Tumour Immunology and Vaccine Therapy

Invited Speakers

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T-CELL IMMUNITY AGAINST TUMORS, A DELICATE BALANCING ACT INVOLVING DENDRITIC CELLS. C.J.M. Melief M.D. PhD. Dept. of Immunohematology and Blood Bank. University Hospital, P.O. Box 9600, 2300 RC LEIDEN, The Netherlands. T-cell immunity occurs naturally against tumors induced by viruses and other causes. In the latter case self antigens are increasingly found to be targets of tumor associated CTL. In all categories of tumors the T cell response usually falls short of the maximally possible response. This situation calls for vaccination, primarily in situations of low tumor burden and adoptive transfer with tumor specific T cells in case of higher tumor burden. We recently observed that the outcome of immunization with vaccines containing tumor virus CTL epitopes strongly depends on mode of epitope delivery. Surprisingly, vaccination with MHC class I binding peptides cause CTL tolerance associated with enhanced tumor outgrowth rather than immunity. Such specific CTL tolerance can be induced by a single injection of 1 µg of peptide in adjuvant. However, in vivo presentation of the same peptides on dendritic cells or in viral vector (adenovirus) causes strong antitumor protection. Thus tumors may escape from immune attack by specific tolerance induction. Tumor specificity of autoreactive CTL can be achieved by T cells directed against tumor associated self antigens of limited tissue distribution. Alternatively useful CTL can be directed against strongly overexpressed self antigens, as illustrated in our lab by the successful eradication of tumors overexpressing wild type p53 tumor suppressor protein, by the adoptive transfer of a wild p53-specific CTL clone. Apparently the low expression of p53 in many tissues does not cause the CTL clone to inflict tissue damage, while the p53 overexpressing tumor cells are specifically targeted and eradicated. Recently we showed that CD40 signalling can replace CD4+ T-cells in priming of helper dependent tumor-specific CD8+ responses. Blockade of CD40L results in profound inhibition of CTL priming that is overcome by CD40 signalling. CD40 signalling converted CTL tolerization by a tolerogenic peptide into strong CTL priming. Moreover, supplementation of an already protective tumor-specific peptide vaccine with a CD40 activating antibody endows this vaccine with therapeutic CTL activity in tumor-bearing mice. The CD40- CD40L pair acts as a switch determining CTL priming or tolerance. This supports the clinical use of CD40 stimulating agents as components of anti- cancer vaccines.

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2

Cellular and molecular mechanisms leading to tumor immunity

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It is established that most experimental and probably many human tumors express antigens against which an immune response can be induced. Systemic tumor immunity usually relies on T cells. As it is known so far, the sequence of events leading to tumor immunity can be summarized as follows: Upon exposure of the host with cancer cells in an immunogenic, non-tumorigenic form antigens are liberated to be taken up by specialized antigen presenting cell (APC) of the host. Probably, this is a MHC class II^+ APC, because for immunity against MHC class I^+/II^- tumors both, $CD4^+$ and $CD8^+$ cells need to be induced. Exceptionally, the tumor cells directly can activate T cells, e.g. if they express B7. The contribution of $CD4^+$ T cells to tumor immunity consists in providing help to the activation of $CD8^+$ cytotoxic T cells (CTL). Thus, recognition of an antigenic peptide presented by MHC class II molecule on the APC is the key event for tumor immunity and the type of MHC class II^+ APC critically determines the nature of the anti-tumor response. The relative outcome of the T cell phenotype depends on whether tumor-derived antigens are presented by B cells or macrophages/dendritic cells. Generation of tumor immunity requires a coordinated and time-dependent expression of IFNγ and surprisingly also IL-4.

3

Quantification of T cell responses

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(Dir.: Ch. Huber)

During the last years techniques have been developed that allow to detect, quantify and monitor antigen-specific T cell responses without prior lymphocyte expansion *in vitro*. Theseassays are based on antigen-triggered lymphokine release [enzyme-linked immunospot (ELISPOT) and intracytoplasmic immunofluorescence assays] and staining of lymphocytes with major histocompatibility complex (MHC) tetramers complexed with peptides. They are applied to detect specific T cells at the single-cell level even when present at low frequencies. It was convincingly shown that results obtained with these assays reflect previous antigen exposure *in vivo* and correlate well with clinical events in infectious and malignant diseases. MHC tetramers containing peptides allow fluorescence-activated cell sorting and direct cloning of rare antigen-specific T lymphocytes, which will provide important informations about immunological memory, the quality of CTL responses against given antigens and opens up new possibilities for generating CTLs for adoptive immunotherapy.

We focus on spot assay techniques. By combining spot assays with computer-assisted video image analysis to automatically determine the number and size of cytokine spots formed by single T cells after antigen contact, we facilitated the spot evaluation and improved its objectivity. Spot assays offer advantages in (a) determining the type of T cell response (Tc1/Tc2), (b) in analysing for specific T cell reactivity against whole proteins, cells and cell lysates, if peptide epitopes are unknown, and (c) in helping to identify new T cell epitopes.

4

Therapy of Melanoma with Interleukin-12 and Interferon-alpha.
William Carson, Mirella Anghelina, Julie Dierksheide, Sara Dierksheide, Poongothai Sundaram, Pierre Triozzi.

Interferon-alpha (IFN-α) has shown promise as an adjuvant therapy following surgical resection of high-risk melanoma tumors, however, its mechanism of action remains undefined. We have recently shown that IFN-α directly stimulates the signal transducer and activator of transcription (STAT) signaling pathway in tumor samples obtained directly from melanoma patients, and that pre-treatment of melanoma cells with IFN-γ results in a 10,000-fold decrease in the concentration of IFN-α required for STAT activation. We have initiated a phase I trial in which pre-treatments of interleukin-12 (IL-12) are used to stimulate the endogenous production of IFN-γ prior to the administration of low-dose IFN-α, in the hope that sensitization of the patient to the effects of IFN-α may lead to increased efficacy and decreased toxicity. The biweekly treatment consists of intravenous IL-12 (100 or 300 ng/kg) administered via the intravenous route on day 1, followed by IFN-α on days 2-6 (1,3,5,7,or 10 million units). Mild hematologic and hepatic toxicities have predominated. IFN-γ serum levels average 1724 ± 125 pg/ml 24 hours following the higher dose of IL-12. Analysis of patient peripheral blood mononuclear cells reveals significant increases in levels of STAT1 and increased sensitivity to low-dose IFN-α in the electrophoretic mobility shift assay. These data suggest that modulation of signal transduction pathways in melanoma patients may represent a strategy to improve the response to cytokine therapies.

5

Cancer immunotherapy with peptides derived from differentiation antigens

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The characterization of tumor-associated antigens recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Several categories of cancer-associated antigens have been described as targets for cytotoxic T lymphocytes (CTL) in vitro and in vivo: 'Cancer Testis' (CT) antigens expressed in different tumors and normal testis, melanocyte differentiation antigens, point mutations of normal genes, antigens that are overexpressed in malignant tissues, and viral antigens. Clinical studies with peptides derived from these antigens have been initiated to induce specific CTL responses in vivo. Immunological and clinical parameters for the assessment of peptide-specific reactions have been defined, i.e. induction of DTH- , CTL-, autoimmmune-, and tumor regression responses. Preliminary results demonstrate that tumor-associated peptides alone elicit specific DTH- and CTL responses leading to tumor regression after intradermal injection. GM-CSF was proven effective to enhance peptide-specific immune reactions by amplification of dermal peptide-presenting dendritic cells. Long lasting complete tumor regressions have been observed after induction of CTL by peptide immunization. However, in single cases with disease progression after an initial tumor response either a loss of the respective tumor antigen targeted by CTL or of the presenting MHC class I molecule was detected as mechanisms of immune escape under immunization in vivo. Based on these observations, cytokines to enhance antigen- and MHC class I expression in vivo are being evaluated to prevent immunoselection. Based on a strategy utilizing spontaneous antibody responses to tumor-associated antigens (SEREX), a new CT antigen, NY-ESO-1, was identified. In a melanoma patient with high titer antibody against NY-ESO-1 also a strong HLA-A2 restricted CTL reactivity against the same antigen was found. A clinical trial is currently being performed to evaluate immune responses to NY-ESO-1 derived peptides injected intradermally and combined with GM-CSF. Since spontaneous NY-ESO-1 serum antibody responses occur frequently in patients with NY-ESO-1 expressing tumors, the immunological and clinical outcome of immunization will be correlated with the patients humoral immunity to NY-ESO-1.

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"Surgery Branch Experience with Melanoma Vaccines"

Francesco M. Marincola and Steven A. Rosenberg. National Cancer Institute, NIH, Bethesda, MD U.S.A.

In recent years significant progress in the understanding of the immune biology of melanoma has evolved from the identification of Melanoma Antigens (MA) recognized by T cells. MA consist of intracellular proteins that are expressed on the surface of cancer cells in association with HLA class I molecules and, therefore, are suitable targets for Cytotoxic T Cells (CTL). The peptide sequences (epitopes) from MA responsible for recognition by CTL in association with different HLA class I alleles have been also identified and have been used for the vaccination of patients with advanced melanoma. Pilot clinical trials performed at the National Cancer Institute, Bethesda, MD, have shown that epitope-specific vaccination is extremely effective in eliciting expansion of melanoma-specific CTL reactivity *in vivo*. However, inexplicably, the amplification of immune responses caused by these vaccines is, in most cases, not sufficient to cause cancer regression. Thus, paradoxically, anti-cancer CTL can coexist with their target cells within the same organism. Not presently clear is whether the immune response induced by the vaccine is quantitatively insufficient or lacks some unknown qualities that could sustain it when started. Present efforts are aimed at testing both hypotheses either by modifying the schedule of administration and/or by administration of adjuvant strategies such as the use of professional antigen presenting cells. Furthermore, several new monitoring strategies have been implemented to evaluate the status of activation and localization of vaccine-induced T cells in the peripheral circulation as well as the tumor site. Among those are in vitro sensitization, use of soluble HLA/peptide complex tetramers, intra-cellular FACS analysis and Real-Time PCR.

7

HPV as target for immunotherapy of cervical cancer

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Human Papilloma Virus (HPV) infection is the most common sexually transmitted disease in the world. More than 80 different HPV types are identified and categorized into low risk and high risk types, according to their malignant potential. HPV infection is controlled by the immune system as evidenced by protective and curative humoral and cellular immune responses in animal models, increased incidence in immuno-compromised individuals, and lymphocytic infiltration during spontaneous regression of warts. Immunotherapy involving unspecific and specific active immunization is currently under investigation. Vaccination strategies are aiming i) at the induction of prophylactic humoral responses for prevention of infection and ii) at induction of cytotoxic cellular responses for a therapeutic approach, particularly for high grade cervical lesions and cervical cancer. Target antigen for prevention of infection is the major capsid protein L1. The HPV early proteins E6 and E7 of high risk types 16 and18 (the most prevalent types in cervical cancer) possess immortalizing activity for keratinocytes and are continuously expressed in transformed cells. Therefore, they are ideal specific antigens on cervical carcinoma cells for cytotoxic T lymphocytes. Immunization strategies include peptides, proteins, recombinant viruses, virus-like particles and chimeric virus-like particles as vaccines.

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Mutant Ras Peptides vaccination-from basic research to clinical application*

G. GAUDERNACK , Section for Immunotherapy, Department of Immunology, The Norwegian Radium Hospital, Oslo, Norway.

Vaccination against molecularly defined cancer specific/associated antigens has now become a clinical reality, and is currently tried out as an alternative treatment modality. We have concentrated on mutations in the RAS family of proto-oncogenes, which occur in many cancer types, the most common being K-RAS mutations (90 % of pancreatic and 40-50% of colorectal cancers). In melanomas 10-20% of the patients have a ras mutation, mainly in H-RAS or N-RAS. Protocols were designed individually for each patient group, and in no instance required HLA typing as inclusion criteria. Out of the more than 100 patient vaccinated, 50-80% has given a positive DTH response. The specificity of the DTH response for the vaccine peptides could be verified by in vitro tests. Cloning of the responding cells from several patients have demonstrated that the majority of the specific clones are CD4+, but also a number of new Class I restricted CTL epitopes have been defined using CD8+ clones derived from the patients. These CTL epitopes are nested within the promiscuous class II binding epitopes used for vaccination. Furthermore, all mutations (in hotspots at position 12, 13 and 61) have been shown to be recognized. These data demonstrate that mutant ras peptides are highly immunogenic in cancer patients regardless of their HLA haplotypes.

In patients with inoperable pancreatic cancer a highly significant prolonged survival was correlated with an immune response to the vaccine. **Collaborative study from The Norwegian Radium Hospital, The National Hospital, Ullevaal Hospital and Akershus Central Hospital. Sponsor: HYDRO ASA.*

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STIMULATION OF TUMOR REACTIVE T-CELLS BY TUMOR-DERIVED STRESS PROTEINS - IMPLICATIONS FOR VACCINE DEVELOPMENT

Preparations of the stress proteins hsp70, hsp90 and gp96 from murine tumors cause unique immunity against the tumor from which the stress protein vaccine was derived. This has been explained by diverse arrays of peptide antigens complexed with these stress proteins. According to the current model, stress proteins act as adjuvants by channeling peptide antigens complexed to them into the MHC class I restricted antigen presentation pathway of professional antigen presenting cells. We investigate the concept of tumor stress proteins as tumor vaccines in human tumor models. We demonstrated that different human melanoma reactive CTL clones with defined peptide specificities were preferentially stimulated by gp96 purified from the autologous melanoma cells. This CTL stimulation was dependent on professional antigen presenting cells, monocytes or dendritic cells. Direct binding of gp96 to human antigen presenting cells could be demonstrated. Preliminary experiments show in vitro priming of melanoma peptide specific T cells stimulated by gp96 preparations from melanoma cells in presence of dendritic cells. The preclinical evidence for the concept of tumor stress proteins as autologous cancer vaccines led to ongoing clinical phase I studies in renal cancer, melanoma, pancreatic cancer gastric cancer and colon cancer in which the frequency of tumor reactive T cells in patients is monitored.

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Breast and Prostate Cancer Immunotherapy using Vaccinia Viral Vectors.

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Breast and prostate cancer are common diseases. The identification and cloning of several common tumor antigens such as MUC1 or, in the prostate PSA, together with the improvement in our understanding of immune response mechanisms, have led to the development of immunotherapy protocols. Results from clinical evaluations in phase I and phase II trials using MUC1 as a target and vaccinia virus as vector and adjuvant, demonstrate an excellent tolerance profile as well as clinical and immunological evidence of response in metastatic breast cancer patients.

Still, innovative methods for patient evaluation and follow up are needed. The patients' inherent capability to mount an effective immune response as well as the heterogeneity of tumor antigen expression are still difficult to evaluate a priori. Furthermore, the complexity and the delay in the laboratory assessment of an effective immune response as well as the low clinical response rates in patients with advanced disease, render the follow up of vaccine trials with a chemotherapy-type design difficult.

More recently, a protocol using PSA as a target for prostate cancer has been developed under a joint NCI and EORTC-btdg sponsorship. The logistics of this ongoing project will be presented.

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GM-CSF – in vivo modulation of the immune response

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GM-CSF is a pleiotropic cytokine activating non-specific cytotoxic cells and APC, stimulates naive T cells, up-regulates various cell surface structures on immune cells etc. Thus, GM-CSF might be a useful agent to combine with monoclonal antibodies as well as with cancer vaccines to increase the therapeutic efficacy. In combination with monoclonal antibodies ADCC was augmented and a strong humoral and cellular idiotypic immune network response (anti-tumor immunity) was induced. Lower doses of GM-CSF might be more effective than higher doses in augmenting ADCC. However, also side effects against the Mab were increased. A low dose of soluble GM-CSF given at the same site as a cancer vaccine preparation was a very effective adjuvant cytokine in inducing a strong humoral and cellular immune response against recombinant CEA, GA733, anti-idiotypic antibodies (mimicking GA733) and B cell tumor derived idiotypic immunoglobulin used as immunogens. Mainly, type I T cells were induced. A substantial fraction of the T cell response was MHC class I restricted. Multiple T cell epitopes could be identified in the external domain of the CEA and GA733 molecules respectively. However, neutralizing antibodies against GM-CSF might be induced hampering the effects of GM-CSF. High dose schedules more frequently induced neutralizing antibodies than low dose schedules. The immune augmenting effect of GM-CSF might be further enhanced by adjuvant administration of soluble IL-12.

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CD40 Ligand (CD40L): Biology and Clinical Use. R. Ghalie, D. Caron, W. Fanslow, R. Armitage, E. Thomas. Immunex. Seattle, USA

CD40L, a membrane-bound glycoprotein primarily expressed on the surface of activated T lymphocytes, signals through binding to its receptor CD40. Functionally active CD40 has been demonstrated on B cells, monocytes, dendritic cells, CD34 positive cells, endothelial cells, and fibroblasts. CD40 has also been demonstrated on all tumor types of B-cell origin and a significant proportion of carcinomas. CD40L functions as the major component of T-cell "help" for B cells in the process of T-cell dependent antibody isotope switching, the formation of memory B cells and germinal centers. Engagement of CD40 on macrophages and dendritic cells by CD40L induces IL-12 production and co-stimulatory molecule expression, and hence cell-mediated immune response. In contrast to its stimulatory effects on normal cells, CD40L was shown to inhibit growth or induce cell death in a proportion of established high grade human B cell lymphoma and solid tumors both in vitro and in SCID mouse models. Based on these experiments, a Phase I dose escalation trial of rhuCD40L was conducted in patients with lymphoma or solid tumors who have failed prior therapy. RhuCD40L was administered SC daily for five days, with cycles repeated every 4-6 weeks. Using the experience gained in the Phase I trial, a Phase II study was recently initiated in patients with Stage IV renal cell cancer.

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Soluble B7-IgG as vaccine adjuvant for therapy of cancer

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The costimulatory B7/ CD28/ CTLA-4 pathway is important for T cell activation and therefore an interesting target for cancer immunotherapy. We have generated soluble fusion proteins comprised of the extracellular domain of either B7.1 or B7.2 and the "Hinge-CH2-CH3" region of an IgG2a antibody. We tested B7.2-IgG in multiple murine tumor models. Vaccination with irradiated tumor cells mixed with B7-IgG of mice bearing day 7 established tumors induced tumor regression and increased survival in all models tested. Even in the poorly immunogenic B16/F10 model, mice survived for more than 90 days compared to only 35 days in the untreated group. Most importantly, therapeutic administration of B7-IgG alone had similar anti-tumor efficacy. The anti-tumor effect of B7-IgG is immune-mediated. No anti-tumor activity of B7-IgG was observed in SCID mice or mice depleted of CD8 T cells. Rechallenge experiments demonstrated that mice cured by B7-IgG therapy were resistant to tumor rechallenge, suggesting the generation of memory responses. Unexpectedly, B7-IgG anti-tumor activity was not dependent on IFN-γ. B7-IgG cured established tumors in IFN-γ KO mice or cured tumors that are IFN-γ insensitive.

Our preclinical data suggest great clinical potential for B7-IgG. Additionally, preliminary studies demonstrated that B7-IgG is safe and that it can readily be manufactured. Currently, human B7.2-IgG is being evaluated as a vaccine adjuvant in non-human primates and we hope to move this promising immunotherapy approach rapidly into the clinic.

