ROLE OF PROGNOSTIC MARKERS BCL2/BAX/ CD40/CD40L/P53 IN MUSCLE INVASIVE AND NON-INVASIVE TCC BLADDER

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Purpose: We have previously shown that over-expression of the anti-apoptotic gene BCL2 predicted a poor response to neoadjuvant chemotherapy but had no prognostic significance in patients receiving radiotherapy alone although data in this area is sometimes conflicting. We therefore set out to investigate other pro-apoptotic genes as observed biological effects may depend not just on absolute but also relative protein expression levels.

Method: We examined expression of a range of genes including BCL2, BAX, P53, CD40 and CD40L by immunohistochemical analysis using archive tissue samples taken from patients included in various treatment trials for transitional cell carcinoma (TCC). An altered phenotype for markers was defined as follows: strong expression (S) vs. weak expression (W) and diffuse (D) (at least 30% malignant cells positive or confluent) versus focal (F) (<30% cells positive or non-confluent). No malignant cell staining was recorded as negative [Table]. An independent pathologist, blind to clinical outcomes, scored the samples. Clinical stage (T1 versus T2 versus [greater than or equal to] T3) and grade were also evaluated along with date of recurrence, date last seen, date and cause of death (if applicable) and other patient’s characteristics. Survival times were calculated as the date of primary tumour to date of death, or date of censor if alive. Survival curves were constructed using the method of Kaplan & Meier and the log-rank test was used to assess the differences between groups.

Results: Data were collected on 94 patients who presented with either invasive or superficial bladder cancer to the Queen Elizabeth Hospital between October 1984 and January 2001. Median follow-up for alive patients was 37 (range 9 – 146) months (m). Median survival for all patients was 61 (95% CI 47 - 80) m. Survival was compared in terms of tumour characteristics. Median survival for the various prognostic markers was as follows: BAX (SD) 77m versus (SF or WD) 45m versus (WF or Neg) 11m, p = 0.0003. BCL2 (SD, SF WD) 77m versus (WF, negative) 56m, p = 0.01. For CD40 the median survival was SD 80m, SF or WD 45m; WF or negative 24m, p = 0.55. For CD40L and p53 respectively, median survivals were 74/57/70m, p = 0.48 and 80/70/26m, p = 0.43.

Conclusion: This study shows BAX as a statistically significant indicator of better survival. Detailed analysis will be presented in the meeting.

| CD40, CD40L, BAX, BCL2 | P53 |
|------------------------|------|
| Diffuse                | Confluent positivity >30% of nuclei |
| Focal                  | Non confluent islands of positivity <30% of nuclei |
| Strong                 | Intensely brown |
| Weak                   | Weakly brown |

P132
IDENTIFICATION OF CANDIDATE MARKERS FOR MELANOMA PROGRESSION
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Advanced malignant melanoma has a poor prognosis. The genetic changes involved in melanoma tumour progression are largely undetermined. We have carried out DNA microarray-based gene expression profile analysis of an isogenic cell line model system that is representative of melanoma progression. The parental cell line, WM793, is established from an early stage human melanoma and is poorly tumourigenic when introduced into nude mice. Using Affymetrix HuGeneFL arrays containing probe sets for 7,129 transcripts, the gene expression profile in the tumourigenic, tumourigenic derivative, WM793-P1 and -P2, were compared with that of the parental cell line. Biological replicates for each of the three cell lines under study displayed low variation (Pearson’s correlation coefficients > 0.96). Comparative analysis revealed 131 genes as being differentially expressed between WM793 and WM793-P1 cells, with 44 of these being up-regulated. One hundred and thirteen genes were differentially expressed between the parental cell line and the second derivative, WM793-P2, with 30 of these showing increasing expression patterns. Cross-comparison revealed 68 genes as being changed in terms of expression between the parental cell line and both derivatives. Moreover, all of these genes displayed similar expression patterns. Interestingly, only 14 genes were differentially expressed between the first and second derivative, which is in keeping with the more analogous phenotypes of the derivatives compared to the parental cell line. Many of the differentially expressed genes identified are known tumour markers, with some of these being specific for melanoma. We have validated the expression of a panel of known and novel genes by semi-quantitative reverse transcriptase-PCR and Northern blotting. This work provides a better understanding of the molecular determinants of melanoma progression, and may offer novel therapeutic avenues. [Funding is acknowledged from the Irish Cancer Society (DJE) and the Health Research Board (WMG)].

P133
CHARACTERISATION OF EFFECT ON COLORECTAL TUMOUR CELLS OF CD44 ACTIVATION
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The process whereby different families of molecules critical in metastasis interact with each other is poorly understood. We have previously reported the results of our research on how activation of the CD44 adhesion molecule on colorectal tumour cell lines induces upregulation of matrix metalloproteinases(MMP)-2 and -9 and is involved in regulation of CD44v6 and ICAM-1 expression. We wished to further explore these interactions by investigating the effect of CD44 activation on CD44 variant mRNA transcript expression and on specific protein production. The effect on adhesion molecule expression by supplementing media with soluble extracellular matrix (ECM) components instead of specific antibody was also assessed. Colorectal cell lines SW480 and SW480-9 (genetically modified to produce MMP-9) were used in the study. CD44 variant mRNA expression in total RNA extracts from cell lines grown with and without specific CD44 antibody was analysed using RT-PCR. Protein extracts were also prepared from these cell lines, separated by SDS-PAGE and investigated for specific protein expression using Western immunoblotting. Cell lines were supplemented with soluble hyaluronate, fibronectin or BSA(control) and adhesion molecule expression analysed by flow cytometry in order to compare with effect of specific CD44 antibody. Soluble hyaluronate caused a marked upregulation in ICAM-1 levels in SW480 cells (30%-57%) in contrast to the minor alterations seen using specific antibody to activate. No difference in expression of CD44 variant mRNA transcripts was observed between control and CD44-crosslinked cells. This would suggest that CD44 exerts its effect at the protein translation level rather than transcriptionally. When Western blots with protein extracts of CD44 or p1 integrin crosslinked cells were probed for CD44-specific protein,
a dominant band at 80kD and a weaker band at 120kD were detected. CD44v6 protein expression was not altered in activated cells. Expression profiles of other adhesion molecules are under investigation. Thus natural ligands of CD44 is hyaluronate, have a marked effect on expression of other families of adhesion molecules whereas activation by specific antibody, while causing upregulation of MMP’s, does not have as profound an effect on the adhesion molecules investigated to date.

P134
ERK5 EXPRESSION IN HUMAN PROSTATE CANCER
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Introduction
ERK5 is required for EGF-induced cellular proliferation and progression through the cell cycle5. We have recently shown that MEK5, the specific activator of ERK5, is over-expressed in prostate cancer and is associated with metastatic disease and poor survival. ERK5 has three splice variants, namely a, b, and c, encoding for different amino termini6. The full length ERK5a is catalytically active, whereas the b and c forms are dominant negative kinases. We studied the expression of ERK5 isoforms, to further characterise the function of the MEK5/ERK5 pathway in human prostate cancer.

Materials and Methods
Primers flanking the region in which ERK5 varies in the three splice isoforms were designed. RNA was extracted from a panel of human prostate, bladder, breast and colon cancer cell lines. It was then subjected to reverse transcription PCR analysis. Snap frozen resected prostatic (7 BPH, 9 Cancer) tissue were also studied. Western blotting was performed on four of the cell lines to confirm ERK5 expression.

