480 Implications of cellular ERalpha/ERbeta ratio in uterine smooth-muscle cells carcinogenesis

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Background: Uterine LM are very common benign tumours of uterine muscle cells. The types in terms of histological criteria consist of ordinary leiomyoma (LM), bizarre or atypical leiomyoma (AL), and cellular leiomyoma (CL). In contrast, malignant tumours or leiomyosarcomas are rare but with a high risk of local recurrence and metastasis. Although the etiology of uterine LM is unknown, their development is considered to be estrogen dependent. It was also shown that the ratio of estrogen receptor a (ERa) to estrogen receptor b (ERb) expression, rather than the individual expression levels, possibly determines the growth potential of LM. The factors affecting transformation of uterine smooth-muscle cells to LMS is unknown although the estrogen may have effect on this process.
The estrogen receptors ratio is a determinant factor in several estrogen-dependent cancers such as in breast, colon, prostate and more recently, endometrium. There are no previous data about ERb in malignant smooth-muscle uterine neoplasms. Our aim was to determine the cellular ratio of ERa to ERb expression in three types of uterine smooth muscle tumours, LM, AL, LMS, and compare it with healthy myometrium. This was contrasted with the expression of the proliferation factor, Ki67.
Methods: We used a significant number of formalin-fixed and paraffin-embedded tumoural and healthy uterine smooth muscle tissue samples, from Hospital Universitario de Canarias and Hospital La Colina, to perform immunohistochemical detection.
Results: In healthy myometrium and LM, ERa is predominantly expressed but LM show and increase of ERb-ir. Interestingly in AL the ratio ERa/ERb is reverse and greater expression of ERb compared to ERa is observed. Even more, all leiomyosarcomas studied were ERb-ir, while only a low percentage of them were ERa-ir This inversion of the ratio ERa/ERb observed during tumour progression was statistically significant.
Conclusions: These results are correlated with Ki67 expression, also suggesting that the ERb/ERa ratio is a useful index of progression in myometrial tumours and that ERb has an important role in uterine smooth-muscle malignancy.

481 Dissection of the NG2 involvement in extravasation phenomena simulated in vitro

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Background: The metastatic cascade entails an initial step in which tumour cells intravasate lymphatic or haematic circuits and develop the ability to interact with the endothelium of distant sites, followed by a subsequent interaction with the underlying ECM to pursue their tissue infiltration. To explore more in detail some of these metastatic passages and address the role played by membrane-bound proteoglycans (PGs), we have devised an experimental paradigm that allows us to investigate how these PGs) may directly or indirectly affect cell-cell and cell-ECM interactions in settings that mimic *in vitro* the rheological conditions encountered by tumour cells in peripheral blood.
Materials and Methods: A perfusion system that mimics the microcirculation was devised and exploited to explore the interactions of sarcoma cells harbouring diverse constitutive expression of NG2 or manipulated levels of the PG through RNAi, with activated and non-activated endothelial cells (HUVEC) or with native ECM isolated according to a specifically devised protocol from vascular smooth muscle cells. To investigate the extravasation capabilities of the same cells, we have combined our protocol for deriving native, cell-free ECM with transmigration assays involving porous membranes.
Results and Conclusions: In perfusion experiments cells enriched by immunosorting for high surface levels of NG2, bound significantly more avidly to activated HUVEC and, most strikingly, clustered in the proximity of the endothelium. Chondroitin sulphate chains of NG2, and possibly other unidentified cell surface PGs, seemed to play a key role in these cell-cell interactions. Antagonists of known cell-cell adhesion molecules and signal transduction probes are currently adopted to dissect the involved molecular mechanisms. In similar experiments with cell-free native ECM, we observed a differential capability of tumour cells to interact with it and even in this case the presence of NG2 modulated their adhesion to the underlying matrix.
Preliminary transmigration data show that expression of NG2 also modulate the capability of cells to extravasate in presence of native ECM, especially in the initial phases of the process. The findings highlight a crucial role of sarcoma NG2 in mediating the tumour cell-endothelium binding and the cells interaction with vascular matrix suggesting a fundamental role of the PG in the control of intra- and extravasation events.