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APPLICATIONS OF IMMUNE STIMULATORY CpG DNA FOR ANTIGEN-SPECIFIC AND ANTIGEN-NONSPECIFIC CANCER IMMUNOTHERAPY

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Bacterial DNA contains unmethylated CpG dinucleotides in particular base contexts which are detected by pattern recognition receptors in B cells, NK cells, and DCs. These cell types are activated by these "CpG motifs" to express increased levels of costimulatory molecules and to secrete Th1-like cytokines such as IL-12, IFN-α, IFN-γ, and TNF. Synthetic oligodeoxynucleotides (ODN) containing CpG motifs activate innate immune responses in mice that protect against subsequent lethal challenge with intracellular bacteria, viruses, or parasites. This nonspecific innate immune activation also protects against challenge with melanoma and can even be used for successful immunotherapy of established murine melanoma or sarcoma. This immunotherapeutic approach is not dependent on the presence of T or B cells and does not lead to the generation of memory responses. On the other hand, CpG ODN can also function as outstanding adjuvants for tumor vaccines, providing protection against tumor challenge or prolonged survival in the case of established tumor. The innate immune activation triggered by CpG DNA leads to enhanced antibody dependent cellular cytotoxicity (ADCC) mechanisms. The addition of a CpG ODN to a passive immunotherapy regimen using a monoclonal anti-tumor antibody improved survival from 10% to 80% in established B cell lymphoma. Therapeutic doses of CpG ODN are well tolerated in rodents and primates. Human clinical trials using CpG ODN are currently underway. CpG ODN appear to offer several potential approaches for tumor immunotherapy.

Tumour Immunology and Vaccine Therapy

Poster Presentations

15

Urokinase-receptor expression in colon cancer is transcriptionally induced by Src via an upstream element (-152/-135) bound with Sp1.
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The urokinase-receptor (uPA-R) promotes the invasive phenotype and is associated with a poor prognosis in diverse human cancers. As the *c-src* oncogene has also been implicated in colon cancer progression, the present study was undertaken to determine a role for this oncogene in the regulation of uPA-R gene expression. Increased uPA-R protein and uPA-R-mediated proteolysis was evident in stable SW480 subclones transfected with a constitutively active *c-src* (Y-c-src527F), the uPA-R increase corresponding with the extent of increased Src-activity. Treatment of the Src transfectants with a specific Src-inhibitor diminished the amount of uPA-R promoter activity and protein as well as uPA-R-mediated proteolysis. Conversely, transient transfection of SW480 cells with Y-c-src527F strongly induced the uPA-R-promoter. Site-directed mutagenesis abolishing the binding of Sp1 to region -152/-135 of the promoter inhibited this induction. Electrophoretic mobility shift assays (EMSA) comparing nuclear extracts of SW480 and the Src transfectants revealed an increase of Sp1 binding to region -152/ -135 in the latter. These data suggest that uPA-R gene expression and -proteolysis are induced by Src in colon cancer via transactivation of a motif (-152/-135) bound with Sp1, this being the first report to implicate Sp1 in Src-induced gene expression. It further raises the exciting possibility of Src inhibition as an anti-invasive therapy in colon cancer.

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Overexpression of p73 protein is a prognostic factor in patients with colorectal adenocarcinoma.
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The function of p73 protein in carcinogenesis is not clarified although the p73 is considered as a novel p53-like tumor-suppressor gene. In the present study, overexpression of p73 protein was examined by immunohistochemistry in normal tissue, primary tumor and metastasis in lymph nodes from patients with colorectal adenocarcinoma. One hundred and ninety five out of 227 tumors (83%) had positive expression of p73 protein. In the matched-samples, the frequency and intensity of immunostaining were dramatically increased from normal mucosa (21%) to tumor tissue (79%) and to metastasis in lymph nodes (90%, $p<0.05$). Overexpression of the p73 was related to non-mucinous carcinoma, DNA aneuploidy, higher s-phase fraction and K-ras mutations. The 5-year survival rate was significantly lower in the patients with p73-positive tumor than those with negative one (50% vs 71%, $p<0.05$). The data suggest that overexpression of p73 may serve as a prognostic implication in the patients with colorectal adenocarcinoma.

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Tumor Cells Express The Ligand For The Tumor Necrosis Factor Receptor Family Member 4-1BB (CD137/ILA)

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The expression of members of the tumor necrosis factor superfamily like Fas (CD95/APO-1), Fas ligand or CD 40 on cancer cells and the possible role in the immune escape of tumors has recently been subject of multiple investigations. We looked at several different tumor cell lines including A 2780, COLO 205, HCT 116, HT 29, LX 1, LS 174T, PC 3 and SKBR 3 for expression of members of this family and also of another, the ligand for the 4-1BB receptor. Most of the cell lines expressed both Fas ligand and CD40 to differing extents. In addition, using a 4-1BB fusion protein (human 4-1BB with a mouse IgG2b tail) for cytofluorometric detection, we found that all of the tumor cells expressed 4-1BB ligand on the surface with the highest level detectable on HCT 116 and Colo 205. Total RNA was extracted from tumor cells and analysed by RT-PCR. All of the cell lines showed bands that corresponded to the 4-1BB ligand. Expression was confirmed by Northern blot analysis of mRNA using a recombinant extracellular domain of 4-1BB ligand for hybridization. In agreement with FACS analysis, expression was highest in HCT 116. Recently the role of the 4-1BB molecule as a costimulatory receptor and as a potent survival signal on T cells after antigen recognition has been established. A possible modulation of 4-1BB ligand, especially in concert with Fas ligand expressed on the same tumor cells might provide promising targets for therapeutic option in cancer therapy.

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Direct visualization of antigen-specific T cells using MHC-Ig Dimers

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Multivalent MHC constructs have been shown to be a powerful new tool for the analysis of antigen-specific T cells. Using immunoglobulin as a molecular scaffold, we produced dimeric peptide-MHC complexes, which can be used to directly analyze antigen-specific T cells *ex vivo*. These dimeric MHC-Ig molecules, when loaded with the correct peptide, bind stably and specifically to antigen-specific T cells. We have used these molecules to analyze the immune response of HLA-A2 positive HTLV-1 infected patients with a neurological disorder and could demonstrate that up to 12 % of the CD8+ T cells in the patients peripheral blood recognized the HTLV-1 derived Tax peptide. We have also genetically engineered several peptide MHC-Ig constructs to enhance specific loading of the HLA-A2/Ig molecule. Plasmids containing a 13 aminoacid linker between the peptide and β2m were constructed to enhance loading efficiency of the peptide onto the dimeric protein. Preliminary data suggests that the covalent linkage of the peptide to β2m enhances the loading efficiency of the dimeric molecule. We have now begun to explore the different chimeric molecules in infectious diseases as well as tumor models. These chimeric molecules cannot only be used to evaluate antigen-specific T cell responses, but they can also activate the T cells for therapeutic purposes.

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Mechanisms of Prostaglandin-Mediated Immunosuppression in Patients with Head and Neck Cancer and Successful Immune Restoration by Cyclooxygenase-Inhibition In Vitro

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Prostaglandins, especially Prostaglandin E_2, i.e., PGE_2, as metabolites of the cyclooxygenase pathway have been demonstrated to suppress the function of antitumoral immune effector populations in a variety of cancers *in vitro* and *in vivo*. However, the mechanisms of this phenomenon have not been clearly identified yet. That prompted us to investigate PGE_2-mediated immunosuppressive mechanisms in squamous cell carcinomas of the head and neck (SCCHN) and to analyze immunorestaurative strategies via cyclooxygenase inhibition *in vitro*. Here, we could demonstrate that SCCHN lines produced significant amounts of PGE_2 but no IL-10 or TNFα. Supernatants (SN) of PGE_2-secreting SCCHN lines strongly induced the synthesis of IL-10 and TNFα in primary monocytes. In addition, PGE_2-containing SCCHN-SN as well as exogenous PGE_2 significantly reduced (i) the expression of the chemokine receptor CCR5 and the β2-integrin adhesion molecule Mac-1 on monocytes, (ii) the ability of monocytes to migrate towards the chemoattractant MIP-1β, (iii) and the ability to adhere to endothelial cells. These effects could be completely abolished by adding the cyclooxygenase inhibitors Aspirin or Indomethacin to the cell cultures. In conclusion, our results provide –to the best of our knowledge- the first explanation of a possible mechanism of PGE-induced immunosuppression in cancer patients due to downregulation of CCR5 and Mac-1 on monocytes. Further studies are in progress for the analysis and restoration of CCR5 and Mac-1 expression by cyclooxygenase inhibition *in vivo*.

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Modulation Of Fas (CD95/APO-1) Ligand Surface Expression and Release on Tumor Cells.

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Many recent reports have addressed the expression of Fas ligand (FasL) on tumor cells and its possible role in the escape of tumors from immune surveillance. However, few studies have addressed mechanisms that regulate the expression of messenger RNA, protein expression, cell surface expression and release of soluble FasL in these tumor cells. Changes in any of these might influence the immune-reaction against tumors. We screened various tumor cell lines including A 2780, COLO 205, HCT 116, HT 29, LX 1, LS 174T, PC 3, SKBR 3 and SW 620 for FasL surface expression and release by FACS, ELISA and Western Blot. Subsequently we investigated the effect of vitamin E succinate (VES), retinoic acid (RA), phorbol-myristate-acetate (PMA), ionomycin and interferon γ (IFN-γ) on selected tumor cells in vitro. These agents have previously been reported to influence growth and invasiveness of cancer cells. VES and RA reduced FasL surface expression and increased the release of soluble FasL in a dose dependent way. Effects could be antagonized by addition of metalloprotcinase-inhibitors to the culture medium. PMA, ionomycin and IFN-γ caused minor but reproducable changes of regulation of FasL expression depending on the tumor cell line. Thus we have found it is possible to regulate FasL expression and release from tumor cells. These pathways may play a vital role in determining the outcome of tumor sensitivity or resistance to host immune mechanisms.

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Tumor regression after adoptive transfer of effector T cells is independent of perforin or Fas-ligand

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The adoptive transfer of tumor specific effector T cells can result in complete regression and cure mice with systemic tumor. Perforin and FasL mediated cytotoxicity have been proposed as the major mechanisms for T cell-dependent tumor destruction. To determine the role of perforin and FasL in T cell-mediated tumor regression in a murine melanoma model, B16BL6-D5 (D5), we generated D5 specific effector T cells from tumor vaccine-draining lymph nodes (TVDLN) of wild type (wt), perforin k/o (PKO) or FasL mutant (*gld*) mice and treated established D5 pulmonary metastases in mice with the same genotype. The tumor specific cytokine release of the effector cells was determined in vitro by ELISA.

Effector T cells from wt, PKO and *gld* mice induced complete regression of pulonary metastases and significantly prolonged survival of the treated animals regardless of their genotype ($p < 0,05$). Furthermore, adoptive transfer of PKO and wt effector T cells provided long-term immunity to D5. Therapeutic T cells from wt, PKO or *gld* mice exhibit a significant, tumor-specific type 1 cytokine profile, secreting IFN-γ ($p< 0,05$), but not IL-4.

These results suggest that the antitumor effect of transferred T-cells is not due to direct cell-mediated cytotoxicity, but that the tumor specific release of IFN-γ seems to be critical to therapeutic effecacy.

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Expression of Mage Genes in occult disseminated tumor cells: A RT - PCR study on bone marrow aspirates of patients with localized prostate cancer

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Background: Despite local curative therapy, patients with prostate cancer (CaP) frequently relapse with metastases derived from early disseminated cells. To diagnose such occult dissemination, we developed a sensitive multimarker RT-PCR assay for bone marrow aspirates relying on the tumor restricted expression.

Methods: Bilateral pelvic bone marrow aspirates were obtained from 30 patients with localized CaP and from 30 healthy bone marrow donors. For the nested RT-PCR assay, we selected pairs of unique primers specific for MAGE genes 1,2,3/6,4 and 12. In parallel, we used a sensitive RT-PCR assay for the detection of PSA.

Results: Transcripts of one or more MAGE genes were detected in 18 of 30 patients with localized CaP, but in none of the healthy donors. Ten of 11 patients (91%) carrying a high risk for metastatic relapse were MAGE positive, in comparison to 12 of 19 patients (58%) with no such risk factors ($p = 0,02$, Fisher's exact test). All 8 patients exhibiting a positive PSA PCR were also positive for MAGE.

Conclusion: A multimarker nested RT-PCR assay for MAGE 1, 2, 3/6, 4 and 12 applied to bone marrow aspirates enables the specific detection of rare disseminated tumor cells in patients with localized CaP. The MAGE assay was twice as sensitive as a conventional PSA PCR. Preliminary findings indicate that MAGE expression is positively correlated with established prognostic factors.

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c-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease system

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The *c-erbB-2* gene (encoding the protein p185) is overexpressed in many human cancers. It has been implicated to be of prognostic value in gastric cancer and to promote the invasive capacity of tumor cells. Therefore, the present study was conducted 1. to support the prognostic value of *c-erbB-2* in gastric cancer in a large prospective series (n=203, median follow-up 42 months), 2. to determine the association of *e-erbB-2* expression with the expression of invasion-related genes 3. to perform the first overall multivariate analysis including *c-erbB-2* and the invasion-related tumor-associated protease systems. Expression of c-erbB-2 and a pattern of tumor-associated proteases and inhibitors were evaluated semiquantitatively (score 0-3) with immunohistochemistry. Kaplan-Meier analysis (log-rank) revealed a significant association of increasing expression of *e-erbB-2* with poorer disease-free (p=0.0023) and overall survival (p=0.0160). High amounts of p185 were significantly associated with a high expression of uPA (p<0.0001), uPA-receptor (p=0.015), PAI-1 (p<0.001), PAI-2 (p=0.003), cathepsin D (p=0.012), MMP-2 (p=0.004), alpha-1-antichymotrypsin (p=0.005), and alpha-2-macroglobulin (p=0.017). Multivariate analysis considering these factors in addition to alpha-1-antitrypsin, t-PA, plasminogen, alpha-2-antiplasmin, antithrombin III and established prognostic parameters revealed that *c-erbB-2* is an independent prognostic factor for overall survival (p=0.028, rel. risk 1.33, 95% CI 1.28-1.38)besides surgical curability, pT, pN and PAI-1. In conclusion, *c-erbB-2* is confirmed as a new and independent prognostic parameter in gastric cancer, even in consideration of a pattern of invasion-related factors. The significant correlation of p185 with serveral tumor-associated proteases supports the hypothesis of *c-erbB-2* as a promotor of tumor progression and proposes *c-erbB-2* as a promising target for therapy in gastric cancer.

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MUTATIONS OF E-CADHERIN: A NOVEL TARGET FOR CANCER SPECIFIC IMMUNOTHERAPY

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Somatic mutations of the homophilic cell adhesion molecule E-cadherin are characteristic for diffuse-type gastric cancer. In-frame deletion of exon 9 from the E-cadherin messenger RNA due to different splice site gene mutations is a mutational hot spot. The aim of our investigation was to generate and analyse a functional immunotoxin against the exon-9-deleted E-cadherin variant. Modified *Pseudomonas* exotoxin was coupled to a mutation-specific monoclonal antibody reacting with exon-9-deleted E-cadherin. Various concentrations of the resultant immunotoxin (6H8-PE38) were added to L929 fibroblasts stably transfected with mutant or wild type E-cadherin cDNA, respectively. The immunotoxin was shown to be exclusively cytotoxic for cells expressing mutant E-cadherin (LD_{50} of appr. 220 ng/ml) but not to cells expressing wild-type E-cadherin as determined by XTT-metabolism of viable cells. Furthermore this effect was demonstrated to be concentration dependent and could be inhibited by adding a surplus of free mutation-specific antibody. The toxin induced apoptosis, as shown by Annexin-V / propidium iodide staining prior to FACS analysis. Our study indicates that immunotoxins targeting mutant E-cadherin, expressed exclusively on the surface of tumor cells, may be used as a novel, highly specific approach to treat small tumor deposits.

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LARGE SCALE ISOLATION OF CD56+ DONOR LYMPHOCYTES FOR CELL THERAPY AFTER ALLOGENEIC TRANSPLANTATION - P. Lang*, M. Schumm, M. Pfeiffer, B. Demirdelen, D. Niethammer and R. Handgretinger

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Transplantation of highly purified CD 34+ stem cells from mismatched related and matched unrelated donors is a well established method in our institution, which makes it possible to prevent Graft versus Host Disease (GvHD) without enhanced risk of graft failure.

However, after successful transplantation relapses still represent a major problem in children with high risk leukemias. Donor lymphocyte infusion without alloreactivity might be an approach to control the minimal residual disease immediately after transplantation.

Therefore, we investigated a time-saving and efficient clinical upscaled method for immunomagnetic separation of CD56+ Donor Natural Killer (NK)-cells, which offer an antileukemic effect without inducing GvHD. Apheresis products from healthy donors were enriched for CD56+ lymphocytes using the CLINIMACS method. In a second step, CD56+/CD3+ cells, which were copurified in the first step, were depleted with Dynabeads, directly coated with anti-CD3 antibodies. The purity of CD56+/CD3- NK cells was 96.1%, and only 0.03% contaminating CD3+ cells could be detected. The recovery was 40%. The complete procedure can be done within 4-5 hours. A high cytotoxic activity was measured against the erythroleukemic cell line K562 and could be increased by over-night incubation with Interleukin 2. Furthermore, fresh leukemic blasts could be lysed by antibody dependent cellular cytotoxicity (ADCC), using an anti-CD19 antibody. No proliferative activity was detected in mixed lymphocyte cultures (MLC) against irradiated allogeneic stimulator cells and after mitogen stimulation (PHA) due to the absence of CD3+ T-cells.

In conclusion, this method allows to isolate large numbers of highly purified NK-cells for clinical use with anti-leukemic activity and low anti-host proliferative potential. The cytotoxic activity can be further increased by Interleukin 2 and antibodies. This cell population might be infused after matched and mismatched allogeneic transplantation in order to treat a minimal residual disease without inducing GvHD.

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Xenogenization of human leukemia and neuroblastoma cells by tetanus toxoid loading results in enhanced in vitro anti-tumor immunity.

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We studied induction of anti-tumor immunity by utilization of the xenogenization concept. Primary human leukemia cells, culture adapted primary human neuroblastoma cells, and human lymphoblastoid cell lines (LCLs) were loaded with tetanus toxoid (TT) molecules. To mediate loading we used polyarginine (pArg) molecules of various degrees of polymerization, cationic liposomes, or chimeric molecules of transferrin (Tf) and the polycation polyethylenimine (PEI). All primary human tumor cells and cell lines could be loaded with high efficiency by all procedures as determined by flow cytometric detection of fluorescein labeled TT and western blot analysis of cells loaded with unlabeled TT. Trypsin treatment of loaded cells provided evidence that liposomes and Tf-PEI mediated internalization of TT. In a human in vitro tumor model, MNCs were pre-incubated with TT-xenogenized autologous LCLs and challenged with unmodified LCLs. In xenogenized pre-stimulation cultures increased IFNγ secretion was observed compared to MNCs derived from not xenogenized pre-stimulation cultures. Together, these data indicate the functional utility of the xenogenization strategy for induction of anti-tumor immunity.

