451 POSTER

Irradiation-driven tumor selectivity of liposome-based gene therapy targeting Akt activation

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The vascular network is a highly accessible target for tumor therapy. However, as for any cancer treatment, the primary goal is to deliver sufficient amounts of drug to the targeted tissue while minimizing damage to healthy organs. Cationic liposomes have been identified as delivery systems significantly more effective at targeting tumor *versus* normal vascular networks. Though, the liposome uptake by the liver restricts their potential as shuttles to selectively target the tumor endothelium. Here, we reasoned that additional selectivity could be found in the nature of the delivered gene and by combining another anti-tumor therapy. Accordingly, the pro-survival PI3K /Akt pathway is thought to be activated by ionizing radiations and a dominant-negative Akt would therefore mostly target tumors (versus non-irradiated organs). Furthermore, we have recently documented that irradiation led to NO-mediated tumor vessel dilation which could thereby enhance the access of liposomes to the tumor. In this study, we have therefore examined whether radiotherapy and the use of dominant-negative Akt plasmid delivered by cationic liposomes could mutually improve their efficacies.
We first used cultured endothelial cells and isolated tumor microvessels to demonstrate that low dose irradiation led to the stimulation of both Ser^{473} Akt and Ser^{1177} eNOS phosphorylations (e.g., activation); the use of a PI3K inhibitor further indicated that the former largely accounted for the increased NO/cGMP production. Using a reporter-encoding plasmid, we then showed that irradiation dramatically enhanced the *in vivo* expression of the tagged protein in the tumor endothelium. Also, using $eNOS^{-/-}$ mice, we documented the key role of NO in mediating the adjuvant effects of X-Ray on plasmid delivery, likely through an increase in tumor blood flow. We then combined local irradiation to the liposome-dominant negative Akt DNA complex administration. In two different protocols associating gene therapy with either a single 6 Gy dose or a 5×2 Gy fractionated scheme, we consistently observed synergistic effects of the combinatory treatment. In fact, the transgene when administered alone, did not reveal any tumor response and the tumor growth delay after irradiation represented less than 50% of the gain obtained when combining both approaches (n=8; $P<0.01$); these findings were obtained in two mouse tumor models.
In conclusion, the combination of low dose radiotherapy and liposome-cargoed dominant-negative Akt gene therapy appears particularly well suited to selectively target tumor vasculature. Besides the intrinsic tumor specificity of local X-Ray administration and the propensity of cationic liposomes to bind tumor vessels, we have further identified a "treatment-driven" selectivity, e.g. the ability of radiotherapy to induce Akt activation in tumor vasculature and to increase the liposome access to the tumor.

452 POSTER

The oncolytic reovirus, Reolysin, augments the anticancer effects of cytotoxic agents in vitro against the ras-mutated human colon cancer cell line HCT116

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The type 3 reovirus, Reolysin, is an oncolytic virus with selectivity for ras-activated cells both in vitro and in vivo. In order to better define the activity of Reolysin in vitro and specifically to study interactions between Reolysin and cytotoxic agents, cell proliferation studies were undertaken in HCT116 cells. Reolysin was highly cytopathic against HCT116 with 60% reduction in cell number at doses as low as 1×10^{-5} pfu and nearly 100% at doses of 0.1 pfu at 7 days exposure. With as little as 2 days of exposure to virus, there was 50% cytotoxicity with doses of 1 pfu. Of interest, Reolysin was equally active against cells that were 80% confluent or at confluence as low as 50%. Despite enhanced cytotoxic activity in ras-mutated cells, previous studies have also demonstrated enhancement of activity in cells with a disrupted ras pathway. In order to test this hypothesis, we studied HCT116 variants in which ras was disrupted by homologous recombination. Of interest, these cells demonstrated equivalent sensitivity to Reolysin on days 2–7 and at doses of $1x\ 10^{-4}$ to 10 pfu. To determine whether Reolysin augmented the effects of various cytotoxic agents, cells were exposed to cytotoxic agents for 3 days with or without Reolysin, 0.1 pfu. The reovirus enhanced the cytopathic effects of fluorouracil at every concentration tested. Similar effects were observed with gemcitabine. Enhancement of the cytotoxicity of doxorubicin and cisplatin was observed, but only at concentrations approaching the IC50 for these agents. No synergy was observed with paclitaxel. Reolysin has in vitro activity against ras-mutated HCT116 and its ras-disrupted variant, and enhanced the cytotoxicity of fluorouracil, gemcitabine, doxorubicin and cisplatin against HCT116, making it a promising agent for clinical trials.

