

Specialized Program of Research Excellence (SPORE) in Ovarian Cancer

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Ovarian cancer is the number one gynecologic killer in the United States. New opportunities for diagnosis, prevention, and treatment will be dependent upon an expanded translational research effort. Fox Chase Cancer Center's Specialized Program of Research Excellence (SPORE) in Ovarian Cancer consists of six translational research projects, a Developmental Research Program, a Career Development Program and five specialized Cores to support the research programs. These research projects represent a diversity of translational research objectives, including diagnosis, prevention, and treatment: 1) Mechanisms of COX-2 inhibition in ovarian cancer prevention; 2) AKT as a Biomarker of Ovarian Cancer Progression and a Target for Therapeutic Intervention; 3) Anti-Müllerian Inhibiting Substance Type II Receptor (MISIIR) Immunoconjugates to Detect and Treat Ovarian Cancer; 4) Molecular-targeted Therapy in Ovarian Cancer; 5) Immunotherapy of Ovarian Cancer: Taking Down the Barriers; and 6) Methyloomics for Detection and Profiling of Ovarian Cancer in Serum. The fifth Project is in collaboration with Abramson Cancer Center of the University of Pennsylvania. A Biological Scientist and an Applied Scientist are Co-Principal Investigators on each Project. The specialized Cores include an Administrative Core, a Biosample and Tissue Procurement Core, an Ovarian Cancer Consortium for Research and Surveillance Core, a Mouse Engineering Core, and a Biostatistics and Data Management Core. The Ovarian SPORE Program is a multi-disciplinary collaboration of laboratory researchers and clinicians focused on decreasing morbidity and mortality from this disease. This goal will be accomplished by prevention strategies based on an understanding of ovarian oncogenesis, coupled with novel scientifically based therapeutic approaches.

Mechanism of Cyclooxygenase Inhibition in Ovarian Cancer Prevention

Xiang-Xi (Mike) Xu, Ph.D., *Project Leader*; Mary B. Daly, M.D., Ph.D., *Project Co-Leader*

Ovarian cancer often develops in women of peri-menopausal age, when ovulation ceases but gonadotropin levels are increased. The gonadotropin stimulation hypothesis suggests that the surges of gonadotropins associated with ovulation and the elevated gonadotropins

in the peri-menopausal period are risk factors. This theory is supported by epidemiological data that ovulation frequency and reproductive history are etiological factors for ovarian cancer risk. The surges of gonadotropins during ovulation stimulate inflammatory-like reactions in

ovaries, and we hypothesize that the postmenopausal increase in gonadotropins provokes an inflammatory environment in ovaries that resembles reactions during ovulation. These inflammatory reactions promote ovarian remodeling and accelerate the selection of cancer-prone cells, leading to an increased ovarian cancer risk. This idea is a theoretical framework for considering an approach for preventive intervention of ovarian cancer. Since cyclooxygenases COX-1 and COX-2 play critical roles in gonadotropin-stimulated ovulation, we investigated the possibility of inhibiting COX enzymatic activity to suppress ovulation and gonadotropin-stimulated ovarian inflammation and cancer risk. We designed studies in tissues, cell lines, mouse models, and women to explore the approaches and biological mechanisms for targeting COX enzymes in ovarian cancer prevention.

In tissue studies, we investigated the ovarian tissues from prophylactic oophorectomies for clues of early neoplastic changes and the potential correlation of markers related to ovulation and inflammation. We found that expression of matrix metalloproteinase MMP-9 and COX-2 and loss of epithelial basement membrane are associated with ovarian surface epithelial morphological changes. Unexpectedly, we discovered that COX-2 is not commonly expressed in established ovarian cancer. In ovarian tissues and tumors, we also observed that MMP-2 and MMP-9 are often highly expressed in areas of ovarian epithelia contiguously adjacent to neoplastic lesions, suggesting these proteolytic enzymes are causative factors in the basement membrane loss. We concluded that MMP-2 and MMP-9 are often expressed in morphologically transformed lesions, but not in malignant tumors.

Accordingly, MMP-2 and MMP-9 are found expressed in primary human ovarian surface epithelial cells but reduced in cancer cells. These results suggest that MMP-2 and MMP-9 play important roles in the early stages of tumor development and the MMP expression is reduced later on due to loss of the differentiation-determining GATA-6 transcription factor. We have also investigated the expression and regulation of COX-1 and COX-2 enzymes in ovarian epithelial and cancer cells. Using human ovarian surface epithelial (HOSE) cells in culture, we found that among the reproductive hormones and local mediators in ovulation

tumor necrosis factor α (TNF- α) is a strong inducer of COX-2 expression. However, TNF- α -induced COX-2 expression is lost in most ovarian cancer cells. These results lead us to speculate that expression of COX-2 may contribute to an early step of ovarian epithelial neoplastic transformation but play no role in the malignancy of established cancer.

In mouse model studies, we evaluated the potential of the white-spotting locus mutant mice (Wv) mouse to study the molecular mechanism for gonadotropin-induced ovarian epithelial morphological transformation and ovarian cancer risk. The Wv mice harbor a point mutation in the kinase domain of the c-kit gene, which results in the early loss of ovarian follicles and elevation of gonadotropin level, mimicking the menopausal biology in human. In our Wv inbred colony, Wv/Wv females start at around 8 weeks of age to have progressive ovarian alterations that resemble the preneoplastic lesions seen in human ovarian tumors and ultimately develop ovarian tubular adenomas. The Wv mice appear to be a useful model for studying the postmenopausal phenotypes for ovarian biology and pathology. By crossing Wv with COX-2 deficient mice, we found that COX-2 deficiency partially rescued the adenoma phenotype of the Wv/Wv mice. This study indicates that COX-2 plays a role in ovarian surface epithelial morphological remodeling and contributes partially to the epithelial alterations and formation of tubular adenomas in germ cell-deficient Wv/Wv mice. The study also suggests that COX-1 may be redundant and compensatory for COX-2 and also contribute to ovarian tumorigenicity. Additionally, COX-1 inhibition seems to preserve ovarian follicles and reduce the tumor phenotype of the Wv mice.

