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Comparison of ID8 MOSE and VEGF-modified ID8 Cell Lines in an Immunocompetent Animal Model for Human Ovarian Cancer

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Abstract. *Attempts to develop novel immunotherapeutic mouse models have been hampered by the lack of an adequate in vivo system. This study was performed to establish an immunocompetent mouse model for the testing of immunotherapy concepts. The in vivo system was based on a syngeneic mouse ovarian surface epithelium (MOSE) cancer, physiologically and biologically closely resembling human epithelial ovarian cancer. In addition, a more aggressive variant containing a mutated form of vascular epithelial growth factor was also evaluated. The growth patterns of these ovarian cancer cells in mice were compared to the established, highly aggressive 4T1 breast cancer model. A clinically-relevant tool for the study of different growth patterns in ovarian cancer, with potential significance for the development of novel immunological methods, was successfully developed.*

In 2004, an estimated 25,500 women in the United States were diagnosed with ovarian cancer and 16,000 women died from this disease (1). It is the fourth leading cause of cancer death in women in Western countries and has the highest mortality among female reproductive tract malignancies, with a 5-year survival rate of only 43%.

Most ovarian tumors originate from the surface epithelial lining and can be categorized into four major types: serous, endometrioid, mucinous, or clear cell tumors (2, 3), with the serous subtype being by far the most prevalent. However, to date gene expression patterns only allow for the distinction of mucinous and clear cell carcinomas from serous tumors (4).

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Human OSE (ovarian surface epithelial) cells are often found in the form of inclusion cysts and clefts, which may represent the earliest stage of neoplastic transformation leading to primary ovarian tumors (2). Dissemination of ovarian cancer cells within the peritoneal cavity is the result of the exfoliation of cells from the primary tumor, which subsequently disperse in the whole abdomen by the physiological flow of peritoneal fluid. These cells will attach to the serosa to begin forming solid secondary lesions and can lead to ascites production. The ability of ovarian tumor cells to metastasize requires reorganization of the actin cytoskeletal network, particularly at sites of focal adhesion complexes, and changes in cellular adhesion molecules, growth factor receptors and intracellular signaling kinases (5, 6). These changes appear to be common events in an otherwise very heterogeneous disease.

Increased levels of vascular epithelial growth factor (VEGF) have been reported in invasive ovarian carcinoma as compared to benign tumors or tumors of low malignant potential (7). Besides its role in tumor growth, VEGF has been implicated in the pathogenesis of ovarian cysts and ascites, where markedly elevated levels of VEGF were found (8). The serum levels of VEGF were 10-fold higher in patients with advanced ovarian cancer than in healthy controls, increases that were associated with poor clinical outcome (9). Therefore, ovarian cancer also offers an opportunity to investigate the multifaceted functions of VEGF.

Materials and Methods

Cell culture. ID8 represents a cell line derived from spontaneous malignant transformation of C57BL/6 MOSE cells *in vitro* (10).

Two variants of a syngeneic model were developed: regular, unmodified ID8 mouse ovarian epithelial cells, resembling well-differentiated slow tumor proliferation and the more aggressive VEGF-mutated ID8 strain, representing the more aggressive variants of ovarian cancer. This strain of tumor cells had been generated by transfection with a retroviral vector containing green fluorescent protein (GFP) and VEGF164 (11).

Characterization of the ID8 / ID8-VEGF cell lines. In order to establish a syngeneic mouse model for ovarian cancer mimicking distinct states of neoplastic progression, normal primary syngeneic mouse ovarian surface epithelium (MOSE) and VEGF-transformed cells were kept in permanent cell culture. The malignant phenotype was defined by the ability of the cells to form colonies in soft agar, to grow as multicellular tumor nodules on organotypic raft culture, and to induce intraperitoneal tumor formation in immunocompetent C57BL/6 mice. As cancer cells make a transition to a more aggressive phenotype, their growth rates increase significantly, as has frequently been described.