453 POSTER

Validation of pharmacodynamic assays to determine the clinical effect of an antisense to the XIAP inhibitor of apoptosis

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XIAP is a potent endogenous inhibitor of caspases shown to be overexpressed in human tumours. Recently, a 19-mer second generation antisense oligonucleotide targeting the protein was approved for Phase I evaluation in the UK. Validation strategies and performance data on 4 different assays are presented that will be incorporated as endpoints during this trial. Immunohistochemistry (IHC) of XIAP in tumour biopsies was validated using fixed sections of tissues obtained from XIAP-null mice and matched (wild-type) control animals. These functioned as both positive and negative controls to determine the specificity of the methodology and as replicate quality controls (QCs) in order to assess reproducibility, within-day and between-day precision and stability of the protein. An immunostaining method producing a weighted mean that takes account of both staining intensity and percentage of expressing cells was adopted to score sections. The M30-Apoptosense™ Elisa detects a caspase cleaved fragment of cytokeratin 18 that is released into the circulation after apoptotic cell death and is believed to represent a plasma surrogate marker enabling a quantitative assessment of apoptosis occurring in the patient's tumour. Validation of this technique focussed on generating an independent positive quality control to perform measurements on precision, kit-to-kit QC, and stability studies. Between-day precision data obtained using this control ranged from 3.6–6.7%. Kit to kit variability was less than 10%, while samples stored at −80ºC were demonstrated to be stable for at least four months. To facilitate validation of a Western blotting method for XIAP, we employed replicate cell pellets consisting of a genetically-engineered breast cancer cell line (MDA-MB-231/X-G4) stably expressing an RNAi to silence XIAP as a negative control and 2 positive control cell lines. XIAP was quantitated by densitometry as a ratio to GAPDH and by reference to a 5 point calibration curve (1.8–147 pg/ug) constructed using recombinant GST-XIAP protein added to a surrogate protein matrix. Calibration curves were linear with a mean r^2 value of 0.997. Between day precision was 29% for the low XIAP expressing control and 23% for the high XIAP expressing control. Finally, real time RT-PCR to quantitate XIAP mRNA was validated using two different housekeeping genes (GAPDH and B2M), high and low expressing cell lines as QCs and an 8 point calibration curve constructed from a XIAP cDNA plasmid.

454 POSTER

Highly active, cancer specific promoters for gene therapy of small cell lung cancer

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One of the major problems in conventional, non-invasive cancer treatment is the lack of specific targeting to the cancer cells. For targeted gene therapy, the lack of cancer specific receptors has proven a major obstacle. Less specific receptors can be used for targeting by having the expression of a therapeutic gene regulated by a cancer specific promoter. The identification of novel highly active, cancer specific promoters would improve the potential of cancer gene therapy.
For small cell lung cancer (SCLC) there is currently no satisfactory treatment, wherefore development of novel modalities, such as gene therapy, is highly in demand. Using global gene expression analyses (oligonucleotide arrays), we have identified a number of highly and specifically expressed genes in SCLC (Pedersen N. *et al*., *Cancer Res.* **63**, p1943, 2003). Several of the genes identified by the array analysis are known to be transcriptionally regulated in a developmental manner and several reflect the neuroendocrine origin of SCLC.
The gastrin releasing peptide (GRP) gene is highly expressed in SCLC tumors and cell lines and the GRP promoter has been suggested for gene therapy for SCLC. We found expression levels of a reporter gene from the GRP promoter was as strong as the SV40 promoter in a number of SCLC cell lines. However, as GRP is expressed in some normal adult tissues, this may limit its use for gene therapy.
The insulinoma-associated antigen-1 (INSM1), in contrast, is exclusively expressed during early embryonal development, but has been found re-

activated in a number of neuroendocrine tumors. The array analysis showed very high expression in most SCLC cell lines. Transfection with a reporter gene regulated by a 1.7 kb region of the INSM1 promoter (Li Q, *et al., BBRC.* **236**, p776, 1997) showed very high expression in most SCLC cell lines (3–12 times stronger than SV40) and no expression in other cell lines. Expression of the Herpes Simplex Thymidine kinase gene from the INSM1 promoter conferred cell death after treatment with the prodrug ganciclovir in SCLC cell lines with high expression of INSM1, but not in cell lines with low or no expression of INSM1. We have therefore demonstrated that the activity of INSM1 is sufficient for gene therapy approaches and that the cloned promoter region retains the SCLC cancer specificity.