We proposed a clinical study to investigate the effect of the COX-2 inhibitor, Celecoxib, on human ovaries, to verify whether COX-2 inhibition reduces the biological processes that promote ovarian cancer. However, because of the recent findings of potential cardiac risk of COX-2 inhibitors, we suspended a clinical trial of using Celecoxib. Currently, we are considering a lower dosage of Celecoxib that has a minimal risk of cardiac problem, or use of inhibitors for both COX-1 and COX-2 that have no associated cardiac risk. Preliminary studies suggest that partial suppression of both COX-1 and

COX-2 simultaneously may be a better strategy for prevention of ovarian cancer. Additionally, our animal study suggests that inhibition of Cox-1 may preserve ovarian follicle and delay menopause. Thus, we are considering a general Cox inhibitor (Aspirin) as a preventive agent to be used during peri-menopausal period that reduces the conditions caused by elevated levels of gonadotropins. Additionally, we are considering testing if COX-1/COX-2 inhibition (by Aspirin) may delay menopause timing and reducing the potential tumor-promoting effect of gonadotropin stimulation in peri-menopausal women.

The experimental results obtained in the last period are consistent with our hypothesis that gonadotropin-stimulated ovarian inflamma-

tory activities, mediated by TNF- α , and the induced COX-2 and MMPs, that stimulate degradation and remodeling of basement membrane, may promote the selection and transformation of epithelial cells with oncogenic potential (containing genetic and/or epigenetic alterations). Thus, interfering with the inflammatory process, such as by targeting TNF- α , COX-2, or MMPs, may reduce ovarian cancer risk. Further experiments will be carried out to study the molecular details that will help to understand the etiology of ovarian cancer and provide a biological rationale for the use of COX inhibitors and additional chemopreventive agents to reduce the incidence of ovarian cancer in high-risk women and in the general population.

AKT as a Biomarker of Ovarian Cancer Progression and a Target for Therapeutic Intervention

Joseph R. Testa, Ph.D., *Project Leader*; Robert F. Ozols, M.D., Ph.D., *Project Co-Leader*, in collaboration with Deborah Altomare,[§] Ph.D.

Among its multiple effects, AKT/protein kinase B activation promotes cell survival, cell proliferation, and tumor invasiveness, suggesting that AKT plays a key role in tumorigenesis and therapeutic response. AKT is often activated in ovarian cancer and, thus, AKT signaling represents an important potential target for selective anti-cancer therapy in this disease. Rapamycin and its analog RAD001 target AKT signaling via inhibition of a downstream substrate dubbed mTOR (mammalian target of rapamycin), and such inhibition leads to G1 arrest in tumor cells exhibiting activation of the AKT pathway. Similarly, enzymatic inhibition of fatty acid synthase (FAS), whose expression is regulated at least in part by AKT signaling, by specific pharmacologic agents induces apoptosis in cancer cells. Overexpression of FAS is observed in most ovarian carcinomas, suggesting that FAS might serve as yet another target for therapeutic intervention in ovarian cancer.

The primary goal of this project is to determine the value of AKT as a biomarker for predicting ovarian cancer development, progression and drug sensitivity, and to determine if targeting mTOR and/or FAS has efficacy as a chemotherapeutic strategy in human ovarian cancer. Based on the strong association between AKT and mTOR activity, we analyzed ovarian cancer cell lines with high or low levels of AKT

activity for potential sensitivity to rapamycin or RAD001. Inhibition of mTOR activity resulted in G1 arrest and inhibited phosphorylation of downstream substrates 4E-BP1, p70S6 kinase and S6 ribosomal protein in ovarian cancer cells with high AKT activity, but not in ovarian cancer cells with basal levels of AKT activity. Furthermore, various *in vivo* studies demonstrated that RAD001 inhibits invasiveness, ascites formation, and angiogenesis in ovarian cancers with high AKT activity. One such recent study, using *in vivo* micro-imaging in a genetically defined mouse model of ovarian cancer showed that mTOR inhibition could delay significantly the onset and progression of ovarian cancer.

In other *in vitro* studies, inhibition of FAS activity by cerulenin or C75 induced apoptosis in ovarian cancer cells with high, but not low AKT activity. In addition, the tumor forming ability of ovarian cancer cells with active AKT was markedly diminished in an ovarian cancer xenograft model treated with a FAS inhibitor. Furthermore, inhibition of AKT activity potentiated the cell killing effects induced by FAS inhibitors, indicating that elevated AKT activity protects cells against FAS inhibition-induced apoptosis. Taken together, these experiments provide strong rationale for conducting clinical trials using pharmacologic inhibitors of mTOR or FAS.

Anti-Müllerian Inhibiting Substance Type II Receptor (MISIIR) Immunoconjugates to Detect and Treat Ovarian Cancer

Gregory P. Adams, Ph.D., *Project Leader*; Louis M. Weiner, M.D., *Project Co-Leader*

Development of anti-MISIIR antibody-based molecules. Adams, Simmons,[§] Yuan,[§] in collaboration with Marasco^a

The Müllerian Inhibiting Substance Type II Receptor (MISIIR) is an attractive target antigen for ovarian cancer treatment and detection. This receptor is expressed in a large percentage of ovarian cancer cell lines and cells isolated from ascites fluid collected from ovarian cancer patients. Binding of Müllerian Inhibiting Substance (MIS) to MISIIR on the surface of these cells initiates a cytotoxic effect and has led to the proposed development of MIS as a therapeutic agent for the treatment of ovarian cancer. We postulated that antibody-based molecules directed against the ligand-binding site on MISIIR could exhibit a similar cytotoxic effect with a significantly prolonged bioavailability. Additionally, anti-MISIIR antibodies could be used as vehicles to selectively target agents to ovarian tumors.

We have isolated the gene for the MISIIR extracellular domain (ECD) and have expressed the ECD in mammalian cell lines. The MISIIR ECD was then used as a target to isolate (pan) anti-MISIIR human single-chain Fv (scFv) molecules from a large non-immune phage display library. Genes encoding 12 distinct anti-MISIIR scFv molecules were isolated and used to engineer higher avidity dimeric scFv molecules known as diabodies and fusion proteins with human IgG1 Fc domains (scFv-Fc). The anti-MISIIR scFv and diabodies were expressed in *E. coli* and were secreted, fully folded and functional, into the periplasm. The genes encoding the scFv-Fc were ligated into an expression vector and used to stably transfect HEK cells.