Results
The amplicons seen were related to the presence and size of the intron insert at the region of interest; a-115bp, b-175bp, c-251bp. In J82 bladder cancer cells, all three ERK5 isoforms were detected with the ‘a’ and ‘c’ forms predominant. In the other cell lines examined, only the ERK5’s ‘a’ variant was detected. Uniform ERK5a’ expression was also seen in the benign and malignant prostate tissue throughout all the samples. Western blotting of the cell lines exhibited the same expression pattern as was seen in the PCR results.

Discussion and conclusion
In murine tissue all three ERK5 isoforms are expressed in testis, brain, kidney, lung and heart. The expression of the ‘a’ and ‘c’ forms are much stronger than the ‘b’ form. We have shown that ERK5 ‘a’ is expressed in human prostatic tissue, and the dominant negative proteins and were not detected. The ‘b’ and ‘c’ isoforms may be suppressed in active tissue allowing excessive growth seen in both BPH and prostate cancer. It is possible that manipulation of the expression of these splice variants could be performed to inhibit excessive growth...[1] Kato Y, Tapping Rl, Huang S et al. 1998 Nature 395: 713 [2] Yan C, Luo H, Lee JD et al. 2001 J Biol Chem 276: p10870

P135
MOLECULAR AND MUTATION ANALYSIS OF HEREDITARY MULTIPLE EXOSTOSES
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Wing-Sum Hui1, Kathryn S.E. Cheah1, Jing-Dong Huang1, Kenneth M.C. Lee JD et al. 2001

P136
IDENTIFICATION OF NOVEL CANDIDATE GENES WITHIN THE 250KB TYLOSIS WITH OESOPHAGEAL CANCER (TOC) MINIMAL REGION ON CHROMOSOME 17q25
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The association of autosomal dominant palmoplantar keratoderma (tylosis) and oesophageal cancer has been recognised in three families. Linkage analysis has mapped the TOC gene to a 250kb region on chromosome 17q25. The tissue-specific expression profile of four known genes within this region was investigated by RT-PCR, and all of these genes were shown to be active in the tissues of interest, namely skin, oesophagus, and squamous cell oesophageal cancer.

The entire 250kb region has been examined using the online bioinformatics package NIX (Nucleotide Identification X) available through the UK Human Genome Mapping Project Resource Centre. Expression of regions of interest (predicted exons and ESTs) identified by NIX was investigated by RT-PCR, and evidence for seven novel genes within the TOC region was obtained. All of these genes appear to be expressed in the tissues of interest. One of these genes has since appeared in Genbank and has been screened for mutations in the TOC families, and mutation analysis is underway on a further single-exon gene that is registered in Genbank.

Further characterisation of the remaining RT-PCR gene fragments, by IMAGE clone sequencing and cDNA library screening, will aid in the identification of the TOC gene by providing full-length candidate genes for mutation analysis. The TOC minimal region has been shown to be implicated in sporadic as well as familial oesophageal cancer. The prognosis for patients with squamous cell oesophageal cancer is generally poor, often due to late presentation of the tumour. Identification of the TOC gene could potentially have applications in novel diagnostic and therapeutic strategies for oesophageal cancer.
VARIATIONS IN THE EXPRESSION OF P53 AND DOWNSTREAM TARGET GENES P21 AND MDM2 IN BREAST CANCER TREATMENT

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Background: In the treatment of breast cancer, clinical studies have shown that tumours which are clinically and histologically similar vary in their response to chemotherapeutic drugs. The mechanism of action of cytotoxic drugs is complex but in general mediated through induction of DNA damage and hence activation of p53 and its downstream pathway. Two key gene products of p53, p21WAF1 and MDM2, are known to be transcriptionally induced by p53. p21 mediates the tumour suppressing effects of p53 and the MDM2 protein controls the biological activity of p53 and targets it for destruction.

Aims: To examine the relationship between p53 and its key effector genes p21 and MDM2 in primary breast cancers treated with neo-adjuvant chemotherapy.

Methods: For 26 patients with breast cancer, p53, p21 and MDM2 protein expressions in response to chemotherapy treatment were determined by western blot analysis using highly sensitive human specific monoclonal antibodies. The results were compared with pathological data available during a minimum three years follow up with full approval of the Local Ethical Committee.

Results: Evidence of activity in the p53-MDM2 pathway was observed in tumours that showed a more favourable response to treatment and overall prognosis. No distinct relationship was seen between tumour behaviour/final outcome and p53-p21 axis as presence or absence of activity in this pathway had no clear link to the pathological/variable elements.

Conclusion: The data suggest the p53-MDM2 pathway is activated in breast cancers with more favourable prognosis. More detailed analysis may provide the framework for novel anti-cancer drugs that target the specific proteins involved.

COMBINATION OF MICRODISSECTION AND MICROARRAY ANALYSIS TO STUDY GENE EXPRESSION CHANGES IN BREAST CANCER INVASION

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Gene expression changes may play an important role in cancer invasion. To evaluate this possibility, the PALM micro-laser dissection and capture system was employed to isolate pure populations of tumour cells from two breast cancer types; invasive cancer and ductal carcinoma in situ (DCIS). We dissected the central and peripheral areas of these tumour lesions separately and pooled cancer cells from 10 sections, which were obtained from 5 different patients. By using the TRZol reagent, total RNA was extracted from microdissected tissue. The Atlas SMARTTM Probe Amplification Kit was used to synthesise and amplify cDNA. Probes from the periphery and from the centre were hybridised to duplicates of Atlas Human Cancer 1.2 Arrays which contain 1,176 known genes. After comparing images by AtlasImageTM 2.0, we have found that 22 genes changed their expression levels in the periphery of tumour lesions relative to the central regions of these tumours. 15 genes are up-regulated, and 7 genes are down-regulated (arbitrary threshold of 1.5-fold or greater). Verification that this procedure was identifying genes likely to be of importance in tumour invasion was provided by the observed up-regulation of the mesenchymal marker vimentin at the periphery of the tumours. Equally there was up-regulation of Rho C, which already has been shown to have higher expression levels in metastases relative to the primary tumour. TSG 101 and EF-1 α were shown by microarray analysis, to be expressed differentially between central and periphery. Using immunohistochemistry, we have been able to confirm these findings at the protein level.

The precision of the PALM microdissection technique allows the resolution of cDNA microarray gene expression analysis to be increased from the level of tissue analysis to analysis of one or more of its component cell populations. Using this method we have identified changes in gene expression associated with varying micro-anatomical locations within the developing tumour mass.
may represent a mechanism for disassembly of these complexes and subsequent loss of cell-cell and cell-matrix adhesions. The calpains represent a highly conserved family of non-lysosomal calcium dependent cysteine proteases. *In vivo* calpain activity is tightly regulated by its highly specific endogenous inhibitor, calpastatin. We have recently demonstrated that calpain mediated proteolytic cleavage of the focal adhesion kinase (FAK) and subsequent disassembly of the focal adhesion complex accompanies v-Src induced morphological transformation of fibroblasts contributing to increased cell motility (Carrragher et al., 2001. *J Biol Chem. 276*;4270). Increased calpain activity in v-Src transformed fibroblasts also promotes cell cycle progression and anchorage-independent growth (Carrragher et al., 2002. *Mol. Cell.Biol. 22*:257). To further establish the role of calpain in tumour cell metastasis we have examined the role that calpain plays in regulating the turnover of the adhesive complexes of metastatic epithelial cells. We have demonstrated that the protein levels of calpain are often upregulated in parallel with decreased levels of calpastatin in metastatic epithelial cell lines relative to non-metastatic controls. Furthermore, using pharmacological inhibitors we have demonstrated that inhibition of calpain activity stabilizes integrin-linked focal adhesion complexes of both non-metastatic and metastatic epithelial cells. Inhibiting calpain activity also suppresses the disassembly of cadherin-dependent cell-cell adhesions in response to culture of the cells in low calcium conditions. These studies indicate that modulation of calpain activity may contribute to the turnover of integrin-linked focal adhesions and cadherin-dependent adherens junctions in metastatic epithelial cells.