ID8 cell culture. ID8 cells were isolated as described by Roby *et al*. (10). Briefly, ovaries from female breeder mice (C57BL/6) were resected and incubated for 20 minutes in Dulbecco's modified Eagle's medium (DMEM) supplemented with trypsin following removal of the residual remnants of the oviducts and bursa. Single cells and clumps of MOSE cells were collected, pelleted by mild centrifugation, resuspended in a MOSE growth medium and seeded onto collagen-coated tissue culture dishes. The MOSE cell culture medium consisted of DMEM supplemented with 4% fetal bovine serum (FBS), 100 mg/ml each of penicillin and streptomycin, 5 mg/ml insulin, 5 mg/ml transferrin and 5 ng/ml sodium selenite (Invitrogen, Carlsbad, CA, USA). During early passage of the cells, the medium was further supplemented with mouse epidermal growth factor (mEGF) (2 ng/ml) and hydrocortisone (0.5 mg/ml). Collagen-coated flasks were discontinued after passage 5. The cells were routinely transferred at a 1:6 to 1:12 ratio, depending on their growth rates. The ID8 cells were generously provided by Dr. G. Coukos (University of Wisconsin, USA) (12). For growth rate analyses, the cells were seeded at densities of $1x10^4$ and $5x10^4$ cells, and subconfluent cell counts were determined at different times post seeding. The cell doubling-times were estimated according to the formula: T*ln2/ln(Xe/Xb), where Xe is the cell number determined at the end-point, Xb is the cell number at the beginning time- point, and T is the total elapsed time (in hours).

ID8-VEGF-mutated cell culture. ID8-VEGF-mutated cells were maintained in DMEM supplemented with 4% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Invitrogen) in a 5% CO_2 atmosphere at 37˚C.

In some experiments, the ID8 cells were cultured in serum-free and insulin-free media overnight, or in serum-free conditions in the presence or absence of Genexol-PM (Paclitaxel formulated in polymeric micells) for 24 hours.

Dose-response experiments were performed to define the sensitivity of ID8 cells to the drug, the final experiments being performed at a 50 μmol/l concentration. Select cells treated with Paclitaxel were exposed to recombinant murine VEGF (100 ng/ml; R&D Systems, Minneapolis, MN, USA).

All the experiments were repeated three times. Paclitaxel was kindly provided by Samyang Corp, Seoul, Korea, unless otherwise specified.

In vivo tumor inoculation. Subconfluent ID8 cells were trypsinized, washed twice and harvested by centrifugation at 1000 xg for 5 minutes. A single-cell suspension was prepared in phosphate-buffered saline (PBS), or PBS mixed with an equal volume of cold Matrigel (BD Biosciences, Bedford, MA, USA) at 10 mg/ml.

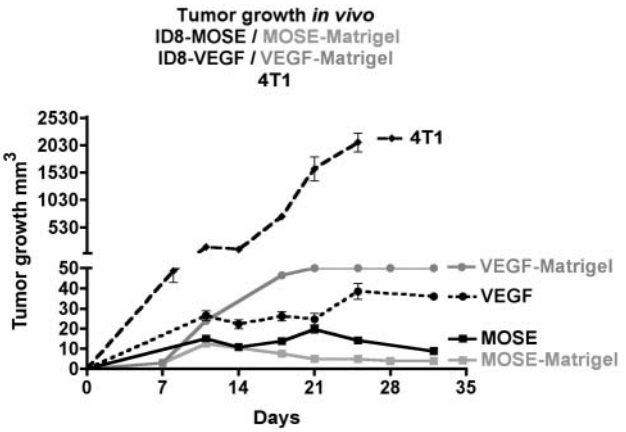

Figure 1. *Comparison of subcutaneous tumor growth after injection of unmodified and VEGF-modified ID8 cells on addition of Matrigel and 4T1 cells. 4T1 cells showed the most rapid growth with larger tumor sizes when compared to the ID8 cell lines. VEGF-modified ID8 cells exhibited more rapid growth and larger tumor size when compared to unmodified ID8 MOSE cells. Using Matrigel did not cause significant differences in the time and size of tumor growth for ID8 MOSE, but led to larger tumor size and more rapid tumor growth in ID8 VEGF-modified cells.*

For flanks injections, a total volume of 0.5 ml containing $5x10^6$ MOSE or VEGF/GFP-transfected cells were injected subcutaneously into the flanks of 6 to 8-week-old C57BL6 mice. The tumor size was measured weekly thereafter using a digital Vernier caliper. Tumor volumes were calculated by the formula: $V=1/2\ (L \times W)^2$, where L is length (longest dimension) and W is width (shortest dimension) (13). The mice were sacrificed 5 weeks after the flank injection.