The ability of the expressed proteins to specifically bind to recombinant MISIIR ECD on the BIAcore instrument and to native MISIIR expressed on the surface of ovarian cancer cells growing in culture was assessed. scFv and scFv-Fc forms of the two lead clones, #17 and #7, were radioiodinated and their ability to target established s.c. human ovarian cancer tumors growing in immunodeficient mice was determined. In these studies we found that there was no detectable tumor retention of the scFv molecules at 24 hours after i.v. administration. In contrast, the scFv-Fc forms of both molecules exhibited substantial retention in the tumors 48 hours after administration.

Developing anti-MISIIR antibodies as agents for the immunodetection and immunotherapy of ovarian cancer. Adams, Simmons,[§] Yuan,[§] Robinson,[§] Weiner

We hypothesize that high-affinity engineered anti-MISIIR antibodies will efficiently target ovarian cancer, and that these molecules could directly inhibit the growth of ovarian tumors. Furthermore, these antibodies could be effectively employed to deliver imaging radionuclides, therapeutic radionuclides, drugs and catalytic toxins to sites of disease. We are currently screening all of the anti-MISIIR scFv molecules to identify the clones that are efficiently and specifically internalized into tumor cells that express the target. These will be employed in the development of anti-MISIIR immunoconjugates based upon these scFv. The antibodies that do not internalize will be labeled with positron-emitting radioisotopes and developed as ImmunoPET imaging agents.

Molecular-Targeted Therapy of Ovarian Cancer

Andrew K. Godwin, Ph.D., *Project Leader*; Russell J. Schilder, M.D., *Project Co-Leader*, in collaboration with Louis M. Weiner, M.D., Gregory P. Adams, Ph.D., Anthony T. Yeung,[§] Ph.D., Michael Ochs,[§] Ph.D., Albert Wong,^b M.D.

An Inter-SPORE phase II clinical trial employing single-agent cetuximab in women with persistent or recurrent epithelial ovarian or primary peritoneal carcinoma. Schilder, Godwin

The ability of the epidermal growth factor receptor (EGFR) to transform epithelial cells,

the over-expression of EGFR and its ligands in several human carcinomas, and the causal association of the receptor network with accelerated tumor progression provided a rationale for targeting this signaling system with tumor-selective strategies. Two of these anti-receptor

approaches, both based on the known structure/function of the EGFR have been recently exploited in clinical trials. The first strategy involved the development of humanized monoclonal antibodies against the non-conserved receptor's extracellular domain. These antibodies block ligand binding and can induce receptor down-regulation. The second approach was to inhibit the generation of adenosine triphosphate for binding to the receptor's kinase pocket and disable the ability of the EGFR to transduce intracellular signals. Early clinical studies already suggest that both of these approaches, either alone or in combination with standard anticancer therapies, can alter the natural history of EGFR-expressing cancers with little toxicity to the tumor-bearing host.

Cetuximab has garnered much attention and has shown promise in several solid tumors that express EGFR. Clinical efficacy of cetuximab appears to involve multiple mechanisms, including inhibition of cell cycle progression, induction of apoptosis, inhibition of angiogenesis, inhibition of metastasis, and enhancement of the response to chemotherapy and radiation therapy. Phase I studies of cetuximab combined with chemotherapy or radiation showed promising response rates in patients with recurrent or refractory squamous cell carcinoma of the head and neck. Phase II and III trials to examine the efficacy and safety of these combinations are currently underway in several solid tumor types. To date, cetuximab has been well tolerated, with skin rashes and allergic reactions being the most clinically important adverse events reported. Cetuximab displays dose-dependent elimination characteristics and a half-life of approximately seven days. The Inter-Ovarian SPORE trial (including Fox Chase Cancer Center and five other institutions) in ovarian cancer patients is designed to test the hypothesis that cetuximab exerts its anti-tumor effects through a mechanism that involves the perturbation of HER1/EGFR signaling via the AKT and/or MAPK pathways, leading to impaired tumor cell proliferation and apoptosis. In addition, we will establish the response rates, duration of response, and time-to-progression in patients given Cetuximab as an intravenous infusion at a loading dose on Day 1 followed by weekly maintenance doses which will be titrated every other week until Grade 2 acneform skin rash toxicity is reached. Prior data

suggested that a lack of a rash is a good predictor for no response; however, it is not clear if the lack of rash in these patients is due to an inadequate exposure, i.e., dose or a true lack of activity. Therefore, titration to a grade 2 rash will insure delivery of a biologically active dose.

We are evaluating newer targeted therapy agents against ovarian cancer in preclinical models. AMG 706 is a multi-targeted TK inhibitor. It inhibits all known VEGFRs, PDGFR, c-KIT and c-RET. VEGF and PDGF are known key growth factors for ovarian cancer. We are testing this agent alone and in combination with chemotherapy and biological agents such as Cetuximab. This drug will soon enter clinical trials.

Similar preclinical studies are being initiated with dasatinib. This compound is a SRC kinase inhibitor which is crucial to signal transduction and is commonly activated in ovarian cancer. There appears to be at least additive if not synergistic activity with this class of drug and paclitaxel. Ultimately, this agent also will enter clinical trials.

Gene expression patterns of ovarian cancer cells in response to cetuximab. Godwin, in collaboration with Zhang,[§] Pan,^c Vanderveer,[§] Birrer^d

The efficacy of anticancer drugs varies among individual patients. A large proportion of cancer patients suffer adverse effects of anti-cancer therapy while showing no effective response in terms of tumor regression. Prediction of effectiveness before treatment cannot be done at present, with some exceptions, such as tamoxifen treatment for patients with estrogen receptor positive breast cancer. Numerous investigators have attempted to establish a diagnostic method for predicting chemosensitivity, and a few markers have been identified. However, properties of cancer cells are determined by complicated interactions among all gene products expressed in cancer cells, and it is certain that many proteins, including enzymes involved in apoptosis, DNA repair, and metabolism and detoxification of drugs, affect individual responses. Hence, to distinguish responders from nonresponders before starting treatment, i.e., to offer a patient-specific, "personalized" medical treatment and also to relieve patients from unnecessary side effects, an informative panel of genes must be identified to serve as accurate predictive markers.

