Results: Cell cycle analysis after treatment with CPT-11 in G0/G1 synchronized cells demonstrated the activation of transfected wild-type p53 and a consequent $p21^{WAF1/CIP1}$-dependent cell cycle blockage in S phase. Activated wt-p53 also increased apoptosis, leading to enhanced sensitivity to CPT-11. DNA microarray analysis showed that, in p53-deficient cells, the cell cycle regulatory machinery did not respond to CPT-11, leading to the accumulation of the G2/M cdk1/cyclin B complex. We found subsequent p53-independent activation of the cdk-inhibitor $p21^{WAF1/CIP1}$, which prevented cell cycle progression. We further exploited cdk1 induction in p53-deficient cells to improve the sensitivity to CPT-11 by additional treatment with the cdk-inhibitor roscovitine.

Conclusions: We demonstrate a gain of sensitivity to CPT-11 in a p53 mutated colon cancer cell line both by restoring wild-type p53 function or by additional treatment with a cdk-inhibitor. Considering that mutations in p53 are among the most common genetic alterations in colorectal cancer, a therapeutic approach that specifically targets tumors with mutated p53 could greatly improve the treatment outcomes.

965 POSTER

Molecular therapy for peritoneal dissemination of gastric cancer with adenovirus-mediated Bax gene transfer

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Gene therapy is one of the candidates for an innovative therapeutic approach against cancer. An adenoviral vector expressing the tumor suppressor p53 gene (Ad/p53) is currently under clinical evaluation in various cancers. We have recently developed a binary adenoviral vector system that can express the strong apoptotic Bax gene (Ad/PGK-GV16+Ad/GT-Bax: Ad/Bax). To evaluate the potential of Bax gene therapy for gastric cancer, we assessed the antitumor effect of the Bax gene in comparison with the p53 gene. The responses of human gastric cancer cell lines, MKN-1, MKN-7, MKN-28 and MKN-45, to recombinant adenoviruses, Ad/Bax or Ad/p53 were assessed in vitro. Cell viability was measured by XTT assay; transgene expression and caspase activation were analyzed by western blotting; and morphological feature of apoptosis was observed by Hoechst staining. The treatment with Ad/Bax or Ad/p53 resulted in the marked Bax or p53 protein expression and effective apoptosis induction in MKN-1, MKN-7, and MKN-28 cells. In contrast, MKN-45 cells showed resistance to Ad/p53 and only the treatment with Ad/Bax resulted in massive apoptosis. To compare antitumor effects between the Ad/Bax and Ad/p53 treatment in vivo, MKN-45 subcutaneous tumors were generated by inoculation of 2×10^6 MKN-45 cells into the dorsal flank of nude mice. When tumor had reached a diameter of about 3-5mm, each mouse was given intratumoral injection of 100 μl of 2×10^{10} particles of each virus. Mean tumor volume of the Ad/p53 group was 515.2± 151.9 mm^3, while that of Ad/Bax was only 236.5± 83.8 mm^3, as of 35 days after inoculation. Furthermore, peritoneal dissemination of MKN-45 cells were generated in nude mice, and each mouse was treated by intraperitoneal injection of 200 μl of 2×10^{10} particles of each virus. Disseminated tumor numbers and weights were assessed 24 days after inoculation. Similarly, mean total tumor weight of the Ad/p53 group was 371.8± 44.0 μg, while that of Ad/Bax was 161.9± 96.9 μg. The treatment with Ad/Bax significantly inhibited the growth of p53-resistant gastric cancer in vitro and in vivo. Therefore, our results suggest that Adenovirus-mediated Bax gene transfer may be useful in gene therapy for gastric cancers.

966 POSTER

Activation of a plasma membrane-cationic channel and apoptosis in prostate cancer cells overexpressing Bax

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Background: Our group has previously identified and characterized a 23 pS non selective-cation channel (NSC channel) in prostatic cancer-LNCaP cells undergoing apoptosis (J Physiol, 1999, 517:95-107). The activation of the channel was only induced by pro-apoptotic stimuli including ionomycin, thapsigargin, staurosporine or serum depletion. Accordingly, channel activity was never registered in intact cells. Further studies using an anti-Bax antibody suggested that the channel opening could be mediated by Bax proteins (Biophys J 2001, 80:2764). To test this hypothesis, we have induced cell death in prostatic cancer LNCaP cells using an inducible Bax adenoviral vector generated by a Cre/loxp system.