For intraperitoneal injections, a total volume of 0.7 ml of PBS containing $7x10^6$ VEGF/GFP-transfected cells (n=10), GFP-transfected cells (n=10), or wild-type cells (n=10) cells was inoculated into the mouse peritoneal cavity. The animals were followed for survival or were sacrificed based upon their tumor/ascites burden. All the animal studies were approved by the Institutional Animal Care and Use Committee of the University of Utah, USA.

Results

In vivo growth properties of ID8 / ID8-VEGF cells. The tumor-igenic potential of the model was confirmed *in vivo* in the immunocompetent C57BL/6 mouse.

Mice injected with MOSE cells died after approximately 60 days, whereas mice injected with the more rapidly growing VEGF-transfected strain died after 35 days. At necropsy, the mice showed tumor nodule formation throughout the abdominal cavity, as well as peritoneal tumor nodule formation.

In the subcutaneous model, VEGF/GFP-transfected ID8 cells were injected subcutaneously into one flank in the presence of Matrigel. The tumor volume of the VEGF-mutated cells 4 weeks after inoculation was significantly

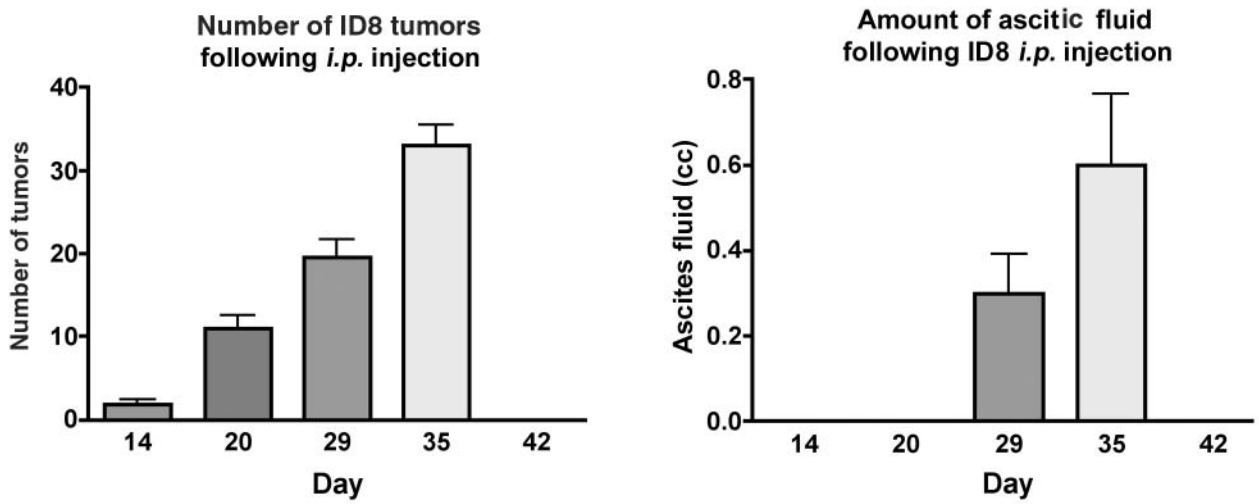

Figure 2. *a) Visible number of tumors following intraperitoneal injection of ID8 cells, showing a maximum within the intraperitoneal cavity after 35 days of inoculation. b) Amount of ascitic fluid collected at certain time-points (29 and 35 days after inoculation with ID8 cells) in cc's, showing a maximum of ascitic fluid of 0.6 cc on day 35.*

larger (39.07±7.01 mm^3) compared to the MOSE cells (6.33±1.34 cm^3, $p<0.0014$). Injecting the same cells without Matrigel led to slower, more disseminated growth and slightly smaller tumor size for the VEGF-transfected cells (30.33±1.87 without compared to 39.07±7.01 with Matrigel, $p<0.2224$). Injection of unmodified ID8 cells initially led to slower flank tumor growth as well, but the addition of Matrigel did not lead to differences in tumor size (Figure 1).

Tumor and ascites formation of ID8-VEGF in vivo. The number of tumors appearing after intraperitoneal inoculation with ID8-VEGF-modified cells in C57BL/6 mice increased over time from three nodules on day 14 to eleven nodules on day 20, 20 nodules on day 29 and 32 nodules on day 35. The same increasing trend could be observed for the ascitic fluid formation, from 0.35 cc on day 29 to 0.6 cc on day 35 (Figure 2).