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Prostate cancer is one of the most commonly diagnosed neoplasm in men. Due to the common metastatic diseases, usual treatment options have no durable response. The aim of this study was the development of a new approach for treatment of prostate-cancer. This approach is based on the observation that hypoxia leads to necrosis and killing of tumor cells. In this study, the blood coagulation factor X was engineered in a way that it is specifically activated by tumor-associated proteinases. This should result in induction of blood coagulation in the tumor vasculature. Prostate-specific antigen (PSA), a serine proteinase, which is overexpressed by prostate cancer cells, was selected as tumor specific proteinase. By insertion of different sequences from the natural substrate of PSA, semenogelin I, into the activation peptide of factor X, several FX-variants were generated that could be activated by PSA. One FX-variant (FX-V4) was further optimized by site-directed mutagenesis of the P2-position (FX-V4-P2Y). FX-V4-P2Y was about four times more active than FX-V4. After preincubation with PSA, FX-V4-P2Y was able to induce coagulation *in vitro*. Further studies have to show whether the FX-variants are activated in the vasculature of PSA-secreting tumors and whether this leads to thrombosis and tumor regression *in vivo*.

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IL-12 generated immune activity in gastrointestinal tumor patients: Evidence for intrinsic capacity against autologous tumor cells

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Immunological mechanisms against disseminated tumor cells are presumably critical for tumor recurrence and prognosis. After tumor cell presentation by DC, IL-12 is a central cytokine for the induction of an effective immune response. We tried to induce an immune response against autologous tumor cells (auTu) by IL-12 in order to examine "intrinsic potential" in curatively resected tumor patients.

From 14 patients with gastrointesinal carcinoma PBMC were incubated without stimulation, with 100 I.U. IL-12/ml, and c) with 1000 I.U. IL-2/ml without contact to tumor cells for 36 h. Specific cytotoxic activity of PBMC against auTu, NK-sensible cells (K 562) and allogeneic tumor cells (RF 48/HT 29) was determined by a fluroescence assay. The auTu of 5 patients were incubated with mAb FMC 16 and W6/32 in order to inhibit specific lysis by CTL via the MHC I system.

Specific cytotoxic activity of PBMC without stimulation was not different among the three targets. IL-12 caused a 3.2 fold elevation of activity against auTu ($p<0.01$). In contrast, after stimulation with IL-2, only a slight increase was seen. After identical IL-12 stimulation, cytotoxic activity against auTu was 2.5 / 2.7 fold higher than corresponding activity against K 562 / allogeneic tumor cells ($p<0.01$). After mAb blocking of the MHC I complex on auTu, a 75% reduction of specific cytotoxic activity of IL-12 stimulated PBMC was seen indicating the participation of specific CTL.

A de novo generation of the observed activity in 36 h without antigen contact seems hardly possible. Therefore, IL-12 triggered activation of a specific preexisting immune response may be supposed. These findings give evidence for "intrinsic immunological potential" against auTu in curatively resected patients, which might be used for adjuvant therapy strategies following curative surgery.

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Xenografted Human Colon Carcinoma in Nude Mice Inhibited by Means of Xenografting the Pateins' Colon Mucosa.

Zhang Hying, Peng Hong, Shi Xin-Pu, Wang Hua, Gao Jie-Ying.

Objection. One of big problems in immunotherapy of cancer is that we don't know which kind of immunotherapy is benefit to which kind of pateins. Some active factors of gastrointestinal Mucosa remains unknown in it's affection on carcinoma growth. To explore new therapy, we induced xenografted tumors inhibition by means of xenografting the pateins' colon mucosa. **Method.** Morphologically normal colon mucosa and fragment of the tumor from surgical samples of fourteen patients with different colon carcinoma were transplanted to nude mice (four xenografted tumors per case) with controls of sham operation, sample-washing solution injection and non-mucosal tissue xenografting groups. **Results.** The original structure of the transplanted mucosal could be seen for 5 days and their contour remained for more than four weeks. Forty xenografted tumors of adenocarcinoma from ten pateins were inhibited completely. The other sixteen of mucinous carcinoma from four donors grew up in different degree, among which two out of the four pateints suffered from right colon mucinous carcinoma and their xenografted tumors did not show any growth inhibition. The paraffin sections of the inhibited show fibrosis with few carcinoma cells in the center. Lymphocytes infiltration was not found in the xenografted tumors. ConA enhanced proliferation of spleen lymphocyte was higher in mucosal transplanted groups than in controls. Flow cytometry of peripheral lymphocyte did not show obviouse change in CD3 subset. But the ratio of CD4/CD8 decreased when the animals were transplanted with human colonic mucosa, which was because of CD8 increasing. Plasma protein electrophoresis by SDS-PAGE displayed an abnormal band about 40kD(P40) in mucosa xenograft group. P40 has a novel sequence in the first 15 animo-acid from N-terminal. Blood concentration of P40 did not show any different in left colon mucosa xenografted animals than in right colon ones($P<0.05$). The plasma administration inhibited xenografted human colon carcinoma cell line HT29 though it did not show cyto-toxicity in vitro. Plasma IL-1, TNFα and LPS level of the mucosa xenografted mice were significantly higher than controls in 3 days after operation. **Conclusions.** The xenograftion of human mucosa caused the tumor inhibition because of it raising the animals immunity. The pathologic stage and the degree of cellular differentiation in adenocarcinoma did not affect therapeutic result. Our experiment either did not show therapeutic efficacy to the mucinous carcinoma(especially those from right colon), which suggested histological classificaton and location be the most important prognostic factors. To open out the mechanism of the results and P40 molecule might be interesting topics in the future.

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Xenografted Hepatic Metastasis of Colon Carcinoma in nude mice Inhibited by Means of xenografting the Patients' Colon Mucosa.

Zhang Hying, Peng Hong, Shi Xin-Pu, Wang Hua, Gao Jie-Ying.

Objection. hepatic metastasis of colon carcinoma remains the biggest problem after surgery. To explore new prevention, we induced inhibition of xenografted hepatic metastasis of colon carcinoma by means of xenografting the patients' colon mucosa. **Method.** Morphologically normal colon mucosa from surgical samples of six patients with colon adenocarcinoma of different pathologic stage and degree of cellular differentiation were implanted subcutaneously to BALB/C nu/nu mice(four animals per case) with controls of sham operation, sample-washing solution injection and mesentery xenograft groups. Tumor cells were isolated from the same surgical samples and cultured for three days in vitro, then implanted into the spleens of the nude mouse four animals per case. Transplanted mucosa, the livers, spleens, lungs, kidneys and peripheral blood of the animals were collected at pointed time for various examination and analysis. **Results.** Hepatic metastasis was not found in all animals with mucosa xenografted(0/24), which was of significant value($P<0.01$) compared with controls of 14/18 for sham, 12/18 for washing solution and 15/18 for mesentery groups respectively. The liver also show the portal track and intra-lobule inflammatory infiltrate of lymphocytes and neutrophils. Immuno-histochemistry examination show infiltration of CD4+ and CD8+ T cells. Acidal bodies and small necrosis were also seen. There were no parasites, bacteria or virus found under electronic scope. Blood tests for glutamic pyruvic transaminase increased in the mucosa xenografted group. Plasma IL-1, TNFα and LPS level of the mucosa xenografted mice were not significantly higher than the controls three days after operation. Blood culture for bacteria counting was negative. There were no parasites, bacteria or virus found under electronic scope in the plasma sediment after centrifuging. **Conclusions.** The transplanted human mucosa prevent the livers from hepatic metastasis because of causing hepatitis. But we did not know why the mucosal transplantation caused hepatitis. The results gave us some revelation which might be useful to investigate the relation between the mucosal immunity and intrahepatic immunity.

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A phase II study of tumour response rates and tumour HLA expression during treatment of metastatic malignant melanoma with low dose γ-interferon. Chao D[1], Propper DJ[1], Braybrooke J[1], Bahl P[1], Thavasu P[2], Balkwill F[2], Talbot DC[1], Harris AL[1] & Ganesan TS[1]. [1]ICRF Oncology Unit, Oxford & [2]ICRF Laboratories, Lincolns Inn Fields, London, UK.
Tumours evade immunotherapy by different mechanisms, including downregulation of Class I pathway. γ-interferon is the most potent inducer of Class I and II expression and has a bell-shaped dose response curve. We have re-evaluated low dose γ-interferon for clinical response and induction of Class I and II in tumours in vivo. Eligible patients had melanoma with skin deposits suitable for biopsy. 100μg/m^2 human recombinant γ-interferon (Immukin, Boehringer Ingelheim) was given s/c weekly for 6 months. FNAs, or core biopsies, and blood tests were taken pre-treatment, 1 day, 7 days, 3 and 6 months. 20 patients are assessable to date, with 2CR and 1PR, all had skin and lung metastases, and CR were durable with disease-free survivals of 20 and 37 months. Biopsies were evaluated for Class I and II expression in tumours by immunohistochemistry. 2/16 patients showed reduction of Class I expression pre-treatment, either due to loss of heavy chains or $\beta_2$microglobulin, and in both cases increased after γ-interferon. In contrast, 14/16 patients showed no Class II pre-treatment and in 7/14 increased post-treatment. Surrogate immunomodulatory markers, neopterin and $\beta_2$microglobulin, increased at 1 day but fell by 7 days. In summary, low dose γ-interferon had clinical efficacy (15%response rate) with very low toxicity and we have shown immunomodulatory activity, including upregulation of Class I and II in tumours in vivo.

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Mode of action of rML: triggering of specific signalling pathways is probably due to intracellular ribosome-inactivation

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Recombinant mistletoe lectin (rML) is a potent apoptosis inducing drug with immunomodulatory activity in vitro and in vivo. The prime activity of ribosome inactivating proteins (RIP) like ML consists of irreversible inhibition of translation. However, it is not known by which mechanism RIP activity induces apoptosis. The availability of the pure and homogenous recombinant protein enabled studies on its mechanism of action with respect to intracellular signal transduction.
We tested the ability of rML to induce apoptosis in different human blood and cancer cell lines by a number of methods which consistently showed that rML mediated cell killing is due to apoptosis in a concentration dependent manner. Additionally, signal transduction pathways were investigated in response to rML treatment, showing that the MAPKinases SAPK/JNK and p38 are activated by phosphorylation, whereas the erk pathway seems to be irrelevant. Thus, activation of the SAPK/JNK and p38 pathways is probably due to ribotoxic stress. Transcription factors are induced by the MAPK which in turn regulate gene expression, e.g. cytokine genes. We therefore speculate that the immunostimulatory effect of rML is tightly linked to intracellular signal transduction mechanisms like the stress kinase pathway and the caspase cascade. According to our results the effect of rML on immune cells is not restricted to inhibition of translation by virtue of its RIP activity but a triggering of a strictly regulated network of intra- and intercellular signals resulting in a local and systemic anti-tumor response.
BMBF grant 0311183

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Assessing the biochemical properties of mistletoe lectin by means of genetic engineering

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Mistletoe lectin (ML), used for adjuvant tumor therapy, is a heterodimeric plant protein consisting of an enzymatically active A- and a lectin like B-chain. Recombinant production of biochemically defined ML (rML) was achieved by cloning and separate expression of the single chains in *E. coli*. In a co-association process, the unsoluble A- and B-chain proteins were naturated in a single step yielding 10% of active rML. The biochemical and biological activities of wildtype rML, a mutated form of rML with defined amino acid exchanges in the active site and plant derived ML (pML) were compared. In a coupled transcription/translation assay for rRNA N-glycosidase activity of the A-chain 50% inactivation was obtained with 20.5 pM rMLA, 3.5 pM pMLA and 2,900 pM of rMLAmut. The IC_{50} values of the two wildtype proteins were also similar (30 pg/ml for rML and 20 pg/ml for pML) in a cytotoxicity assay with MOLT-4 cells. rMLmut with the mutant A-chain showed a reduced cytotoxic activity (factor 30). Carbohydrate binding specificity of the B-chain was analysed in a competitive binding assay. 50% competition of lectin binding was achieved at lactose concentrations of 1.6 mM (rML) and 1.8 mM (pML). Variant forms of rML consisting of an active A-chain and mutated carbohydrate binding sites had a reduced cytotoxic effect on cells. Using three different assays no significant differences in the activities of the *E. coli* derived rML and glycosylated pML were found. Furthermore, from the results with the mutant proteins we deduce that both the lectin and the rRNA N-glycosidase activity are a prerequisite for the cytotoxic and immunomodulating action against target cells.
BMBF grant no. 0311183

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Recombinant mistletoe lectin: A non-immunosuppressive cytotoxic agent in vitro

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The recombinant mistletoe lectin (rML) is a new biological entity being developed for cancer immunotherapy. The objective of this study was to analyze, firstly, immunostimulatory actions of rML which can initiate immune signaling cascades and responses against cancer cells and, secondly, the putative relationship with its cytotoxic potency. rML increased the release of IFN-γ from human PBMC, of IL-1β from PBMC and THP-1 cells as well as of IL-6 and IL-15 from the human HaCaT keratinocyte cell line. The expression of IL-1β mRNA in THP-1 and IIL-60 cells and of mRNA for IL-6 was enhanced in HaCaT, THP-1 and HL-60 cells by rML. Furthermore, the lectinic cytotoxin up-regulated the expression of CD25 on human PBMC surface and primed the granulocyte oxidative burst in an human whole blood bioassay. The natural killer cell activity of mouse splenocytes against YAC-1 target cells was augmented by rML. In most of these bioassays, rML was immunopharmacologically active at cytotoxic concentrations only. The agent induced apoptosis at low and necrosis at high concentrations. In conclusion, these findings indicate a relationship between the cytotoxic and the immunostimulatory effects of rML.
Supported by the grant 0311183 of BMBF, Germany.

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Recombinant mistletoe lectin (rML): A potent anticancer agent in vivo

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The β-galactoside-specific recombinant mistletoe lectin (rML) is a type II ribosome-inactivating protein with cytotoxic and immunostimulating potencies. In the present studies, the anticancer activity of locally or systemically applied rML was investigated in different animal tumor models. rML significantly inhibited tumor growth or malignant transformation in two different urinary bladder carcinoma models (MB49, NMU-induced) when given intravesically to mice or rats at concentrations of 3 to 300 ng/0.1 ml. Additionally, significant antitumor activity was observed dose-dependently in immunocompetent mice at repeated i.p. doses ranging from 3 to 3000 ng/kg against four subcutaneously implanted syngeneic tumor cell types (Lewis Lung-, Renca renal-, C8 colon 38- and F9 testicular carcinoma) and in athymic nude mice against human LXFS 538 (lung carcinoma) xenografts. Antimetastatic activity of rML was shown against different i.v. inoculated syngeneic sarcoma cell types (B16 melanoma, L-1 sarcoma and RAW117-H10P lymphosarcoma) in immunocompetent mice treated repeatedly by the i.v. or s.c. route at doses ranging from 0.3 to 300 ng/kg. These results suggest that rML is a new therapeutic entity and a promising candidate for clinical development in cancer therapy.
Supported by the grant 0311183 of BMBF, Germany.

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DETECTION OF MEMBRANE ASSOCIATED PROTEINS BY SCREENING A MAMMARY CARCINOMA SPECIFIC cDNA EXPRESSION LIBRARY WITH SERA FROM IMMUNIZED MICE

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Serological analysis of antigens by recombinant expression screening (SEREX) provides a new tool to identify immunogenic tumor antigens. We have applied this technology introducing the screening of λ phage expression libraries with sera from membrane immunized mice to characterize membrane associated antigens. Balb/c mice were immunized with membrane preparations from human breast cancer cell lines 8701-BC and BT-474. A λ phage cDNA expression library was constructed using RNA from the same cell lines. Screening of 2.1×10^5 plaques with sera from immunized mice yielded one positive clone mBT/BC 1 which showed 98.3 % amino acid homology with human Mitofilin, a mitochondrial transmembrane protein. The insert of a second weakly positive clone mBT/BC 2 was not in frame with the vector sequence. Nevertheless the insert represented an interesting human gene called Rheb (Ras homolog enriched in brain). This result gives evidence that Rheb is expressed in breast cancer and might contribute to its transformation and aberrant growth properties.

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ANALYSIS OF AN IN VITRO SYSTEM TO CHARACTERIZE THE IMMUNOLOGICAL MECHANISM OF THE IMMUNOTHERAPY OF BLADDER CANCER WITH BACILLUS CALMETTE-GUERIN (BCG)

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BCG-immunotherapy is the treatment of choice for superficial bladder cancer. From animal models and studies on patients it is known that effective therapy depends on cell-mediated immunity and involves the induction of proinflammatory cytokines. We developed an in vitro system to analyze cellular and humoral factors, which contribute to BCG-induced cellular cytotoxicity directed against bladder tumor cells. MNCs stimulated with BCG kill bladder tumor cells of short term and long term culture. By depletion experiments we could show that induction of cytotoxicity depends on monocytes and CD4+ cells as accessory cells. Effector cells were characterized as CD3-/CD8+/CD56+ NK cells using magnetic cell separation and FACS. Cytotoxicity was primarily mediated via perforin without a significant contribution of the FasLigand pathway even in the killing of Fas receptor expressing targets. Activation of BCG-induced effector cells by monocytes is independent of MHC-restriction. Inhibition experiments showed that IL-2, IFN-γ and IL-12 are crucial for NK cell activation. In conclusion, BCG stimulates NK cells to exert perforin-mediated cytotoxicity against bladder tumor cells not susceptible to unstimulated MNCs. This cytotoxicity strongly depends on the secretion of IL-2, IFN-γ and IL-12 and the accessory function of CD4 cells and monocytes.

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Does the superoxide/peroxide ($O_2^{\cdot-}$/ O_2^{2-})- ratio within mitochondrial, microsomal and/or nuclear compartment decide about cell proliferation versus cell differentiation ?

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According to our hypothesis, a high intracellular (intranuclear) $O_2^{\cdot-}/O_2^{2-}$-ratio induces cell proliferation whereas a low $O_2^{\cdot-}/O_2^{2-}$-quotient results in cell differentiation. Arguments: (a) Cell differentiation-inducing IFNgamma induces MnSOD, dismutating HO_2 to H_2O_2. (b) It also generates HO_2-neutralizing NO. (c) Phorbol esters, known as proliferation-stimulating „tumor promotors" cause downregulation of SOD and upregulation of HO_2-generating NADPH oxidase.(d) They induce ornithine decarboxylase (OD) and putrescine synthesis, 2 early markers of proliferation. (e)Arginase, secreted by APCs, converts the iNOS-substrate L-arginine to the OD substrate L-ornithine, resulting in depressed differentiation and deblocked proliferation.(f) H_2O_2 induces - via NFkB transcription - differentiation-promoting type1 cytokines and inhibits c-jun and c-fos (AP-1) genes. (g) These are stimulated by antioxidants, e.g.H_2O_2-scavengers NAC and PDTC . (h) The HO_2-generating LTB4 and lipoxin A4 stimulate c-fos and c-jun genes and herewith the cell proliferation. (i) The cell proliferation-supporting PKC-activator FTT stimulates the HO_2-release. (j) The superoxide anion shows chemotactic properties („chemotaxin") which can be prevented by MnSOD or CuZnSOD. (k) The auxins (plant growth hormones) activate the HO_2-generating NADHoxidase. (l) Various differentiation-inducing agents are HO_2 scavengers, acting, in some analogy to glucocorticoids (cortisol), by reducing lipid hydroperoxides (leukotriene precursors), formed in the presence of HO_2.