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**EXCISION REPAIR CROSS-COMPLEMENTING GENE 1 (ERCC1) PROTEIN EXPRESSION IN COLORECTAL CANCER**

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**Introduction:** The ERCC1 gene is located on chromosome 19 and encodes one of the major enzymes involved in nucleotide excision repair (NER). NER is the mechanism of repair of DNA adducts caused by ultraviolet radiation and platinum-based chemotherapies. High levels of tumour ERCC1 mRNA expression have been linked with resistance to cisplatin in gastric cancer and oxaliplatin in colorectal cancer. Determining the levels of mRNA expression is a complicated technique not available to most histology departments. We have developed a protocol for determining ERCC1 protein expression using immunohistochemistry (IHC) which may be a more practical alternative.

**Methods:** Archival formalin-fixed, paraffin-embedded specimens were retrieved from 224 consenting patients in the MRC CR08 clinical trial of chemotherapy for advanced colorectal cancer. Tissue microarrays (TMAs) were produced containing 0.6mm cores of tumour and where available, corresponding normal colonic mucosa. 0.5micron sections of TMAs were cut for IHC. Very small biopsies were examined as individual whole sections. A reproducible protocol was developed for IHC using a mouse monoclonal antibody to the human ERCC1 protein (Lab Vision-Neomarkers, Fremont, California). Staining was scored by two independent assessors as positive or negative, and the pattern of expression was recorded.

**Results:** 224 colorectal cancers and 138 corresponding normal colonic mucosa specimens were assessed. 28% of normal mucosa and 33% of cancers were positive for ERCC1 protein expression. 76% of normal tissues stained with a nuclear pattern, 21% with a cytoplasmic pattern and 3% with both nuclear and cytoplasmic staining. For tumour tissue, the rates were 36%, 59% and 5% respectively. Staining of whole sections, including both normal and malignant tissue, identified cases where normal tissue showed nuclear staining and adjacent tumour tissue showed cytoplasmic staining. Different patterns of cytoplasmic staining were observed in cancers, varying from a diffuse pattern to very discrete granules.

**Conclusions:** Immunohistochemical staining for ERCC1 using this technique gives positive staining for a third of colorectal cancers. We noted a tendency for positive normal colonic tissue to show predominantly nuclear staining and for cancers to show cytoplasmic staining. ERCC1 protein is produced in the cytoplasm and transported to the nucleus. Some colorectal cancers may have dysregulation of ERCC1 protein production or transport. We are continuing to assess the role of ERCC1 protein expression as a predictor for response to oxaliplatin chemotherapy.

**References:**

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**P142**

**THE MRC CR08 (FOCUS) TRIAL MOLECULAR PATHOLOGY PROJECT. IDENTIFYING PREDICTIVE FACTORS FOR CHEMOTHERAPY RESPONSE.**

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**Introduction:** After over 40 years of 5-Fluorouracil (5FU)-based chemotherapy for colorectal cancer, new agents are now available, including irinotecan and oxaliplatin. However, we are still unable to reliably identify factors that will allow selection of a chemotherapy regimen for an individual patient that will definitely be of benefit. Reported data on molecular predictive factors are so far based mainly on small numbers of patients, outside the setting of randomised clinical trials. Even with the most studied marker for 5FU response, thymidylate synthase (TS), there are conflicting accounts of its value. The MRC CR08 (FOCUS) Trial is investigating the role of irinotecan and oxaliplatin and their sequencing, in addition to 5FU-based chemotherapy for 2100 patients with advanced colorectal cancer and is therefore an excellent opportunity to study predictive molecular variables in a controlled clinical setting. FOCUS is still recruiting and includes a request to retrieve stored pathological material for bowel cancer research.

**Methods:** Over 95% of patients entering FOCUS have consented to this study. Archival primary tumour and when available, metastasis biopsy specimens are retrieved in anonymised form. Tissue microarrays (TMAs) are produced with multiple tumour and normal tissue cores from each patient and these are used for immunohistochemistry (IHC) to assess protein expression. Potential markers being tested by IHC include: for 5FU, TS, thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), for irinotecan; topoisomerase 1 and for oxaliplatin; excision repair cross-complementing gene 1 (ERCC1). We are also investigating RNA expression of the same markers using real-time reverse transcriptase (RT)-PCR. Candidate DNA markers for allelic imbalance, microsatellite instability and gene polymorphisms are also being examined. The role of CDNA microarrays in identifying novel predictive factors is being investigated.

**Results:** To date 300 sets of tumour blocks have been retrieved for study. Techniques being used and a report of the progress of the project will be presented at the meeting. An initial assessment of TS expression using IHC in 229 cases, all receiving 5FU (67% alone; 33% in combination with irinotecan or oxaliplatin), has shown that 28% of tumours are negative (Grade 0/1) and 72% are positive (Grade 2/3). In this preliminary group, TS staining does not correlate with the clinical response status after the first 3 months of chemotherapy (P=0.37).

**Conclusions:** This study is demonstrating that large-scale retrieval and processing of surplus pathological material in association with randomised clinical trials is feasible, and gains almost universal patient consent. The large number of patients in FOCUS will allow chemopredictive association to be presented at the meeting. An initial assessment of TS expression using IHC in 229 cases, all receiving 5FU (67% alone; 33% in combination with irinotecan or oxaliplatin), has shown that 28% of tumours are negative (Grade 0/1) and 72% are positive (Grade 2/3). In this preliminary group, TS staining does not correlate with the clinical response status after the first 3 months of chemotherapy (P=0.37).

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[2] MRC CR08 Clinical Protocol (Version 5) June 2001.

**P143**

**IMMUNOHISTOCHEMICAL PROFILING OF PATHWAY ACTIVATION IN TUMORS USING ACTIVATION-SPECIFIC ANTIBODIES.**

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We have used phospho-specific antibodies to characterize the activation status of a broad range of therapeutically relevant signal transduction pathways in human breast cancer tissues by immunohistochemical
assessment of tissue arrays. We validated the phospho-specific antibody immunohistochemical (IHC) results with methods including Western blot analysis, antigen absorption and staining of embedded cells. Akt kinase pathway activation was demonstrated by IHC with prostate tumor-derived LNCaP cultured cells, which lack PTEN phosphatase. IHC analysis of LNCaP cells also demonstrated the induction of apoptosis following inhibition of the Akt pathway. Multi-pathway IHC analysis of human breast tumor tissue arrays showed significant correlations among activation states of diverse signaling proteins and implicated multiple pathways underlying the neoplastic phenotype. Cluster analysis of protein activation suggests that key signaling pathways cluster with EGFR overexpression. In contrast, HER2 overexpression failed to cluster with any of the pathways tested. The activation state of specific signaling proteins was also found to correlate with pathological indices and patient prognosis. This is illustrated by the significant correlation between the activation states of Erk/MAPK, estrogen receptor, and breast cancer pathological indices of tumor grade and lymph node status. The ability to profile the activation status of a specific therapeutic target, as well as those of multiple other signaling proteins will be an important complement to the new generation of targeted therapeutics and will enable effective and precise diagnosis of the molecular basis of disease.