Material and Methods: Adenoviruses were replicated, purified and tittered by plaque assay, as described elsewhere (). For overexpressing Bax protein, cells were coinfected with a Bax recombinant adenovirus (Ad/Bax) and the inducing adenovirus Ad/Cre in a 5:1 relation (kind gifts of Dr D. Curiel's group, Mol Ther 2000, 1:545-554). LNCaP cells (2×10^5/ well) were infected at an m.o.i. of 5. The transgene encodes a fusion protein containing Bax and a hemaglutinin (HA) tag. Patch clamp in the cell-attached configuration was used to evaluate the opening of the 23 pS NSC channel at 20 to 24 hours post infection. Protein extraction and Western Blot analysis were carried out at 24 hours for evaluating the expression of endogenous Bax and Bax-HA proteins. Cell death was studied by fluorescence activated cell sorter analysis of annexin 5/ iodide propidium and by crystal violet staining at 72 hrs.

Results: The 23 pS NSC channel was only registered in cells co-infected with AdBax/Ad Cre but neither in control cells nor in cells co-infected with AdBax/AdTK. Overexpression of the Bax-HA fusion protein (27 kDa) was confirmed by WB in the former cells. Cell death occurred in over 90% of Ad Bax/AdCre cells at 72 hours.

Conclusions: The sole overexpression of Bax induces apoptosis and activates a 23 pS non selective-cation channel in LNCaP cells. The significance of the opening of this channel in early stages of apoptosis is under study.

967 POSTER

The detection of metastatic cancer cells in peripheral blood using reverse transcriptase polymerase chain reaction for CK 19

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Metastatic cancer cells spread is not detectable by conventional staging methods, but the combination of immunomagnetic cell enrichment and reverse transcription (RT-PCR) is an efficient method to identify small numbers of disseminating tumour cells in blood or bone marrow of patients with cancer. Our aim is to determine whether RT-PCR for cytokeratin 19 (CK19) provides a sensitive method for the detection of a single metastatic cell in peripheral blood.

In several spiking experiments, cell-line (ZR-75) derived tumour cells (2-100 cells) were added to 5ml of human peripheral blood of a healthy donor. We designed a semi-junctional CK19 specific primer set. Human peripheral blood without added tumour cells was used as our specificity control. After enrichment, mRNA was extracted using Oligobead mRNA extraction and a silica-based total mRNA extraction method. Products were amplified by a single-enzyme RT-PCR for CK-19 mRNA. Amplicon were visualised on 2% agarose gel.

Total mRNA extraction from unspiked blood samples followed by RT-PCR yielded in falsepositive amplification due to circulating hemaptopoietic elements. Immunomagnetic enrichment drastically improved the specificity for the CK-19 RT-PCR assay. Both mRNA extraction methods demonstrated similar sensitivity levels. Our detection sensitivity for the combined method including cell enrichment, RNA extraction and subsequent RT-PCR is less than 3 cells.

Immunomagnetic enrichment combined with CK19 RT-PCR is a very sensitive and specific method to detect disseminating tumour cell in peripheral blood of breast cancer patients. Further evaluation by real time quantitative PCR and using other specific breast markers is needed.

968 POSTER

FCU1: a highly potent suicide gene therapy based on 5-FU

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Background: Direct transfer of pro-drug activation systems into tumours was demonstrated to be an attractive method for the selective in vivo elimination of tumour cells. Besides its local cytotoxic impact, this strategy was further demonstrated to enhance the host anti-tumour immune response through the local release of cellular debris that can be presented by the antigen presenting cells.

Material and methods: We describe a novel and highly potent suicide gene derived from the Saccharomyces cerevisiae cytosine deaminase (FCY1) and uracil phosphoribosyltransferase genes (FUR1). This suicide

gene, designated FCU1, encodes a bifunctional chimeric protein that combines the enzymatic activities of FCY1 and FUR1 and efficiently catalyses the direct conversion of 5-fluorocytosine (5-FC), a non-toxic prodrug, into the cytotoxic metabolites 5-fluorouracil (5-FU) and 5-fluorouridine-5'-monophosphate (5-FUMP). Interestingly, the cytosine deaminase activity is 10-fold higher in the chimeric protein compared to the natural protein.

