Results: The use of AF was associated with a significantly less mucositis in the patients with tumours of the pharyngeal tract (30% reduction if compared with the group of patients treated with the same doses without AF). In the group of patients with NSCLC the use of subcutaneous AF was associated with a dramatic decrease of Grade III-IV side effects and of nausea and vomiting. Moreover AF allowed the treatment with higher doses of chemotherapy. In the cervix cancer group at the moment is not showed an improvement in patients conditions during EBRT.

Conclusions: The subcutaneous administration of AF during EBRT is an extremely simple procedure that reduces, in selected patients, the incidence of severe side-effects linked to the radio-chemotherapy association and probably allows the administration of higher doses of chemotherapy, without increasing the side effects. We need further studies to identify the subgroups of patients that can obtain better results.

Cancer cell biology & angiogenesis

357 ORAL

Antiangiogenic and antitumor effect of VEGF antisense oligonucleotide in combination with anti-EGFR C225 monoclonal antibody in human colon cancer

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Angiogenesis plays a key role in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is secreted by cancer cells to stimulate endothelial cell growth through paracrine mechanisms. The transforming growth factor alpha (TGFalpha)-epidermal growth factor receptor (EGFR) autocrine pathway controls the production of VEGF and other angiogenic factors by cancer cells. We evaluated the antiangiogenic and antitumor activity of HYB 676, a novel human VEGF antisense 21-mer oligonucleotide with modified backbone structure, alone and in combination with MAb C225, an anti-EGFR chimeric human-mouse monoclonal antibody, in a human colon cancer xenograft model. The effect of HYB 676 on VEGF production by GEO cells was evaluated in vitro and in vivo by Western blotting and immunocytochemistry. The in vivo antitumor activity of HYB 676 and/or MAb C225 was determined in athymic mice bearing established human GEO colon cancer xenografts. The Student's t test and the Mantel-Cox logrank test were used for statistical evaluation. HYB 676 determined a dose-dependent inhibition of VEGF production by GEO cells in vitro. Treatment of mice bearing established GEO xenografts for three weeks with HYB 676 or with MAb C225 determined a reversible inhibition of tumor growth. In contrast, a prolonged inhibition of tumor growth was observed in all mice treated with the two agents in combination with a significant improvement in mice survival compared to controls ($P < 0.001$), to MAb C225 group ($P < 0.001$), or to HYB 676 group ($P < 0.001$). All mice died within 5, 7 and 10 weeks following tumor cell injection in the control, HYB 676 and MAb C225 groups, respectively. In contrast, 50% of mice treated with the combination of HYB 676 and MAb C225 were alive at 15 weeks. Immunohistochemical analysis of GEO xenografts demonstrated a significant reduction of VEGF expression after treatment with HYB 676 with a parallel reduction in microvessel count. A potentiation in VEGF inhibition in GEO tumors with little or no microvessels was observed following the combined treatment with the two agents. These results demonstrate that anti-EGFR MAbs and VEGF antisense oligonucleotides have a cooperative antiangiogenic and antitumor activity.

358 ORAL

Free circulating vascular endothelial growth factor (VEGF) is detectable only in small tumours of the WAG/Rij rat rhabdomyosarcoma tumour model

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Background: VEGF is a glycoprotein with potent angiogenic, mitogenic and vascular permeability enhancing activities specific for endothelial cells, synthesised and secreted by a variety of tumours and seems to be the major inducer of angiogenesis. We investigated the detection of free circulating VEGF in 11 normal WAG/Rij rats and in 19 WAG/Rij rats bearing a syngeneic rhabdomyosarcoma subcutaneously implanted and randomly selected for these measurements (tumour volume: 0–50 cm3).

Methods: A blood sample of 1 ml was obtained by intracardial puncture under a short general anaesthesia with ether. A plasma separator gel and a 1:10 anticoagulant mix of sodium citrate, theophylin, adenosine and dipyridamole to achieve maximal platelet stabilisation were used. Samples were immediately placed on ice and centrifuged at 2500 G at 4°C and were measured with a human VEGF ELISA (Citymmune®, Maryland, US) with cross reactivity against rat VEGF (detection range: 0.195–50 ng/ml).

Results: The following VEGF plasma concentrations (ng/ml; range; median; SEM) were measured: controls (n = 11): 0–0.420; 0; 0.046 // tumours (n = 19): 0–2.823; 0; 0.209 // tumours < 4.5 cm3 (n = 8): 0–2.823; 1.178; 0.353 // tumours > 4.5 cm3 (n = 11): 0–0.180; 0; 0.016.

Overview: In this rat rhabdomyosarcoma tumour model, free circulating VEGF levels could only be detected in small tumours < 4.5 cm3, not in larger tumours or in control rats. Apparently, in this pilot study, an excess of VEGF is measurable in the plasma until a certain tumour volume is established. This in vivo observation emphasises the role of VEGF as a major inducer of angiogenesis and can be of potential importance for the preclinical studies investigating angiogenesis inhibitors.

