In contrast, drug treatment had no significant effect on CD-31 microvessel density or on the induction of apoptosis as assessed by TUNEL staining. Western blot analysis of flank tumor specimens revealed that erlotinib treatment consistently suppressed phosphorylation of S473-Akt in all lines, while there were minimal effects on phospho-Erk.
Conclusions: These data are consistent with the idea that inhibition of downstream signaling through the PI3K/Akt signaling pathway in PTEN wild-type tumors leads to suppression of tumor proliferation and are consistent with the importance of specific GBM genotypes for the anti-proliferative effects of erlotinib in human GBMs.

594 POSTER

Combined targeting of mTOR and ILK for maximal suppression of cancer cell growth, Akt activation and cell survival

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Constitutive activation of the PI3Kinase signaling pathway is a frequent event in many types of cancers and results in increased cell growth and survival. There is currently intense interest in this pathway, and Pi3Kinases, mTOR and Akt are the major therapeutic targets. mTOR exists in two different complexes: TORC1, which is rapamycin sensitive, regulates protein translation and cell growth, and TORC2 which is rapamycin-insensitive and apparently regulates Akt phosphorylation and activation. Although rapamycin and its analogs are in clinical trials, a major conundrum of TORC1 inhibition is that Akt phosphorylation and cell survival are stimulated due to the inhibition of the normal feedback inhibition of PI3Kinase activation by TORC1. Effective targeting of this pathway therefore requires inhibition of both TORC1 (to block cell growth), and also Akt activation to block cell survival.
Using a combination of siRNA-mediated knock-down and pharmacological inhibitors, we have found that knock-down of Integrin-Linked Kinase (ILK) (an upstream regulator of Akt Serine-473 phosphorylation), or Rictor, a component of TORC2, suppress Akt phosphorylation and induce apoptosis. However, complete functional knock-down of mTOR in breast or prostate cancer cell lines results in a stimulation of Akt phosphorylation. Similarly, treatment with rapamycin also results in increased Akt phosphorylation. Knock-down of either Rictor or ILK, or both, completely suppresses this increased Akt phosphorylation resulting from mTOR inhibition, suggesting that ILK and Rictor regulate Akt phosphorylation in an mTOR-independent manner. Interestingly, ILK and Rictor can be co-immunoprecipitated suggesting a potential interaction between these two proteins. Importantly, treatment of breast and prostate cancer cells with a combination of rapamycin and a highly selective small molecule ILK inhibitor, QLT-0267, results in the complete suppression of Akt and p70S6Kinase, inhibition of cell growth and survival in vitro, and significant tumor growth delay in in vivo xenograft models.
Our results suggest that combined mTOR and ILK targeting effectively shuts down activated PI3Kinase pathway and suppresses tumor growth.

595 POSTER

Insulin-like growth factor-I secreted from prostate stromal cells mediates tumor-stromal cell interactions of prostate cancer

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Solid tumors are composed of tumor cells and surrounding stroma including extracellular matrix, fibroblasts, macrophages, and endothelial cells. The growth of tumor cells is regulated by the stromal cells through diffusible factors and their adhesion. These tumor-stromal cell interactions significantly contribute to the growth and metastasis of some cancers such as the breast and prostate cancers. Prostate cancer shows high expression of type I insulin-like growth factor receptor (IGF-IR) and prostate stromal cells produce insulin-like growth factor-I (IGF-I). Although high plasma level of IGF-I is related to the development of prostate cancer, the significance of prostate stromal IGF-I in the regulation of the prostate cancer remains elusive. Here we show that the stromal IGF-I certainly regulates the development of prostate cancer. Coinoculation of prostate stromal cells (PrSC) increased the growth of human prostate cancer LNCaP and DU-145 tumors in SCID mice. The conditioned medium of PrSC, as well as IGF-I, induced phosphorylation of IGF-IR and increased the growth of LNCaP and DU-145 cells. PrSC, but not LNCaP and DU-145 cells, secreted significant amounts of IGF-I. Coculture with PrSC increased the growth of DU-145 cells in vitro, but the pretreatment of PrSC with siRNA of IGF-I did not enhance it. Furthermore, various chemical inhibitors consisting of 79 compounds with about 60 different targets led to the finding that only IGF-IR inhibitor suppressed the PrSC-induced growth enhancement of DU-145 cells. Thus, these results show that prostate stromal IGF-I mediates tumor-stromal cell interactions of the prostate cancer to accelerate tumor growth, supporting the idea that the IGF-I signaling is a valuable target for the treatment of the prostate cancer.