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Influence of the Hypoxic Subvolume on the Survival of Patients with Head and Neck Cancer

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Tumor hypoxia is regarded as an important factor influencing radiation response, disease-free, and overall survival of patients with squamous cell carcinoma of the head and neck (SCCHN). This study was performed to reevaluate the prognostic significance of the „classical oxygenation parameters" hypoxic fraction and median pO2, and to determine the influence of a new radiobiological factor. This factor was termed the „hypoxic subvolume" (HSV) and was defined as percentage of pO2-values below 5 mmHg multiplied by the total tumor volume. The rationale of this parameter was to quantify approximately the amount of hypoxic tissue which should be correlated to the number of hypoxic cells in the tumor. Pretreatment pO2 was assessed in 59 patients with SCCHN with the Eppendorf histograph, and pretreatment volume was determined by ultrasonography (lymphnode metastases) and computer tomography (primaries). All patients were referred to our departments for radiotherapy (n = 27, median dose 70 Gy) or radiochemotherapy (n = 32, 5-FU, mitomycin C, median dose 70 Gy), respectively. All parameters were evaluated using the Kaplan-Meier analysis, and significance was assumed at a p-value of < 0,05 (log-ranktest, Cox-Mantel). A multivariate analysis was performed to control for confounding factors. The median follow-up was 233 days. At the time of the evaluation, 34 of the 59 patients were dead.
In univariate analyses, the hypoxic fraction (pO2 < 5 mmHg, pO2 < 2,5 mmHg [p < 0,05]), the hemoglobin concentration (p < 0,05), and the hypoxic subvolume (p < 0,01) were of prognostic significance for overall survival. In multivariate analysis, the hemoglobin concentration and the hypoxic subvolume (p = 0,01) were significant prognosticators. We found no significant correlation between tumor volume or median pO2 and overall survival. No clear correlation was found between tumor volume and hypoxic fraction.
These data suggest that the total amount of hypoxic tissue, as determined by the hypoxic subvolume, influences the prognosis of patients suffering from SCCHN. In addition, our data confirm the statements of previous studies that low pretherapy pO2-values indicate a worse prognosis.

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Influence of adjuvant active specific immunotherapy (asi) on disseminated tumorcells in bone marrow (dtbm) of patients with gastrointestinal malignancies

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Although many gastrointestinal cancers can be detected in early stages, relapses frequently occur due to early dissemination of tumor cells. There is evidence that detection of dtbm is a good prognostic predictor for later relapse in many malignancies.
We treat patients (pat.) with completely resected gastric cancer, pancreatic and colon cancer Dukes B with asi, according to the modified method of Prof. Dr. Schirrmacher (described elsewhere), in the intention to reduce the relapse-rate. Bone marrow biopsy was performed before and after asi.
So far, we vaccinated 10 pat. (7 gastric cancer, 1 pancreatic cancer and 2 colon cancer Dukes B). Median follow up is 25 months (20 - 33 months; 1996-1999).
Before ASI we detected dtbm in 7 pat. (5 pat. with gastric cancer). After asi there was only one pat. with a borderline positive test. 2 pat. had suffered a relapse: 1 pat. with pancreatic cancer (pT2pN1G1L1) relapsed 16 months after asi (peritoneal wall, but no metastases in liver or lung); 1 pat. with gastric cancer (pT2pN2G3) had a local relapse even without liver or lung metastases after 19 months. 6 of our pat. with gastric cancer had stage II or III, but so far we have only 1 relapse after a median follow up of 25 months; the common 5-year-survival in stage II is 50-60 %, in stage III 20-30%

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The Effective Screening for Anticancer Traditional Chinese Medicines, Foodstuffs and Their Curative Effects for Early and Rather Too Advanced Stage's Tumor, Cancer of Mice

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Abstract Anticarcinogen and anticancer foodstuffs have been screened by a direct, quick, easy and contact killing method using Hepa and S_{180} cells of ascites type of mice as model and dyeing dead cells, in vivo and in vitro, by Trypan Blue as indicator, as compared with control. The method comes true after the dyeing cells of 0.1ml or more were injected into abdomen of health mice, then, they don't have cancer or tumor. But the mice injected live cells control all died. If the screened lower doxicity anticarcinogen or foodstuffs are injected early using suitable concentration and quantity(2 or 3 times a day in combine with per os) into abdomen or tumor body of primary stage of cancer for mice, then, some of the treated mice can be cured, whereas control mice died in a limited days. If high doxicity or side-effect and high concentration or unsuitable quantity drugs, especially the antimetabolite's carcinogenicitical anticarcinogen, are used as injections, although the mice have no any body form change, yet, they died ahead of control mice which already bear a big belly or a tumor. Perhaps, this is why cancer, tumor could not be cured by so many kinds of anticarciogen which can not prevail over the quick cancer cells proliferation and development of cancer or tumor, except early find and timely operated them thoroughly.

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Molecular analysis of the activation status of human NK cells – a method screening cancer patients before immune therapy

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Natural killer (NK) cells build the first barrier in the cellular defense of tumor cells in peripheral blood killing target cells by two main mechanisms, namely, the perforin/granzyme and the Fas ligand (FasL) pathways. These mechanisms are augmented by the stimulation of NK cells with interleukin 2 (IL-2). Because cancer patients often exhibit an impaired immune system it is important to know whether their NK cells can be activated before immune therapy. Our aim was to analyze the activation of NK-associated gene expression using the real-time quantitative RT-PCR TaqMan technology. The stimulation of purfied NK cells (>90%) from peripheral blood of healthy donors with IL-2 led to an upregulation of IFN-γ, CD69, TNFα, Bcl-2, perforin, and granzyme B mRNA expression, whereas FasL expression was only weakly affected. Using this system it will be possible to determine the actual status of NK cells with respect to the regulation of genes involved in cytolysis, self-protection and apoptosis. Additionally, 17 patients with breast carcinoma were tested; the results will be presented.
This method in combination with the detection and characterization of disseminated tumor cells seems to be a new diagnostic tool controlling the efficacy of a (immune)therapy and the NK activation status of cancer patients.

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CHOICE OF UKRAIN AND INTERFERON-ALPHA DOSES FOR THE CANCER PATIENTS THERAPY

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We have studied *in vitro* the effects of ukrain (semisynthetic compound from *Chelidonium majus L.* alkaloids and thiophosphoric acid triaziridide, "Nowicky Pharma", Austria) and reaferon (recombinant human interferon alpha-2b, "Vector", Russia) on the state of thiol-disulfide ratio (SH/SS) of the blood by amperometric titration method. There were examined 14 cancer and chronic viral hepatitis patients. Ukrain was tested in doses 0.1, 0.5, 1.0 и 2.0 μg/ml of blood, and reaferon in doses 20, 50, 100, 200, 400, 600, 1000 ME/ml of blood. It was revealed that the control proofs of SH/SS ratio were decreased in all patients. The increasing of SH/SS ratio under influence of preparations was estimated as positive effect and decreasing – as negative one. First group of patients have shown positive reaction to all doses of ukrain and reaferon with one or two peaks of optimal doses. The second group of patients have demonstrated positive reaction to one doses and negative to another doses of ukrain and reaferon, also with one or two peaks of positive effective doses. And at least, some of patients have shown negative reaction both to ukrain, and reaferon in all tested doses. Eleven of these patients were treated by ukrain or reaferon. In the cases when the dose for therapy coinsided optimal one, treatment had a positive clinical effect. On the basis of these results we can offer original trial design for the biological response modifiers including ukrain and interferon-alpha.

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POSTSURGICAL LYMPHOCYTE BLAST-TRANSFORMATION ACTIVATION IN PATIENTS WITH ADVANCED SOLID MALIGNANT TUMORS OF CHEST AND ABDOMINAL ORGANS

Low effect of biological therapy of advanced malignancies is connected in certain extent with inability of T-lymphocyte proliferation in response to antigen stimulation. Previously, we demonstrated (Hybridoma, 1999, v.18, No.1, P.99-102) that radical surgery even in stage IV disease facilitates the possibilities of immunotherapy due to the elimination of immunodepressive effect of the tumor. Yet, in the majority of patients prolonged immunodepression remains after surgery, the key moment being cutoff of the co-stimulating function of monocytes, which is necessary for induction of lymphocyte proliferation. Addition of monocytes from healthy donors *in vitro* to the lymphocytes of the patient in the blast transformation reaction leads to recovery of proliferative activity of the lymphocytes to the phytohaemagglutinin. 50 patients who survived different kinds of surgery for metastatic thoracic and abdominal malignant tumors received intracutaneous injections of autologous lymphocytes activated *in vitro* with consecutive check of blast-transformation. When using repeated injections of autulogous lymphocytes activated *in vitro* all the patients presented with systemic effect which duration and extent depended upon surgical radicalism and frequency of lymphocyte injections We hope to increase effectiveness of immunotherapy in this category of patients by this approach.

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PROPHYLAXIS OF LIVER METASTASIS BY SPECIFIC LECTIN BLOCKADE

The prognosis of colorectal cancer is mainly determined by liver metastasis. In 40% of all cases the liver is the only location of metastases. The process of metastasis depends in a varying degree on the growth rate of tumor cells, the capacity of migration, secretion of lytic enzymes, tumor angiogenesis, and the immunological reactions against tumor cells.
Since membrane-integrated beta-D-galactose lectins on liver cells and freely exposed galactose residues on tumor cells have been detected, a specific receptor-lectin binding as an important step in the process of metastasis was proposed. Therefore the idea was born to reduce liver metastasis by blockade of the liver lectins using galactose or galactans. The usefulness of this idea was confirmed by several studies:
1. The binding of a variety of human tumor cells to human hepatocytes in vitro was shown. The binding was specifically inhibited by galactose and arabinogalactan, but not by mannan.
2. Galactose and arabinogalactan but not mannan significantly reduced liver metastasis after injection of L-1 sarcoma cells in BALB/c mice. The same was true for ESB cells in DBA/2 mice.
3. Patients with colorectal cancer and liver cirrhosis developed liver metastases in only 7% compared to 68% of patients with normal liver. This is interpreted as a lack of lectins in the cases of liver cirrhosis.
4. 93 patients with resected colorectal cancer were treated with perioperative infusions of galactose compared to 100 patients who got glucose. The incidence of liver metastases was 7,5% in the verum group and 12% in the control group, which is not a significant difference. 48 patients with stage II colon cancer treated with galactose developed liver metastases in 8/48 cases compared to 15/48 in the control group. 40 patients with stage II gastric cancer treated with galactose developed liver metastases in 15/40 cases compared to 25/40 patients in the control group.
In a prospective randomized double blind multicenter trial the previous results will be critically examined.

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New models for the action of some type2-cytokines (IL4/IL13 and IL10),based on the postulated increased H_2O_2-level in Th1 vs. Th2 cells: (1) IL4 and IL13 act, in contrast to type 1-cytokines (IFNgamma),(a) by upregulation of catalase/glutathione peroxidase and/or (b) by downregulation of superoxide dismutase(s). (2) One of IL-10 actions is inhibition of cAMP-PDE(s).

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According to our hypothesis, a minimal intracellular H_2O_2(and HO_2) concentration is critical for a normal cell function. We suppose that this critical H_2O_2 treshold value is higher in Th1 than in Th2 subset of helper T cells, but also in the Tc/CTL subset, when compared with the Ts subset. In addition, we predict a higher H_2O_2 level in T4 than in T8 cells. The supposed upregulation of H_2O_2-degrading catalase and GSH-peroxidase by IL4 and IL13 in Th2 cells, supported by the PC-PLC stimulated, SOD-downregulating PKC (without a concomitant Ca_i-increase, as observed in Th1 cells through the PI-PLC activation) would result in the H_2O_2 drop under the treshold value for Th1 and Tc cells, respectively, and would strongly increase the proliferation-stimulating HO_2/H_2O_2-ratio. The transcription of type 1 vs. type 2 cytokines is based, according to our hypothesis, on the redox regulation within the nuclear compartment. We suppose that the type1 transcription factors are redox-upregulated via critical disulfide bonds, as (a)H_2O_2 direct activates NFkB and (b) since NFkB transcription can be prevented by antioxidants, such as NAC and PDTC and by an increased $cAMP_i$ level which both strongly inhibit PLA2 and herewith the NADPH : P450 oxidoreductase-generated H_2O_2. The transcription of type 2 cytokines seems to be inhibited by oxidants such as H_2O_2 and promoted or tolerated by increased $cAMP_i$.

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Two competing,ROSgenerating systems (plasmalemma NADPH oxidase and mitochondrial/microsomal NADPH: cytochrome P450 oxidoreductase) differently modulate the superoxide versus peroxide generation. A possible role in atherogenesis

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Mn SOD is upregulated by IFN, IL1 and TNFalpha and downregulated by PKC stimulators (DAG, TPA), DAG being a product of both, the PI-PLC,stimulated in Th1 cells and PC-PLC, stimulated in Th2 cells upon activation of TCR. (a) As the plasmalemma-associated NADPH oxidase is activated by the same,SOD-inhibiting PKC, so the PKC upregulation results in the HO_2/H_2O_2- shift towards HO_2 and herewith towards proliferation. (b) The HO_2, generated by the mitochondrial NADPH : P450 oxidoreductase, dismutates to H_2O_2 by the mitochondrial MnSOD, which is coinduced by type 1 cytokines, decreasing in this way the HO_2/H_2O_2-ratio and favouring the cell differentiation.This competition between both ROS-generating enzymes may also play a crucial role in atherogenesis. The control function of macrophages in prevention of plasma (micro)destabilization by OxLDL is impaired during chronic infections and protracted immunosuppression. The uptake of opsonized microbes or IC : LDL/VLDL complexes via Fc gamma-R induces (a) ROI generation by plasmalemma NADPH oxidase, and (b) $cAMP_i$ increase. The latter inhibits, via PLA2 downregulation, the glycosylation of apoAI-R and herewith the cholesterol removal by HDL and upregulates the cholesterol-esterifying enzyme ACAT and apoE synthesis, so that CE is released in a less stable form as a CE:apoE complex.The downregulated NADPH:P450 reductase seems to be responsible for the impaired HDL:apoAI-R transport mechanism,leading to foam cell formation.

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The novel MIS/MIT strategy opens new ways in restoring the pre-disease state by the in vivo depletion of disease inducing and maintaining immunocyte subclones, with a broad spectrum of potential clinical applications.

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Advantages of MIS/MIT: (a) low rate of complications due to the relative selectivity, and (b) broad spectrum of potential clinical applications due to some common phenotypical markers on the deregulated effector cells.The antitumor effect of MIS/MIT effectors is based (a)on a strong GvHD-free GvT/GvL effect, (b) on depletion of tumor-protecting Ts on humoral plus cellular level, and (c) on type1 (Th1) reprogramming of patient's APCs. (a) Solide tumors: A 94,6% long -term survival rate by a single and a 100% (!) survival by a repeated MIS/MIT treatment. Reinoculation of 1.10^7(!) tumor (B16) cells into long-term survivors had minimal impact on the excellent survival rate (tumor-specific resistance by MIS/MIT).Up to 67% survivors in non-excised mice; considerable survival even in the terminal disease group. (b)Autoimmune disorders: A modified MIS/MIT prevented the RA-mimicking AA establishment in rats even at a 3x increased standard MBP/CFA challenge dose and at a twice repeated AA challenge. MIS/MIT was also successful in the treatment of MS-mimicking EAE in rats. (c) BMT: A further MIS/MIT modification was able to prevent the GvHD induction even across MHC I and MHC II barriers (C57Bl6 and CBA2 strains). In addition, different strategies in the treatment of established GvHD as well as various approaches, aimed at the replacement of BMT by T cell exchange, were also successfully tested.

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A study of the effects of an antitumor drug Ukrain on some physiological parameters.

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We studied the influence of a new antitumor drug Ukrain on cardiovasculary and renal function. Ukrain is a semi-synthetic compound made of thiophosphoric acid and alkaloids obtained from a herb *Chelidonium Majus L*, which possesses antitumor, immunomodulatory and radioprotector (RP) properties. RP effects were established in earlier studies. 60 male Wistar rats weighting 200-250 g were used in the experiments. Each experiment group was matched to an appropriate control group. Intravenous Ukrain doses of 0.2 mg/kg, 1.4 mg/kg, and 10 mg/kg were used to study the effects of the drug on blood pressure (BP) and heart rate (HR). In studies of the influence of Ukrain on diuresis, it was administered intraperitoneally (4mg/kg). 30 min prior to measurements, a 2ml water load was given to each animal intraperitoneally. The urine was collected over 24h after water administration, after which the total amounts of water and protein excreted were determined. In order to established the acute toxicity of Ukrain, it was administered to the experimental animals at doses of 0.1, 1.0, 10 and 100 mg/kg. In no case any lethality could be observed within the time span specified by the method used which suggests that the drug is virtually non-toxic. Conclusion: Ukrain administered to animals at the doses of 0.2 and 1.4 mg/kg do not cause any changes in BP over the 4 h study period. When a 10 mg/kg dose of the drug was given, a transient (2-3 min) reduction of BP by 15-20 mm Hg could be observed within 10 min, BP level restored and was stable.

Dendritic Cells

Invited Speakers

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Plasmacytoid monocytes migrate to inflamed lymph nodes and produce high levels of type I IFN

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We have identified two cell subsets in human blood based on the lack of lineage markers (lin$^-$) and the differential expression of Ig-like transcript receptor 1 (ILT1) and ILT3. One subset (lin$^-$/ILT1$^+$/ILT3$^+$) is related to myeloid immature dendritic cells. The other subset (lin$^-$/ILT1$^-$/ILT3$^+$) corresponds to "plasmacytoid monocytes" (PMs). These cells are found in inflamed lymph nodes within and around the high endothelial venules (HEV). They express CD62L and CXCR3, and produce extremely high levels of type I IFN upon stimulation with influenza virus or CD40L. These results, together with the distinct cell phenotype, suggest that PMs represent a specialized cell lineage that enters inflamed lymph nodes at HEV, where it produces type I IFN. The role of PMs may be to protect other cells from viral infections and promote survival of antigen-activated T cells.

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Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells

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No cure is currently available for advanced melanoma. Recent progress in the understanding of mechanisms of immune activation and immune escape during an anti melanoma specific immune response has resulted in new concepts for immunotherapeutic intervention in this disease.
Dendritic cells seem to be a potent adjuvant to induce an anti-melanoma specific immune response. In a clinical pilot trial metastatic melanoma patients were vaccinated with peptide and/or tumor lysate-pulsed DC. Patients developped a strong DTH-reaction to the tracer molecule KLH. Peptide-specific immune response could be detected by DTH to peptide-pulsed DC and was correlated to response to therapy. Induction of peptide specific CTL were detected by various methods. Clinical responses were induced in one third of the patients. Correlation of durable clinical and immunological responses over time as well as the induction of antigen loss variants in relapsing tumors proofs the principle of DC vaccination. Immune escape mechanisms were evident at various levels of antigen-presentation including defects in expression of proteasomal antigens, TAP-deficiency, melanoma antigen loss variants and absent expression of relevant HLA surface molecules. DC-vaccination for induction of an anti-tumor response in melanoma patients is safe and promising. However we believe that aside from the optimal strategy for the induction of an immune response also factors like tumor-immune escape mechanism have to be considered as limitations for therapy.

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ANTITUMOR VACCINES USING AUTOLOGOUS DENDRITIC CELLS, PULSED WITH MAGE PEPTIDES. M. Toungouz, L. Faid, M. Libin, F. Lehmann, F. Branle, M Laporte, P Vereecken, C. Bruyns, M. Lambermont, D. Gangji, M. Goldman, T. Velu. Dept of Erasme-Bordet Medical Oncology, UTCM, Dept of Immunology, IRIBHN, Hôpital Erasme, Université Libre de Bruxelles, Belgium.