EGFR is over-expressed in approximately 50% of ovarian cancers and EGFR targeted therapies such as cetuximab are currently being evaluated in phase II clinical trials. As a first step to further understand the molecular mechanism of tumor response to EGFR-based therapy, we have employed a xenograft animal model to study the gene profiling of ovarian cancer cell lines before and after drug treatment. Over 600 genes with statistically significant alterations at mRNA level have been identified by microarray analysis, including PPAR binding protein (PPARBP) and insulin-like growth factor binding protein 6 (IGFBP6). These genes are components of various signaling pathways and many of them have been demonstrated to be involved in tumor development and progression. Our present focus is to validate interesting targets by real-time PCR and explore their tumorigenic roles *in vitro* and *in vivo*. In the near future we hope to be able to identify essential pathways that mediate EGFR-induced ovarian tumor growth and distinguish the genes that are predictive of response prior to therapy.

EGFR mutations correlate with therapeutic response. Schilder, Godwin, in collaboration with Chen,[§] Armstrong,[§] Arciero,[§] Wu,[§] Gynecologic Oncology Group (GOG)^e

Tyrosine kinases (TKs) regulate signaling pathways that control critical cellular activities. When overexpressed or activated by mutations, TKs can contribute to the development of cancers. If tumor cells depend on a mutant TK for survival then the mutated enzyme can fortuitously serve as an Achilles' heel for cancer therapy. Recent studies have shown that somatic mutations in the tyrosine kinase domain of the *EGFR* gene are associated with sensitivity of lung cancers to gefitinib (ZD1839, IressaTM), an anilinoquinazoline that reversibly competes with ATP at a critical ATP-binding site (lysine 745) in EGFR. What is not currently known is whether other cancers, including ovarian, possess certain EGFR mutations that contribute to tumorigenesis and can thereby be a target for therapy. Therefore, we examined archived tumor tissue from a phase II trial designed to assess the activity and tolerability of gefitinib in patients with recurrent or persistent ovarian carcinoma or primary peritoneal cancer. Four of the 27 patients on trial had a progression-free survival (PFS) \geq six months including one partial responder. We hypothe-

sized that mutations in EGFR may exist that are associated with response. In this blinded study, we found that the patient who experienced the only objective response on this trial, which lasted 23 months, had a mutation in the catalytic domain of EGFR. By comparison, no mutations were observed in 23 of the cases for which DNA was retrievable and who had no measurable response to gefitinib ($P < 0.05$ by an exploratory one-sided Fisher's exact test). Given the apparent clustering of *EGFR* mutations based on previous studies of NSCLC, we sequenced exons 18–21 in an additional 32 ovarian tumors not treated with gefitinib. The majority of the tumors were late stage serous adenocarcinomas (21 papillary serous, 4 mucinous, 2 endometrioid, 2 undifferentiated, 2 mixed Müllerian, and 1 clear cell). One additional sample was found to have a mutation (3.1%; 1 of 32). What was particularly unique in comparison with the data from the reports regarding lung cancer was our observation that the ovarian tumors were homozygous for their mutations. This was the first report that found such a mutation in ovarian cancer. Overall, we detected mutations in the tyrosine kinase domain region in 2 of 56 (3.6%) of ovarian adenocarcinomas and observed that a patient on the clinical trial with a mutation in the catalytic domain of EGFR responded to gefitinib, suggesting a method to preselect a subset of patients whose tumors may be more responsive to this EGF receptor-targeted therapy. We are currently evaluating tumor specimens from several phase II clinical trials of ovarian and endometrial cancer in which EGFR is the primary therapeutic target (gefitinib-E. Kohn, NCI; gefitinib-K. Leslie, GOG-0229C; lapatinib-A. Garcia, GOG-0170G; taxol/carboplatin and tarceva-S. Blank, New York Medical Center) for EGFR gene amplification and/or mutations.

Expression of EGFRvIII in human ovarian and therapeutic targeting. Godwin, in collaboration with Chen,[§] Slater,[§] Connolly,[§] Klein-Szanto, Hamilton, Wong^b

Many malignant cells have been shown to express mutated epidermal growth factor receptor (EGFR). EGFRvIII is a naturally occurring and tumor specific variant of the EGF receptor (EGFRvIII) that was initially identified in glioblastoma multiforme. This mutation is the result of the in-frame deletion of

exons 2–7 producing a juxtaposition of ordinarily distant sequences and is due to a gene rearrangement in these brain tumors. This specific mutation has been reported to be expressed only in tumor tissue. In comparison to EGFR, the tumor specific expression character makes EGFRvIII an ideal target for tumor therapies. Previous studies have shown that expression of EGFRvIII promoted aggressive growth of tumor cells *in vitro* and *in vivo*. A relationship between expression of EGFRvIII and poor prognosis in glioblastoma has been reported. Clinical trials based on a peptide vaccine have shown statistically significant results for time to progression and survival in glioblastoma patients.

EGFRvIII has been reported in several other types of cancer, including breast, ovary, prostate and lung cancer. Studies to confirm the presence and significance of EGFRvIII transcript and protein in these cancers, however, are still controversial. Two recent studies have reported conflicting results in regards to the EGFRvIII expression in breast cancer. To confirm our early immunohistochemistry results and ascertain EGFRvIII mRNA expression in human ovarian tumors and also further explore the EGFR and EGFRvIII expression in colon tumors, we applied multiple transcript and protein assays to detect and evaluate the expression of EGFR in a tissue microarray of human ovarian and colon tumor specimen. These results confirmed the presence of EGFRvIII in both ovarian and colon cancers. Several factors that impede identification of the EGFRvIII in RT-PCR were identified and a novel one step RT-PCR method was developed to rapidly identify this variant. We believe that these findings will have significant implications in the studies of EGFRvIII in cancer research, diagnosis, and treatment. The fact that vaccination has been effective in glioblastoma patients raises the prospect of effectively treating ovarian cancer patients with the same vaccine.

