastases, and death from disease. In vitro findings of potential therapeutic significance can be evaluated in this in vivo system where results can be monitored by the clear-cut parameter of increased survival.

The pattern of metastases and the human derivation suggest that the model may be useful in the evaluation of the therapeutic effect of natural killer cells (25, 27), mullerian regression substance (6), bacterial toxins (15), and monoclonal antibodies alone or conjugated to toxic agents (7). Likewise, the continued presence of available androgen and estrogen receptors in the ascites cells obtained from intact animals (ovaries not removed) suggests the relevance of this model for the evaluation of hormonal and antihormonal therapy in a well-controlled in vivo setting. The evaluation of therapy with conjugates of steroids and cytotoxic effectors should also be feasible in the model. The presence of the CA125 antigen also offers the opportunity to correlate anti- gen levels with experimental therapies in living animals.

The rapid development of drug resistance frequently limits the effectiveness of chemotherapy in the treatment of patients with advanced ovarian cancer. We have recently demonstrated in human ovarian cancer cell lines that resistance to Adriamycin and melphalan can be reversed by calcium channel blockers (19, 23) and depletion of glutathione (9), respectively. The i.p. model

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**Chart 1.** Cytosol (protein, 14 mg/ml) prepared from OVCAR-3-induced malignant ascites cells was subjected to sucrose density gradient analysis following incubation (3 hr, 4°) with 1 nM [3H]R1881 alone (O) or estrogen-specific sites (diethylstilbestrol compatible) per cell, = 6.9S), and bovine serum albumin (c, SM, = 4.6S). bovine catalase (a, SM, = 11.35), human IgG (b, SM, = 8.05), and bovine serum albumin (c, SM, = 4.85).

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5 R. Bast (Dana-Farber Cancer Center), personal communication.
6 M. Zweig (Clinical Pathology, Clinical Center, NIH), personal communication.
of ovarian cancer described here appears to be well suited to determine whether these in vitro manipulations can also be successfully performed in vivo. The demonstration that survival can be improved by using pharmacological means to reverse drug resistance or increase chemotherapeutic agent efficacy would indicate that clinically exploitable differential effects exist between normal tissues and tumor cells. Studies with Adriamycin plus verapamil and of the effects of glutathione depletion upon melphan cytotoxicity are currently in progress in this model.

REFERENCES

Fig. 1. A, female nude athymic mouse with abdominal distention apparent 45 days after injection of 10 million cells prepared as described in "Materials and Methods." B, presence of milky ascites (arrow) in animal shown in A. C, presence of bulky tumor and small foci of tumor throughout peritoneal cavity. D, malignant-appearing sheets of cells in the ascites. Papanicolaou, × 220. E, surface invasion of the liver; note numerous mitotic figures. H & E, × 54. F, metastatic lesion in lung of animal shown in A. H & E, × 130.
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