452 POSTER

The effects of oestrogen and tamoxifen on purified normal and malignant human primary breast epithelial cells

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In order to investigate the effects of oestradiol (E2) and 4-hydroxy tamoxifen (4OHT) in epithelial cells from normal and malignant breast tissue, we have devised a system to purify these cells and study them in vitro.

Fresh pathological discard tissue from reduction mammoplasties and primary breast cancers were enzymatically digested and after appropriate filtration epithelial cells were isolated using the epithelial specific Ber-Ep4 immunolabelled beads (Dynal) and cultured. The purity of malignant cells from primary tumours was confirmed using conventional cytology and fluorescent in situ hybridization (FISH) and shown to be >95% and >90% respectively. Cytokeratin 8 and 18 immunostaining confirmed the epithelial nature of both, normal and malignant samples.

Oestrogen receptor alpha (ER) and progesterone receptor (PR) were examined by immunostaining and we have demonstrated that ER and PR expression were retained in malignant cells in culture. In contrast, ER expression was rapidly attenuated, in normal cells, resulting in loss of expression within the first 24-36 hours of culture. A similar reduction was noted in PR expression, although very low levels could be detected for upto 60 hours in culture. Further, contra-intuitively, E2 induced a proliferative response in normal cells and none in cancer cells. 4OHT had an inhibitory effect on proliferation in normal cells and decreased cell survival in some malignant cultures.

We conclude: (1) This novel system is an effective methodology for isolating malignant epithelial cells to a high degree of purity, (2) Oestrogenic responses are different in normal cells compared to cancer cells and (3) 4OHT treatment results in a decrease in cell numbers in some ER positive malignant cultures. This suggests that this can be a way of selecting tamoxifen sensitive patients from the ER+ group of patients.

453 POSTER

Multifluorescent labelling of immunmagnetic enriched circulating colon tumour cells and cell clusters

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Purpose: Detection and characterisation of circulating tumour cells may contribute to the better understanding of metastatic process in human. With a recently developed intracellular cytokeratin immunmagnetic microbead cell separation method intact cells and mixed cell clusters were detected in patients with different stage and duration of colorectal cancer. Development and evaluation of a fluorescent cytokeratin/haemopoetic labelling technique with immunmagnetic microbead cell separation method for the characterisation of enriched tumour cell and cell clusters in patients with colorectal cancer.

Methods: 16 colon cancer patients (stage TNM II, III, IV) and 20 healthy donor samples were evaluated. The colon cancer patients were evaluated before adjuvant chemotherapy. Immunmagnetic cell separation was performed from whole blood/ficoll separated cell blood fractions by the surface epithelial markers (HEA 125, Ber-EP4). The enriched cell fraction (400microliter from 5ml blood) was immuncytochemically labelled using an FITC labelled cytokeratin antibody (CAM 5.2) and a Texasred labelled CD45. Cell death was detected by Propidium Iodide nuclear staining. Spiking experiments were performed using the HT29 colon cancer cell line. The lowest detectable concentration was 1 cell/ml blood. A fluorescent inverse microscope was used to evaluate and manipulate the labelled cell fraction.

Results: Two of the twenty healthy donor samples contained CK+CD45+ cells (3 and 4 such cells in 5ml blood). In cancer patients the lowest cell number was 9 CK+CD45- cells/ml. Cell clusters containing CK+CD45-, CK-CD45+ and CK+CD45+ cells were detected only in colon cancer patients.

Conclusions: Immunmagnetic cell separation with immuncytochemical labelling is a useful method for detection and characterisation of circulating colon cancer cells and clusters. CD45+CK+ cells appear both in healthy and colon cancer patients in low number, as well. Their further characterisation and evaluation needs additional fluorescent markers and studies.

454 POSTER

ZD0473 exhibits marked in vitro anticancer activity in human tumor specimens

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ZD0473 is a new generation platinum drug that shows evidence of an extended spectrum of antitumor activity and overcomes platinum resistance mechanisms. ZD0473 has demonstrated in vivo activity against cisplatin-resistant human ovarian xenografts. The present study tested ZD0473 in a soft agar cloning assay to determine its in vitro activity against human tumor specimens taken directly from patients. One hundred and five patient tumor specimens were exposed to ZD0473 at 1, 4, and 16 μg/ml for 2 hours versus 24 hours, at 37°C. Approximately 35% of these specimens were evaluable, falling within negative and positive control parameters. Following exposure, cells were washed with medium and plated into a two-layer soft agar cloning system then incubated at 37°C for 14 days. Specimens were removed on Day 14 for colony counting. The number of colonies formed in the treated plates was compared with that from untreated control plates, and the percent colonies surviving at each concentration was calculated. Negative and positive controls were sterile saline (0.9% NaCl) and orthosodium vanadate (200 μg/ml), respectively. After 2-hour exposure, ZD0473 demonstrated activity in 15% (6/40), 30% (12/40), and 47% (15/32) of the specimens tested at 1, 4, and 16 μg/ml, respectively. After 24-hour exposure, ZD0473 demonstrated activity in 27% (10/37), 65% (24/37), and 87% (27/31) of the specimens tested at 1, 4, and 16 μg/ml, respectively. Notable responses to 2-hour ZD0473 16 μg/ml exposure were seen in breast (29%), ovarian (78%), colon (33%) and NSCL (60%) cancer specimens. Ongoing trials in these tumour types are designed to further investigate these results. Phase I studies have indicated that ZD0473 has a predictable and favorable toxicity profile, comparable to carboplatin (Trigo et al., Proc ASCO 1999; 18: 169a[abs 648]). ZD0473 is currently undergoing Phase II monotherapy and Phase I combination studies, Phase III studies are in planning.