Monoclonal antibodies that specifically target

EGFRvIII were also evaluated for their anti-tumor efficacy and compared against IMC-225. When used against cells that only express EGFRvIII, none of these agents showed any significant effect on cell growth in tissue culture. However, when these antibodies were used against tumors in animals, all of the antibodies were effective with those directed against EGFRvIII being more effective than IMC-225. An analysis of the effect on EGFRvIII expression and signal transduction proteins revealed that these antibodies did not have a distinct effect on reducing levels of these proteins. Instead, the mechanism of action seemed to be related to antibody dependent cellular cytotoxicity.

To further these studies we are deriving transgenic mice that express EGFR or EGFRvIII cDNA under the transcriptional control of the MISII receptor promoter in order to obtain ovarian epithelial specific expression. These mice are being derived as part of the transgenic core of this Ovarian Cancer SPORE. Seven pMISIIR-EGFRvIII transgenic founders have been identified and are currently being bred for further analyses. When fully developed, this model will be a powerful tool for examining potentially relevant therapies in the treatment of ovarian cancer. Our initial focus is on LEEK (ALT-110), a peptide vaccine to the mutant form of EGFRvIII. LEEK is a 14 amino acid peptide that corresponds to the deletion junction seen in EGFRvIII and an IND was approved by the Food and Drug Administration in June 2002 for the treatment of solid tumors that express EGFRvIII, which includes ovarian, brain, breast, lung, and prostate tumors. Recent studies have shown efficacy in a phase I trial of glioblastoma (G.E. Archer et al., *Neuro-Oncology* 6:341, 2004).

Overall, these studies should shed light on clinically relevant aspects of tumor biology, tumor immunology and on the clinical pharmacology of potentially important therapies in the treatment of ovarian cancer.

Immunotherapy of Ovarian Cancer: Breaking Down the Barriers

George Coukos,^f M.D., Ph.D., *Project Leader*; Carl June,^f M.D., *Project Co-Leader*,
in collaboration with Christina Chu,[§] M.D., Michael Bookman,[§] M.D.

Central to the success of immunotherapy is the abrogation of tumor-initiated immune evasion mechanisms. We found that most patients with advanced ovarian carcinoma have expanded

populations of regulatory T cells (T_{REG}) in the blood, tumor and ascites, and we demonstrated that T_{REG} cells profoundly suppress the ability of effector T cells to mount an antitumor

immune response *ex vivo*. Furthermore, we found that vascular endothelial growth factor (VEGF) inversely correlates with the presence of intratumoral T cells in ovarian carcinoma both in the human and in the mouse; it induces paralysis of antigen presentation mechanisms and prevents the engraftment of effector T cells in the tumor. We hypothesize that these two barrier mechanisms need to be circumvented in order to enhance the efficacy of T cell-mediated immunotherapy

To fully understand the therapeutic potential of tumor-associated T cells in ovarian cancer, we undertook a detailed characterization of T cells in ovarian cancer and examined the supporting tumor recognition, and the trafficking and accumulation of T cells in ovarian cancer. We previously described a novel mechanism by which ovarian carcinoma promotes the intratumoral expansion of T cells through the expression of ligands to the NKG2D immune receptor (J.R. Conejo-Garcia et al., *Cancer Biol. Ther.* 2:446, 2003; J.R. Conejo-Garcia et al., *Cancer Res.* 64:2175, 2004). Recently we found that intratumoral T cells are associated with a significantly higher frequency of tumor-infiltrating CD45RO⁺ memory T cells and higher mRNA levels of IFN- γ (10-fold \uparrow) and IL-2 (26-fold \uparrow), suggesting T cell activation. In addition, a significant correlation between the presence of intratumoral T cells and the detection of peripheral blood T cells recognizing tumor antigens in 10 consecutive patients with ovarian cancer studied. We compared the TCR V β repertoire detected in tumor islets to matched surrounding peritumoral stroma, in 10 tumors with intratumoral T cells. Laser capture microdissection (LCM) was used to procure pure specimens from each compartment. PCR-based TCR spectratyping demonstrated the presence of oligoclonal expansion of intratumoral T cells, strongly suggesting antigen-induced local expansion of TILs in tumor islets. We also demonstrated a significant correlation between the presence of intratumoral T cells in tumors and the detection of MHC-I restricted, IFN- γ secreting, TAA-specific T cell precursors in peripheral blood in chemotherapy-naïve patients with Stage-III ovarian cancer. Collectively, these data suggest that tumor-infiltrating T cells in select patients with ovarian cancer exhibit antitumor activity *in vivo*.

To further explore the feasibility of adoptive

cell transfer therapy for ovarian carcinoma using TILs or tumor associate lymphocytes (TALs), we examined blood, ascites, and tumor tissue from 21 patients with Stage III ovarian cancer. Phenotypes of lymphocyte subpopulations of PBL, TAL and TIL were analyzed with FACSCanto and FlowJo. IFN- γ ELISpot and ELISA were used to assess T cell status.

Nearly all TALs and TILs in this study were CD45RA⁻ and CCR7^{hi} or CCR7^{lo}, consistent with central memory and effector memory phenotypes. Around 40% of TALs expressed high levels of CD25, CD69, CD107a and low levels of CD27, indicating antigen-experienced cells. A significant frequency of HER-2-specific (up to 4% for specific nonamers) and CD107a⁺ TALs (up to 7%) were detected in many specimens, indicating TAA-specific T cells with cytolytic activity. To investigate the possibility of *in vitro* expansion of tumor-infiltrating lymphocytes, mixed ascites cells were incubated in high dose rhuIL-2. Significant T cell expansion was noted in these cultures with 1-2 log amplification within two weeks. Significant IFN- γ expression was found in these expanded lymphocytes by ELISpot and ELISA compared to normal lymphocytes. Expanded lymphocytes also showed IL-2 expression in 20–50% of CD4⁺ and 20–30% of CD8⁺ T cells. Approximately 50% of amplified CD4⁺ and CD8⁺ T cells exhibited a central memory phenotype. These preliminary data provide encouraging information for the development and optimization of adoptive T cell therapy in human ovarian cancer. To that end we are planning a trial with adoptive lymphocyte therapy.