In cancer immunotherapy, the injection of dendritic cells (DC) loaded with tumor antigens emerged as a promising strategy. Monitoring of immune responses elicited by such treatments is mandatory to improve their efficiency. We initiated a clinical trial using Mage-1 and/or -3 peptide-pulsed DC to treat patients bearing Mage-positive tumors and HLA A1, A2 and/or B44. DC were generated in clinical grade-closed system from leukapheresis PBMC using IL-4 and GM-CSF. They were harvested after a 7-day culture and loaded with the relevant Mage peptide(s). 10 patients received at least 3 series of sc and iv DC injections (16 to 60.10^6 DC/series): 1x/3 weeks for the 3 first series, and then 1x/6-8 weeks. Cytometry and Elispot analysis of blood samples disclosed a clear-cut but transient increase in the number of peripheral blood CD3+ T lymphocytes secreting IFN-γ in response to the peptides. In some patients, this increase was already apparent after the first DC injection. These responses were seen after the 1-3, but not after the 4th (or more), series of DC injection. The transient nature of these responses is compatible with the hypothesis that the induction of long-lasting T cell responses to MHC class I-restricted tumor peptides presented by DC requires helper T cell-derived signals. To test this hypothesis, 4 of these patients and 12 additional patients received DC pulsed with both Mage-peptide(s) and KLH as a source of non-specific help. Immune responses elicited in these patients, and clinical responses, are currently examined and will be presented and discussed.
Our data suggest that the induction of long-lasting T cell responses to MHC class I-restricted tumor peptides requires helper T cell-derived signals.

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Novel Immunologic And Therapeutic Attributes of Dendritic Cells (DC): Modulation of T and NK cell -Mediated- Antitumor Immune Responses.

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Vaccination with mucin gene (MUC1) transfected dendritic cells in patients with breast or pancreatic cancer

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We performed a clinical phase I / II trial using mucin-gene (MUC1) transfected dendritic cells as tumor vaccine in patients with breast or pancreatic cancer. Due to an underglycosylation of tumor cells mucin core protein epitopes are exposed to the immune system and can be recognized by cytotoxic T-cells (CTL) and monoclonal antibodies. We transfected MUC1 into autologous dendritic cells expressing costimulatory molecules necessary for T-cell activation. Dendritic cells were isolated from human peripheral blood using IL-4 and GM-CSF. MUC1 was transfected into dendritic cells by lipofection. The transfection rate was determined by flow cytometry using three different mucin core protein specific antibodies. After treatment of the cells with a glycosylation inhibitor (*Phenyl-GalNac*), they exposed the relevant mucin epitopes. One million transfected and with *Phenyl-GalNac* treated dendritic cells were injected subcutaneously as vaccine in patients every three weeks. Antigen specific T cell responses, specific antibody production, Delayed Type Hypersensitivity and tumor responses were monitored in the patients.

Dendritic Cells

Poster Presentations

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mDC8+ versus monocyte derived dendritic cells: which one is the best antigen presenting cell?

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Recently, a new class of dendritic cell (DC) precursor has been described in the peripheral blood which carries a distinct surface marker that is recognized by the monoclonal antibody mDC8. These cells, which represent about 1% of PBMC, aquire several characteristics of myeloid derived DC after 48h of in vitro culture.

In this study we determined the phenotypical and functional characteristics of DC derived in vitro from mDC8+ cells as compared to monocyte derived DC.

Since mDC8+ cells die rapidly under culture conditions without added growth factors we tested different cytokines for their ability to promote their survival. We could show that mDC8+ cells develop into a very homogenous population of cells with DC phenotype in the presence of IL-3 or GM-CSF and IL-4 and can thus be easily maintained in vitro for more than 10 days. Under these culture conditions mDC8 DC showed a up to four fold higher expression of MHC class I and class II molecules than monocyte derived DC. Upon induction of maturation with LPS, mDC8+ DC as well as freshly isolated DC8+ cells secreted higher levels of TNFα and lower levels of IL-10 as compared to monocyte derived DC. Antigen uptake by mDC8 DC via macropinocytosis and the mannose receptor as well as T cell stimulatory capacity as tested by MLR and antigen-specific T cell clones was at least as efficient as by „conventional" DC. Priming of naive T cells by mDC8 DC induced a strong TH1 immune response.

The unique possibility to target mDC8+ cells in vivo offers the opportunity to develop a new strategy for an efficient DC-based immunotherapy.

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Development of a tumor vaccine based on cell-lysate pulsed human dendritic cells: an *in vitro* model

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Background: Using autologous, tumor-lysate pulsed dendritic cells (DC) as a cancer vaccine offers the advantage of inducing an immune response to known as well as yet unknown tumor-associated antigens expressed by the individual tumor. The aim of our study is to develop an *in vitro* model to monitor the potency of tumor-lysate based DC vaccines. **Methods:** After 5 days of culture in the presence of GM-CSF (800 U/ml) and IL-4 (1000 U/ml) monocyte-derived DC are loaded with the cell lysate of pancreatic carcinoma cell lines for 3 h. Subsequently the DC are incubated with or without TNF-α (1000 U/ml) and PGE_2 (1μM) for 24 h. DC are tested for their phagocytic activity, expression of surface markers, IL-12 secretion and the stimulation of T-cell proliferation. Their capacity to induce CTL-mediated cytotoxicity is assessed in a ^{51}Cr-release assay after repeated stimulations of autologous T-cells with lysate-pulsed dendritic cells. Furthermore the tumor cell lysis is correlated to the expression of HLA-DR and CD69 on CD3+ cells. **Results:** After 5 days of culture DC express CD40, CD54, CD80, CD86 and MHC-II, are highly phagocytic and are potent inducers of T-cell proliferation. Upon activation with TNF-α and PGE_2 they express the maturation marker CD83, upregulate the above-mentioned surface markers and induce a markedly higher T-cell proliferation. After weekly restimulations of T-cells with lysate-pulsed and matured DC the highest levels of IL-12 and IFN-γ can be detected in the supernatant, indicating a Th1 type response. This correlates with the expression of activation markers on the CD3+ cells and tumor cell lysis of up to 20% in a 18 h assay. **Conclusions:** From our *in vitro* model we conclude that the potency of a tumor-lysate based DC vaccine is enhanced if maturation is induced after antigen loading.

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Dendritic cells (DC) generated from hematopoietic progenitor cells express endothelial cell markers and can be converted into endothelial-like cells (ELC) in the presence of angiogenic growth factors.

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$CD34^+$ cells were isolated from leukapheresis products of G-CSF mobilized donors and were cultured with GM-CSF/IL-4. During the early culture phase immature dendritic cells (DCs) develop, expressing CD34, HLA-DR and CD1a as well as low levels of CD86 and CD83. Further maturation was achieved by cultivation with GM-CSF/IL-4/TNFa. These cells show typical DC morphology and like professional antigen presenting cells (APC) express high levels of HLA-DR, CD86 and CD83. However, these cells also express some typical endothelial cell markers for example von Willebrand factor, VEGF receptor-2 and VE-cadherin. Culturing of DCs in the presence of angiogenic growth factors (VEGF, bFGF, etc) on fibronectin coated culture dishes leads to the development of endothelial like cells (ELCs). A change in morphology of DCs into caudated or spindle cells could be observed. These cells were characterized by a marked increase of von Willebrand factor, VE-cadherin and CD36 and also by a decreased expression of HLA-DR and CD86, showing a typical expression pattern for microvascular endothelial cells. Mature DCs were much more potent APCs than ELC in mixed lymphocyte culture (MLC) assays. Our results may suggest that this could be due to the conversion of DC into endothelial cells by tumor derived angiogenic factors.They might represent a circulating pool of endothelial progenitors which may repopulate the capillary bed in vivo and are involved in antigen responses of T lymphocytes.

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Induction of a T Helper Cell Response against the Tumor-associated Antigen HER-2/neu using Dendritic Cells

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Antigen-specific CD4+ T helper cells are critical for the tumor rejection. The goal of our work is to generate and augment T helper cell immunity to the tumor-associated antigen HER-2/neu which is overexpressed in a variety of human adenocarcinomas. We have developed an *in vitro* priming model using monocyte-derived dendritic cells (DC) as professional antigen-presenting cells (APC).

Immature DC, known to phagocytose and present exogenous protein antigens best, were pulsed with the recombinant protein of the intracellular domain of HER-2/neu (H2N-ICD). Following antigen uptake, immature DC were further matured into CD83+ DC and used as APC for the induction of T helper responses. Protein-pulsed DC from healthy donors stimulated primary responses of naive CD4+/CD45RA+ T lymphocytes. In contrast, monocytes were not able to induce a primary response of autologous T cells. Antigen-specificity of sensitized T cells was confiremd by evaluating secondary responses. CD4+ T cells primed to H2N-ICD responded to H2N-ICD, but not to the irrelevant protein antigen KLH. Furthermore, these CD4+ T were also capable to produce interferon-gamma in response to DC loaded with ICD-H2N. Current experiments focus on the generation of HER-2/neu-specific CD4+ T cell clones following repetitive stimulations with protein-pulsed DC.

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MURINE DENDRITIC CELLS (DC) PULSED WITH A NOVEL HYDROPHOBIZED POLYSACCHARIDE/HER2 ONCO-PROTEIN COMPLEX VACCINE INDUCE SPECIFIC CELLULAR AND HUMORAL IMMUNE RESPONSES IN VITRO AND IN VIVO

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To elicit specific cellular immune responses against HER2$^+$ cancers, we used DC as professional antigen presenting cells in concert with a novel delivery system for HER2 peptides to the MHC class I pathway. A truncated erbB-2/neu/HER2 (HER2) oncogene protein (p1-147) was complexed with two kinds of hydrophobized polysaccharides, cholesteryl group-bearing mannan (CHM) and pullulan (CHP), to form nanoparticles (CHM-HER2 and CHP-HER2) inducing specific CD3$^+$/CD8$^+$ CTLs against HER2$^-$ cell lines. In contrast, HER2 protein alone failed to do so. Vaccination by CHM-HER2 complexes also strongly enhanced the production of IgG antibodies against HER2. Mice immunized with CHM-HER2 or CHP-HER2 before tumor challenge successfully rejected HER2-transfected tumors. Complete rejection of tumors also occurred when CHM-HER2 was applied 3 days after tumor implantation. The complete rejection of tumors also occured when CHP-HER2 pretreated DC was administered as vaccine10 days after tumor inoculation. Therefore, bone marrow-derived DC are a powerful tool the enhance the effectiveness of complexed oncoprotein for anti-tumor vaccination, opening new options for immune cell therapy.

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Activation of Antitumor Cytotoxic T Lymphocytes by Fusions of Human Dendritic Cells and Breast Carcinoma Cells

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Dendritic cells (DC) are potent antigen-presenting cells that sensitize CD4$^+$ T cells and generate cytotoxic T lymphocytes (CTLs) from naive T cells. DC express costimulatory and adhesion molecules necessary for the initiation of these primary responses. We have recently reported the fusion of DC with murine carcinoma cells in which the resulting heterokaryons coexpressed tumor-associated antigens and DC-derived costimulatory signals. Fusions of DC with tumor cells reversed unresponsiveness to tumor-associated antigens and induced the rejection of established metastases. In the present study, successful fusions were generated with DC and human breast carcinoma cell lines. Fusion cells coexpressed DC and tumor derived antigens, and retained the functional capacity of DC. Fusions were also generated from patient-derived DC and autologous primary cultures of breast carcinoma cells. The autologous fusion cells stimulate T cells in allogeneic and autologous mixed lymphocyte reactions (MLRs). Fusions of human DCs with autologous primary or metastatic breast tumor cells efficiently present antigen derived from tumor cells and stimulate autologous CTL cells. Importantly, the primed CTLs are MHC class I-restricted and functional in the lysis of autologous tumor cells. These findings demonstrate that fusions of human DC and breast cancer cells activate T cell responses against autologous tumor.

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Human cancer vaccination with Dendritic Cells (DC) pulsed with autologous tumor Heat Shock Proteins (HSP).

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DC are antigen-presenting cells capable of inducing immune response in vivo.HSP function as intracellular carriers of antigenic peptides and can be used as a source of tumor-associated antigens.A phase I-II vaccination protocol for advanced melanoma and renal carcinoma with DC pulsed with HSP obtained from autologous tumor tissue was begun in April '99.DC are differentiated from PBL obtained by leukapheresis immediately before the first treatment cycle (7-day culture of post ficoll adherent cells in AIMV+IL-4 and GM-CSF 1000 U/ml).A part of PBL are frozen for subsequent treatment cycles.HSP are extracted from the autologous tumor with affinity chromatography.Five patients (4 melanoma, 1 renal ca.) have entered the clinical protocol.Three patients (2 melanoma, 1 renal ca.) have received more than 1 cycle: 2 with DC pulsed with HSP (3μg/ml) and 1 with tumor lysate (50μg/ml). Two melanoma patients are currently being vaccinated with DC pulsed with HSP.Total median WBC recovery is $10.1 \cdot 10^9$ (3.2-15).Median post ficoll cells in culture are $0.9 \cdot 10^9$ (0.2-3).Median DC obtained after culture are $49.97 \cdot 10^6$ (2-317.7) with a differentiation percentage varying from 0.7 to10 (median 5.7).In the 2 patients treated with thawed cells, it was observed that the percentage of DC differentiation from PBL was lower for thawed than for fresh PBL.No side-effects have been observed.Therapeutic responses are being evaluated.

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TNF-α– and CpG-oligodeoxynucleotide-enhanced dendritic cell maturation and protection against experimental murine colon carcinoma

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Aim: We studied whether dendritic cell maturation improves induction of tumor immunity *in vivo*. To identify cellular aspects influencing this process, dendritic cells were characterised by three function-related assays *in vitro*. **Methods:** Following differential maturation of murine bone marrow-derived dendritic cells with GM-CSF and IL-4 only or with further addition of TNF-α or of a CpG motive-containing oligo-deoxynucleotide (CpG-ODN), the cells were characterised by flow cytometry, by interleukin (IL)-12 production and by mixed leukocyte reaction. Tumor immunity was induced in Balb/c mice (Th-2-biassed) by injec-tion of dendritic cells cocultured with irradiated colon carcino-ma cells (Colon-26) before or after tumor challenge with vital Colon-26 cells. **Results:** CpG-ODN-stimulated dendritic cells expressed highest rates of the function-related molecules CD40, ICAM-1, CD80, CD86 and MHC II, secreted 19-fold more IL-12 and induced an 11-fold stronger alloreactive T-cell proliferation compared to maturation with GM-CSF and IL-4 only. TNF-α was intermediately effective in enhancing these functional parameters. CpG-ODN-stimulated dendritic cells also had the highest potential to prevent tumor formation. Injection of dendritic cells was superior to combined injection of irradiated tumor cells with CpG-ODN as adjuvant (without dendritic cells). **Conclusion:** We find a strong correlation of three *in vitro* function-related parameters and the *in vivo* potential to induce tumor immunity. Thus *in vitro* maturation may improve dendritic cell-based clinical tumor vaccination.

Gene Therapy and Antisense Therapy

Invited Speakers

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Adenoviral p53 Gene Therapy for Cancer: Pre-clinical summary and status of the clinical program.

Scott Freeman, Daniel Maneval, JoAnn Horowitz, Lorietta Nielsen, Mary Ellen Rybak, and the p53 Gene Therapy Group.
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A replication-deficient recombinant human adenovirus (rAd-p53) encoding wild-type p53 has been developed to assess the therapeutic potential of p53 gene therapy for cancer. Mutations in the p53 tumor suppressor genes are, to date, the most common genetic alterations in human malignancy, occurring in approximately half of all tumors. Therapeutic strategies based on introduction of p53 are being considered. Pre-clinical studies with rAd-p53 using p53 altered human tumor cell lines have demonstrated inhibition of cell cycle, induction of apoptosis, and increased sensitivity to chemotherapeutic agents. Studies in animals show the p53-specific anti-tumor effects of rAd-p53, but highlight the challenges for efficient delivery to tumors in vivo. We have developed clinical strategies to maximize p53 gene delivery to tumors focusing on regional delivery to ovarian cancer and liver malignancies. Over 100 patients have been treated in phase I clinical trials, and safe doses for future investigations have been identified. Based on pre-clinical models of the disease, an initial clinical strategy has been proposed to treat malignancies in the liver via hepatic artery administration of rAd-p53. Analysis of tissue biopsies demonstrated transgene expression in both normal and tumor tissue that was not abrogated by serum adenoviral antibodies. This presentation will review pre-clinical data with rAd-p53 to highlight the issues and challenges associated with in vivo gene therapy of cancer.

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Adenoviral p53 gene therapy with RPR/INGN201: from the laboratory to clinical validation

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P53 is a nuclear protein that controls the apoptotic pathway and regulates cell cycle progression. Alterations in the p53 gene have been implicated in the progression of many common human cancers. RPR/INGN201, an E1, partially E3 deleted, replication-impaired adenoviral vector that encodes a wild type p53 gene driven by the CMV promoter, has demonstrated broad spectrum antitumoral activity in rodent models of human cancer. Combining adenoviral p53 transduction of established tumors with chemotherapy or external beam radiotherapy in these models has resulted in enhanced activity with no apparent increase in toxicity. Clinical development of RPR/INGN201 by the intratumoral route has expanded into multiple phase 2 studies, with objective activity demonstrated in head and neck cancer, non-small cell lung cancer and prostate cancer. Phase 1 evaluations are proceeding in additional tumor types by a variety of routes of administration, including intravenous. While the earliest commercialization and clinical adoption of this technology is likely to be in palliation of advanced locally aggressive cancers, gene-based tumor suppressor therapy will ultimately be expanded into combination primary treatment regimens, administered before cancers have metastasized, to improve local tumor control and to increase overall survival rates.

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Clinical Trials with p53 Tumor Suppressor Gene for p53-mutant Ovarian Cancer

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The p53 tumor suppressor gene encodes a 393 amino acid nuclear transcription factor which binds to sequence-specific DNA elements in the promoter regions of its target genes which are involved in cell cycle control (P21/WAF1/CIP1), DNA repair pathways (GADD45), and apoptosis (BAX/BCL). Using full-length sequence analysis, we have demonstrated p53 gene mutations in 62/108 (57%) of consecutive ovarian cancers. Transfection of p53-mutant ovarian cancer cells with wild-type p53 results in apoptosis and/or cell cycle arrest; and treatment of human ovarian xenografts with adenoviral vector containing p53 alone or in combination with paclitaxel, or cisplatin, results in decreased tumor burden *in vivo* suggesting that further study of these combinations is warranted. A phase I/II trial of recombinant adenoviral vector containing human p53 gene (SCH58500) was initiated for treatment of p53-mutant recurrent ovarian cancer. The objectives were to assess safety, gene transfer in tumor biopsy specimens, immune response, vector pharmacokinetics, and tumor response. Thirty-six patients have received 263 doses of SCH58500 alone or in combination with chemotherapy. Intraperitoneal doses of SCH58500 ranged from 7.5 X 10^{10} to 7.5 X 10^{13} particles/dose for up to five days per cycle. Major toxicities included anemia, fever, hypotension, fatigue, non-specific abdominal complaints, nausea and emesis. One grade 4 event (elevated alkaline phosphatase) was observed in a patient with extensive hepatic metastasis. Vector-specific transgene expression was documented by RT-PCR at doses >7.5 X 10^{11}. The host immune response did not preclude repetitive adenoviral-mediated gene transfer. CA125 responses were documented in 41/76 (54%) evaluable cycles. The mean CA125 decrease among responders was 29% with SCH58500 alone and 45% in combination with chemotherapy. Based upon safety, tolerability, and encouraging CA125 response data, a randomized phase II trial of carboplatin/taxol chemotherapy with and without SCH58500 has been initiated.