482 Characterising the role of the metastasis associated cell surface glycoprotein CDCP1 in cancer cell lines – possible roles in cell adhesion and survival

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Background: CUB domains are immunoglobulin-like folds often involved in protein-protein interactions. Expression of CUB Domain Containing Protein 1 (CDCP1) in cancer is associated with an increased frequency of metastasis. Our investigations have focussed on CDCP1's role in colon cancer cell line adhesion and survival.
Methods: The CDCP1 negative Colo320 cell line was stably transfected with an expression vector encoding a CDCP1-FLAG fusion protein, yielding the Colo320-CDCP1 cell line. Apoptosis assays, cell cycle analysis, cell-cell aggregation assays and CDCP1 cell surface expression assays were performed by flow cytometry. Antibody-mediated CDCP1 endocytosis was assessed by immunocytochemistry. Cell adhesion assays were conducted in 24 well plates +/− a coating of 10 μg/ml Matrigel.
Results: In fixed A549 cells, CDCP1 was enriched along points of cell-cell contact. The rate of CDCP1 antibody-mediated endocytosis was reduced at points of cell-cell contact in both SW480 and A549 cell lines. Calcium supplementation of growth media preferentially increased aggregation of Colo320-CDCP1 cells and reduced binding to tissue culture plates. Matrigel reduced the binding of the Colo320-CDCP1 cell line to tissue culture plates. Reduction of CDCP1 protein by RNA interference (RNAi) in SW480 cells decreased cell binding to Matrigel coated tissue culture plates.
CDCP1 RNAi reduced the growth rate of SW480 cells forced into suspension, whereas it had negligible effects on adherent SW480 cells. SW480 cells forced into suspension for 20 hours displayed a lower level of sub-G1 propidium iodide (PI) staining (an indicator of apoptosis) than cell lines known to undergo anoikis (cell death induced by loss of adherence to substrate) such as HaCaT and 3T3. However the apoptotic frequency (assayed by sub-G1 PI stain) of SW480 cells forced into suspension for 20h following 48h CDCP1 RNAi was not altered.
Conclusions: CDCP1 modulates the adhesive properties of colon cancer cell lines. Some CUB domains have been demonstrated to bind Ca^{2+}. Given that calcium affected the adhesive properties of Colo320-CDCP1 cells it is possible that one or more of CDCP1's CUB domains bind Ca^{2+}. It remains to be investigated whether CDCP1 regulates cell adhesion directly or through association with other Ca^{2+} binding proteins such as Cadherins. Further work is required to determine the role of CDCP1 in the prevention of anoikis in SW480 cells.

483 Dual role of the extracellular matrix glycoprotein EMILIN2 in the tumour microenvironment

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Background: Elastin Microfibril Interface Located ProteINs (EMILINs) are a family of extracellular matrix (ECM) glycoproteins characterized by the presence of an N-terminal cystein rich EMI-domain and a coiled-coil region. We have recently demonstrated that EMILIN2 is a pro-apoptotic molecule that significantly reduces the viability of different tumour cell lines resulting not toxic for normal cells. The peculiar mechanism of action involves a direct binding of EMILIN2 to TRAIL receptors and the activation of the extrinsic apoptotic pathway. The binding to death receptors triggers receptor clustering, colocalization with lipid rafts, DISC assembly and caspase activation. The aim of this study was to identify the EMILIN2 pro-apoptotic region and to verify its antitumourigenic potential *in vivo*.
Material and Methods: Cell viabilty and apoptosis were evaluated by MTT and TUNEL assays, respectively. The interaction between EMILIN2 and its deletion mutant with TRAIL receptors was analyzed by GST pulldown using *in vitro* transcribed and translated proteins. Tumourigenicity in the presence of EMILIN2 or the deletion mutants was analyzed by soft agar colony and clonogenic assays. For *in vivo* experiments fibrosarcoma or malignant glioblastoma cells were subcutaneously injected in nude mice and pharmacologically treated every other day. Tumours apoptosis was quantified by TUNEL assay and analysis of caspases-8 and -3 activity while tumour vasculature was analyzed by immunofluorescence.