455 POSTER

ARHI gene therapy and paclitaxel exert additive cytotoxicity for breast cancer cells through caspase-independent and caspase-dependent apoptotic mechanisms

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Background: ARHI is an imprinted tumor suppressor gene encoding a 26kD GTPase with 50–60% homology to ras and rap, whose expression is downregulated in breast and ovarian cancers. Re-expression of ARHI inhibits growth, decreases invasiveness, and induces apoptosis. Our recent work demonstrated that ARHI could induce apoptosis through a caspase-independent and calpain-dependent mechanism. Re-expression of ARHI can reduce tubulin expression and significantly inhibit mitosis in breast cancer cells. More effective and less toxic therapy is needed. Consequently, we have combined ARHI gene therapy with paclitaxel that induces apoptosis through caspase-dependent pathways.
Intratumoral injection of ARHI in an inducible binary adenoviral vector produced significant regression of established human breast cancer xenografts. To develop tumor specific expression, we have modified the first binary vector to contain a human telomerase reverse transcriptase (hTERT) promoter. As over 90% of breast cancers are positive for telomerase activity, more specific tumor cell killing might be established by this new delivery approach.
Material and Methods: Using a dual adenovirus system (Ad/ARHI/PGK-GV16, Ad/ARHI/hTERT-GV16), ARHI was re-expressed in breast cancer cells (MCF-7, SKBr3) that have lost ARHI expression. Cells were treated with paclitaxel alone and in combination with virus. Limiting dilution clonogenic assays and isobolographic analysis were used to measure clonogenic growth. For studies with ARHI driven by hTERT promoter, the sulforhodamine B (SRB) assay was used to measure cell growth.
Results: We have tested the effects of a combination of Ad/ARHI/GV16 and paclitaxel on breast cancer cell lines MCF-7 and SKBr3 by limiting dilution clonogenic assay. Additive cytotoxicity was produced with a combination of paclitaxel (5–10nM) and Ad/ARHI/PGK-GV16 (1000–2000 particles/cell). Clonogenic growth was only inhibited 40 to 90% by Ad/ARHI/GV16, and 90 to 99.9% by pacitaxel. Inhibition was greatly increased to more than 99.99% by a combination with both reagents.
Given the limited specificity of the adenovirus system and the need to develop strategies that could be used systemically, we have used the hTERT promoter to target ovarian and breast cancer cells. When ARHI gene was induced by Ad/hTERT-GV16, high levels of transgene expression and significant cell growth inhibition were observed in breast cancer cells. In contrast, only minimal toxicity was detected in normal human primary mammary epithelial cells after treatment with this vector.
Conclusion: A combination of paclitaxel and ARHI gene therapy is more effective than either agent alone for inhibiting breast cancer cells growth through a combination of caspase-dependent and caspase-independent mechanisms. Ad/hTERT-GV16 vector facilitates specific expression of ARHI gene in breast cancer cells.

