Preclinical Science

Oral presentations (Mon, 24 Sep, 10.45−12.15)
Preclinical science

300 ORAL

Lipid rafts as novel targets for anti-cancer therapy

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Background: For long time the plasma membrane has been undervalued as a relevant target for radiation-induced cytotoxicity. It has become evident, however, that this subcellular compartment is a source of bioactive molecules that can be generated upon exposure to radiation and may act as (apoptotic) second messengers. In addition, liquid-ordered dynamic membrane micro-domains enriched in sphingolipids and cholesterol, also known as lipid rafts, have been implicated in the recruitment and internalization of receptors, signaling molecules and certain (anti-cancer) drugs. To engage these membrane functions, rafts need to cluster into larger platforms. This process can be facilitated by radiation-induced hydrolysis of sphingomyelin (SM) into ceramide.
Material and Methods: We have recently established a crucial role for lipid rafts in targeting apoptotic pathways by alkyl-phospholipids (APL). APL (Perifosine, Miltefosine, Edelfosine) comprise a group of synthetic anticancer agents that are used for various clinical indications. Perifosine, for example, has recently been evaluated in combination with radiotherapy in a clinical phase I study. Its role as radiosensitizer is currently tested in a multicenter randomized phase II study. APL induce apoptosis in tumor cells and strongly enhance the radiation response in vitro and in vivo. Unlike conventional chemotherapeutic drugs, APL act at the level of cell membranes where they accumulate in lipid rafts. Following internalization, APL interfere with de novo phosphatidylcholine (PC) biosynthesis, which is essential for membrane homeostasis and cell survival. Disruption of raft integrity by cholesterol extraction or SM degradation, inhibits APL uptake and apoptosis induction.
Results: We have generated an APL-resistant cell variant, S49AR, that is unable to internalize APL via lipid rafts and reveals no impaired PC metabolism after APL treatment. Furthermore, these cells were found to be cross-resistant to apoptosis induction by other stimuli, including DNA-damaging agents (ionizing radiation, etoposide) and death receptor stimulation (CD95/Fas). Intriguingly, S49AR cells had lost the ability to synthesise sphingomyelin as a result of an impaired expression of the enzyme sphingomyelin synthase 1 (SMS1). In these S49AR cells, survival signaling pathways (MAPK/ERK, Akt/PKB) are upregulated, whereas pro-apoptotic signaling (SAPK/JNK) is reduced. Knock-down of SMS1 in S49WT cells by siRNA recapitulates the SM deficiency, impaired APL uptake and apoptosis (cross-)resistance. Conversely, normalization of cellular SM levels restored raft function, APL uptake and apoptosis sensitivity, including to radiation.
Conclusion: Our data point to a causal relationship between sphingomyelin synthesis, lipid raft function and susceptibility to apoptosis induction to a variety of anti-cancer agents. These studies define lipid raft as novel targets for anti-cancer therapy and apoptosis induction.

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TROP2 is a novel, potent stimulator of tumour growth and of metastatic spreading of human cancer