P144
TARGETED INTERRUPTION OF THE CDK4-CYCLIN D1 COMPLEX
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Cyclin dependent protein kinases (CDKs) control cell cycle progression and thus provide a good target for the development of inhibitory molecules for cancer therapy. To date inhibitors have been directed against the kinase domain of each CDK. We aim to develop inhibitors that target the binding of CDK4 to cyclin D, an interaction which is essential for CDK4’s activity. In addition to inhibiting CDK4’s ability to support cell cycle progression, our inhibitors are also likely to facilitate CDK4-independent activities of cyclin D, which are in general themselves thought to be anti-proliferative. This two pronged approach should have broad applicability in the clinic. To screen for small molecules that would inhibit the formation of the cyclin D/CDK4 complex, we are using a peptide aptamer that defines the cyclin D binding site. Peptide aptamers are themselves small molecules, but ones that can be readily screened in a yeast two-hybrid format for those that bind to a protein of interest. Because of their biological (peptidic) nature, peptide aptamers are particularly useful in disrupting protein-protein interactions in vivo. A random peptide aptamer library consisting of peptides (20 amino acids long) was screened against cyclin D (see accompanying abstract). One of the biologically active peptide aptamers was then used as the basis for the following screen. We are establishing a small molecule screen in a cell free system using bioluminescence resonance energy transfer (BRET) technology. We intend to screen a small diverse compound library (10, 000 compounds) to identify inhibitors of the cyclin D/CDK4 complex. Results will be presented.

P146
THE DETERMINATION OF HISTONE DEACETYLASE ACTIVITY IN INTACT CELLS USING A NON-ISOTOPIC ASSAY
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Inhibition of histone deacetylases (HDACs) may have therapeutic potential in patients with malignant disease. A rapid in vitro HDAC activity assay, based on the deacetylation of an acetylated lysine (MAL), has recently been described (Hoffmann et al, Nuc Acids Res 1999; 27: 2057-8), with substrate and product (ML) concentrations determined by specific HPLC analysis with fluorescence detection. We have further developed this assay for use in intact cells with a single time-point reaction. For isolated rat liver, enzyme, substrate (5 µg/mL MAL) and inhibitor at 6 conc2 were incubated at 37°C for 60 minutes, after which the reaction was stopped with aceticnitrile, and MAL and the deacetylated product ML determined in the supernatant. For intact cells, 1x106 CEM cells in 1 ml medium were exposed to inhibitors at 6 conc2 for 60 minutes, after which MAL (20 µg/mL) was added for a further 30 minutes, all at 37°C. Cells were then rapidly washed at 4°C, lysed by sonication, the reaction stopped and MAL and ML determined in the supernatant. The IC50 values for % viability were determined over a 3-day continuous exposure in CEM cells. Results are presented in the table for phenylbutyrate (NaPB), phenylbutyrate hydroxamic acid (NaPBHA) and Trichostatin (TSA).

| TREATMENT | %VIABILITY DAY 4 (mean ±SD) |
|-----------|-----------------------------|
| Control   | 98 ± 1                      |
| A         | 90 ± 4                      |
| C         | 87 ± 6                      |
| I+A       | 67 ± 3                      |
| T         | 41 ± 4                      |
| I+A       | 21 ± 6                      |
| E         | 43 ± 4                      |
| E+A       | 36 ± 10                     |

P145
THE ADDITION OF ALL-TRANS RETINOIC ACID (ATRA) COUNTERACTS THE INCREASE IN BCL-2 LEVELS INDUCED BY CYTOTOXIC AGENTS
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INTRODUCTION: Over-expression of BCL-2 is common in AML and is associated with increased resistance to cytotoxic drugs. ATRA has been shown to down regulate BCL-2 levels and may thus improve chemosensitivity. We investigated the effects of combining ATRA with existing anti-leukaemic agents on BCL-2 expression and cytotoxic response in a panel of human leukaemic cell lines.

METHODS: CEM, HL60, K562 and ML-1 cells were exposed for 4 days to IC50 concentrations of cytarabine (C), idarubicin (I) or etoposide (E) alone and in combination with 10 µM ATRA (A, previously shown to be optimal for BCL-2 downregulation). Cell viability and % apoptosis were assessed daily. BCL-2 status was assessed by flow cytometry, immunoblotting and ‘real-time’ PCR.

RESULTS: Exposure to low concentrations of A, I or E alone typically raised BCL-2 levels (in CEM cells on day 4, with 10 nM C or 3 nM I: +20 ± 9% and +22 ± 4%, respectively; p<0.025). Conversely, A alone resulted in decreased BCL-2 by day 2, which was maintained through to day 4 (-20 ± 6% on day 1 and -14 ± 8% on day 4; p<0.02). The combination of ATRA with cytotoxic agents typically resulted in BCL-2 levels that were lower than those seen with cytotoxic agents alone (at day 4 in HL60 cells: +1 ± 15% with C alone; -52 ± 6% with C+A; p=0.038). The changes in BCL-2 protein correlated with changes in bcl-2 mRNA and were similar for CEM and ML-1 cells. % viability for each cytotoxic agent ± A are shown in the table. Flow cytometric analysis indicated cell death to be by apoptosis.

CONCLUSIONS: These data confirm the increased cell kill reported when ATRA is used with cytotoxic agents, but this increased cell kill is no greater than additive. Whilst ATRA prevents the rise in BCL-2 seen with chemotherapy alone this is insufficient to cause a synergistic increase in cell kill. As ATRA effects the expression of many other genes, agents that target BCL-2 more specifically, such as Antimycin A1 may help to determine whether targeting BCL-2 sensitises cells to conventional agents.
The relative HDAC inhibitory activities of NaPB and NaPBHA are in line with the proposed structure of the enzyme active site. Although more time intensive, this modification of the HDAC activity assay for use in intact cells may provide a better indication of the potential biological activity of selected compounds.

### Table: HDAC inhibitory activity IC50

| Compound | Rat liver | Intact CEM cells | CEM cells |
|----------|-----------|------------------|-----------|
| NaPB     | 405 µM    | 9.5 nM           | 8.4 nM    |
| NaPBHA   | 3.1 µM    | 269 µM           | 244 µM    |
| TSA      | 19 nm     | 23 nM            | 119 nM    |

The relative HDAC inhibitory activities of NaPB and NaPBHA are in line with the proposed structure of the enzyme active site. Although more time intensive, this modification of the HDAC activity assay for use in intact cells may provide a better indication of the potential biological activity of selected compounds.

### PI47
**A CELL-BASED ELISA IS A USEFUL PHARMACODYNAMIC AND SCREENING TOOL TO EVALUATE CHANGES IN CELLULAR ACETYLATION**

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Acetylation of specific lysine residues in histone tails is an essential element (with phosphorylation and methylation) of an intricate histone code that regulates gene transcription in a temporal and context dependent manner. Acetylation is regulated by two classes of enzyme, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Several HDAC inhibitors have been described and some are presently in pre-clinical and clinical development. There is also evidence that deregulated HAT enzymes play a role in the progression of some cancers. No drug-like inhibitors of HATs have yet been described. High-throughput screening is being used in the Centre to screen a ~60,000 compound collection for compounds that inhibit HATs. Such compounds would be useful chemical probes to investigate the effects of HAT inhibition on cellular behaviour and could form the basis of a new type of chemotherapy. It is important that a mechanism-based assay with relatively high throughput is available to compare compounds identified by screening. A cell-based ELISA (cytoblot) has been used as an alternative to Western blotting for determining the effects of HDAC and HAT inhibition. Trichostatin A (TSA), a natural product HDAC inhibitor was used as a positive control. Treated human colon tumour cell lines (HCT116 and HT29) (8000 cells/well seeded) were fixed and permeabilised in 96-well plates and primary antibodies with different specificity for histone acetylation added. Following washing, a europium-labelled second antibody (PerkinElmer Life Sciences) was added and time-resolved fluorescence measured. Fluorescent counts were normalised to protein measured in the same well using BCA reagents. Cellular acetylation increased in response to TSA (0.01 – 2.2µM) compared to DMSO treated controls within as little as 15 minutes and results were consistent with those obtained by Western blotting. An acetylated protein antibody (Abcam Ltd.) has been used to determine the extent of cellular acetylation in cells cultured over 5 days. Acetylation peaked 24 and 48hrs following plating out before decreasing again 72hrs - 120hrs later. The relationship between cellular acetylation and proliferation is being studied further using antibodies that recognise different histones and selective sites of acetylation. The ELISA is proving useful as a pharmacodynamic measure of HDAC inhibition in vitro and to evaluate and rank HAT inhibitors identified by high-throughput screening. The ELISA is also being used as a cell-based high throughput screen to identify compounds that either increase or decrease acetylation. Supported by Cancer Research UK Programme Grant SP2330/0203 and the Institute of Cancer Research.