We have begun exploring biological therapeutics to maximize accumulation of T cells in tumors through expression of inflammatory chemokines. We employed replication restricted oncolytic herpes virus HSV-1716. Intratumor injection of HSV-1716 induced expression of IFN- γ , MIG and IP-10 both at the levels of RNA and protein. This was accompanied by an increase in the numbers of tumor-associated NK and T CD8⁺. These cells expressed high levels of CXCR3 and CD25, indicating an activated status. Murine monocytes and dendritic cells (DCs) were responsible for inflammatory cytokine and chemokine production upon HSV-1716 infection. This was partially abrogated by neutralizing antibodies against IFN α and β , thus indicating a role of

type-1 IFNs in the reported effect. Moreover, ascites from HSV-1716-treated animals efficiently induced *in vitro* migration of NK and CD8⁺ T cells, and this effect was dependent on the presence of MIG and IP-10 in the supernatants. Finally, human ovarian carcinomas showed high levels of monocytes and DC infiltration. Upon HSV-1716 infection, human monocyte-derived DCs produced large amounts of IFN- γ and upregulated MIG and IP-10 expression (F. Benencia et al., *Mol. Ther.* 12(5):789, 2005).

During the past year we have constructed and begun to characterize lentiviral vectors expressing Chimeric Immunoreceptors (CIR) composed of a high-affinity single-chain Fv specific for mesothelin, and the cytoplasmic signaling modules of TCR, CD28 and 4-1BB. The constructs have been authenticated by sequencing, and by transfection into 293 T cells. In initial testing, we have determined the ability of primary T cells expressing the CIR to kill A431 cells that express mesothelin. Approximately 99% killing is observed at an

E:T ratio of 1:2 by the construct that expresses the ScFv:4-1BB: TCRzeta tail. This is encouraging, and we are currently comparing this CIR to the CD28:TCRzeta construct.

We have previously reported on the presence of CD4⁺CD25⁺ regulatory T (T_{REG}) cells in ovarian cancer. We showed, in detailed studies of CD4⁺CD25⁺FOXP3⁺T_{REG} cells in 104 individuals affected with ovarian carcinoma, that human tumor T_{REG} cells suppress tumor-specific T cell immunity and contribute to growth of human tumors *in vivo* (T.J. Curiel et al., *Nat. Med.* 10:942, 2004). A trial was designed to test the hypothesis that systemic cyclophosphamide depletes T_{REG} cells and generates a favorable immunological status for therapeutic dendritic cell (DC) vaccine therapy. We will answer this question by testing in a clinical trial (in progress) whether cyclophosphamide amplifies the anti-self response to her2/neu and the human telomerase reverse transcriptase (hTERT) in patients vaccinated with mature dendritic cells and well defined tumor-associated antigenic peptides.

Methylomics for Detection and Profiling of Ovarian Cancer in Serum

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Promoter hypermethylation is a valid target for sensitive and specific early detection of ovarian cancer in serum. We have obtained matched tumor, pre-operative serum or plasma and peritoneal fluid (washes or ascites) DNAs via the Biosample and Tissue Procurement Core from 50 patients with ovarian or primary peritoneal tumors and 10 patients with benign cysts of the ovary. Microdissected tissue DNA and body fluid DNA was analyzed by conventional gel-based methylation specific PCR for hypermethylation status of the normally unmethylated *BRCA1* and *RAS association domain family protein 1A (RASSF1A)*, *adenomatous polyposis coli (APC)*, *p14^{ARF}*, *p16^{INK4a}* or *death associated protein-kinase (DAP-Kinase)* tumor suppressor genes. Hypermethylation of one or more of the gene panel was found in all 50 tumor DNAs (100% diagnostic coverage) and in all histological cell types, grades and stages of ovarian tumor examined. An identical pattern of gene hypermethylation was found in the matched serum DNA from 41 of 50 patients (82% sensitivity) including 8 of 8 cases of Stage I disease and 5 of 9 patients with tumors of low

malignant potential. Hypermethylation was detected in 27 of 29 peritoneal fluid DNAs from Stage IC-IV patients, including 3 cases with negative or atypical cytology. In contrast, no hypermethylation was observed in non-neoplastic tissue, peritoneal fluid or serum from control women. (I. Ibanez de Caceres et al., *Cancer Res.* 64:6476, 2004). We have identified additional Stage I disease patients as well as age-matched, healthy normal controls and patients with benign disease and will screen tissue and matched serum DNAs for hypermethylation of the panel of tumor suppressor genes. We propose to use the more powerful quantitative real time MSP also known as "Taqman" or "Methyl-light" for detection of hypermethylation in serum. This technology can increase the level of detection to 1 in 10,000 and will establish a threshold value for positive methylation that can be used as a reference in other laboratories thereby facilitating independent testing of the assay. The combined diagnostic coverage and sensitivity of the panel will be calculated using this technology in a larger cohort of Stage I patients and controls. We will also collect

a follow-up serum from 25 of the 100 patients with no clinical or biochemical evidence of disease several weeks or months after undergoing oophorectomy for organ-confined disease and whose tumor and pre-operative serum showed hypermethylation. We will test whether the clinical absence of disease is mirrored by absence of hypermethylation in the follow-up serum.

Global signature of genes silenced by epigenetic mechanisms in ovarian cancer. To generate a global signature of genes silenced by hypermethylation in epithelial ovarian cancer, we performed an expression microarray-based analysis of genes reactivated in tumor cell lines after treatment with the demethylating drug 5Aza-2 deoxycytidine (5Aza-dC) and histone deacetylation inhibiting drug trichostatin A (TSA). We first determined the optimal drug treatment level, then analyzed the global expression pattern of the Agilent 44k whole genome oligoarray in drug-treated versus untreated ovarian tumor lines A2780, SKOV3, OVCAR 3, 5 and 10. Between 100 to 150 genes were found to have at least 3-fold upregulation of expression after treatment in each cell line. Gene Ontology was used to interrogate the upregulated gene data by signaling pathways and function. Our tested and successful algorithm was employed for validation. Briefly, upregulated genes were examined for evidence of expression in normal ovary by analysis of the

Cancer Genome Anatomy Project (CGAP) Serial Analysis Gene Expression (SAGE) database and presence of a promoter CpG island proximal to the transcription start site. RT-PCR was used to confirm the upregulation seen on the array. Methylation in the ovarian cell lines was then confirmed by bisulfite sequencing and methylation in primary tumors confirmed by MSP analysis of primary tumor DNAs. To date, we have identified five candidate tumor suppressor genes with promoter methylation in tumor cell line DNA but which were unmethylated in normal cell DNA. We will determine the frequency of methylation of each new gene in at least 50 (15 LMP, 15 Stage I, 20 advanced stage) primary tumors. Further study of the genes identified by our screen will provide insight into the biology of the disease and facilitate molecular diagnostic, prognostic and therapeutic studies in ovarian cancer.