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Background: Trop-2 is a transmembrane calcium signal transducer, that plays a role in cell-cell and cell-substrate adhesion.
Material and Methods: We investigated the expression pattern of Trop-2 by DNA array, SAGE, Northern and Western blotting, flow cytometry, confocal microscopy and IHC analysis in experimental systems and in man. We explored its functional role by overexpression or down-regulation and by directed mutagenesis in transformed and/or metastatic cells in vivo.
Results: DNA array, EST, SAGE, RT-PCR and Northern blot analysis of human tumors revealed expression of the TROP2 gene in the vast majority of human cancers. A corresponding overexpression of the Trop-2 protein was demonstrated by a large scale IHC analysis of human tumors (1755 cases). Trop-2 was demonstrated to potently stimulate the growth of tumor cells, and its down-regulation by siRNA inhibited it. Deletion of the cytoplasmic region of Trop-2 abolished its growth stimulatory capacity, as mutagenesis of the S303 PKC phosphorylation site did. Proteomic and phosphoproteomic analysis demonstrated a Trop-2-dependent activation of PKCα, FAK and Raf1, modulation of ERK, MEK and p38 MAPK, and upregulation of NF-κB. In vivo imaging demonstrated a dynamic colocalization of PKCα and Trop-2 in membrane ruffles and podosomes. Dominant negative PKCα and PKCα siRNAs selectively abolished the Trop-2-induced growth demonstrating that PKCα plays a key role in Trop-2 signaling. Strikingly, comparative global gene expression analysis revealed that TROP2 was the only gene up-regulated across different metastatic models, tumor types and animal species. IHC analysis revealed a dramatic up-regulation in metastases from colon, stomach, breast and ovary tumors in man. To assess if Trop-2 plays a causal role in metastatic spreading, the metastatic potential of TROP2-transfected KM12SM colon cancer cells, orthotopically injected in nude mice, was assessed. TROP2-overexpressing cells indeed demonstrated a profoundly increased metastatic potential to the liver. Deletion of the HIKE domain of Trop-2 severely diminished, whereas that of the whole cytoplasmic region vastly increased metastatic diffusion, indicating the existence of metastatic enhancers and silencers in the Trop-2 cytoplasmic tail.
Conclusions: Our findings demonstrate the existence of a previously unsuspected, strikingly widespread mechanism of stimulation of tumor growth and of metastatic spreading in man, and candidate Trop-2 for novel diagnostic and therapeutic procedures.

302 ORAL

PCAF plays a key role in the regulation of the cellular fate in hypoxia

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Background: The p53 tumour suppressor is one of the most extensively studied transcription factors involved in several crucial cellular functions such as cell cycle arrest, apoptosis, differentiation, or senescence. Although in hypoxic conditions p53 is stabilised the same way as under DNA damage it is incapable of inducing the expression of its pro-apoptotic target genes, including members of the Bcl-2 family. The aim of this study is to investigate the p53 transcriptional activity and specifically its inability to induce expression of several of its target genes including pro-apoptotic members of the Bcl-2 family under hypoxia.
Materials and Methods: In order to approach this question we used transcriptional regulation and gene expression methodology such as chromatin immunoprecipitation, luciferase reporter assays and cell cycle analysis.
Results: In this study we present evidence that p53 is hypo-acetylated at K320 site, which is targeted by PCAF, whereas efficiently acetylated at K382 by p300/CBP under hypoxia-mimicking conditions. Using several transcription assays we demonstrate that the acetylated p53 at K320 is not recruited to the BID promoter and hence p53 is incapable of activating this pro-apoptotic Bcl-2 family target. On the contrary, the limited amounts of acetylated p53 at K320 are still recruited to the promoter of the cell cycle arrest gene p21WAF and the expression of this cyclin/cdk inhibitor appears to be unaffected by hypoxic conditions.
Conclusions: Since the K320 p53 acetylation is the mainly affected site in hypoxia we conclude that PCAF HAT activity is the key regulator of the cellular fate under these conditions. Furthermore, the decision between apoptosis or cell cycle arrest is determined by the selective recruitment of a higher proportion of the K320-acetylated p53 to the p21WAF promoter in hypoxia. This provides an additional molecular mechanism explaining cell survival in hypoxic conditions.