Paclitaxel cytotoxicity in vitro in ID 8-VEGF cells. Chemosensitivity to Paclitaxel was established. The cytotoxicity of Paclitaxel is of particular significance since this drug is frequently used for current chemotherapeutic regimens against ovarian cancer (14, 15). Fifty percent of the cells were observed to be viable at a concentration between 6 and 0.6 ng/well Paclitaxel on a 96-well plate (Figure 3).

Comparison of ID8 / ID8-VEGF growth to 4T1 growth in vivo. 4T1 cells, from a highly aggressive stage IV breast cancer cell line, showed a significantly greater tumor size

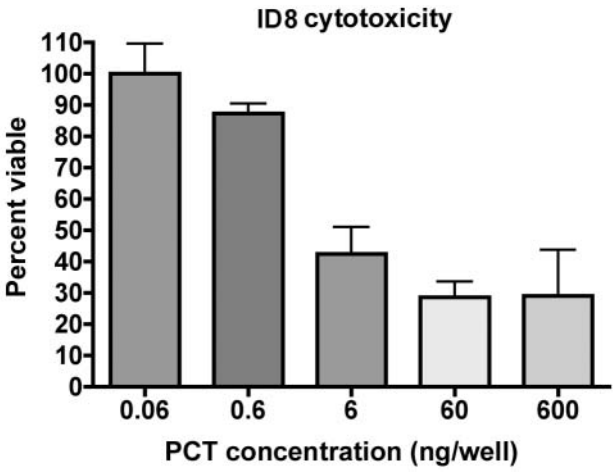

Figure 3. *Cytotoxicity of ID8 cells tested in a 96-well plate against different concentrations of Genexol-PM (polymeric-micelle-formulated Paclitaxel) showed 50 percent viability for ID8 cells at a dose range of between 0.6 and 6 ng of Genexol-PM per well.*

($p<0.0001$) and more rapid growth (Figure 1) than both ID8 subtypes .

Discussion

Various models have been developed to study ovarian epithelial surface cells. However, to date no immunocompetent model has been established that would permit the delineation of different states of ovarian cancerogenesis .

Genetically-modified adenoviruses have been used to illustrate that inactivation of both p53 and RB1 led to the

induction of tumors in mice (16). More recently, a mouse ovarian cancer model, using transgenic mice expressing the SV40 T antigen under the control of the MSIIR promoter, has been described (17). A rat model of spontaneous ovarian tumor progression provided interesting insights into progressive cytogenetic changes during tumorigenesis (18,19). In other existing rodent models, human cancer cells have been used as xenografts in immunodeficient mice (20). These models lack critical site-specific interactions between the tumor and stromal cells and, possibly, regulatory stimuli from the immune system. This is especially critical because ovarian cancer is a disease of older women, and changes in the immune response have been implied in the carcinogenesis of the ovaries and other sites (21). These existing models provide important insights into ovarian tumor biology, but may or may not be representative of the heterogeneous and spontaneous nature of human ovarian cancer and, most importantly, of the host-tumor interaction in the clinical setting.

The genetic make-up of established cell lines represents only a limited number of advanced human malignancies. In the present study, an animal model of ovarian cancer, mimicking different stages of ovarian cancer, was identified. This system lends itself to efficacy studies of chemotherapeutic treatment regimens and immunotherapy-based strategies in the immunocompetent host, which is significant because it enables the inclusion of immune surveillance to studies into the progression of ovarian cancer. The unmodified ID8 cells in C57BL/6 mice in our model represent less aggressive disease, characterized by a slower growth rate. ID8-VEGF-mutated cells are similar to a more aggressive tumor phenotype, with rapid growth rate and rapid tumorigenic potential *in vivo* being characteristic.

Many of the clinical features typical of ovarian cancer, including the presence of tumor nodules throughout the omentum and lymphogenic metastasis in the lungs, were also seen in our model, as was the formation of hemorrhagic ascites.

Conclusion

We established a syngeneic mouse ovarian cancer model that mimicks various stages of human disease. Different growth patterns typically seen in human ovarian cancer were observed.

This model can be used to study mechanisms of neoplastic progression *in vivo*. Importantly, *in vivo* analysis can be conducted in the immunocompetent host, allowing for an evaluation of immunological mechanisms in ovarian carcinogenesis. The unmodified and VEGF-mutated ID8 cell lines should provide new insights into genes involved in ovarian cancer progression and which are suitable targets for the development of novel methods for prevention and treatment.

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