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Development and Targeting of Recombinant Adeno-Associated Virus (rAAV) Vectors

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Adeno-associated virus (AAV) is a single-stranded DNA parvovirus with some advantageous features as a gene transfer vector such as its ability to transduce of terminally differentiated and non-dividing cells, lack of apparent pathogenicity, low immunogenicity, and a relatively high stability of transgene expression. There are, however, some limitations for using rAAV: 1. The packaging methods are relatively cumbersome. 2. In contrast to wild-type AAV, rAAV vectors do not integrate into a specific site, AAVS1, at chromosome 19, because they do not contain the viral rep gene, which is essential for this function. 3. The gene transfer of rAAV has a low specificity (broad host range). 4. rAAV is not efficient in transducing hematopoietic cells.

To overcome some of these limitations, our group has done the following: 1. We improved the methods of rAAV packaging and purification, thus achieving approximately 10^{12} genomic rAAV particles/ml. 2. We have genetically modified the viral capsid in order to achieve a vector retargeting. We could show that a ligand could be inserted into the viral capsid, which allowed redirecting rAAV to target cells resistant to wild-type AAV infection. With these improvements, we hope to be able to develop a new generation of rAAV vectors, which is more efficient in transducing hematopoietic cells.

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Lentiviral Vectors for Gene Therapy

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Hybrid lentiviral vectors are novel gene transfer tools made by the core of a lentivirus, most often HIV-1, and the envelope of a different virus, such as VSV. They mediate efficient delivery and sustained expression of marker genes in several rodent tissues in vivo, and in primitive human hematopoietic stem cells ex vivo. To exploit the potential of this gene transfer system, its biosafety and manufacturing must be improved, and the control of transgene expression advanced. We have now built safer vectors that inactivate upon transduction and are produced by a minimal set of viral genes: *gag, pol* and *rev*. By studying vector-virus interaction in culture, we showed that self-inactivated vectors are not mobilized by wild-type HIV. Novel cis-acting sequences that enhance vector integration and transgene expression were identified, and the use of tissue specific promoters demonstrated. Inducible producer cell lines were generated that yield significant output of vector of high transducing efficiency. A sensitive assay was developed to detect contamination by recombinants in vector batches independent on the envelope of the particle. The availability of efficient vectors made from a minimal set of HIV sequences, of stable producer systems, and of sensitive assays to screen recombinants should facilitate testing of lentivirus vectors.

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CLINICAL DEVELOPMENT OF G3139 ANTISENSE DRUG (OLIGONUCLEOTIDE) TARGETING BCL-2

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Preclinical studies demonstrate that modulation of BCL-2 protein in cancer can impact treatment response to radiation or chemotherapies, or to biologic agents. G3139 is a phosphorothioate oligonucleotide, antisense to the *bcl-2* mRNA, selected to optimize specificity and potency (90% inhibition of BCL-2 protein expression at 30 nanomolar). Radiolabeled G3139 showed wide tissue distribution in major organs except brain; and potent biologic activity by G3139 has been demonstrated in disseminated and solid tumor xenografts. Clinical programs are in development to study G3139 therapy alone, or combined with anticancer agents in lymphoma, melanoma, prostate, breast, lung, gastrointestinal, and other malignancies. A clinical study in lymphoma demonstrated tolerance to systemic monotherapy, responses of measurable disease, and effective reduction of BCL-2 protein in target tumor cells (Webb et al, Lancet 349:1137, 1997). A phase I-II study in melanoma demonstrated tolerance to systemic G3139 therapy when combined with full-dose chemotherapy, major responses of measurable metastases in some patients, and reduction of BCL-2 protein in serial biopsies of metastatic lesions (Jansen et al, Proc. ASCO 18:531a, 1999). PK analyses demonstrate that steady-state plasma levels of G3139 are tolerable and maintained after repeat cycles, exceeding the concentrations predicted to be bioactive from preclinical models, but below the levels associated with clinical toxicity. In sum, initial clinical results support the safety and feasibility of G3139 to modulate BCL-2 protein levels in cancer therapy.

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Clinical development of antisense oligonucleotides against protein kinase C-alpha (ISIS 3521/ISI 641A), C-*raf* (ISIS 5132/ODN 698A), and H-*ras* (ISIS 2503) to treat patients with cancer. Jon T. Holmlund, M.D., and F. Andrew Dorr, M.D., Isis Pharmaceuticals, Carlsbad, CA, USA.

Antisense oligonucleotides are capable of selectively inhibiting the expression of a single gene product, offering the potential for achieving therapeutic benefit while minimizing non-specific toxic effects. The phosphorothioate oligonucleotides ISIS 3521, ISIS 5132, and ISIS 2503 have demonstrated antitumor activity and minimal toxicity in early clinical trials. These drugs hybridize to a 20-base sequence in their target mRNAs by Watson-Crick base pairing, forming a heteroduplex region amenable to degradation by RNase H and leading to quantitative reduction of the mRNA. In Phase 1 trials, antitumor activity has been observed in ovarian cancer and non-Hodgkin's lymphoma, with prolonged stable disease observed in non-small cell lung cancer, pancreatic cancer, colon cancer, and renal cell cancer. Mild thrombocytopenia and constitutional symptoms, which appear to be characteristic of phosphorothioates as a chemical class, are the principal toxicities observed in patients. Complement activation, observed in primate toxicology studies, is preventable with careful dose selection and the use of a continuous intravenous infusion schedule. Addition of ISIS 3521 to standard chemotherapy regimens is possible at full doses of all drugs, with promising activity in non-small cell lung cancer. Phase 2 trials are in progress. New oligonucleotides, with further chemical modifications to enhance pharmacologic properties, and new formulations, for topical and oral delivery, are under development.

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THYMIDINE KINASE GENE THERAPY IN OVARIAN CANCER

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Purpose: This is an FDA-approved Phase I study of the delivery of a replication-deficient recombinant adenovirus containing the herpes simplex virus (HSV) thymidine kinase (TK) gene (ADV-HSV-TK) to the peritoneal cavity of patients with recurrent ovarian cancer after optimal tumor debulking followed by concomitant administration of Acyclovir and Topotecan.The purpose of this study was to evaluate the safety and toxicity profile of this therapy.

Patients and Methods: Informed consent was obtained from ten patients with recurrent stage IIIc epithelial ovarian cancer who subsequently underwent optimal secondary tumor debulking to ≤0.5 cm tumor residual and intraperitoneal delivery of adenovirus if peritoneal flow study showed unimpaired fluid distribution in the abdomen. Two patients each were treated on dose level 1 (2 x 10^{10} vector particles = VP), dose level 2 (2 x 10^{11} VP), dose level 3 (2 x 10^{12}VP), and four patients on dose level 4 (2 x 10^{13} VP), a dose intensity 100-fold higher than used in humans so far. Treatment with an antiherpetic prodrug and Topotecan was started 24 hours after virus injection.

Results: The most common adverse event after gene therapy and Topotecan was myelosuppression. Grade 3 and 4 thrombocytopenia and anemia were seen in two patients each, and grade 3 and 4 neutropenia in eight patients. Three patients showed thrombocytosis and three patients had a mild elevation of serum SGPT/ALT. Temperature elevations not associated with an infection occurred in two patients. All side-effects were reversible.

Conclusions: Intraperitoneal vector delivery after optimal tumor debulking surgery with concomitant Topotecan chemotherapy was well tolerated and did not result in any significant lasting toxicities even at the highest dose used in humans so far. Side-effects were independent of the maximum dose of adenovirus.

Gene Therapy and Antisense Therapy

Poster Presentations

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EFFICIENT RETARGETING OF AAV2 BY GENETIC MODIFICATION OF THE VIRAL CAPSID PROTEIN

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Because the broad host range of the adeno-associated virus type 2 (AAV2) represent a limitation for *in vivo* AAV2-based gene transfer applications, we developed a strategy for targeting AAV2-based.
On the basis of a theoretical study of the Canine Parvovirus 3-dimensional capsid structure, and an alignment of the CPV and AAV2 capsid protein sequences, we constructed six different capsid mutants, in which we inserted at the above six sites a 14-mer RGD-containing peptide (L14).
Our results strongly suggested that the L14 sequence was not inserted in any capsid region critical for the correct folding of the capsid protein, or the capsid assembly, or for the packaging of the genome.
Moreover, AAV2 particles carrying a LacZ viral vector produced with the mutated capsid at site 587 of the VP1 capsid protein could infect cells by using the L14-specific receptor. By this way, we were able to enhance the susceptibility of AAV2-resistant cells to be infected by AAV2 by at least four orders of magnitude. Our results show for the first time the feasibility of a genetic receptor targeting of AAV2 vectors.

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Requirement of DOPE as "Helper Lipid" in novel cationic liposomes depends strongly on DNA size and cationic lipid structure

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The transfection efficiencies of novel cationic lipids in the presence and absence of DOPE have been compared using standard assay conditions. For the transfection with Plasmid-DNA (P-DNA) or oligonucleotides (ODNs), the efficiencies of the cationic lipids with or without DOPE have been determined for 3.5 kbp EGFP-Plasmid or ODNs in mouse fibroblasts. After 24 hrs of incubation in the presence of 10% FCS, the yield of transfected cells was measured using FACS analysis of EGFP fluorescence and digital epifluorescence microscopy for FITC-ODNs. We have used novel cationic lipids designed and prepared in our laboratory. The cationic lipids with one structurally consistent head group contained different types of fatty acid chains. The transfection efficiencies of these lipids have been analysed in liposomal mixtures, molar ratios of cationic lipids to DOPE of 1:1 or with cationic lipids alone. In the case of P-DNA, DOPE was required for high transfection efficiency. For ODNs, however, the requirement for DOPE depended on the fatty acid composition of the cationic lipids. In the case of myristoyl-chains, the transfection activity was even higher in the absence of DOPE with values of > 90% for transfected cells. In contrast, the transfection efficiency of this new type of cationic lipids with oleoyl-chains was strongly improved by DOPE. To summarize: New cationic lipids have been prepared and have been used successfully for the transfection of Plasmid-DNA and oligonucleotides. For high transfection efficiency, the cationic lipid complex had to be optimized with respect to the fatty acid composition of the cationic lipids and surprisingly with respect to the presence or absence of "Helper Lipids", such as DOPE.

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Immunotherapy of colorectal cancer: T-cell activation induced by gene transfer of B7.1 and 4-1BB-ligand and combined effects of IL-12 and IL-18

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Introduction: T-cell activation and proliferation requires antigen-presentation by MHC molecules and costimulatory signals such as B7.1 and 4-1-BB-L provided by antigen-presenting cells. The cytokines IL-12 and IL-18 induce synergistically IFN-γ production by T cells and augment NK cell activity. We are investigating the combined effect of IL-12 and IL-18 on T-cell activation through B7.1- and 4-1-BB-L-transduced human colorectal cell lines. **Methods**: The cDNAs for B7.1, B7.2 and 4-1-BB-L were amplified by PCR from human monocyte-derived dendritic cell RNA. The respective fragments are ligated into the expression vector pTracerCMV2 to transfect tumor cells. The proliferation of T cells (99% pure) incubated with irradiated transfected tumor cells in the presence of various IL-12 and IL-18 concentrations is measured by quantification of radioactive thymidin incorporation. **Results**: Transfection of B7.1 enhances T-cell proliferation induced by human colorectal cell line HT29 in the presence of submitogen concanavalin A concentration. IL-12, but not IL-18, increases B7.1-associated T-cell proliferation. Combination of IL-12 and IL-18 significantly augments T cell proliferation induced by IL-12 alone indicating a synergistic effect of these cytokines. **Conclusion**: T cell activation and proliferation through tumor cells is enhanced by combination of IL-12, IL-18 and by gene transfer of costimulatory molecules *in vitro*.

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Preclinical and clinical studies of combinations of chemotherapy with p53 tumor suppressor gene for ovarian cancer. Konecny G[1], Pegram MD[1], Buller R[2], Nielsen LL[3], Horowitz J[4], and Karlan BY[1], Slamon DJ[1]. [1]*UCLA School of Medicine;* [2]*University of Iowa; Departments of* [3]*Tumor Biology and* [4]*Clinical Oncology, Schering-Plough Research Institute*

The function of p53 is central in control of the cell cycle and DNA repair. Transfection of p53-mutant ovarian carcinoma cells with wild-type p53 cDNA results in apoptosis and/or cell cycle arrest. To extend the preclinical observation of synergy of rAd p53 in combination with cisplatin and paclitaxel rAd p53 was analyzed in combination with topotecan, liposomal doxorubicin, or gemcitabine. Multiple drug effect analysis of these drug/rAd p53 combinations on SK-OV-3 ($p53^{null}$) ovarian carcinoma cells indicate a synergistic *in vitro* interaction for all three drugs. For preclinical *in vivo* studies we developed an ovarian cancer model using intraperitoneal (IP) SK-OV-3 ($p53^{null}$) xenografts in SCID mice. Groups of 6-7 mice were randomized to treatment with rAd p53 alone (5 X 10^9 particles/0.2mL IP 3 times/week X 8 doses), rAd control vector devoid of p53 sequence at an identical dose, rAd control + topotecan, rAd p53 + topotecan, rAd control + gemcitabine, and rAd p53 + gemcitabine. In this experiment, treatment with rAd p53 alone did not result in improved survival compared to rAd control vector alone (P = 0.29). Combination of rAd p53 with topotecan or gemcitabine resulted in significant increase in survival compared to the same chemotherapy treatment with control vector (P = 0.047). In contrast, treatment with liposomal doxorubicin (3 mg/kg IP days 0 and 5) either alone or in combination with rAd p53 did not result in improved survival compared vector alone controls in this model. In a phase I trial of rAd p53, to date, 12 patients with p53-mutant, relapsed ovarian cancer have been dosed with rAd p53 (dose range 2.5 X 10^{13} particles/dose X 3 days - 7.5 X 10^{13} particles/dose X 5 days) as a single agent (cycle 1) and combined with topotecan or liposomal doxorubicin (cycles 2 - 6). Treatment has been well tolerated with the most common side effects of grade I-II abdominal distention or pain, grade II fever, grade I - II nausea, and grade II flu-like syndrome with malaise. These data suggest the combination of rAd p53 with topotecan, liposomal doxorubicin, or gemcitabine may have therapeutic potential.

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HEAT INDUCED EXPRESSION OF REPORTER MOLECULES IN HUMAN EWING SARCOMA CELL LINES UNDER THE CONTROL OF HEAT SHOCK PROMOTERS

Conditional expression of therapeutically relevant genes in tumor tissues is an important goal in the gene therapy of cancer. Preliminary results of others have shown that the expression of the IL-2 gene under heat shock can enhance antitumor immune responses. Evidence is accumulating that heat induced HSP72 on human Ewing sarcoma tumor cells is also involved in this response. We have engineered constructs consisting of the GFP gene as a reporter gene and fragments of the human HSP70B promoter to analyze the characteristics and optimize the parameters of heat induced gene expression in human Ewing sarcoma cell lines (CADO-ES) which we use as tumor model system. To determine heat shock parameters (time, temperature) and drug concentrations that allow high expression of HSP70B promoter constructs we investigated the GFP expression after heat shock. A ~500 bp fragment of the human HSP70B promoter was sufficient for a high and strictly heat controlled gene expression. Isophosphorylation of HSP27 and heat induced expression of HSP72 was used as control for the stress response. In the future we will analyze the effects of heat induced expression of cytokines and chemokines in human Ewing sarcoma and a panel of other tumor cell lines on anti-tumor immune response in SCID/beige mouse model systems. Strictly heat controlled expression of e.g. IL-2 locally defined in tumor tissues will help to circumvent side effects often observed during systemic application.

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MOBILIZATION OF CD34+ HEMATOPOIETIC PROGENITOR CELLS *IN VITRO* AND *IN VIVO* BY VLA-4-DIRECTED ANTISENSE OLIGONUCLEOTIDES

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The β1α4 integrin very late antigen-4 (VLA-4) plays a central role in mobilization of CD34+ hematopoietic stem cells. In this study, we examined antisense oligodesoxyribonucleotides (ODN) directed against the mRNA of the α4 chain of VLA-4 as a new modality to mobilize hematopoietic progenitor cells. Following transfection of ODN in immunomagnetically enriched CD34+ cells from leukapheresis products using cationic lipids a significant antisense-mediated downregulation of the VLA-4 surface expression of 29% (SD: 13.1%) was observed. Downregulation of VLA-4 resulted in an inhibition of adhesion to IL-1β-stimulated endothelial cells of 32% (SD: 11.4%). Using Dexter-type long-term bone marrow cultures (LTBMC) as *in vitro* model for stem cell mobilization, a 29-fold increase of colony-forming units (CFU) in the non-adherent cell fraction of antisense-treated cultures was found in comparison to controls, indicating that antisense-mediated downregulation of VLA-4 results in a mobilization of progenitor cells from bone marrow stroma *in vitro*. Moreover, mice were treated with VLA-4 antisense ODN or scrambled control ODN for 5 days by daily intravenous injections of the ODN. Treatment with antisense ODN as well as control ODN led to a significant increase in CD34+ cells and of CFU in the spleen. Thus, mobilization of HPC in mice may be unspecific and probably due to stimulation of the immune system by the phosphorothioate modification of the ODN. This is supported by the finding that cytokine production (Il-2, IL-4, IL-10, IFN-γ) by spleen cells was significantly increased. So far, the results of our *in vitro* data support antisense ODN as a novel approach to improve progenitor cell mobilization for autologous or allogeneic transplantation.

Haematopoiesis

Invited Speakers

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c-myc activation severely impairs immune recognition of Burkitt lymphoma cells

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Burkitt's lymphoma (BL) is characterized by its frequent association with Epstein-Barr virus and the invariable presence of chromosomal translocations activating the c-myc gene. Paradoxically, all viral gene products except EBNA1 playing a crucial role in B cell immortalization by EBV are not expressed in Burkitt's lymphoma cells. We have established a cellular *in vitro* system to recapitulate important features of the pathogenesis of BL. To this end, we have (i) developed a cell line conditionally immortalized by EBV using a hormone regulated EBNA2-estrogen receptor fusion protein, and (ii) introduced into these cells a plasmid carrying the reconstructed breakpoint of a chromosomal t(2;8) translocation. The myc-tranfected cells, in contrast to its parental cells, proliferate in the absence of estrogen and adopt many features of BL cells. They grow in single cell suspension, have downregulated adhesion and activation molecules like CD21, CD23, CD39, CD54, CD58, CD80 and CD86 and have upregulated CD10 and CD38 similarly to BL cells. They have completely lost the ability to stimulate allogeneic T cells in a mixed lymphocyte reaction and cannot be recognized by antigen-specific T-cells even after expression of the respective antigen using recombinant vaccinia virus. Using model antigens and substrates we shown that this failure of immune recognition is caused by defects in several cellular functions relevant for a cytotoxic T cell response: cleavage of peptides in the proteasome, peptide transport into the ER, selective down-regulation of HLA-A11, and downregulation of adhesion molecules.

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Stimulation of early hematopoietic progenitor cells by signaling of the gp130 pathway

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The complex of soluble IL-6R and IL-6 induces biological signals by binding to gp130 in cells lacking transmembrane IL-6R, such as early hematopoietic progenitor cells. We studied the influence of the designer molecule H-IL-6, which is several orders of magnitude more effective than the natural complex of IL-6/sIL-6R, on the proliferation and differentiation of purified CD34+ progenitor cells in combination with other early acting growth factors and stromal cell support. Cell number, colony forming units (CFU) and cobblestone area forming cells (CAFC) were determined weakly in long-term suspension cultures and stromal cell cultures of CD34+ cells stimulated with combinations of various hematopoietic growth factors.