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Intestinal inactivation of canonical Notch signaling by removal of protein O-fucosyltransferase 1 triggers secretory cell fate differentiation

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The intestinal epithelium is a continuously differentiating tissue in which the high rate of cell self-renewal contributes to the susceptibility of intestinal epithelial cells to malignant transformation. Therefore understanding mechanisms that regulate cell proliferation and maturation towards the different intestinal cell lineages is critical to understand intestinal tumorigenesis. Here, we report that active canonical Notch signaling in

vivo marks the uncommitted, proliferative cells in the small intestine of the mouse.
To address the physiological role of Notch signaling in intestinal homeostasis, the gene encoding Pofut1, a fucosyltransferase required for the activity of all Notch receptors, was deleted in the mouse intestinal epithelium, through Villin-Cre-mediated recombination.
Inhibition of canonical Notch signaling was confirmed by concomittant down- and up-regulation of Hes1 and Math1 mRNA levels, respectively. The body weight of Pofut1F/F:VillinCre mice was dramatically reduced compared to control littermates. Removal of Pofut1 led to a massive increase in commitment to the secretory cell lineage characterized by increased numbers of goblet cells, Paneth cells and enteroendocrine cells, as revealed by alcian blue, lysosyme and chromogranin A/B immunostaining, respectively. Consistent with this, the levels of mRNA encoding gut hormones (CCK, GIP, Glucagon), Paneth cell markers (MMP7) and mucus-secreting cell markers (MUC2, TFF3, FIZZ2) were enhanced, as determined by quantitative RT-PCR. Whereas, the specific allocation of Paneth and enteroendocrine cells was unchanged, goblet cells accumulated in the crypts. In parallel, microarray gene expression data revealed that absorptive cell markers (L-FABP, DPP4, ApoB) were repressed in intestinal epithelium lacking Pofut1. Interestingly, determination of cell renewal capacity in the intestinal mucosa, through Ki67 and BrdU immunostaining, revealed that the transit amplifying compartment was maintained in the upper crypts of the intestinal mucosa whereas decreased proliferating cells were detected in the colonic epithelium. In vitro, in human colon carcinoma HT29 Cl16E cells that spontaneously differentiate along the goblet cell lineage in culture, inactivation of Notch receptors activation led to cell cycle arrest in G1, and concomitant induction of expression of the MUC2 and TFF3 goblet cell markers. This later effect was mediated by Hath1 since its targeted down-regulation by specific siRNAs also inhibited MUC2 and TFF3 expression.
Therefore, we conclude that Notch signaling participates in the maintenance of intestinal progenitors and cancer cells in a highly proliferative state. Moreover, this study provides novel insight into the molecular mechanisms involved in intestinal cell fate specification, and maturation of secretory cell types in particular, induced by Notch.

304 ORAL

Early normalization of elevated baseline bone resorption marker levels by zoledronic acid and improved survival in patients with bone metastases from solid tumors

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Introduction: In patients with malignant bone disease, elevated N-telopeptide of type I collagen (NTX) levels are associated with significantly increased risks of skeletal-related events (SREs), disease progression, and death compared with normal NTX levels. Zoledronic acid reduces the risk of SREs and levels of NTX, parameters that have been associated with survival, in patients with malignant bone disease. This exploratory analysis investigated whether early normalization of urinary NTX correlated with a reduced mortality in patients with bone metastases from solid tumors.
Material and Methods: In this subset analysis, urinary NTX was measured at baseline and at 3 months in 3 randomized trials in patients with bone metastases from breast cancer (n = 379), hormone-refractory prostate cancer (n = 314), or lung cancer and other solid tumors (n = 204) who received zoledronic acid for up to 24 months. Patients were classified by baseline NTX levels (normal, <64 nmol/mmol creatinine; elevated, $\geqslant$64 nmol/mmol creatinine).
Results: Approximately 55% of patients had elevated NTX at baseline. Levels of NTX normalized within 3 months of zoledronic acid treatment in 76.2% of patients with elevated baseline NTX levels. Moreover, zoledronic acid-mediated NTX normalization reduced the risk of death by 48% in patients with breast cancer (risk ratio [RR] = 0.517; P = 0.002), 59% in patients with prostate cancer (RR = 0.410; P < 0.0001), and 58% in patients with lung cancer and other solid tumors (RR = 0.427; P = 0.012). Zoledronic acid-mediated normalization of NTX also significantly prolonged SRE-free survival in patients with breast cancer or prostate cancer (P < 0.001 for both) compared with persistently elevated NTX at 3 months. Further analyses revealed that there was a continuum of benefit in all cancer types tested dependent on the percentage decrease of NTX levels at 3 months, with the greatest survival benefit occurring in patients whose NTX levels decreased $\geqslant$ 75% (P < 0.01 for comparison between percentage reduction quartiles in all tumor types tested).
Conclusions: Among patients with elevated baseline NTX receiving zoledronic acid, those whose levels normalized by 3 months had better clinical outcomes, including prolonged SRE-free survival and overall survival, compared with patients whose NTX levels remained elevated. This finding held for all tumor types studied. New treatment strategies should be investigated in patients with persistently elevated NTX levels.