### PI48
**IN EXPERIMENTAL METASTASES INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-4 (IGFBP-4) IS EXPRESSED AT HIGH LEVELS**

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A murine model of metastatic breast cancer has been established. 4T1.2 murine mammary carcinoma cells are a subclone of the spontaneously arising BALB/c 4T1 cell line (a gift from Robin Anderson, University of Melbourne). Lung metastases were established by tail vein injection of 50,000 4T1.2 cells, bone metastases by inter-tibial injection of 10,000 4T1.2 cells. Immunohistochemistry and X-ray imaging were used to confirm that the tumours were bone associated. Tumour and normal lung and bone tissues were harvested and flash frozen in liquid nitrogen. RNA from the tissues was used to produce radiolabelled cDNA. The labelled cDNA was hybridised against a commercially available gene array containing 588 genes (Clontech), and the arrays were then exposed to X-ray film. Differential gene expression was identified using specialised software (Atlasimage 1.01, Clontech). Housekeeping genes were used on each array to normalise expression patterns between them. A number of genes were putatively identified as being differentially expressed between the bone and lung tumours. These genes were selected for further analysis. IGFBP-4 was found to be up-regulated in both metastases relative to normal tissues. These results were confirmed through Northern hybridisation and reverse transcription polymerase chain reaction (RT-PCR). IGFBP-4 is involved in the regulation of bone homeostasis. In breast cancer metastasis to bone there is associated bone degradation. Parathyroid hormone, normally released when calcium levels in the blood drop, coincides with an up-regulation of IGFBP-4 in osteoclasts. This IGFBP-4 binds to IGFs, preventing the normal stimulation of osteoblasts by IGFs, and therefore decreases osteoblast activity.

### PI49
**THE TUMOUR SUPPRESSOR PTEN AND THE CELL CYCLE INHIBITOR p27KIP1 IN PROSTATE CARCINOMA AND PROSTATIC INTRAEPITHELIAL NEOPLASIA (PIN) - AN IMMUNOHISTOCHEMICAL STUDY**

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Genetic abnormalities involving the PTEN locus (10q23) and loss of p27KIP1 expression are frequently observed in prostate carcinoma, especially in advanced stage disease and metastases. Results of recent investigations on gliblastoma cell lines demonstrated that PTEN determines cell cycle arrest in G1 by activating p27KIP1. However, it is currently unknown if a functional relationship exists between PTEN and p27KIP1 in the human prostate. Furthermore, inhibition of HATs could provide a basis for the development of therapies for prostate cancers. In this study PTEN and p27KIP1 expression status were immunohistochemically assessed on serial slides in 46 prostate carcinomas (23 prostatectomies and 23 transurethral resections), 20 prostatic intraepithelial neoplasias associated to the carcinomas and 15 separate metastases. The results were correlated with the Gleason score (7 = low Gleason score, >7 = high Gleason score). We defined a cut-off level for p27KIP1 of 40% positive nuclei.

Ten of the 20 PIN lesions (50%) showed loss of at least one marker and p27KIP1 was more frequently affected than PTEN (p<0,05). Simultaneous PTEN and p27KIP1 deletion was observed in only one case (5%). The disease progression from PIN to cancer (assessed by comparing the staining pattern in PIN and cancer lesions of the same case) associated loss of PTEN in 39% of cases and loss of p27KIP1 in 15,8% of cases, but no concomitant PTEN and p27KIP1 loss. Nine of 19 carcinomas with low Gleason score (47,3%) showed loss of at least one marker; the same pattern was noticed in 18 of 27 high Gleason scores (66,7%). Seven of 27 high-Gleason scores (25,9%) associated concomitant deletion of both markers, whereas only 2 low-Gleason carcinomas showed this pattern (10,5%). Similar to PIN, the low-Gleason scores correlated with loss of p27KIP1 but not with loss of PTEN (p>0,05). Surprisingly, 10 of the 15 metastases (66,7%) showed positive signals for PTEN, whereas p27KIP1 was under 40% (mean 14,2%) in all but one case. These data taken together suggest that: 1) loss of p27KIP1 occurs earlier and is more frequent than PTEN protein loss in the prostate carcinoma 2) the progression of PIN to invasive carcinoma could be determined by separate loss of PTEN or p27KIP1 proteins, but not by a negative regulation of p27KIP1 through PTEN protein deletion, 3) the low-differentiated carcinomas and the metastases maintain a surprisingly high PTEN expression, associated with
low p27^KIP1 levels, 4) p27^KIP1 seems to be down-regulated in prostate carcinoma by other pathways than those involving PTEN.

**P150**

**REGULATION AND INTRACELLULAR LOCATION OF PROTEIN KINASE B/AKT IN HUMAN PROSTATIC CELL LINES**

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Protein Kinase B (PKB, also known as Akt) is a key intracellular signalling enzyme which regulates cell survival, motility, and gene transcription via the apoptotic regulator protein BAD, the Forkhead transcriptional regulators, GSK-3, and caspase 9. PKB is activated by phosphorylation at thr308 and ser473 by phosphatidylinositol-dependent kinases (PDKs). PKB and the PDKs are activated at intracellular membranes by PI3P generated by phosphatidylinositol 3-kinase (PI3K) in response to soluble growth factors (GFs) and integrin- or cadherin-mediated cell adhesion. PKB is downregulated by PTEN tumour-suppressor protein, which inactivates PI3P. We studied PKB activation in cell lines derived from human non-tumour prostate epithelium (PNT2, PNT1a) and from prostate tumour metastases (LNCap, PC3). Phosphorylation of PKB was assayed using antibodies specific to the ser473- and thr308-phosphorylated protein forms in Western blot probing and immunocytochemistry. The non-tumour cells showed strong dependence on soluble GFs for maintenance of PKB activation. EGF (5 ng/ml) caused a rapid (<5 min) peak of activation followed by a gradual (>30 min) decline, while 5 ng/ml IGF-I or IGF-II caused a slower (>30 min) increase to higher final levels of phosphorylation. The tumour cells maintained high levels of PKB activation in the absence of exogenous GFs. Inhibition of PI3K by 10 μM LY294002 resulted in rapid loss of PKB activation (<30 min) in the non-tumour cells, followed by partial recovery over 2-3 hours. In contrast, the tumour cells showed a slower (3 hours) response to LY294002 which did not reverse. PKB activation in the different cell lines was paralleled by levels of phosphorylation of GSK-3 and by levels of S and G2/M phases of cell growth. Confocal microscopy showed that phosphorylated PKB was located at the plasma membrane in the tumour cells, while in the non-tumour cells it was largely associated with particulate or vesicular cytoplasmic structures, where it was resistant to LY294002. Cells transfected with myc-tagged PDK-1 or with the pleckstrin homology domain of PKB fused to green fluorescent protein revealed a similar pattern, indicating that PKB activation and location is PI3P-dependent.