455 POSTER

Immunmagnetic detection and characterisation of circulating tumour cells and cell clusters in patients with colorectal cancer

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Purpose: To produce metastases, tumour cells must enter into the circulation and survive the transport. Although most tumour cells are destroyed in the bloodstream, animal models seem to demonstrate that the greater number of cells and cell clusters and larger clusters released by a primary tumour, the greater the probability that some cells will survive and form metastases. The latest methods using RT-PCR techniques proved to be useful, but intact cells and cell clusters could not be detected. Our aim was the immunmagnetic (IM) detection and characterisation of circulating tumour cells and cell clusters in patients with different stages (TNM II, III, IV) of colorectal cancer.

Methods: 25 patients with colon cancer (test samples) and 7 healthy donors (control samples) were evaluated. Follow-up investigations were done in some cases. IM cell separation was performed from the buffy coat of peripheral blood samples by the Carcinoma Enrichment Kit (Miltenyi Biotech, Germany) avoiding any filtering steps. The enriched cell fraction was cytocentrifuged. The cytospins were immuncytochemically labelled using a pancytokeratin antibody (Dako MNF116) and biotin-streptavidin-peroxidase technique. Spiking experiments were performed using the HT29 colon cancer cell line.

Results: From the control samples only once did one of the samples contain a cytokeratin positive cell. However, from the 25 patients 20 showed cytokeratin positive cells. Besides single cytokeratin positive cells, tumour cell clusters (>2 cells), mixed cell doublets (one cytokeratin positive and one negative cell) and mixed cell clusters (>1 cytokeratin positive and negative cells) were detected in 15 of 25 patients. Most (74%) of the circulating cancer cells were found in clusters. The mean number of circulating tumour cells, single cells and the average size of the clusters correlated with the

stage ($p<0.05$). In 3 cases cytokeratin positive dendritic-like cells were detected.

Conclusions: IM cell separation with cytocentrifugation and immuncytochemical labelling is a useful method in the detection of circulating colon cancer cells and clusters. Further studies are in progress to reveal the significance of the cytokeratin negative cluster cells and the dendritic-like cells. Our results showed an association between the size of circulating cell clusters and the TNM stages. Our human data are supported by the animal models about the importance of the circulating cell clusters.

456 POSTER

Detection of circulating tumour cells in the peripheral blood of colorectal cancer patients using cytokeratin 20 RT-PCR and immuncytochemistry

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Background: The detection of circulating tumour cells and micrometastases may have important therapeutic and prognostic implications. Our objective was to compare the two gold stndard techniques, such as RT-PCR and immuncytochemistry (ICC), of circulating tumour cell detection in clinical samples with advanced disease.

Methods: 30ml of peripheral blood was obtained from 11 Dukes D colorectal cancer patients at two separate times within a month. The first 5ml after puncturing the skin were discarded. Five ml of the sample was used for mRNA isolation and subsequent RT-PCR with cytokeratin 20 primers. Twenty ml was subjected to immunmagnetic tumour cell enrichment with positive selection of cytokeratin 7/8 expressing cells on high-gradient magnetic columns (Carcinoma Cell Enrichment Kit, Miltenyi Biotech, Germany). The enriched cell fraction was cytocentrifuged on poly-L lysin coated slides. The cytospins were immuncytochemically labelled using a pancytokeratin antibody (DAKO, MNF116) and biotin-streptavidin-peroxidase technique (DAKO, LSAB2). In spiking experiments the sensitivity of both assays were in the 1 cell/ml range.

Results: Of the 22 samples in this study, 19 (86.36%) showed concordance between the two methodologies. The 3 disparity cases were due to RT-PCR positivity, but ICC negativity. With using RT-PCR 9/22 (40.90%) samples were positive, while with ICC 6/22 (27.27%) samples showed positive results. In 5 cases (45.45%) with RT-PCR and in 4 (36.36%) with ICC the result of the second sample conflicted with the first one.

Conclusion: Our results indicate that both RT-PCR and immunmagnetic enrichment with subsequent ICC are sensitive and reliable methods for circulating tumour cell detection. The concordance rate in our study is high, but because both techniques have significant limitations it may be advisable to use them complementary in further investigations. Even in advanced staged patients with a high probability of circulating tumour cells, multiple sampling can increase the risk of positive finding.