Results: In this study we demonstrate that a MVA (Modified Vaccinia Virus of Ankara) engineered to express the FCU1 gene significantly enhances the sensitivity of numerous human tumour cells to 5-FC (LD50 5-FC = 1μM in the FCU1 treated cells compared to LD50 5-FC = 10mM in the CDase treated cells; $p<0.01$). Moreover, passive diffusion of the 5-FU ensures an impressive bystander effect with the ability to kill 100% of a in vitro tumour cell population with only 1% FCU1-transduced cells. Intratumoral injections of MVA-FCU1 into human tumour-bearing mice, with concomitant systemic administration of 5-FC, led to a sustained control of tumour growth. The FCU1-induced tumour growth suppression was observed in different human colorectal tumour models whereas 5-FU administered IP at the maximum tolerated dose did not show any anti-tumor effect in the same models. Finally, a 10-fold higher concentration of 5-FU is detected inside the tumour compared to a systemic administration of 5-FU while no detectable 5-FU is found in the circulation, ensuring a higher safety profile with no systemic toxicity.

Conclusions: The FCU1 suicide gene is a unique combination of an innovative approach and a validate and secure chemotherapy that makes it a novel and powerful candidate for treating all 5-FU sensitive tumours.

969 POSTER

Pre-clinical safety and immunogenicity studies of a HER-2 protein vaccine in cynomolgus monkeys

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Background: The HER-2 growth factor receptor has emerged as an attractive target for immunotherapy of breast cancer. Pharmexa A/S has developed a modified recombinant HER-2 protein vaccine (HER-2 AutoVacTM protein) that includes potent helper T cell epitopes to enhance immunogenicity. Pre-clinical safety and immunogenicity studies were performed in cynomolgus monkeys to support clinical trials in patients with HER-2 positive breast cancer.

Methods: A pilot immunogenicity study and a two armed 3-9 month repeated dose toxicity study were performed. In the pilot study, groups of 6 monkeys were injected with 100mg HER-2 protein vaccine formulated in three different adjuvants: two commercial adjuvants (A and B) and a standard aluminum adjuvant The monkeys received either 5 immunizations at 2 week intervals, or 3 immunizations at weeks 0, 2 and 6. In the 3-9 month repeated dose study, groups of 6 monkeys received 20, 100 or 500mg HER-2 protein vaccine formulated in the aluminum adjuvant at weeks 0, 2 and then every 4 weeks for the duration of the trial. A recovery period was included for one group of the 3 month arm of the study. Sera were collected at regular intervals and evaluated for HER-2 specific antibodies by direct ELISA. At the conclusion of treatment, monkeys in the toxicology study were sacrificed and subjected to full necropsy and histological analyses.

Results: Significant HER-2 specific antibody titers were measured in all monkeys in the pilot study. The highest response was seen with adjuvant A after 5 immunizations at 2 week intervals. The responses after 3 immunizations on weeks 0, 2 and 6 were similar between adjuvant A and aluminum. In the 3-9 month study, all animals receiving 500mg HER-2 protein vaccine had significant antibody responses after one immunization, while the 20mg and 100mg dose groups developed titers after 2 immunizations. Titers were maintained by continued boosting and declined rapidly in the recovery group upon cessation of treatment. No vaccine associated toxicity was observed after 3 months treatment (9 month results pending). It was concluded that immunization with the HER-2 AutoVac protein induced significant HER-2 specific antibody titers and was safe in cynomolgus monkeys.

970 POSTER

Increased gap junctional intercellular communication in esophageal cancer augments the bystander effect in HSV-tk/GCV suicide gene therapy

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Background: There is accumulating evidence indicating that gap junction is important in induction of the bystander effect (BE) in a gene therapy through herpes simplex virus thymidine kinase (HSV-tk)/ganciclovir (GCV). We previously reported that substantial BE was observed in esophageal cancer cells with gap junctional intercellular communication (GJIC). Here, we investigated the effect of chemicals, such as all-trans retinoic acid (ATRA), cAMP and glucocorticoid, and transduction of gap junction protein, connexin (Cx), gene on GJIC and BE in esophageal cancer cells.