PKB activation in normal prostatic epithelial cells thus appears to be maintained by GF-dependent, LY294002-resistant generation of cytoplasmic vesicle-associated PI3P by PI3K, while in tumour cells the PI3P is largely generated at the plasma membrane by PI3K which is independent of exogenous GFs, but extremely sensitive to LY294002. These studies suggest that drugs which inhibit PI3K and its products may be selectively therapeutic in treatment of advanced prostate cancer. Supported by YCR and MRC.

**P152**

** METHYLATION OF GSTP-1, CD44, E-CADHERIN AND RASSF1A IN NORMAL AND MALIGNANT PROSTATE TISSUE**

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Sensitive methods for the detection of prostate cancer may be useful in defining prognosis and in directing future therapeutic strategies. RT-PCR for PSA mRNA in the peripheral blood of cancer patients has been studied by our group and others and found to be sensitive but lacking in absolute specificity. Identification of tumour specific molecular abnormalities should circumvent this problem, however consistent tumour specific abnormalities have not been identified in prostate cancer. Abnormal methylation of 5’ CpG islands within promoter regions of tumour suppressor genes have been identified in prostate cancer (GSTP1, CD44 and e-cadherin) and proposed as a mechanism of inactivation of these genes. These tumour specific abnormalities within GSTP1, CD44 and e-cadherin were studied further in prostate tumours as well as RASSF1A a putative tumour suppressor gene in bladder and nasopharyngeal cancers. DNA from eleven laser-microdissected prostate tumour needle biopsies and 5 non-malignant prostate biopsies were modified with bisulphite and methylation examined using a sensitive nested methylation-specific PCR.

GSTP1 methylation was detected in all tumours, CD44 methylation in 5/11 tumours, e-cadherin methylation in 1/11 tumours and RASSF1A methylation in 10/11 tumours. Non-malignant prostate biopsies from men with elevated serum PSA levels also showed methylation patterns for all 4 genes.

The methylation status of GSTP1 and CD44 concurs with other studies in prostate cancer, however e-cadherin methylation was rarely seen. RASSF1A was also methylated in prostate cancer (novel observation).

Interestingly, methylation of these genes was also found in age-matched non-malignant prostate tissue. We hypothesise that the methylation patterns seen in normal tissues may be an age-related change or be part of a spectrum of ‘background’ changes seen prior to the malignant phenotype. Tumour specificity for methylation of the 4 genes studied was not demonstrated.

**P151**

**HUMAN SPROUTY2 (A NOVEL INHIBITOR OF FGF RECEPTOR SIGNALLING) IN HUMAN PROSTATE CANCER**

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INTRODUCTION:

The Fibroblast Growth Factors (FGFs) and their receptors (FGFRs) are important in prostate cancer pathogenesis. Over-expression of multiple members of the FGF family (FGF1, FGF2, FGF7 and FGF8), occur in prostate cancer. FGFs activate FGF Receptors. FGR1 and FGR2 are over-expressed in prostate cancer.

Sprouty protein was first described in Drosophila to regulate airway development. It functions in a competitive fashion to block FGF mediated signalling. It has similar activities in vertebrates. The expression of Sprouty is induced by activation of FGFR and therefore serves as a physiologic negative feedback to control the function of activated receptors. Over-expression of Sprouty blocks FGF mediated signalling enough to result in an altered phenotype. We aimed to examine expression levels of hSprouty2 in human prostate cancer.

**MATERIALS AND METHODS:**

RNA was extracted from 62 BPH and malignant prostate chips. Expression of hSprouty2 was examined within prostate samples using Reverse Transcription (RT)-PCR and Western Blot analysis. Sprouty mRNA expression was quantitated by Real Time RT-PCR, which is currently the most sensitive method for mRNA quantitation.

**RESULTS:**

Expression of hSprouty2 was compared between benign prostate hyperplasia (BPH), low grade (Gleason 2-4), moderate grade (Gleason 5-7) and high grade (Gleason 8-10) prostate carcinoma. Levels in BPH and low grade carcinoma were similar, and although moderate grade carcinoma showed a downward trend in Sprouty expression, this was not significant. When compared to high grade BPH group, hSprouty2 mRNA expression was significantly reduced in high grade tumours (p=0.005).

**CONCLUSIONS:**

Expression of hSprouty2 (a physiologic inhibitor of FGFR) is significantly down-regulated in high grade prostate cancer. The functional significance of such hSprouty2 down-regulation warrants further investigation.
P153
INVESTIGATION OF THE INVASIVE CAPACITY OF BASAL CELL CARCINOMA USING AN INVASION ASSAY MODEL
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Basal cell carcinoma (BCC) is the commonest cancer in humans and presents major problems in terms of surgery for the patient and workload for the NHS with over 40000 cases of BCC each year in the UK. Despite this there has been relatively little research into BCC compared to other skin cancers. We have previously shown that in aggressive BCC there is an increased expression of matrix metalloproteinase-2 (MMP-2) and reduced expression of its inhibitor TIMP-2. MMPs have been implicated in the invasion and metastasis process in a number of different tumours. We are examining if the invasive properties of BCC and investigating if these can be modified with inhibitors of MMPs.

Invasion studies were performed using the cell line of BCC, KMC-1/BCC and primary cell lines isolated from surgical specimens. Suspensions of the BCC cells were placed in the upper compartment of the invasion chamber and these were attracted to conditioned media in the lower chamber. Invasive cell migration was quantified using a nigratract-coated polycarbonate filter. It has been possible to use this experimental model to examine the effect of a number of substances on BCC’s including TIMP-2 and synthetic MMP inhibitors such as ilomastat. Provisional results suggest that the invasive capacity of BCC can be reduced by MMP inhibitors.

P154
CHANGES IN THE APOPTOTIC PHENOTYPE OF PROSTATIC EPITHELIAL CELLS DURING THEIR DIFFERENTIATION FROM BASAL TO LUMINAL SECRETORY
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Basal prostate epithelial cells differentiate into an endstage luminal cell which is dependent on the presence of androgen. Why these cells become androgen dependent and susceptible to cell death via apoptosis remains unknown. We hypothesise that alterations in the apoptotic phenotype of the luminal prostate epithelial cells ultimately leads to its dependence on androgen and susceptibility to apoptosis. The NRP-152 cell line is a benign basal cell line derived from the dorsal-lateral prostate of the rat. Under mitogen-deficient conditions, NRP-152 cells were differentiated from a basal to a luminal prostate epithelial cell. Using Western Blotting immunofluorescence, this change in phenotype was confirmed by the progressive loss of cytokeratin 14 expression and the increased expression of cytokeratin 18, over an 8 day time-course. The expression of the apoptotic proteins Bcl-2, MCL-1, cIAP-1, cIAP-2 and caspase 3 were then characterised. Western blotting demonstrated that the anti-apoptotic proteins Bcl-2, MCL-1, cIAP-1 and cIAP-2 all decreased in expression in a time dependent manner. However, the expression of the pro-apoptotic protein, caspase-3, was unchanged.

These findings suggest that prostatic luminal cells become more susceptible to apoptosis during differentiation as a result of decreased expression of anti-apoptotic proteins. Further characterisation of these apoptotic changes will help define the potential areas in the differentiation process where carcinogenesis and hormone-independence may occur.