305 ORAL

Mapping of interstitial fluid pressure in solid tumours using dynamic contrast enhanced MRI – dream or reality?

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Background: Interstitial fluid pressure (IFP) of most solid tumours is increased relative to normal tissues creating a barrier for transvascular transport, thus compromising the delivery and efficacy of chemotherapy and macromolecules. Here we demonstrate that bevacizumab decreases IFP in HT29 (human rectal cancer xenografts) and assess if infusion dynamic contrast enhanced MRI (iDCE-MRI) kinetic parameters correlate with IFP or changes induced by bevacizumab.
Materials and Methods: 29 SCID mice bearing subcutaneous HT29 tumours of ~8.5 mm diameter received a single dose of 10 mg/kg bevacizumab intraperitoneally; controls received saline. iDCE-MRI was performed on days 1, 3 & 5, using a slow infusion rate (5.5 microlitres/min for 60 mins) of contrast agent [Gadopentetate dimeglumine; Gd-DTPA], sequential images before and during the infusion were acquired. The kinetic parameters (inflow rate (Kinf); max enhancement (Minf) and total Gd-DTPA delivery for 60 mins (AUC60) were estimated from changing T1 relaxation rates. Immediately after MRI, the IFP was measured directly using wick-in-needle technique.
Results: There was no correlation of IFP measurements and any kinetic MRI parameter. IFP was significantly lowered (p < 0.001) on day 5 only in treated tumours (mean $\pm$ SD 15.1$\pm$4.7 cf 36.9$\pm$5.6 mmHg). There were no significant differences in any kinetic MRI parameters between treated and control animals at day 1, 3 & 5.
Conclusions: Tumour IFP cannot be directly related to iDCE-MRI. Changes in IFP induced by bevacizumab on day 5 were not reflected by alterations in MRI parameters.

Poster presentations (Tue, 25 Sep, 14:00−17:00)
Basic science

306 POSTER

Methylnaltrexone-induced receptor tyrosine phosphatase mu (RPTP mu) activation regulates inhibition of VEGF-induced angiogenesis

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Angiogenesis or the formation of new blood vessels is important in the growth and metastatic potential of various cancers. Therefore, agents that inhibit angiogenesis have important therapeutic implications. We have previously shown that methylnaltrexone (MNTX), a peripheral mu opiate receptor (mOP-R) antagonist which has completed phase 3 trials for opioid-induced constipation in advanced illness, inhibits VEGF and opioid-induced endothelial cell (EC) proliferation and migration, two key components in tumor-associated angiogenesis (Microvasc Res 2006; 72(1−2): 3−11).
In this study, we examined the mechanism by which MNTX inhibits VEGF-induced angiogenic events. Our results indicate that treatment of human pulmonary microvascular EC with MNTX (100 nM), but not the uncharged mOP-R inhibitor, naloxone, increased Receptor Protein Tyrosine Phosphatase mu (RPTP mu) activity which was independent of mOP-R expression. Silencing RPTP mu expression (siRNA) in human EC inhibited MNTX protection from VEGF-induced proliferation and migration. Mechanistically, silencing RPTP mu increased VEGF-induced Src and RhoA activation as well as tyrosine phosphorylation (inactivation) of the negative regulator of RhoA, rhoGAP.