596 POSTER

Complexity of constitutive NF-kappaB activity as a therapeutic target in breast cancer cells

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Constitutive activation of NF-κB is common in breast cancer and provides a potential therapeutic target. We compared the mechanisms of activation in un-stimulated breast cancer cell lines. The effect of specific cell signalling inhibitors on NF-κB activity (24 hrs treatment), mRNA levels of NF-κB-regulated genes for IκBα and metastatic biomarkers Bcl-xL, ICAM-1, IL-6 and uPA (30 hrs), viability and apoptosis (48 hrs) were compared in HBL100, MDA-MB-468 and MDA-MB-231 lines. NF-κB activity was measured by reporter gene expression. mRNA levels of downstream targets were examined by real-time PCR. NF-κB subunits were detected by immunoblotting. Nuclear extracts of HBL100 and MDA-MB-231 cells contained NF-κB p65, p50, p105, p52, p100, RelB and RelC proteins, whereas expression of p105 and RelC differed in the extracts of MDA-MB-468 cells. The level of constitutive NF-κB activation was in the order HBL100 $\ll$ MDA-MB-468 < MDA-MB-231, with no correlation to IκBα mRNA levels or nuclear levels of NF-κB family members. Aggressively metastatic MDA-MB-231 cells also showed the highest levels of mRNA expression of the biomarkers Bcl-xL, IL-6 and uPA, consistent with their metastatic potential. Expression of uPA mRNA in all three cell lines correlated with invasiveness. Upstream inhibition using specific inhibitors for PI3K, Src, EGFR, IKKβ or proteasomal degradation resulted in cell line-specific effects on NF-κB-regulated reporter gene activity. Inhibition of PI3K with LY294002 or proteasomal degradation with MG132 had the most prominent effect on NF-κB activity in all cell lines. The altered patterns of reporter gene expression did not correlate with viability in response to any treatment. Nor was there any correlation between expression of the reporter gene and endogenous NF-κB target genes. MG132 increased cell death in all three lines. It increased IL-6 mRNA levels and decreased mRNA levels of uPA and anti-apoptotic Bcl-xL in MDA-MB-231 and HBL100 cells, however NF-κB reporter gene expression was increased in HBL100 cells and decreased in MDA-MB-231 cells. NF-κB activity was inversely correlated with IκBα expression in LY294002-treated HBL100 cells, but showed no correlation in the other cell lines. In MDA-MB-468 cells, PI3K inhibition resulted in increased NF-κB reporter gene activity and decreased IL-6 mRNA, whereas proteasomal inhibition decreased NF-κB reporter gene activity and increased mRNA levels of metastatic biomarkers ICAM-1 and uPA. These data show that the NF-κB pathway is regulated in a complex way, probably, by several members of the NF-κB family, which results in cell-specific modulation of expression of NF-κB–regulated reporter gene and endogenous downstream target genes. Modulation of this pathway has a significant effect on expression of several metastatic biomarkers.

597 POSTER

MDM2 309T>G polymorphism in human sarcomas

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Background: MDM2 protein regulates p53 activity and stability through the inhibition of p53-dependent transcription and its proteosomal degradation. Further, MDM2 regulates the location of p53 by transporting p53 out of the nucleus into the cytoplasm. The presence of the SNP 309T>G in the promoter region of the MDM2 gene, determines an increased level of the MDM2 protein with an attenuation of the p53 tumour suppressor activity. This genetic variant may have an important role in the pathogenesis of sarcomas, either for soft tissue sarcomas (STS) or osteosarcomas. The aim of this study is to investigate the role of the polymorphism 309T>G of the MDM2 gene in the risk of developing sarcomas.
Methods: Three hundred fifty-two patients affected by sarcomas (150 patients with STS and 202 patients with osteosarcomas) have been tested on genomic DNA from peripheral blood mononuclear cells or frozen tumour tissue. The genotype analysis was performed using the Pyrosequencing a mini-sequencing technique. The relative risk (OR, 95% CI) associated with the polymorphism MDM2 309T>G in developing sarcomas was investigated by matching the frequencies among patients with those of a healthy population of 487 individuals.
Results: Among all the cases enrolled in our study, the frequencies were 34.1% for the homozygous wild type TT (120 pts), 44.0% for the heterozygous TG (155 pts) and 21.9% for the homozygous polymorphic variant GG (77 pts). A significant difference was observed in frequencies