359 ORAL

Plasma D-dimer levels, serum VEGF, b-FGF and IL-6 in metastatic breast cancer (MBC): Correlation with tumour load and response to therapy

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Purpose: Plasma levels of D-dimer reflect both coagulation and fibrinolysis. This activation might be associated with progression and angiogenesis. Plasma indices of activated coagulation, serum levels of angiogenic cytokines were determined in a group of patients with MBC.

Methods: Plasma D-dimer levels, routine coagulation tests, serum levels of VEGF, IL-6 and b-FGF were measured and analyzed for any relationship with a series of clinicopathological parameters in two groups Group A consist of 30 patients considered to be free of disease after locoregional treatment for BC, group B of 100 patients with progressive MBC.

Results: In group A D-dimer levels were elevated in 4/30 with a median level of 330. In group B plasma D-dimer levels were elevated in 94/100 pts ($p = 0.001$) with a median level of 1820. D-dimer levels in group B did not correlate with age, tumour type, disease-free interval, ER or PR status, number of sites of disease, fibrinogen level, aPTT or PTT. In group B serum VEGF was increased in 43%, and b-FGF levels were increased in 40%. A positive correlation between platelet count and sVEGF ($r = 0.44$; $p < 0.005$), and fibrinogen level and s IL-6 ($r = 0.78$; $p < 0.0001$) was shown.

Conclusion: Plasma D-dimer levels are nearly always elevated in patients with MBC. Both progression kinetics and volume of disease seem to determine the level of D-dimers. Further analysis of the angiokine levels and D-dimers and progression kinetics will be presented.

360 ORAL

In vitro differentiation of endothelial cells (EC) from AC133-positive progenitor cells

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The formation of new blood vessels can be due to two different processes. The first is vasculogenesis, which implies the primary differentiation of endothelial progenitor cells from haemangioblasts, and their subsequent organization into a primary capillary plexus. The second is angiogenesis, which is the formation of new vessels by a process of sprouting from pre-existing vessels. It is currently assumed that vasculogenesis is limited to early embryogenesis, while angiogenesis occurs both during development and post-natal life. However, the possibility that endothelial stem cells or haemangioblasts persist into adult life where they may differentiate and contribute to the formation of new blood vessels, e.g. in malignant tumours, through circulating EC, remains to be explored. In this study we investigated the ability of various growth factor combinations to generate EC. Human AC133-positive (+) cells that represent a subset of CD34+ stem and progenitor cells with haematopoietic potential were isolated from

G-CSF mobilized peripheral blood and then cultured for 14 days on fibronectin-coated chamber slides with IMDM supplemented with 10% fetal bovine serum and 10% horse serum; at day 0, different growth factors were added to the media, including SCF (100 ng/ml), SCGF (100 ng/ml), and VEGF (50 ng/ml). Cells of endothelial lineage were identified by immunocytochemistry, flow cytometry, and electron microscopy. Additionally, gene expression patterns of the freshly isolated AC133+ cells as well as of the cultured were analyzed by RT-PCR. Within 1–2 hours of culture in these conditions, AC133+ cells became adherent. In the presence of VEGF alone or in combination with SCF, the adherent cells did not proliferate whereas the stimulation with SCGF and VEGF resulted in an up to 8-fold higher cell number after 14 days of culture. In those proliferating cultures, a round non-adherent cell population occurred within 6–7 days of culture, and was transferred to fresh chamber slides to again become adherent. Phenotypic analysis of the adherent cells grown in the presence of SCGF and VEGF revealed that the vast majority displayed characteristics of the endothelial lineage including the expression of CD34, VE-Cadherin, vWF, FLK-1/KDR, TIE-2, and Ulex europaeus agglutinin-1. Furthermore, electron microscopic analysis showed structures similar to Weibel-Palade bodies which are found exclusively in vascular endothelium. These data indicate that the AC133+ cell population contains precursors not only with haematopoietic, but also with endothelial potential. The functional role of circulating EC in vivo was assayed in tumour-bearing mice and will be presented at the meeting.

361 ORAL

Genomic instability in bronchial lavage specimens from individuals with no evidence of lung cancer: An early detection marker?

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Genomic instability (GI) is now considered to be a hallmark of cancer. Analysis of microsatellite markers in tumour tissue compared to its normal counterpart has become the most widely used method to determine genomic instability in the form of microsatellite alterations (MA) or loss of heterozygosity (LOH). We have studied 90 bronchial lavage specimens with 12 microsatellite markers from individuals with suspected lung cancer. GI was found in 15/43 (35%) patients with lung cancer, however, GI was also found in 11/47 (23%) patients with no cytological or radiological evidence of bronchial neoplasia; of whom 9 or 11 individuals had evidence of MA/LOH and two were found to have LOH alone. On comparing LOH with MA based on the cytology review, we found that the prevalent type of instability in specimens with cytological evidence of malignancy, is LOH. In contrast, the individuals with negative cytology show a preponderance of MA (Fisher,s exact P = 0.01). A statistical correlation was found between GI and individuals who smoked more than 2 packs/day (P = 0.02) and in the lung cancer patients (P = 0.009). Using current diagnostic techniques, the detection of lung cancer usually occurs late in the disease when it is beyond effective treatment. Thus, increased attention on earlier detection and intervention management is therefore imperative.