P155
CELL CYCLE CHECKPOINT RESPONSE AND MICRONUCLEUS FORMATION INDUCED BY UV A RADIATION IN CELLS FROM INDIVIDUALS HARBOURING GERMLINE MUTATIONS IN BRCA1 S.E. Tobi1*, J. Shorrocks1, R. Ecles2, J.H.Peacock2 and T.J.McMillan1
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Individuals carrying germline mutant alleles of the BRCA1 gene have an increased risk of developing breast and ovarian cancer. A number of observations point to the participation of the BRCA1 protein in the cellular response to DNA damage and, more specifically, there is evidence for its involvement in regulating cell cycle checkpoints after ionising radiation. Using primary human diploid fibroblasts from individuals either wild-type for BRCA1 or containing a mutant allele (heterozygous), we examined the cell cycle response following exposure of exponentially-growing cells to the oxidative stress induced by U VA radiation (320-400nm). Although cells from all individuals exhibited a G1 arrest after UVA (100kJ/m²), the response was of a shorter duration in the BRCA1 (+/-) heterozygotes. Thus, 14 hours after irradiation, the proportion of cells in S-phase in three wild-type fibroblast strains (expressed as percentages (mean ± range) of corresponding unirradiated controls) was 20.33 ± 5.36, 16.85 ± 2.25 and 7.85 ± 2.41, as compared to values of 33.78 ± 1.52 and 37.53 ± 1.52 for two BRCA1 (+/-) strains. Since a defective G1/S checkpoint in BRCA1 heterozygotes could lead to a greater proportion of S-phase cells with unrepaird DNA damage (strand breaks) and a resultant increase in chromosomal instability, the frequency of micronuclei induced by UVA was examined. Three normal (+/+), one heterozygote (+/-) and one homozygote (-/-) strain of each were irradiated (in the case of the latter experiments) produced mean micronuclei frequencies of 0.080 ± 0.013 and 0.094 ± 0.031/binucleate cell respectively (not statistically significant), 48h after UVA exposure . Our data suggest a defective G1/S checkpoint in cells from BRCA1 heterozygotes in response to UVA radiation although this is not reflected in genomic instability as measured by micronuclei induction.

P156
INTRACELLULAR SIGNALING IN THE NFκB ACTIVATION PATHWAY FOLLOWING OXIDATIVE STRESS MEASURED BY FLUORESCENCE LIFETIME IMAGING OF FLUORESCENT PROTEINS
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Fluorescence resonance energy transfer (FRET) between spectrally different fluorophores has allowed the study of protein-protein interactions to be followed within the cell. In this study, spatially resolved FRET was measured by multiphoton fluorescence lifetime imaging microscopy, which provides a method for following changes in the lifetimes of fluoroscently tagged proteins, indicating a change in protein environment inside both live and fixed cells.

NFkB is a transcription factor that is activated by a wide variety of stimuli including ionizing radiation and many anticancer agents. Its activation has been shown to be cytoprotective and may reduce the efficacy of many anticancer drugs. Oxidative stress has also been shown to activate NFkB, but only in certain cell lines. However, in all cell lines examined NFkB activation was inhibited by the addition of antioxidants. In order to determine whether reactive oxygen species are involved in NFkB activation and where in the NFkB pathway they exert their effect, we have used green fluorescent protein (GFP) tagged fusion proteins of NFkB and other members of the signalling pathway to examine lifetime changes following a variety of stimuli inducing oxidative stress. Measurements were performed by analysis of fluorescence decay kinetics acquired using single photon counting following two-photon excitation (ca 150 fs 890 nm pulses), on a pixel-by-pixel basis. We initially examined the interaction between IkBz (inhibitory subunit of NFkB) and its activating kinase IKK in MCF-7 cells transiently transfected with both GFP fused IkBz and FLAG-tagged IKK. Following treatment, the cells were fixed and the IKK protein was labelled with AlexFluor 532. By stimulating the cells with activators of NFkB, a reduction in the average lifetime of GFP was observed indicating the proteins change in the environment. This reduction was only observed in the cytoplasm of the cell (not in the nucleus) confirming the cytoplasmic localization of the IkBz/IKK interactions. When lifetime data were fitted with biphasic kinetics quantitative measurements of FRET efficiency were obtained which allowed measurements of the distance between the two proteins during interaction. Oxidative stress (100 µM H2O2) was able to stimulate members of the NFkB activation pathway in this MCF-7 cell line as measured by FRET and confirmed by Western Blot analysis of NFkB and IkBz proteins. In conclusion, the results show that analysis of the lifetime images was successful in reporting interactions between the proteins IkBz and IKK.

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THE ROLE OF CD44 AND EZRIN DURING PROSTATE CANCER-ENDOTHELIUM INTERACTION.

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CD44 is a multifunctional cell surface adhesion molecule that has been implicated in tumour cell invasion and metastasis. Many cancer cell types as well as their metastases expresses high levels of CD44. It is known that ezrin, a member of the ERM family of proteins, can bind to CD44 and thus raises the possibility that it is involved in cell migration and metastasis. Therefore we examined the expression and distribution of CD44, its co-localisation and translocation with ezrin in prostate cancer cell lines as they interact with endothelial cells. RT-PCR revealed multiple isoforms of CD44 were expressed in DU-145 and PC-3 and DNA Sequencing identified both the standard and different variant isoforms in both cells. CD44 and ezrin were found to associate with each other in prostate cancer cell lines as seen by immunoprecipitation and Western blotting. In addition, incubation of cells with Hepatocyte growth factor/scatter factor (HGF/SF), a cytokine known to enhance cancer invasion and migration, resulted in the up regulation of CD44 expression in both PC-3 and DU-145 cells as seen by Western blotting. Dual-immunofluorescence and laser scanning confocal microscopy revealed the co-localisation of CD44 and ezrin but also the co-translocation of these proteins in response to HGF/SF exposure. Using a tumour-endothelial adhesion assay, more HGF/SF-activated PC-3 cells were found adhered to endothelial cells than untreated PC-3 cells and adhesion was further increased by pre-activated endothelium with HGF/SF. Results from an in vitro invasion assay confirm a significant increase in the invasive potential of HGF-treated cells. The increased adhesion and invasiveness were reduced or abolished by the inclusion of anti-CD44 antibodies or the addition of an excess hyaluronic acid (HA).

In conclusion, the change in distribution of CD44-Ezrin complex and up-regulation of CD44 suggest CD44 plays a role in the initial capture and subsequent invasion of endothelium by prostate tumour cells.

A SIMPLE MODEL SYSTEM TO STUDY THE MOLECULAR BASIS OF EXT MUTATIONS USING IN VITRO CHONDROGENESIS

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Mutations in Ext1 and Ext2 are associated with Hereditary Multiple Exostoses (HME), which is characterized by benign cartilage-capped outgrowths (exostoses) from ends of long bones. The Ext1 and Ext2 proteins possess a copolymerase activity that participates in heparan sulphate (HS) chain extension on the linker tetrasaccharide to the core protein of proteoglycans (PGs). Cell surface PGs can interact with diffusible growth factors (FGFs) and signaling molecules (Ihh) expressed in the growth plate cartilage and thus play a part in regulating bone development. A hypothesis is that mutations in the Ext1 and Ext2 genes alters HS synthesis, in terms of the length of HS chains and sulphation pattern, leading to defective chondrogenesis as a prelude to the formation of exostoses. Previous studies on Ext1 and Ext2 mutations have demonstrated a number of mutations act in a dominant-negative manner leading to defective HS chain synthesis. However, these studies were carried out in non-chondrogenic cell line. Our objective is to establish tools and a cell based model system required to study the molecular and cellular changes during chondrogenesis. In this system, we will exploit the ability of a mouse chondrogenic progenitor cell line, ATDC5 that undergoes in vitro chondrogenesis in culture. We have initiated stable transfections with expression plasmids for wild type ext1 and ext2 cDNA, and cDNA plasmids with dominant-negative mutations in ext1 (R340C) and ext2 (D227N) genes. This will allow the opportunity to monitor the effect of the mutations on proteoglycan synthesis. In particular, extension of hapanan sulphate chain and sulphation pattern. More importantly, we can monitor and compare the progression of in vitro chondrogenesis and to address the responsiveness of transfected cells to Indian Hedgehog (Ihh) signaling. Experiments are also in progress to establish efficient protocol to monitor hapanan sulphate synthesis using a newly developed fluorescent-assisted carbohydrate electrophoresis.