Hypermethylated genes with utility for profiling the biological behavior of individual tumors. Data from microarray analysis will be interrogated with respect to biological differences in tumor cell line behavior, e.g. cisplatin sensitivity of the cell lines, and identified validated genes. We then plan to examine the predictive utility of the new hypermethylated genes in a retrospective study of patients identified, and stratified by response and outcome, from the Fox Chase ovarian cancer patient databases of drug response and outcome.

Developmental Research Projects

Evaluation of soluble mesothelin related protein (SMRP) as a diagnostic marker of ovarian cancer. Altomare,[§] in collaboration with Testa, Godwin,[§] Daly,[§] Litwin[§]

Our hypothesis is that soluble mesothelin-related protein (SMRP) represents a diagnostically useful marker that is associated with ovarian tumor development and can be used in combination with CA-125 to detect early ovarian cancer. We propose to validate whether a diagnostic ELISA for SMRP can accurately measure, with specificity, sensitivity and reproducibility, elevated SMRP levels in blood from ovarian cancer patients compared to normal controls. Because ovarian cancer and malignant mesothelioma arise from cells from the mesothelial lining, a set of previously tested sera was provided by the manufacturer of the

kit in a blinded study consisting of samples from 20 mesothelioma patients and 20 controls (healthy donors). Converted concentration values of the sera showed a strong linear correlation between our readings and the manufacturers ($R^2=0.9657$). Studies are underway to test the sera of ovarian tumor patients and to establish baseline cut-off values for determining concentrations of SMRP that correlate with a diagnosis of cancer. Following validation studies, we plan to use banked serum collected from individuals at high risk of developing ovarian cancer to determine if SMRP levels correlate with CA-125 levels, and if these tumor markers, alone and in combination, change over time. The long-term objective is to determine whether elevated SMRP levels permit early detection of ovarian cancer. The significance of

these studies is that a finding of a raised SMRP concentration in an at-risk individual could be followed by proactive, aggressive attempts using clinical modulator to find as ovarian cancer.

Vascular leukocytes as novel therapeutic targets against cancer. Coukos,^f Conejo-Garcia^g

We have identified a novel mechanism of tumor vasculogenesis in ovarian cancer. In this process, bone marrow dendritic cell precursors, massively recruited by beta-defensin in the tumor, are induced by tumor VEGF-A to undergo endothelial-like transdifferentiation and build blood vessels. We named these cells "vascular leukocytes" (VLCs) (J.R. Conejo-Garcia et al., *Nature Med.* 10(9):950, 2004; J.R. Conejo-Garcia et al., *Blood* 105(2):679, 2005; G. Coukos et al., *Br. J. Cancer* 92(7):1182, 2005). The central hypothesis of this project is that VLCs play a critical role in ovarian cancer development and growth, and they are a source of important molecular biomarkers and represent optimal therapeutic targets.

To evaluate the relative contribution of VLCs to ovarian tumor vascularization and the efficiency of depleting therapies, we used ITGAX-DTR-GFP transgenic (Tg) BL6 mice (Jackson Laboratories). In these mice, a single intraperitoneal dose of diphtheria toxin (DT) induces rapid reduction of CD11c⁺ cells. Reconstitution of the CD11c⁺ population requires 7 days to complete. ITGAX-DTR-GFP mice were backcrossed with wild-type BL6 females. ID8-Defb29/VEGF tumors were injected in their progeny. GFP-positive dendritic cells were recruited to the tumors and were found to engage in vasculogenesis. ID8-Vegf/Defb29 tumors grew similarly in wt mice receiving DT injection or PBS. We have demonstrated that >90% VLCs express CD11c. Targeting of these cells with a single injection of DT at the time of inoculation of s.c. flank ID8-Vegf/Defb29 tumors was well tolerated and resulted in 65% reduction in tumor growth compared to ITGAX-DTR/GFP Tg mice receiving PBS (p<0.05). Two DT injections on day 2 and 9 following tumor transplantation resulted in death of 60% of the mice. However, surviving animals exhibited tumor rejection at 100%. These results indicate that CD11c cells are critical to tumor development and growth.

To discover markers that are specific to VLCs, we have generated single cell suspen-

sions from 6 different fresh human epithelial ovarian tumor specimens. The specimens were sorted according to their expression of CD45 (pan-leukocyte marker) and VE-Cadherin (endothelial-specific marker). Three clearly distinctive populations were produced from all the specimens: 1) A population of CD45⁺VE-Cadherin⁻ cells (bona fide leukocytes); 2) a subset of CD45⁻VE-Cadherin⁺ cells (canonical endothelial cells); 3) and a population of CD45⁺VE-Cadherin⁺ cells, identified as Vascular Leukocytes (VLCs). cDNA arrays will be performed with the three subsets from the different samples.

The three-dimensional signaling pathway and its implication on the study of ovarian cancer.

Cukierman,[§] Quiros,[§] K.M. Brown[§]

The manner by which tumors overcome the normal stromal barrier inducing stromal changes, which promote, rather than impede, tumor progression is not well understood but presumably involves changes in the predominant stromal cell-type fibroblasts that produce and modify the stromal extracellular matrix. To that end, we proposed to develop an *in vivo*-like 3D stromal system derived from human tumor-associated ovarian fibroblasts. On a related project comparing normal and tumor-associated murine stroma, we have observed structural and biochemical alterations in fibroblasts and their matrices. Thus, we hypothesize that altered stromal signal transduction, contributes to the tumor promoting properties of the ovarian stroma. Tumor-associated fibroblasts obtained from fresh ovarian tumor samples show characteristics reminiscent of desmoplastic matrices observed in another study (M.D. Amatangelo et al., *Am. J. Pathol.* 167:475, 2005). Their stromagenic signal transduction is being characterized, and the resultant matrices will be evaluated for ovarian cancer permissiveness in a novel 3D *in vivo*-like invasion assay.