Materials and Methods: Four esophageal cancer cell lines were selected by the status of Cxs expression and GJIC out of 8 cell lines for subsequent experiments: KE-3, KE-6, KE-8 and KE-10. Expression of Cx26 and Cx43 and GJIC before and after treatment with ATRA, cAMP and glucocorticoid in above cells were examined with Western blot analysis, immunohistochemistry and dye transfer assay. Those chemicals' effect on BE was examined by cell killing assay with GCV treatment by using pretreated mixtures of cells transfected with HSV-tk gene and parental cells at varioius ratios. Finally we transfected cells with Cx32 and HSV-tk gene and then examined the extent of GJIC and BE with the same procudures.

Result: KE series did not express Cx26 mRNA and protein. The localization pattern of Cx43 protein was classified into four types; at cell-cell contact area(KE-8), in the cytoplasm (KE-3), at both cytoplasmic and cell-cell contact area(KE-6), and no expression(KE-10). The excellent GJIC and BE was observed in KE-8, and the moderate in KE-6. Little GJIC and BE was observed in KE-3 and KE- 10. When 10% HSV- tk(+) cells were mixtured, about 50% of cells were killed in KE-8. All three chemicals showed no influence on the Cx expression. Although cAMP and glucocorticoid had no positive effect on GJIC and BE in all cell lines, ATRA enhanced the GJIC in KE-6 by 25%, leading to significant increase of BE by 50%. Mixing experiment with Cx32+tk+ cells and parental cells revealed augmentation of BE, resulting from increase of GJIC; 50% of cell death in the presence of 10% Cx32+tk+ cells.

Conclusion: Our results indicate that the bystander effect in HSV-tk/GCV suicide gene therapy is induced according to the intensity of GJIC in esophageal cancer cells and that enhance of GJIC by ATRA and Cx gene transduction is effective to induce strong bystander effect.

971 POSTER

Additive and synergistic effects on TRAIL/Apo2L induced apoptosis after irradiation and eradication of clonogenic cells in solid tumor cell lines in vitro

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Background: TRAIL (TNF related apoptosis inducing ligand)/Apo2L is a very promising member of the family of death ligands. The ligand preferentially induces apoptotic cell death in a wide range of tumor cells but not in normal cells. TRAIL/Apo2L triggers apoptosis even in cells not undergoing apoptosis in response to radiation, since ionizing radiation induce apoptosis by a different pathway as death ligands although an overlapping set of molecules is involved. Based on the assumption of distinct signalling pathways, combination of TRAIL/APO2L and ionizing radiation seems ideal to increase the therapeutic efficacy. The question in how far the combination of both modalities induces additive or synergistic apoptotic effects and eradication of clonogenic tumor cells is subject of this report.

Material and methods: Tumor cell lines: NCI-H460 (adenocarcinoma of the lung), HCT-15 and Colo-205 (colon carcinoma), MDA-MB-231 (mamma carcinoma), LnCAP (prostate cancer), SCC-4 (squamous cell cancer). Determination of apoptosis was performed 12-48 h after simultaneous application of TRAIL/APO2L and irradiation (2-10 Gy) and after 12h preirradiation of TRAIL/Apo2L treated tumor cells. Rates of apoptosis were determined by flow cytometric evaluation of caspase activation and/or by microscopical observation of morphological changes of Hoechst stained cell nuclei. Clonogenity was determined by colony forming assays.

Results: Both TRAIL/Apo2L and ionizing radiation alone lead to a dose dependent induction of apoptosis in several of the analysed tumor cell lines. Concomitant application of combined treatment leads to an additive effect. Preirradiation (12h prior to TRAIL/Apo2L treatment) leads to supraadditive effects in HCT-15 und Colo-205 tumor cell lines. Clonogenity of the cells was inhibited to a noticeable degree.TRAIL/Apo2L triggers cell death also in cell lines with very low (HCT-15, SCC4, MDA-MB231) rates of irradiation induced apoptosis.

Conclusion: TRAIL/APO2L and irradiation lead to additive and, dependent on the time course of application, partially supraadditive effects after combined treatment. Therefor, combined treatment with TRAIL/Apo2L and irradiation represents a new treatment strategy for tumours based on activation of two distinct cell death mechanisms.