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362 ORAL

Tumour cells can eliminate amplified genes

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Highly malignant tumours are often characterised by the presence of an amplified proto-oncogene providing the cells with a proliferation advantage. Aggressive neuroblastomas (NBs) and their cell lines often show a substantial increase of copies of the MYCN gene, which encodes for a nuclear phosphoprotein involved in proliferation control. NB cell lines are usually characterised by the occurrence of morphologically distinct cell types. The neuronal cells (N-cells) show a neuronal expression pattern and phenotype, whereas the so-called flat cells (F-cells) cease to express neuronal markers and have a more fibroblastoid-like morphology. Our aim was to further characterise the nature of the F-cells with special focus on the genetic features, the proliferative activity and the expression pattern of different antigens. For these purposes, we used fluorescence-based in situ hybridisation techniques, BrdU incorporation and immunocytochemistry in five NB cell lines. F-cells were shown to have a markedly decreased MYCN copy number or even to completely lack amplification and the amplified gene copies were shown to be entrapped by micronuclei. In contrast to the N-cells, F-cells showed a reduction or lack of MYCN expression, a decreased proliferation rate and lack of bcl-2 expression. However, they up-regulated MHC class I molecules and expressed b-galactosidase, an enzyme linked to cellular senescence. Based on these results, we conclude that NB cells in vitro can loose their malignant properties, a process which is accompanied by elimination of the amplified oncogene and which allows the cells to senesce. Moreover, MYCN containing micronuclei were also observed in tumour cells infiltrating the bone marrow and in tumours after exposure to cytotoxic agents.

363 ORAL

Inhibition of NF-kB activation confers sensitivity to TNFa by impairment of cell-cycle progression in human glioma cells

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Tumor necrosis factor a (TNFa) has been shown to exert cytotoxic or cytostatic effect on tumor cells, but the susceptibility varies among the different cells. TNFa activates a transcription factor, nuclear factor kappa B (NF-kB), which induces a wide variety of genes and causes pleiotrophic responses. In this study, the relationship between the susceptibility to TNFa and the activation of NF-kB was investigated in six human malignant glioma cell lines. Cell proliferation analysis revealed that only one cell line, SK-MG-1, was sensitive to TNFa and that the other five including U-251MG were resistant. On electrophoretic mobility shift assay, TNFa strongly activated a subtype of NF-kB, p50–p65 heterodimer, in all the resistant cell lines tested. However, this activation was weak in the sensitive cell line, SK-MG-1. Activation of NF-kB by TNFa in the resistant cell lines resulted in significant increases of a reporter gene expression driven by NF-kB site, suggesting a possibility that activation of p50–p65 confers resistance to TNFa. To test this hypothesis, a stable cell line which expresses an inducible dominant negative NF-kB (p65 DN) protein was established in one of the TNFa resistant cell lines, U-251MG. In the established clone, induction of p65 DN protein decreased TNFa-dependent increase in the DNA binding of p50–p65 heterodimer and NF-kB-dependent reporter gene activity. While no growth inhibition of this clone was observed by TNFa treatment, induction of p65 DN together with TNFa resulted in a significant decrease in cell number. Cell-cycle analysis revealed that this growth inhibition was due to an impairment of cell-cycle progression. These results indicate that the active NF-kB complex, such as p50–p65 heterodimer, plays a crucial role for the progression of cell-cycle in malignant glioma cells. The refractoriness to TNFa treatment could be prevented by inhibiting the NF-kB activation.

364 POSTER

Death receptors in etoposide treated lymphoma cells

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Purpose: Apoptosis induction of tumor cells is a potential aim in cancer therapy. Since tumor cells could be resistant to apoptosis inducing agents, it is a question, how we can circumwent this problem. How many pathways are available in a particular tumor cell population using one inducer?

Methods: target cells (HT58 NHL cell line, in vitro) were treated with etoposide; basic methods were immunocytochemistry, flow cytometry and RTPCR

Results: Etoposide activated three different apoptotic signaling pathways in a human B-cell lymphoma (HT58) line, which were distinguished upon time-kinetics and the participants. The first wave of cell death is rapid (~4 h), does not require new proteins, and dependent on caspases. The second wave is slower (1–3 days), still caspase-dependent. The cells express FasR and FasL, and the former appears on the cell membrane. The third wave (3–5 days) of etoposide induced apoptosis became caspase independent. The role of other newly identified death receptors (DR3, 4, 5, TRAIL) is still uncertain, still the target lymphoma cells express all of them.

Conclusion: A commonly used cytostatics, etoposide, can induce three different pathways in lymphoma cells of a well established in vitro line, suggesting the heterogeneity of tumor cells towards a potentially apoptosis inducing agents.

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