Results: The treatment of tumour cells with the various EMILIN2 deletion mutants led to the identification of the pro-apoptotic region of the molecule. This N-terminal fragment binds to death receptors, induces apoptosis and reduces the clonogenic potential of tumour cells. EMILIN2 and its deletion mutant displayed also an *in vivo* antitumourigenic effect which correlated with a higher activity of both caspase-8 and -3. Unexpectedly, tumours treated with EMILIN2 or the deletion mutant displayed a significant increase of tumour angiogenesis. In view of these findings the co-treatment of the growing tumours with an antiangiogenic drug, resulted in most cases in a complete regression of tumour growth.
Conclusions: Taken together these results unravel the possibility to employ EMILIN2 fragments or peptides in combination with angiogenesis inhibitors as potent antineoplastic tools for cancer treatment.

484 Rapid adherence to collagen IV enriches for tumour initiating cells

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Background: There is now evidence for the existence of cancer stem cells in many solid tumours, including oral squamous cell carcinoma. However, there is still a need to develop robust methods to enrich for cancer stem cells for studying their biological properties. Data from normal epithelia indicate that the rapid adherence to collagen IV is an efficient method to enrich for normal epithelial stem cells.
Objective: To investigate the rapid adherence to collagen IV as a method for enrichment for cancer stemm cells in human oral carcinomas and to characterize the cell populations obtained using this method in terms of their self renewal potential and electrophysiological properties.
Methods: Rapid adherent cells (RAC) and middle adherent cells (MAC) were isolated after 10 and respectively 60 minutes incubation on collagen IV-coated dishes in a panel of oral carcinoma cell lines (H357, DOK and CaLH3). The non-attached cells were designed as late adherent cells (LAC). Their clonogenic ability was investigated in vitro (single cell colony forming assay and 3D organotypic model).and their ability to initiate tumours was investigated in vivo (tongue xenograft NOD/SCID mouse model). The electrophysiological parameters of cells were determined non-invasively, using dielectrophoresis (DEP)-an electrostatic phenomenon defined as the motion of particles resulting from polarisation forces. Parameters such as cytoplasmic conductivity (which relates to the cytoplasm ionic strength), membrane conductance (indicates how well ions are transported across the membrane), and specific membrane capacitance (relates to membrane morphology) were extracted using the single-shell model.
Results: Significantly higher number of cells were found to initiate colonies ($p < 0.05$) and form spheres in vitro ($p < 0.01$) in both RAC and MAC when compared to LAC. MAC tumour formation was the fastest to occur, but both RAC and MAC induced tumour formation at earlier time points and at lower cell numbers than LAC after tongue xenotransplantation in NOD/SCID mice. No difference was observed in 3D cultures in terms of biomatrix invasion, but RAC and Mac gave rise to thicker cultures when compared to LAC. DEP analysis revealed that RAC and MAC exhibited a significantly higher membrane capacitance relative to LAC ($p < 0.001$), indicating a difference in the membrane morphology between these subpopulations of cells.
Conclusion: This study brings evidence for the use of rapid adherence to collagen IV for enriching in cells with increased clonogencity and tumour formation ability in oral cancer cell lines, and indicates that these properties are associated to differences in electrophysiological properties.

485 Checkpoint kinase 1 modulates sensitivity to chemotherapy in aneuploid cell lines

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Gastric cancer (GC) is one of the most frequent causes of death worldwide. Despite many advances in surgery and the diagnosis or the development of new regimens of chemotherapy (QT), after surgery most patients die of recurrent disease due to the presence of disseminated disease at the time of surgery. The main treatment of disseminated disease is chemotherapy, which only benefits a few and cause toxicity in the majority of patients. Therefore, it is necessary to improve the capability of selecting those patients most likely to have clinical benefit with a determined treatment. Our previous studies showed that SW620 cells showed higher BubR1 and Chk1 mRNA levels than control cells under normal conditions. These studies showed that these cells undergo synergistic cell death after spindle checkpoint activation (taxol treatment) followed by cisplatin treatment, suggesting a role of Chk1 in this checkpoint, very likely dependent on BubR1 protein. Importantly, Chk1-depleted SW620 cells lost this synergistic effect. In summary, we proposed that Chk1 could be used as a biomarker predictive of the efficacy of sequential chemotherapy across different types of tumours with aneuploidy. These results encouraged us to deeply study the role of Chk1 protein as a predictive factor of response to this combined chemotherapy in GC. A panel of cell lines derived from GC with and without aneuploidy, will be selected and treated with a combination of 5-Fluorouracil, cisplatin and taxane derivatives, in order to study the viability and the cross-talk between the activation of the checkpoint protein Chk1 and the spindle assembly checkpoint, as these are the main signaling pathways activated by these agents. The results of these studies will be reported at the Meeting.
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486 The sphingosine kinase-1 survival pathway is a molecular target for the tumour-suppressive tea and wine polyphenols in prostate cancer