457 POSTER

Damage-induced Bax N-terminal change and translocation to mitochondria occur regardless of cell fate

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Resistance to chemotherapy is the major obstacle to the successful treatment of neuroblastoma. A model system for the investigation of drug-resistance in vitro is described, exploiting two NB cell lines, SH-EP1 and SH-SY5Y, derived from the same parental background. These subclones show no difference in their sensitivity to the DNA damaging agent cisplatin, but have very different responses to the microtubule stabilising agent paclitaxel; SH-EP1 cells are sensitive, whilst SH-SY5Y cells are resistant. The protein product of the tumour suppressor gene p53 is stabilised to the same extent in SH-SY5Y cells following exposure to cisplatin, which readily engage apoptosis, as in those exposed to paclitaxel, which do not. Stabilised p53 is active in SH-SY5Y cells following paclitaxel exposure as reflected by the transcriptional upregulation of the cyclin dependant kinase inhibitor, p21WAF-1, a downstream effector of p53, after both drug treatments.

The pro-apoptotic Bcl-2 family protein Bax is latent in healthy cells and requires activation by drug-damage signals. Exposure of an epitope in the N-terminus of Bax was observed in both NB cell lines following both types of drug induced damage. This N-terminal exposure occurred to the same extent in settings of drug resistance as in those of drug sensitivity. The exposure of the N-terminus of Bax occurred in the cytosol, and was followed by the translocation of Bax to the mitochondria, again irrespective of cell fate. The exposure of the N-terminus of Bax was also observed following detachment of NB cells into suspension. Thus the N-terminal changes in Bax represent a reversible response to disparate types of damage, and do not commit the cell to death. A model for the activation of Bax by drug-induced damage in NB cells is suggested that must require a second signal, after N-terminal epitope exposure and mitochondrial translocation, which is needed to commit the cell to apoptosis. This damage-induced second signal is suggested to be abrogated in SH-SY5Y cells after treatment with paclitaxel. Lack of the full activation of Bax may represent a novel method of drug resistance in NB cells.

458 POSTER

A new approach for the detection of control sites in DNA

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Purpose: The concept of the consensus sequence of DNA control sites is well established. We have examined the variation of individual sequences about the consensus sequence. With the rapidly rising volume of reported DNA sequences, there is lively interest in automatic methods for detecting control sites or other short specific sub-sequences. We have developed an approach by which the statistical analysis of a reference set of sequences of a particular type of site allows one or more equations to be defined. If such an equation is satisfied by a new sequence then it is highly likely that the sequence corresponds to a site of the particular type. The definition of the equations makes use of the properties of the eigenvalues and vectors of the covariance matrix of suitably encoded sequences. In particular, the existence of one or more zero eigenvalues implies the existence of one or more such equations.

Methods: The approach is illustrated with the sequences of 173 promoters recognized by human RNA polymerase II. Many of these promoters are of particular interest in oncology and the database includes sequences for growth factors (e.g. GM-CSF, erythropoietin, various interleukins etc.), oncogenes and tumour viruses among others. Sub-sequences of 25 bases around the TATA box were extracted. Two bits were used to encode each base and the covariance matrix of the resulting 50 variables was determined. The eigenvalues and eigenvectors of the covariance matrix were calculated.

Results: The eigenvalues of the matrix ranged from 0.787 down to 0.035. This eigenvalue of 0.035 (almost zero) means that there is an equation which is (almost) satisfied by all the promoters in the dataset. A new sequence which (almost) satisfies this equation may be regarded as a putative promoter.

Conclusion: We have shown that promoter sequences contain correlations of such a nature that a rule can be derived which may be applied to detect other putative promoters. This regularity, not previously described, may be a common or indeed universal feature of sets of sequences with a common function.

459 POSTER

Measurement of tumor blood flow using colored dye extraction microspheres in two rat tumor models

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A technique that can measure tumor blood flow easily, accurately and economically is required to study tumor angiogenesis and angiogenesis inhibition. Using dye extraction colored microspheres, we measured tumor blood flow in Sato lung carcinoma (SLC) and ascites hepatoma LY80 in rats. Colored microspheres were infused into tumor-bearing rats via a catheter in the left ventricle. After removal of the tumor and the liver, the tissue samples were dissolved, and the microspheres were isolated. Dye was extracted, and the dye concentration was quantified by spectrophotometry. The dye concentration per gram of tumor was compared with that per gram of liver as follows (AU = absorbency units): [AU per gram of tumor]/[AU per gram of liver] x 100 = (%). Tumor blood flow corrected for wet weight was calculated as follows: [blood flow to tumor] = [AU per gram of tumor] x [reference withdrawal rate]/[AU per gram of reference blood]. Tumor blood flow rate was divided by tumor weight to yield ml/Emin-1g-1. The tumors were also examined histologically, and casts of the tumor vasculature were prepared with silicone rubber. Blood flow 2 weeks after transplantation was equivalent to 1/10 and 1/2 at 1 week in SLC and LY80 tumors, respectively (SLC, $P = 0.009$, $n = 10$; LY80, $P = 0.05$, $n = 10$). These decreases in tumor blood flow were associated with underlying pathological and vascular change. Blood flow in LY80 tumors negatively correlated with tumor volume ($P = 0.009$, $n = 10$). We concluded that the colored microsphere method,