Serum Müllerian inhibiting substance: assay reproducibility and within person variation.

Dorgan,[§] Spittle[§]

Müllerian inhibiting substance (MIS) is a member of the transforming growth factor- β (TGF β) family of growth and differentiation factors that causes regression of the Müllerian ducts in male fetuses. Epithelial ovarian cells derive from the same embryonic tissues as the

Müllerian ducts, and MIS secreted by adult ovarian granulosa cells could potentially inhibit development of epithelial ovarian tumors. We will evaluate the association of prediagnostic serum MIS levels with subsequent development of epithelial ovarian cancer in a prospective cohort study. We hypothesize that women with higher MIS levels are at a decreased risk for development of these tumors. The aims of this study are to: 1) quantify the reproducibility of a commercially available ELISA (enzyme-linked immunosorbent assay) to measure MIS in serum; and 2) determine if a single measurement of serum MIS adequately reflects longer term levels to characterize individuals.

Briefly, to evaluate assay reproducibility, MIS was measured in 4 serum aliquots from the same blood draw from each of 5 women on 4 different days using different MIS ELISA kits.

Serum was collected from 5 healthy women without a history of cancer (other than non-melanoma skin cancer). Because the ovaries cease secreting MIS after menopause, participants were restricted to premenopausal women 30–45 years old with regular menstrual cycles.

MIS was measured in serum samples using a MIS ELISA Kit. Results of this study indicate that the MIS ELISA is reproducible. Furthermore, assay variability is small relative to between person variability and the assay should adequately discriminate individuals in epidemiologic studies.

Evaluating the potential use of vaccines in the treatment of epithelial ovarian carcinoma using a mouse model. R-H. Xu,[§] Sigal[§]

CD8⁺ T cells are capable of killing virus infected cells and tumors. This requires the tumors or infected cells to display at their surface, antigens recognized by specific CD8⁺ T cells. These antigens are small peptides (epitopes) derived from the degradation of a viral or tumor protein bound to MHC class I molecules. In non-immunized mice, there are only a few CD8⁺ T cells specific for each given epitope. Therefore, to exert their function, they must proliferate extensively and reach very high numbers. This expansion of antigen specific CD8⁺ T cells can be induced by immunization and its extent depends of the strength of the antigenic stimulus and of inflammatory signals. In general, the CD8⁺ T cell responses

induced by tumors are weak while those generated by viruses are very strong. The goal of this project is to determine whether Epithelial Ovarian Carcinoma (EOC) can be cured by tumor-specific CD8⁺ T cells and whether the magnitude of the CD8⁺ T cell response is important for the effectiveness of the therapy. For this purpose we need an antigen to which we can generate strong CD8⁺ T cell responses. While we do not know of any EOC specific antigen, we took the alternative approach of experimentally introducing into EOC a CD8⁺ T cell antigen for which a strong CD8⁺ T cell response can be induced. For this purpose we transfected ID8 cells (K.F. Roby et al., *Carcinogenesis* 21:585, 2000) with the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) to generate ID8-GP cells. In this tumor model, GP is used as the tumor antigen and CD8⁺ T cell responses to GP are induced by immunization with either LCMV or recombinant vaccinia virus (VACV) expressing GP (VACV-GP). GP contains one of the immunodominant CD8⁺ T cell epitopes from LCMV (known as GP33) in C57BL/6 (B6) mice. Initial analysis of ID8-GP cells showed that they express GP33-MHC class I complexes at the cell surface. Also, we found that similar to the ID8 parent, ID8-GP formed intraperitoneal tumors when inoculated into B6 mice. We recovered these intraperitoneal tumors and found that they maintained expression of GP33-MHC class I complexes.

Infection of mice with LCMV elicits an extremely strong CD8⁺ T cell response. During the acute phase of the infection, ~30% of the total CD8⁺ T cells in a B6 mouse are specific for GP33. VACV-GP also induces an anti-GP33 response but in this case, the magnitude of the response is 30-60 fold smaller than that induced by LCMV. Initially, we performed experiments to determine whether immunization can prevent the establishment of tumors. Mice were immunized with LCMV or VACV-GP and one month later they were challenged with ID8 or ID8-GP cells that were grown in tissue culture. We found that either LCMV or VACV-GP immunization prevented the growth of ID8-GP but not of ID8 cells. In addition, immunization with wild type VACV did not prevent the growth of ID8-GP cells. Therefore, CD8⁺ T cell immunizations can prevent EOC independently of the strength of the CD8⁺

T cell response. Interestingly, when we challenged the mice with ID8-GP cells rescued from peritoneal tumors, vaccination did not prevent the formation of tumors. This indicates that the cells became resistant to CD8⁺ T cell killing through a mechanism that was different than the loss of MHC class I or antigen expression.

We next determined whether immunization could cure established tumors. Mice were challenged with ID8-GP cells and ten days later were immunized with LCMV or VACV-GP. Regrettably, while immunization induced some delay in the growth of the tumors, all the tumors finally grew and the animals had to be sacrificed. Tumor growth was not due to tumor-induced CD8⁺ T cell tolerance because we could still

detect very strong CD8⁺ T cell responses to GP33 in tumor-bearing mice. Also, the decrease in tumor growth induced by immunization was not antigen-specific because immunization with wild type VACV also resulted in delayed growth. Because we immunized the mice intraperitoneally, we speculate that the delayed growth was due to the inflammation that the viral infection produced in the peritoneal cavity. Therefore, immunization cannot cure established EOC in spite of the tumor not losing expression of the antigen and the very strong CD8⁺ T cell responses induced. This seems to indicate that once established, EOC becomes resistant to the action of CD8⁺ T cells. Future work will determine the mechanism of this resistance.

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