Whereas H-IL-6 alone failed to induce proliferation of CD34+ cells, this compound acted synergistically with flt-3 ligand and stem cell factor. The addition of Thrombopoietin to these cultures did not further enhance proliferation. After 3 – 4 weeks of incubation, in H-IL-6 containing cultures the highest amounts of CFUs and CFACs was detected, suggesting that H-IL-6 exerts a selective proliferative effect on primitive progenitor cells. The stimulatory capacity of H-IL-6 and flt-3L on progenitor cells was preserved in stromal cell supported long-term cultures. Therefore, we expressed H-IL-6 and flt-3L in hematopoietic active stromal cell lines in order to improve expansion of early hematopoeitic progenitor cells in vitro.

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Biology of Stem Cell Mobilization

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Since the observation was made that cytokines are capable of inducing stem cell mobilization, adhesion molecules have been implicated to play a role in this process. From the two major families, the β1 integrins VLA-4(CD49d/CD29), and VLA-5(CD49e/CD29) as well as the β2 integrin LFA-1(CD11a/CD18) have been reported to be expressed on progenitor cells. The prominent role of VLA-4 and its ligand VCAM-1 in retaining progenitor cells in the bone marrow in-vivo is most clearly shown by the ability of anti-VLA-4 or anti-VCAM-1 antibodies to induce mobilization of progenitor cells in primates and mice. Likewise, these molecules have been implicated to play a role in the induction of stem cell mobilization. We have studied the role of β1 and β2 integrins in stem cell mobilization using IL-8 induced stem cell mobilization as a model.
Antibodies against the ß-2 integrin Leukocyte Function Associated Antigen-1 (LFA-1) completely prevented the IL-8 induced mobilization, whereas antibodies against the ß-1 integrin VLA-4 had no effect. The blocking effect was not due to a direct effect on hematopoietic progenitor cells, since LFA-1 was demonstrated not to be expressed on murine hematopoietic progenitor cells (Blood 93:107-12,1999). Additional experiments in rhesus monkeys showed that IL-8 induces the rapid systemic release of the metalloproteinase gelatinase-B (MMP-9), capable of degrading matrix molecules to which hematopoietic progenitor stem cells are attached. Mobilization coincided with MMP-9 release and could be completely prevented by pretreatment of monkeys with an inhibitory anti-gelatinase-B antibody. Since neutrophils express LFA-1 and high affinity IL-8 receptors and also release gelatinase-B upon stimulation with IL-8, these findings support the hypothesis that mature neutrophils serve as key regulators in IL-8 induced mobilization. Indeed, further studies showed that IL-8 induced mobilization was completely absent in mice rendered neutropenic following treatment with anti-Gr-1 antibodies, recognizing a granulocyte differentiation antigen. Taken together, these data are consistent with the hypothesis that neutrophils are the major mediators of IL-8 induced stem cell mobilization. Further studies will focus on a possible role for mature neutrophils in mobilization induced by other cytokines and growth factors.

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The pathogenetic role of TGF-ß and bFGF in the development of hairy cell leukemia (HCL)

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A functional feature invariably associated with HCL is bone marrow fibrosis. Because Transforming Growth Factor ß (TGF-ß) and basic Fibroblast Growth Factor (bFGF) seem to be involved in fibrogenic diseases these cytokines may also play an important role in the pathomechanisms leading to HCL. When serum and bone marrow (BM) aspirates from HCL patients were screened by ELISA, we found that specimen of the patients contained significantly higher levels of TGF-ß and bFGF than those from healthy subjects. Subsequently it could be demonstrated, both on the mRNA as well as protein level, that hairy cells are the main producers of these cytokines. Western blotting analysis showed that hairy cells synthesize at least three isoforms of bFGF, but only the 23-kD isoform is exported. Interestingly, this isoform is mainly produced by tumor cells. A biological function of bFGF in HCL might be mediation of chemoresistance, since expression of the cytokine by hairy cells was not affected by the purine analogue 2-CdA and apoptosis induced by this compound was much weaker in hairy cells than in PBMC from healthy subjects. When BM fibroblasts of HCL patients were investigated, the amount of collagen and reticulin fibers produced under basal conditions as well as upon stimulation with TGF-ß, was considerably higher than in control experiments with healthy donors. The results were confirmed by immunofluorescence staining using procollagen type I and type III antibodies and by RT-PCR analysis. Co-culture studies revealed that hairy cells adhere to BM fibroblasts within few minutes and that the adhesion is further enhanced by TGF-ß and inhibited by neutralizing anti-TGF-ß antibodies.
Taken together the results of this study point to a key role for TGF-ß in regulating the interaction between hairy cells and BM fibroblasts and for the formation of collagen and reticulin fibers. Furthermore, the increased production of bFGF seems not only to result in fibroblast proliferation but might also play a role for the survival of the malignant cells.

Haematopoiesis

Poster Presentations

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A NEW TECHNOLOGY FOR THE EVALUATION OF MINIMAL RESIDUAL DISEASE STATUS IN B-CELL LYMPHOMAS

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Real-time PCR applications will be used as diagnostic tool for the determination of residual tumor cells in malignancies during and after therapy. The aim of this study was the development of a real-time PCR technology (LightCycler™) to quantify residual tumor cells in monoclonal B-cell lymphoma. The assay is based on an immunoglobulin heavy chain (IgVH) specific PCR with allele-specific oligonucleotides (ASO) complementary to hypervariable CDRII and CDRIII regions, which has been introduced by other groups. This patient-specific ASO-PCR was established for the LightCycler™ format. Sample DNA from peripheral mononuclear cells and from the bone marrow of one patient with B-CLL, who received a combined treatment of fludarabine and the monoclonal antibody IDEC-C2B8 (rituximab) was analyzed. After designing a set of framework region IV (FRIV) specific hybridization probes, IgVH copy numbers in the patients DNA samples were quantified in comparison to the patients IgVH sequences as external standard. Tumor DNA was reduced from 2,079 to 176 copies in 1 µg genomic DNA obtained from peripheral blood lymphocytes ($P<0.0001$), and from 5,078 to 2,502 copies in 1 µg genomic DNA obtained from bone marrow ($P=0.003$) after completion of therapy. We conclude that this technology allows the detection and quantification of minimal residual tumor cells in monoclonal B-cell lymphomas over several log steps with high sensitivity.

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Peripheral blood lymphocytes of Non-Hodgkin's lymphoma patients after treatment with TNF-alpha

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TNF-α is a multifunctional cytokine, which induce various immune responses and modulate multi-drug resistance. For this, we evaluated in vitro effect of rh TNF-α on the lactate dehydrogenase (LDH) content and apoptotic index (AI) of peripheral blood lymphocytes (PBL) in Non-Hodgkin's lymphoma (NHL) patients. The diagnosis of NHL was based on histologically proven lymph node biopsy (The Working Formulation) and combination of ultrasonic analysis and computerized tomography scan for clinical staging (Ann Arbor system). TNF-α showed dose-dependent increase of LDH_1 and LDH_5 activity in sonified PBL of healthy persons, while in PBL of NHL patients prior to chemotherapy it induced a significant decrease (Student's *t*-test, $p<0.05$) of LDH_1 activity, but not LDH_5, in 18 h cultures. Contrary to this, in NHL patients treated with chemotherapy, TNF-α, only in dose of 100 U/ml, induced significant increase (Student's *t*-test, $p<0.05$) of LDH_1 activity, but not of LDH_5. AI in PBL of NHL patients receiving chemotherapy without stimulation with TNF-α was significantly higher in comparison to controls and NHL patients prior to therapy. TNF-α stimulation of PBL of lymphoma patients receiving chemotherapy showed significant increase of AI. However, there is no significant difference (Student-*t*-test $p>0.05$) in AI of the high-grade malignancy NHL between patients that received "firs line therapy", high dose chemotherapy including VP-16 or "third-line therapy regiment". These results showed different alterations in metabolism and apoptosis of PBL in NHL patients in the presence of TNF-α

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High-dose chemotherapy followed by autologous transplantation of purged hematopoietic progenitor cells and posttransplant immunotherapy with rHu IL2 in poor-risk neuroblastoma – a report of two cases.

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To improve results of high-dose regimen followed by autologous stem cell rescue in two children with relapsed (n=1) or advanced, poor-risk stage IV (n=1) neuroblastoma, graft purging by positive selection of CD34+ cells together with posttransplant IL-2 immunotherapy were applied. In one case hematopoietic progenitor cells were collected from peripheral blood, in one bone marrow was harvested due to inefficient mobilisation. The grafts underwent subsequent positive selection of CD34+ cells with CellPro affinity columns. Conditioning regimen consisted of oral busulfan 16mg/kg and melfalan 140mg/m^2. After transplantation, to eradicate residual NBL cells that might survive high-dose regimens, immunotherapy with rHu IL-2 (Proleukin) was started from 65 and 84 days after transplantation. The IL-2 protocol consisted of inrevenous IL2 (Proleukin) in escalating doses 2-4-6-8-8x10^{6}U/m^2 administered monthly for 5 consecutive days. Activation of NK cells was measured by standard flow-cytometric cytotoxicity assay.

Results: Both children achieved sustained, tri-lineage hematopoietic recovery. No major toxicity of either transplant regimen or IL-2 administration were observed. One patient is alive but relapsed 150 days after BMT. One patient is still in complete remission 178 days after BMT

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Src-kinases are potential targets for a molecular based therapy of chronic myeloid leukemia

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Chronic myeloid leukemia (CML) is characterized by the expression of Bcr-Abl, a fusion protein with constitutive tyrosine kinase activity. Here we show that Bcr-Abl activates Src family kinases by a complex mechanism which is independent of the tyrosine kinase activity of Bcr-Abl. Deactivating point mutations in Src kinases revealed that Bcr-Abl preferentially binds inactive Src molecules. This interaction involves the SH3 domain of Src kinases, presumably leading to a disruption of the autoinhibitory interaction between the SH3 domain and the SH2-kinase linker. We made use of PP1, a tyrosine kinase inhibitor specific for Src kinases, to further analyze their role in Bcr-Abl induced leukemogenesis. PP1 effectively inhibited the kinase activity of Src-family kinases but not of Bcr-Abl. Moreover, PP1 and a kinase inhibitor specific for Bcr-Abl negatively affected the phosphorylation of different subsets of cellular proteins, showing that Src kinases and Bcr-Abl may act on different downstream substrates. As to the biological effect of PP1 on Bcr-Abl expressing cells, we could show that 1 - 10µM PP1 markedly reduced the proliferation of 32Dp210 cells. At higher concentrations, PP1 induced spontaneous apoptosis in 32Dp210 but not in untransfected 32D cells, indicating specificity of PP1 induced cell death for Bcr-Abl expressing cells. Similar results were obtained from a variety of leukemic cell lines. Coincubation of 32Dp210 with PP1 and a specific Abl inhibitor lead to a fully additive effect with nearly complete apoptosis of 32Dp210 cells even in the presence of IL-3, further suggesting that Bcr-Abl and Src kinases may act on non-overlapping effector pathways. In summary, our data points to an important role of Src kinases in the pathogenesis of CML. Inhibition of Src-kinases may therefore be a useful method for new, molecular-based treatment strategies of CML.

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INTERACTION OF THE BCR-ABL TYROSINE KINASE WITH THE SRC-KINASE HCK

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Bcr-Abl, the transforming agent in chronic myeloid leukemia (CML), is a fusion protein with a constitutively active tyrosine kinase and transforming capacity for hematopoietic cells. The mechanism of Bcr-Abl induced transformation is not fully understood, but it acts at least in part through phosphorylation and activation of signaling proteins which are part of the Ras/MAP-kinase signaling pathway. We could recently demonstrate that Src kinase family members Lyn and Hck interact with Bcr-Abl. To identify the binding domains mediating the interaction of Hck with Bcr-Abl, we constructed different deletion mutants of Bcr-Abl and expressed them together with Hck in COS7 cells. Subsequent coimmunoprecipitation experiments allowed us to identify at least three independent binding regions, one in the Bcr, one in the region comprising the SH3, SH2 and SH1 domain of Abl, and one in the C-terminal domain of Abl. To examine whether the interaction of Bcr-Abl with Hck is direct or mediated by adaptor proteins, we expressed both proteins in Sf9 cells using the baculovirus system. Pull down assays confirmed our results from the COS cell system. By analysing Hck deletion mutants we could show that binding to the Bcr and the Abl part is mediated by the Hck-SH3 domain while the Hck-SH2 domains interacts only with the Bcr part. In conclusion, this results indicate that the interaction of Bcr-Abl with Hck is mediated by a novel, complex mechanism that involves multiple domains of both proteins.

Antibody Therapy

Invited Speakers

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Antibody therapy - the next questions

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Antibody targeted therapy of cancer can cause tumour regression with low toxicity to normal tissues. Antitumour antibodies acting through signal transduction or natural immune effector mechanisms are already licensed for use in breast and colorectal cancer and in lymphoma.. Antibody targeting of therapeutic radionuclides (radioimmunotherapy) has shown response rates of 5-40% in a range of common carcinomas but of up to 89% in malignant lymphoma when given in high dose with autologous stem cell support. Targeted toxins, enzymes and cytotoxic drugs have the potency and potential for high selectivity because they are only effective against cells in which they are internalised and responses are reported in man. Benefits of these treatments are greatest in patients with small volume disease or particularly sensitive tumours but long term control of established common epithelial cancers has still to be shown.

Knowledge of tumour biology and antibody targeting mechanisms is growing rapidly and therapeutic molecules can be genetically engineered for optimal function. What are the key developments likely to give more effective therapy? The challenge is complex, requiring identification of suitable tumour targets, optimisation of antibody molecules, development of more potent and selective therapeutic systems, avoidance of immunogenicity and adaptation to heterogeneous tumour biology. The components then need to be integrated into an effective and feasible therapeutic package.

Tumour Targets

A growing knowledge of the normal human genome leads to a corresponding identification of the mutations that define cancer. The abnormal spectrum of proteins (proteome) that results includes products of mutant genes as well as over or under expression of protein products of unmutated genes. Both mutant and non-mutant cancer-associated proteins may serve as targets for therapy and modern proteomics techniques make it possible to characterise the proteome of individual cancers and match specific antibodies to accessible targets.

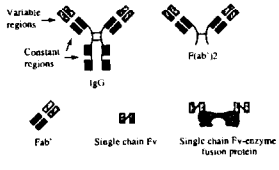
Antitumour antibodies

Purification of antibody genes by PCR from populations of B cells makes it possible to examine very large numbers of antibody clones ($<10^{11}$). Antibody variable regions amplified from cDNA libraries can be cloned as single chain Fvs (scFv) (VH and VL joined by a flexible linker) into filamentous bacteriophage, producing an antibody library. Each phage contains the gene for the scFv and expresses the scFv antibody on its surface. Selection for a defined specificity and characteristics such as high affinity are greatly facilitated. Phage antibody library technology is set to take over from monoclonal antibodies made by the hybridoma technique.

Control of antibody targeting performance

Tumour and normal tissue antibody pharmacokinetics are controllable by the design of targeting molecule. Representative examples of the many possibilities are shown in the figure. The rate of clearance of antibodies from the blood stream is related principally to the molecular mass which can be controlled within limits as shown. The slower the clearance from the circulation, the greater the availability of antibody for tumour binding and the absolute tumour antibody level but the lower the tumour to normal tissue ratio. Systems which accelerate clearance of antibody from the circulation increase tumour to normal tissue ratio. Generally, tumour localisation is superior with divalent rather than monovalent antibodies and the stability of an antibody in the circulation and in the tumour environment are critical factors for good performance.

Antibodies and their derivatives



Tumour heterogeneity

Carcinomas begin to outgrow their blood supply after reaching about 1mm diameter. Although they stimulate vascular growth, this is disorderly resulting in poorly nourished and necrotic areas. Delivery of any drug to poorly vascularised areas is reduced but access of large molecules such as antibodies is even more limited. scFvs penetrate better than whole IgG but do not completely overcome the problem. Drugs causing thrombosis and occlusion of tumour blood vessels can destroy poorly vascularised tumour areas. A rim of viable tumour remains and this is accessible to and can be eradicated by antibody targeted therapy.

Pretargeting therapeutic systems

Systemic targeted therapy is often limited by toxicity. Two stage or pretargeting systems give higher relative concentrations in tumour than single step targeting making this a promising strategy for improving therapeutic ratio. The first component, typically an antibody linked to a molecule which is not toxic in its own right, is given intravenously and concentrates in the tumour. This first component clears from the circulation, sometimes accelerated by a clearing agent. When tumour to normal tissue ratios of the first component are high, a therapeutic component is given which is bound or activated by reaction with the first component. This gives a high concentration of the therapeutic agent in the tumour with lower levels in normal tissues. Examples being investigated include avidin-biotin systems, radionuclide capture bispecific antibodies, and antibody-directed enzyme prodrug therapy (ADEPT)

Conclusion

Eradication of common cancers remains a challenge but the major obstacles to effective therapy are understood. Understanding of tumour targets and biology, ability to engineer antibody-based therapeutic molecules and the design of more potent and selective therapeutic systems give opportunities to develop more effective therapy than the first generation. There are many parameters to manipulate giving a complex task for clinical investigation. Successful development is likely to depend on clinical investigation in which mechanisms of action is elucidated and the therapies optimised using this knowledge.

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IMMUNOTHERAPY VIS-À-VIS GENOMIC INSTABILITY OF HUMAN CANCER

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Despite considerable efforts spent on the development of cancer immunotherapies, the so far reported survival benefits are rather disappointing. Among the many reasons for this blatant failure antigenic heterogeneity of the individual tumor bears a special significance, because it is now recognized as direct function of genomic instability. Chromosomal aberrations increase with tumor progression, specific intervention should be preferentially applied as early as possible. Consequently, minimal residual cancer when diagnosed after removal of the primary tumor offers a unique therapeutic opportunity not only because the absolute number of antigenic cell variants is already smaller within the residual cell population but also because dissemination and micrometastasis are highly inefficient i.e. selective processes. Recently obtained data, however, indicate that early disseminated tumor cells exhibit extensive genomic instability detectable by single-cell comparative genomic hybridisation. Thus, as in advanced tumor stages also in minimal residual disease, genomic instability may pose a formidable barrier for any type of mono-therapy. Therefore, a rationally designed multipronged therapy directed at targets expressed on minimal residual cancer cells, may be applied to decrease the potential precursors of clinical metastases by a substantial i.e. operationally curative degree.