between cases and controls ($p < 0.0001$) that reflects about a two-fold higher relative risk of sarcomas for patients with the homozygous mutated genotype with respect to the other genotypes (OR = 2.199, 95% CI: 1.507–3.209) (two-sided Fisher's Exact Test). The frequencies in blood donors were 43.7% for the homozygous wild type TT (213 individuals), 45.0% for the heterozygous TG (219 individuals) and 11.3% for the homozygous polymorphic variant GG (55 individuals). Considering the STS patients, the GG genotype was associated with an OR of 1.882 (95% CI: 1.150–3.082, $p = 0.0132$), while for osteosarcomas, GG patients have about a 2.5-fold risk (OR = 2.448, 95% CI: 1.595–3.759, $p < 0.0001$).
Conclusion: This data highlights that patients with the GG MDM2 309T>G polymorphism have a higher risk of developing sarcomas.

598 POSTER

Analysis of the mTOR signaling pathway versus proliferative response of tumor cells to the mTOR inhibitor RAD001 (everolimus)

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The mammalian "target of rapamycin" (mTOR) is an intracellular protein kinase controlling cell growth and proliferation, regulated by nutrient/energy availability and the PI3K/AKT pathway. Two mTOR complexes exist: a rapamycin-sensitive complex defined by interaction with raptor (regulatory-associated protein of mTOR) and a rapamycin-insensitive complex defined by interaction with rictor (rapamycin-insensitive companion of mTOR). Both complexes contain GbL, an essential subunit required for optimal mTOR activity.
RAD001 (everolimus), an oral mTOR inhibitor currently in Phase 2 clinical trials in oncology, shows antiproliferative activity in vitro in a wide range of tumor cell lines. Although most lines are sensitive to RAD001 treatment (IC50s for antiproliferative effects in the low/sub nM range; eg. BT474 breast: IC50 = 0.55±0.12 nM), a small number of lines are indifferent (IC50s in the >100 nM – uM range; eg. KB31 epidermoid: IC50 = 1778±800 nM). The reason why RAD001 is effective against some transformed cells but not others remains unclear, as does the role of mTOR-raptor vs. mTOR–rictor signaling in the cellular response to RAD001. Confirming previous data, we demonstrated in a mixed panel of cell lines ($n = 21$), representing lung, breast and colon cancer as well as glioblastoma, an association between higher phospho-(serine 473) AKT levels and the antiproliferative response to RAD001 in vitro ($r = -0.701$, $p < 0.001$; Spearman's Rank correlation). For raptor and rictor protein levels, however, no such correlation has been observed (Raptor: $r = -0.0989$, $p = 0.687$; Rictor: $r = -0.00589$, $p = 0.98$; Spearman's Rank correlation). Molecular analysis has shown that RAD001 treatment can induce AKT phosphorylation in a subset of tumor lines/models as well as in patient tumors. Interestingly, induction of pospho-AKT in vitro following mTOR inhibition does not predict the anti-proliferative response to RAD001. For example, BT474 and KB31 both exhibit induction of AKT phosphorylation, but with quite different sensitivities towards RAD001. As mTOR-rictor was recently shown to directly phosphorylate AKT on serine 473, a role for this mTOR complex in the cellular response to mTOR inhibition is feasible. We are currently investigating this question using siRNA approaches, overexpression of mTOR complex components and specific inhibitors.

599 POSTER

Epidermal Growth Factor Receptor (EGFR) Expression and Mutational Analysis in Synovial Sarcomas and Malignant Peripheral Nerve Sheath Tumors