INTERACTIONS BETWEEN CD44, BETA-1 INTEGRIN AND THE MATRIX METALLOPROTEINASES IN REGULATING COLORECTAL CANCER CELL ADHESION AND INVASION

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The complex process of tumour cell invasion involves the attachment of cells to the extracellular matrix (ECM), degradation of the ECM and subsequent migration and detachment of cells from the ECM. Cell adhesion molecules like CD44 and integrins are key players in such cell-cell, as well as cell-matrix, adhesion interactions. Beta-1 integrin mediated signal transduction is thought to up-regulate matrix metalloproteinase (MMP) expression via the mitogen activated protein kinase (MAPK) pathway. Collectively, members of the MMP family can degrade the ECM completely. Such extracellular proteolytic activities have given the MMPs well established roles in tumour cell migration and invasion. Appreciating the importance of both attachment
to and degradation of the ECM in tumour cell invasion, it is no longer appropriate to consider CD44, integrins and MMPs as acting in isolation. This research aims to elucidate the interactions of these molecules in regulating colorectal tumour metastasis.

The expression of CD44 and beta-1 integrin was examined in the primary human colon carcinoma cell line SW480, its lymph node metastatic derivative SW620 and SW480(Mut-6) which have been transfected with the gene for Matrilysin (MMP-7). The SW480(Mut-6) has been shown to be more invasive than the parental SW480. Western blot analysis showed that the SW480, SW620 and SW480(Mut-6) expressed an 80kDa variant of CD44 with increased expression in the SW620. The expression of a 120kDa variant of CD44 was also observed in SW620 but not in SW480 or SW480(Mut-6). The SW620 cell line also expressed higher levels of beta-1 integrin than the other cell lines. The adhesion of these cell lines on plastic, fibronectin and hyaluronic acid was also examined. Results showed that there was a significant difference in cell attachment between these cell lines. The SW620 and SW480(Mut-6) were more adherent to plastic than the parental SW480. The SW480(Mut-6) were also shown to be more adherent to fibronectin and hyaluronic acid than SW480. In contrast, the SW620 appeared to be less adherent to hyaluronic acid than SW480. These results demonstrate that MMP-7, CD44 and beta-1 integrin interact with each other to regulate colorectal tumour cell attachment and migration. This work will help elucidate the functional relationships of these molecules in controlling invasion and metastasis in colorectal cancer.

**P161**
THE BISPHOSPHONATE ZOLEDRONIC ACID UPREGULATES APOPTOTIC SIGNALS AND IMPAIRS THE ADHESION OF BREAST CANCER CELLS TO MINERALISED MATRICES PRIOR TO THE INDUCTION OF APOPTOSIS
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**AIMS.** Bisphosphonates are of proven efficacy in limiting skeletal related morbidity in patients with advanced breast cancer. The mechanisms by which these compounds exert their clinically important effects remain inadequately understood. We have previously demonstrated that bisphosphonates directly induce apoptosis in the breast cancer cell lines MCF-7 and MDA-MB-231. We aimed to further elucidate the signalling pathways of these effects. We also aimed to investigate the relationship between bisphosphonate induced breast cancer cell apoptosis and effects on adhesion to bone-like dentine matrices.

**PROCEDURES.** Breast cancer cells were incubated in medium supplemented with zoledronic acid or vehicle. Apoptosis was assessed morphologically and by colourimetric assays of cell proliferation and cell viability. Immunoblotting was performed using a standard assay comprising gel electrophoresis, transfer to a nitro-cellulose membrane, protein detection with primary and secondary antibodies and protein visualisation against colour standards by chemiluminescence. Adhesion assays were performed by seeding treated breast cancer cells onto mineralised matrix for 1 – 24 hours. Adherent cells were fixed, stained and counted.

**MAJOR FINDINGS.** Zoledronic acid induced apoptosis in the breast cancer cell lines MDA-MB-231, MCF-7, SKBR3 and HS578T. Treatment of MDA-MB-231 and MCF-7 breast cancer cells with 100 μM zoledronic acid resulted in downregulation of the anti-apoptotic protein bcl-2 and upregulation of the pro-apoptotic protein bax. The importance of bcl-2 as a survival signal was confirmed by the demonstration that forced bcl-2 expression in MDA-MB-231 cells abrogated the effects of zoledronic acid. Pro-caspase 3 cleavage was demonstrated by a FACS based assay in MDA-MB-231 cells but not in MCF-7 cells which constitutively lack caspase 3. However, MCF-7 cells underwent a time-related cleavage of caspase 7 in response to treatment with zoledronic acid. Finally we provide evidence to suggest that zoledronic acid can impair the normally observed adhesion of breast cancer cells to mineralised matrix in a dose and time dependent manner and moreover that this occurs at an earlier time point than that required to observe its apoptotic effects.

**SIGNIFICANCE OF RESEARCH.** Our findings suggest that clinical responses to bisphosphonates in patients with breast skeletal metastasis are related to induction of apoptosis. This may occur through impaired cell-matrix interactions resulting in anoikis.

**P162**
HE66 REGULATES MYOGENIC DIFFERENTIATION
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In rhabdomyosarcoma, tumour cells fail to undergo the cell cycle withdrawal and terminal differentiation that occur in normal muscle differentiation. Candidate regulators of proliferation and differentiation in normal and malignant myogenesis are the Hes proteins, basic helix-loop-helix transcription factors homologous to Enhancer of split (Eos) proteins that regulate differentiation in Drosophila. We identified Hes6 as a member of the Hes family expressed in normal muscle and rhabdomyosarcoma by database searches. Hes6 is known to promote neural differentiation and to bind to Hes1, a related protein that has been implicated in cell cycle regulation, affecting Hes1 function. Here, we show that Hes6 is expressed in human rhabdomyosarcoma cells and that Hes6 is induced on C2C12 myoblast differentiation in vitro. Hes6 binds DNA containing the Enhancer of Split E box (ESE) motif, the preferred binding site of Drosophila Eos proteins, and represses transcription of an ESE box reporter. When overexpressed in C2C12 cells, Hes6 impairs normal differentiation, causing a decrease in the induction of the cyclin dependent kinase inhibitor, p21WAF1, and an increase in the number of cells that can be recruited back into the cell cycle after differentiation in culture. Hes6 is well conserved in Xenopus, so we used this system to study the effect of Hes6 on myogenesis in vivo. In situ hybridisation in embryonic Xenopus reveals that Hes6 is co-expressed with the early muscle marker, MyoD. Microinjection of Hes6 RNA in Xenopus embryos results in an expansion of the myotome, but suppression of terminal muscle differentiation and disruption of somite formation at the tailbud stage, paralleling the phenotype seen in vitro. Analysis of Hes6 mutants indicates that whilst the DNA binding activity of Hes6 is not essential for its myogenic phenotype, protein-protein interactions mediated by the C terminus of the protein are required. Thus we demonstrate a novel role for Hes6 in multiple stages of muscle formation, and that down regulation of Hes6 expression is required for terminal differentiation of muscle. We plan to investigate if Hes6 is highly expressed in rhabdomyosarcoma and identify Hes6 interacting proteins.