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In vitro and *in vivo* studies have reported that dietary polyphenols can affect a wide array of signaling and molecular pathways resulting in cancer cell growth inhibition, apoptosis and inhibition of invasion, angiogenesis and metastasis. Here we provide the first evidence that dietary agents, namely epigallocatechin gallate (EGCg), trans-resveratrol (RV) or a mixture of polyphenols from green tea (Polyphenon E, PPE) or red wine (vineatrol) impede prostate cancer cell growth in vitro and in vivo by inhibiting the SphK1/S1P pathway, which is up-regulated in prostate cancer patients. Our results establish that SphK1 is a downtream effector of the ERK/Phospholipase D (PLD) signaling pathway inhibited by green tea and red wine polyphenols. Enforced expression of SphK1 in both PC-3 and C4−2B prostate cancer cells markedky impaired the efficacy of green tea and red wine polyphenols, as well as pharmacological inhibitors of PLD- and ERK, to induce apoptosis. The inhibitory effects of green tea and red wine polyphenols on tumour growth and the SphK1/S1P pathway were confirmed in an heterotopic PC-3 tumour in place model established in nude mice. SphK1-overexpressing PC-3 cells implanted in animals developed remarkably larger tumours and resistance to treatment with polyphenols. Furthermore, in an orthotopic PC-3/green fluorescent protein model, EGCg and PPE diet induced a marked SphK1 inhibition associated with a pronounced decrease in primary tumour volume and occurrence and number of metastases. These results provide the first demonstration that the SphK1/S1P pathway is a molecular target of dietary polyphenols in prostate cancer.

487 Prostaglandin E2 upregulates ErbB2 and enhances EGF-stimulated DNA synthesis in hepatocytes

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Background: Several G protein-coupled receptor (GPCR) agonists, including prostaglandin E_2 (PGE_2), act as comitogens in hepatocytes, by synergistically enhancing EGF-stimulated DNA synthesis. The underlying mechanism is not clear. In MH1C1 hepatoma cells PGE_2 transactivates the EGF receptor (EGFR), but in normal hepatocytes PGE_2 induces an upregulation of EGF-mediated phosphorylation of Erk and Akt independently of EGFR transactivation. EGFR belongs to the ErbB family, and one factor that may contribute to the diversity of EGFR signaling is the availability of other ErbB members that can engage in heterodimerization with EGFR. In this study we examined the role of PGE_2 on the expression of ErbB2 and ErbB3, and their role in the comitogenic effect.
Methods: Rat hepatocytes were cultured as primary monolayers in a defined medium. Expression and phosphorylation of signalling proteins, including EGFR, ErbB2, ErbB3, Erk, Akt, and cyclin D1, were assessed by Western blotting. ErbB2 and ErbB3 mRNA was measured by quantitative real time PCR. DNA synthesis was determined by incorporation of ^{3}H-thymidine. Transfection with small interfering RNA (siRNA) was used to block the expression of ErbB2.
Results: At plating, the cells expressed EGFR (ErbB1) and ErbB3, but not ErbB2. As they were cultured, traversing G1 with relatively high synchrony, ErbB3 expression decreased, while ErbB2 expression, in contrast, appeared and then increased up to a point in mid/late G1 where the cells are optimally sensitive to EGF. Pretreatment with PGE_2 increased ErbB2 expression and reduced ErbB3 expression. PGE_2 also enhanced and hastened EGF-stimulated cyclin D1 expression and DNA synthesis. Also, blocking of the