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Background: Synovial sarcomas (SnSrc) and malignant peripheral nerve sheath tumors (MPNST) are rare mesenchymal tumors of adolescence and young adulthood. SnSrcs are characterized by either a monophasic or biphasic morphology and a distinct chromosomal translocation t(X;18), which has prognostic significance. MPNSTs are characterized histologically by a monophasic spindle cell histology. Previous work from our laboratory has demonstrated that SnSrcs express the receptor tyrosine kinases (RTKs) EGFR and Her2/neu. This present study extends the work to examine the expression of EGFR in MPNST, the role of the activated EGFR/MAPK pathway in both tumors and the characterization of potential targets of EGFR tyrosine kinase domain.
Methods: A tissue microarray was constructed containing 48 cases of SnSrc and 32 cases of MPNST. Immunohistochemistry (IHC) stains for EGFR, EGFRvIII, activated EGFR (Y1221-P-EGFR) and activated MAPK (T202/T204-P-p44/42 MAPK) were performed. Tumor DNA was extracted from fresh and formalin fixed paraffin-embedded tissue blocks. Exons 17−21 of the EGFR tyrosine kinase domain, as well as, Exon 2, containing codons 12/13 of KRAS, were amplified by PCR and sequenced.
Results: Immunohistochemistry (IHC) demonstrated that the EGFR is expressed in a majority of SnSrcs and MPNSTs. EFGRvIII immunoreactivity was negative. IHC was weakly positive for activated EGFR and MAPK. Sequence analysis of the EGFR genomic DNA did not demonstrate mutations in exons 17−21. However, a presumptive 20 base pair insertion was seen in Exon 18 in 7 of 48 samples of SnSrc (14.5%) and 8 of 32 MPNSTs (25%) respectively. The functional significance of this insertion is not known. Correlation of the 20 bp insertions to clinical outcome is presently being determined. KRAS mutations in codons 12/13 were not identified in either tumor type.
Conclusions: Expression of EGFR in SnSrc and MPNSTs with an intact EGFR/MAPK pathway has been hypothesized to contribute to the malignant potential of these tumors. Our study reveals the relative absence of known activating mutations in EGFR in both tumor types, which suggests that trials of small molecule inhibitors would be of little clinical benefit. A recent Phase II trial of erlotinib in 38 MPNST patients failed to detect a clinical response. A clinical study of treatment with cetuximab is ongoing and may help elucidate whether blockade of EGFR with antibodies is likely to be more active.

600 POSTER

Bone marrow stromal cells modulate cytotoxicity of 8-amino-adenosine in myeloma cell co-culture studies.

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Background: We have previously demonstrated that 8-amino-adenosine (8-NH_2-Ado) is rapidly taken up by human multiple myeloma (MM) cell lines and converted to 8-NH_2-ATP causing cytotoxicity, loss of endogenous ATP and loss of phosphorylation of key signaling kinases. However, myeloma cell growth *in vivo* is supported by the cytokine rich marrow microenvironment and this may modulate the cytotoxicity of 8-NH_2-Ado. To test this, we have co-cultured the MM cell line MM.1S with two well characterized human bone marrow stromal cell lines (Hs5 and Hs27A) and evaluated the cytotoxicity and uptake of 8-NH_2-Ado.
Materials and Methods: We have optimized the use of Hs5 and Hs27A cells in co-culture with MM.1S cells to provide a reproducible system for *ex vivo* drug screening. Cytotoxicity of 8-NH_2-Ado was evaluated by flow cytometric Annexin V staining on bone marrow stromal and MM cells separated by anti-CD38 staining. HPLC was used to quantify intracellular 8-NH_2-ATP and ATP. Alteration in signaling pathway kinases and phosphorylation were evaluated by immunoblotting.
Results: We have determined a dose-dependent specificity of 8-NH_2-Ado induced cytotoxicity towards the MM.1S cell line. The stromal cells undergo significantly less apoptosis than the MM.1S cells when treated with 3 micro molar 8-NH_2-Ado for 24 hrs, a dose that induces more than 85% apoptosis in the co-cultured MM.1S cells. To determine the basis of the selective cytotoxicity towards the MM.1S cells, we measured the uptake of 8-NH_2-Ado and conversion to 8-NH_2-ATP in both cell types. Interestingly, at the 3 micro molar concentration of 8-NH_2-Ado, stromal cells take up drug and generate 8-NH_2-ATP associated with a loss of intracellular ATP. To investigate the differential 8-NH_2-Ado-induced cytotoxicity we evaluated various signaling pathways associated with apoptosis and note a differential regulation of phospho-Akt and phospho-p38.
Conclusions: The purine nucleoside analogue, 8-NH_2-Ado is preferentially cytotoxic towards the MM.1S cells but not the bone marrow stromal cells in an *ex vivo* co-culture system that approximates the cytokine rich bone marrow milieu. This differential sensitivity is not accounted for by a differential uptake or conversion of 8-NH_2-Ado rather through selective expression and regulation of pathways associated with apoptosis in the MM.1S cell line. These results provide insight into the specific cellular targets associated with the therapeutic efficacy of 8-NH_2-Ado.