456 POSTER

Mesothelial cells as target of antiangiogenic therapy for peritoneal metastasis

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Background: Peritoneal mesothelial cells (PMC) have the capacity to produce large amounts of VEGF. Although VEGF in ascites has been considered to mainly originate from tumor cells, the contribution of VEGF derived from host cells including PMC has also been considered. We first assessed the production of VEGF by cultured PMC in vitro. Then, we evaluated the ratio of host-derived VEGF using in a nude mouse model, and finally evaluated the effectiveness of gene transduction of soluble Flt-1 targeted to PMC against peritoneal dissemination.
Methods: VEGF in the culture supernatants of isolated PMC was measured by ELISA. With the same method, human- and mouse-VEGF were measured separately in ascitic fluid of nude mice that had been intraperitoneally inoculated with a human gastric cancer cell line, MKN45, at various time points. Finally, we evaluated whether transfer of the sFlt-1 gene specifically to PMC using an adenovirus vector could inhibit tumor formation in the peritoneal cavity.
Results: PMC produced similar amounts of VEGF to gastric cancer cells, which was significantly augmented by the addition of fibroblast growth factor (FGF) or lysophosphatidic acid (LPA). Of VEGF in ascitic fluid at 3 weeks after MKN45 inoculation, 12.8% was derived from mouse cells. At 6 week, however, the ratio of mouse-derived VEGF was reduced to 5.0%, suggesting that the ratio of host-derived VEGF may be higher in the earlier phase. When adenovirus expressing sFlt-1 was intraperitoneally (ip) administered in vivo, a high level of sFlt-1 protein could be detected in peritoneal lavage for 8 weeks, with a significant reduction in the number of intraperitoneal metastatic nodules and prolonged survival of nude mice that were ip inoculated with MKN45 3 days later.
Conclusion: PMC-derived VEGF has an essential role in the development of peritoneal metastasis and thus could be an attractive target for antiangiogenic gene therapy.

457 POSTER

Interim evaluation of a multi-institution phase I/II study of antisense oligonucleotide GTI-2040 (G) and capecitabine (C) in patients with metastatic renal cell carcinoma (mRCC)

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Introduction: G is a 20-mer phosphorothioate oligonucleotide complementary to the R2 subunit of ribonucleotide reductase that has broad-spectrum activity in human xenograft models including RCC models. 5-fluorouracil (5FU) has some activity in renal cell carcinoma (RCC) and G plus 5FU has cooperative activity against RCC in SCID mice.
Methods: An open label, non-randomized phase **I/II** study of G plus C was conducted to determine the toxicity and objective response rate in patients with metastatic RCC. G was dose escalated in the phase **I** portion and administered continuously via central venous catheter by a portable infusion pump for 21 days with C at 1660 mg/m^2/d orally divided into two daily doses followed by 7 days of rest during each 28 day treatment cycle. Pharmacokinetic samples were collected from all phase **I** pts and 6 additional pts treated at the phase **II** dose. Only toxicities occurring during cycle 1 were considered for determining dose limiting toxicity (DLT).
Results: To date, 29 pts [M: F ratio 18:11; median KPS 80 (70–100); median age 59 (37–73)] have received a total of 88 cycles of therapy. Twenty-six pts had prior systemic therapy (25/26 prior immunotherapy, 14/26 received $\geqslant$ 2 systemic therapies); 13 had prior radiotherapy and 19 had prior nephrectomy. None of the 3 pts treated at dose level 0 (G 145 mg/m^2/d) and only 1 of 6 pts treated at dose level 1 (G 185 mg/m^2/d) experienced DLT (grade 3 diarrhea). Since dose level 1 is also the recommended phase **II** dose for single agent G, the phase **II** portion of the study was conducted at this dose. The major toxicities in both the phase **I** and **II** portions included Grade 4: pancytopenia (1), pulmonary embolism (1) and bone pain (1); Grade 3: thrombocytopenia (4), lymphopenia (3), anemia (2), neutropenia (2), nausea/emesis (2), infections (3), fatigue (2); neuropathy, thrombosis, dehydration, hypophosphatemia, diarrhea (1 each). Additional common grade 1/2 toxicities include: nausea (62%), fatigue (45%), emesis (35%), hematologic toxicity (28%), anorexia (28%), and diarrhea (24%). Amongst the 25 response-evaluable pts at the phase **II** dose; 13 (52%) had stable disease (SD) as best response (median duration: 4 months, range 2–10), and 1 durable (8 months) partial response was observed. At the phase **II** dose, the pt with PR experienced a unidimensional tumor reduction of 39%, and the pt with the longest duration SD had a 23% tumor reduction. One additional pt at dose level 0 also had SD and a 13% decrease in tumor size. Pharmacokinetic studies are ongoing and completed analysis will be presented.
Conclusions: The combination of G and C is tolerated at the recommended phase **II** dose with expected toxicities. This G + C trial is ongoing and overall activity and objective response will be assessed when the Phase **II** efficacy timepoint is achieved. An additional G + cytokine combination study is being planned for mRCC patients.