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Anti-Idiotypes for Breast and Ovarian Cancer

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Anti-idiotypic antibodies, which imitate a tumor-associated antigen by their variable region, offer an elegant method for the induction of a specific immune response, when used as a surrogate antigen for immunization. We generated anti-idiotypic antibodies imitating 2 different tumor-associated antigens. I. CA125 for ovarian carcinomas and II. 14C5, a tumor-associated cell substrate adhesion molecule on breast cancer cells, whereas the first approach could be introduced in a first clinical trial and the second was evaluated in an immunocompetent animal model. For the induction of an immune response against CA125 46 patients with pretreated recurrences from advanced ovarian cancer were immunized with the anti-idiotypic antibody MAb ACA125. Patients were treated with 2mg anti-idiotype antibody every two weeks for 4 injections i.m. and then monthly. 29 of 46 (63.0%) patients demonstrated an anti-anti-idiotypic (Ab3) response, which was to a lower extent also directed against CA125, within 9/18 patients developed a CA125 specific cellular immune response by their peripheral blood lymphocytes. The mean progression free survival showed to be 8.1±4.3 months in anti-anti-idiotype positive patients (3.5±3.9 months in negative pts.). Based on this data a randomized anti-idiotype vaccine trial in recurrent ovarian cancer patients as a consolidation after second line chemotherapy in case of CR/PR will be started to evaluate the effect of the immune reponse on the progression free survival. For immunotherapy of breast cancer, we generated a murine monoclonal anti-idiotypic antibody (MAbACA14C5), which imitates a cell substrate adhesion molecule on breast cancer cells. The anti-idiotype was introduced in an immunocompetent animal to prove his capability on induction of an immune and tumor response. The results showed a highly significant difference in the tumor growth of the ACA14C5 treated group in contrast to the controls starting the immunization on day 6 after tumor cell application with 10 of 12 animals being cured from their tumor burden. Prophylactic immunization against the invasion antigen of breast cancer by anti-idiotypic antibodies showed protection against increasing tumor burden. However, in the situation of established tumors only minor responses could be detected. Vaccination with anti-idiotypic antibodies comprises an effective method for induction of a specific immune response against non-immunogenic tumor-associated antigens and should be therefore considered in immunological approaches to tumor therapy, where the primary structure and sequence of the antigen e.g. CA125 is up to now not available (supported by DFG Wa740/1-3 and Wa 740/2-1).

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Anti tumor activity of the BAT antibody

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We produced a monoclonal antibody (BAT) directed against membranes of Daudi, a Burkitt lymphoma B cell line. BAT was found to bind and stimulate human and murine T lymphocytes. BAT also exhibited anti tumor properties in mice bearing a variety of tumors. A single intravenous administration of BAT to mice resulted in regression of the tumors and prolongation of their survival. BAT also induced regression of human tumors inoculated into SCID mice that have been implanted with human lymphocytes. The anti tumor activity of BAT was related to its immune stimulatory properties as was manifested by regression of tumors in mice that have been transplanted with lymphocytes from BAT treated mice. The immune stimulation of selected lymphocyte sub-populations indicated that CD4 T cells responded to BAT activation by proliferation and by IFN-gamma secretion. This was corroborated by FACS analysis indicating a selective increase in BAT binding to the CD4 positive cells. To elucidate the lymphocyte sub-populations mediating the anti tumor effect of BAT monoclonal antibody, we performed experiments with selective depletion in mice or selective engraftment of T or NK cells into SCID mice. The results indicated a dual role for T and NK cells in mediating the anti tumor activity of BAT.

Our current study establishes a base for the potential therapeutic use of BAT monoclonal antibody in cancer patients.

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CD40 antibodies evoke cytotoxic T-cell responses that eradicate established tumors.

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CD40 is now established as a key molecule for activating antigen-presenting cells (APC) and controlling their ability to process and present antigen effectively to T cells. We have found that when antibodies to CD40 are used to treat mice harbouring syngeneic lymphoma (BCL_1, A31, A20 and EL4) and certain solid tumors (CMT93), a rapid cytotoxic T cell response occurs which protects mice and leaves them immune to further tumor rechallenge. In other tumor models, such as B16 melanoma, anti-CD40 antibody provides only partial protection and delays but does not stop tumor growth. In those tumors studied in depth (BCL_1 and A31), we find that the cytotoxic responses are helper (CD4) independent, and occur over a period of 5-6 days with a >10 fold expansion of $CD8^+$ T cells. While the nature of the target antigens on these lymphomas is unknown, $CD8^+$ T cells recovered from responding mice show powerful cytotoxic activity against the target tumor in vitro. The therapeutic activity of anti-CD40 antibody is critically dependent on the tumor load at the time of treatment. Paradoxically, only mice carrying a large tumor burden can be cured and immunised by the anti-CD40 treatment. We believe this dose phenomenon may relate to the amount of tumor antigen required to generate effective CTL responses. One of the major outstanding questions relates to the nature of the APC. In the case of $CD40^+$ lymphoma cells, the anti-CD40 antibody may stimulate neoplastic cells to present their endogenous antigens to $CD8^+$ T cells, however, in the case of $CD40^-$ tumors it seems more likely that other APC, such as dendritic cells, may be the principal candidates.

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Clinical promise of bispecific molecules and targeting of immunoglobulin receptors.

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Successful immunotherapy of cancer aims at efficient mobilization and stimulation of immune effector cells. Phagocytic cells can easily be increased in number / activity *in vivo* by the use of growth factors such as G-CSF, GM-CSF or Flt3L. Specificity of these immune effector cells for tumor cells can be provided by so-called bispecific molecules (BsAb). BsAb combine specificity for a potent cytolytic trigger molecule on immune effector cells, with that for a tumor associated antigen. We showed the combined use of anti-Fc receptor BsAb and growth factors to result in efficient killing of tumor cells *in vitro*, and *in vivo* in transgenic animal models. This concept is currently clinically evaluated in breast, renal and prostate carcinoma patients, yielding promising results. Another class of diseases -that often occur in immuno compromised patients, such as patients with cancer- represent invasive fungal infections. Since neutrophils represent the primary effector cells against fungi, we evaluated ways to improve targeting of neutrophils towards fungi such as Candida albicans. Our data document targeting of phagocytic effector cells via select Fc receptors, to represent a powerfull way to induce tumor cell destruction or eradication of infectious micro organisms. Targeting to some Fc receptors may, furthermore, trigger a 'vaccine effect' that might positively affect survival of patients following immunotherapy.

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Bispecific antibody MDX-H210 (FcγRI x HER-2/neu) in combination with G-CSF: clinical and biological phase I results

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HER-2/neu is overexpressed in approximately 30 % of breast carcinomas, and is target for immunotherapy in clinical trials. In vitro experiments showed application of G-CSF to significantly enhance killing of HER-2- positive breast cancer cells in the presence of MDX-H210 - a bispecific antibody against HER-2/neu and FcγRI (CD64). In blood from human FcγRI- transgenic mice, MDX-H210 in combination with G-CSF was shown to generate HER-2/neu specific cytotoxic PMN.

Based on these preclinical data, a phase I study was initiated in which 30 patients with advanced metastatic breast cancer were treated with single doses of MDX-H210 during G-CSF therapy. In cohorts of three patients, MDX-H210 doses were escalated from 0.35 to 200 mg/m^2. No dose- limiting toxicity was observed with side effects consisting mainly of fever/chills and nausea/vomiting, which were timely related to elevated IL-6 and TNF-α plasma levels. Pharmacokinetic studies showed effector cell arming for up to four days after MDX-H210. Several patients observed pain or skin flares in metastatic lesions. Tumor biopsies documented infiltration of PMN and monocytes after MDX-H210 infusion. Further clinical studies are warranted to optimize this promising approach.

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RADIOIMMUNOTHERAPY OF SMALL VOLUME DISEASE OF COLORECTAL CANCER: RESULTS OF A CLINICAL PHASE-I/II TRIAL

Whereas radioimmunotherapy (RIT) has shown disappointing results in "bulky disease" of solid tumors, preclinical results in small volume disease are promising. The aim of this study was to evaluate, in a phase-I/II trial, the therapeutic efficacy and dose-limiting toxicity of RIT in colorectal cancer patients with small volume disease. Forty colorectal cancer patients with small volume disease (all lesions ≤ 2.5 cm) were entered in a mCi/m^2-based dose escalation study with the ^{131}I-labeled murine anti-CEA MAb, F023C5, which belongs to the IgG$_1$ subtype. The patients were given single injections, starting at 50 mCi/m^2, and escalating in 10 mCi/m^2 increments (three patients/dose level; six patients, if one of these three patients develops a grade-4 toxicity). The maximum tolerated dose (MTD) was defined as the very dose level where ≤ 1/6 patients develop a grade-4 myelotoxicity. Thirty-one of the 40 patients had lesions known from radiological procedures (CT, MRI), 9 patients suffered from occult disease, as indicated by elevated and/or rising tumor markers (CEA, CA19-9) without radiological correlate. Myelotoxicity was dose-limiting, and a fairly good correlation between the red marrow doses and resulting toxicities was found. At 110 mCi/m^2 (i.e., the MTD), patients regularly developed grade-3, at 120 mCi/m^2 2/6 patients had a grade-4 leuco- or thrombopenia. Tumor doses increased exponentially with decreasing tumor sizes (up to 185 cGy/mCi in a 0.5-cm lung lesion). In the 31 patients with radiologically documented lesions, one had a complete, 7 had partial remissions (corresponding to an objective response rate of 26 %); twelve patients (i.e., 39 %) experienced stabilization of their previously rapidly progressing disease, lasting for up to 18+ months. The majority of patients showed a significant (i.e., > 50%) decrease of tumor marker levels in blood. Myelotoxicity is the only dose-limiting toxicity of the ^{131}I-labeled monoclonal anti-CEA antibody F023C5. Although many patients were treated below the maximum tolerated dose level, the observed anti-tumor effects are encouraging.

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CXC chemokine IP-10 mediated IL-12 gene therapy induced T-cell memory immune response is amplified by targeted IL-2

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We achieved a cell-mediated anti-tumor response followed by a persistent tumor-protective immunity by targeted cytokine therapy with an anti-ganglioside GD2 human/mouse chimeric antibody IL-2 fusion protein (ch14.18-IL-2) following s.c. vaccination with tumor cells genetically engineered to produce single chain interleukin-12 (scIL-12) in an immunocompetent model of murine neuroblastoma. Protective systemic tumor immunity was demonstrated by the complete absence of bone marrow and liver metastases after i.v. challenge with wild-type NXS2 cells at day 7 and the eradication of established bone marrow and liver metastases. This immune response was mediated primarily by CD8+ T-cells in vivo and in vitro and depended on the presence of the CXC chemokine IP-10. A decreased protective immunity following scIL-12 gene therapy over time was re-amplified by tumor-specific ch14.18-IL-2 fusion protein resulting in a re-activation of CD8+-T-cells and subsequent MHC class I-restricted tumor target cell lysis in vitro. The sequential increase in the usage of T-cell receptor chains Vb 11 and 13 in CD8+ T-cells in these mice suggested the initial polyclonal CD8+ T-cell response to be effectively boosted by targeted IL-2. A successful boost of a partially protective memory T-cell immune response induced by scIL-12 gene therapy can thus be generated by tumor-specific targeting of IL-2 with a ch14.18-IL-2 fusion protein. This approach may increase success rates of clinical cancer vaccines.

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ANTITUMOR ACTIVITY OF RECOMBINANT IMMUNOTOXIN anti-Tac(Fv)-PE38 (LMB-2) IN PATIENTS. I. Pastan, T. A. Waldman*, R. J. Kreitman, Lab. Molecular Biology & *Metabolism Branch, NCI, NIH.
Recombinant immunotoxins are composed of the Fv portion of an antibody fused to a protein toxin. The Fv replaces the binding domain of the toxin and directs the toxin to an antigen on a cancer cell resulting in cell death. We use Pseudomonas exotoxin A (PE) to make recombinant immunotoxins. PE has three domains: a cell binding domain, a translocation domain and an ADP-ribosylation domain that inactivates elongation factor 2 and leads to cell death. The aim of the current study was to determine the safety, efficacy and antitumor activity of LMB-2 in patients with IL2 receptor (CD25) positive malignancies. LMB-2 is composed of the Fv portion of the anti-Tac (anti-CD25) monoclonal antibody fused to a 38kDa truncated form of PE. We conducted a Phase I trial in 35 patients. 58 cycles of LMB-2 were given at 8 dose levels ranging from 2 to 63 μg/Kg i.v. QOD x 3. Dose-limiting toxicity at the 63 μg/Kg level was reversible and included transaminase elevations in 1 patient and hypotension and cardiomyopathy in another. LMB-2 was well tolerated in 9 patients at the maximum tolerated dose (40 μg/Kg QOD x 3) where toxicities were minimal and transient. Only 6 of 35 patients developed significant neutralizing antibodies after the first cycle. The T1/2 of LMB-2 was 3-7 hours. We observed 1 complete response in a patient with hairy cell leukemia (HCL) and 7 partial responses in patients with HCL (3), cutaneous lymphocytic leukemia (CTCL) (1), Hodgkin's disease (1), and adult T-cell leukemia (1). Partial responses included 2-3 log reductions of peripheral blood malignant cells (3), improvement in skin lesions (1), and regression of lymphomatous masses (4). All patients with HCl and CTCL responded. Marginal responses were observed in 3 patients with Hodgkin's disease and in 1 patient with mantle cell lymphoma. We conclude the LMB-2 has activity in several forms of CD25+ hematologic malignancies and is relatively non-immunogenic. Phase II trials are planned.

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Anti-CD20 (rituximab) in follicular lymphoma

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Ongoing experience with rituximab continues to be encouraging. An update of the pivotal trial shows that the median remission duration is 11.6 months, quite comparable to results with other good single agents such as the nucleoside analogs. Some traditionally difficult patient subsets do very well with rituximab, including the elderly and those who have received prior high dose therapy programs with marrow or stem cell transplant. Pharmacokinetic data indicate that rapid depletion of serum rituximab levels correlates with lower response rates, a potentially important observation, especially for those with small lymphocytic lymphoma.

New applications to explore for rituximab are numerous, including: (a) its use in conjunction with other biological agents, e.g. interferon, G-CSF, or other cytokines; (b) its use in conjunction with chemotherapy, e.g. CHOP, FND (fludarabine, mitoxantrone, dexamethasone) and others; (c) its use as an "in vivo purging" technique prior to stem cell harvest; (d) its use in other B-cell malignancies, e.g. aggressive lymphoma, AIDS-related lymphoma, lymphocyte predominant Hodgkin's disease, and others.

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Poster Presentations

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PRECLINICAL TARGETED THERAPY OF RENAL CELL CARCINOMA (RCC) WITH CONJUGATES OF MAB 138H11 AND CALICHEAMICIN θ

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Monoclonal antibody 138H11 produced against human renal γGT stained over 99% clear cell and papillary RCC on frozen sections, showing a *membranous* expression of the target antigen. Human tumor-bearing kidneys perfused in an extra-corporeal system with ^{99m}Tc-138H11 revealed a specific uptake into the tumor. In contrast, renal cells were not accessible to the circulating antibody due to the polarized expression of γGT at the brush-border membrane of proximal tubules.

Here we evaluated the cytotoxicity of novel conjugates with the DNA cleaving enediyne calicheamicin θ (Camθ) and mAb 138H11 using different linkers for a prodrug effect. FACS analysis showed binding of mAb 138H11 to RCC cell lines, while squamous cell carcinoma lines, fibroblasts and the murine RENCA were negative. XTT cell proliferation assays revealed efficient killing of Caki-1/2 cell lines by the conjugates using SPDP (EC_{50} = 10^{-11} M) or DTSSP as covalent linkers.

For *in vivo* testing, groups of eight nude mice each were injected with 2,5 x 10^6 cells s.c. and treated with: (1) PBS, (2) 138H11, (3) Camθ, (4) mixture of 138H11 and Camθ, and (5) 138H11-Camθ conjugate. Treatment started on day 1 after tumor induction and was repeated three times. The preliminary data show a significant inhibition of tumor growth with the conjugate versus PBS. Only mice treated with the conjugate showed a tumor shrinkage to minimal residues. These combined data encourage further dose evaluation studies.

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CLINICAL COURSE OF OVARIAN CANCER PATIENTS AFTER STIMULATION OF HAMA FOLLOWING RADIOIMMUNOSCINTIGRAPHY (RIS) USING MOAB B72.2 PRIOR TO REASSESSMENT LAPAROTOMY: Michael W. Method, LtCol, USAF, Keesler Medical Center, MS.

Objective: Monoclonal antibodies (MoAb) offer the potential for high selectivity toward tumor-associated antigens, growth factors, or oncogenes, which could be used for both detection and therapy. Some patients who receive murine MoAb will develop a Human Anti-Murine-Antibody (HAMA) response with associated anti-idiotypic antibodies. This trial was designed as a pilot to determine if the development of this response conferred a survival advantage in patients with ovarian cancer.

Methods: 25 consecutive patients with no clinical evidence of disease after primary therapy were enrolled between 1/94 and 12/95. Each patient underwent RIS using ^{111}Indium-satumomab pendetide (labeled B72.3) prior to reassessment laparotomy. Serum samples were drawn at 4, 12, and 24 weeks for the presence of HAMA and tumor markers Endpoints included progression free interval and overall survival for patients with and without a HAMA response following a single infusion of antibody.

Results: Eleven patients (44%) developed a HAMA response. Six patients (54.5%) are alive, and five patients are without evidence of disease. Five patients (45.5%) have died of disease (DOD). Median survival is >50 months. Average HAMA response at 12 weeks was 213.5ng/ml. Fourteen patients (56%) did not develop HAMA. Five patients (35.7%) are alive, and four patients are without evidence of disease. Median survival is 33mos. Demographics, MoAb dose, and CA125 level were comparable.

Conclusions: Patients who develop a HAMA response to a single infusion of radiolabled MoAb B72.3 have an overall trend toward increased and prolonged survival. Use of this or other agent(s) should be investigated in a prospective, randomized fashion as consolidation therapy in patients without evidence of disease clinically at the completion of primary therapy.

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PROSPECTIVE STUDY OF ADJUVANT THERAPY WITH MONOCLONAL ANTIBODY 17-1A OF DUKES' B2 / B3 - COLON CARCINOMA - INTERIM ANALYSIS OF TOXICITY

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Background: Adjuvant immune therapy with the murine monoclonal antibody (mAb) 17-1A has been shown to be an alternative to chemotherapy in the treatment of stage III colorectal cancer presenting similar efficacy in combination with lower toxicity. Therefore we assumed that also patients with Dukes' B colon cancer could have benefit from adjuvant treatment with mAb 17-1A.

Patients and methods: In 1997 we started this prospective multicentre trial including patients after curative (RO) resection of Dukes' stage B2 / B3 adenocarcinoma of the colon. Patients are randomly assigned to either treatment with mAb 17-1A (arm A) or observation regimen (arm B). In the treatment arm patients are administered 500 mg of mAb 17-1A intravenously followed by four infusions of 100 mg every four weeks. Until July 1999, 260 patients have been entered into the trial.

Results: Our interim data concerning toxicity reveal that of 267 courses of mAb 17-1A eligible we saw a total number of 58 (22%) adverse events. Except one case of severe toxicity (exacerbation of Wegener's granulomatosis) all side effects are of WHO grades 1-2 (17% grade 1, 5% grade 2). Adverse events are most frequent within the first course with 39% events (versus 22% in course 2, 17% in course 3, 20% in course 4, 2% in course 5). Diarrhoea, nausea and vomiting represented the most common side effects.

Conclusions: These data underline the favourable toxicity profile of adjuvant treatment with mAb 17-1A, which migt confirm its role in the therapy of the a priori good risk group of patients with Dukes B colon carcinoma.

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The possibility of antitumor therapy with PANOREX® in patients with additive severe illness – initial results

Purpose: Today efficient chemotherapeutics are needed in adjuvant situations to prevent metastases in Colon cancer Dukes C or pancreatic cancer. However some patients are too ill to undergo this therapy. Aim of this survey is to demonstrate the efficacy and tolerance of the monoklonal antibody PANOREX® in the treatment of such patients.

Methods: We report on 10 patients (2 pancreatic cancer, 8 colorectal cancer Dukes C) who underwent immunotherapy with PANOREX®. All these Ro resected patients were 17-1-A AG positiv on the disseminated tumor cells in the postoperative examination of the bone marrow. Additional illness were infectious disease (n=4), cardiac failure (n=3), renal failure (n=2) and severe psychosis (n=1). Immunotherapy was performed with PANOREX® i.v. (initial dose 500 mg, followed by 100 mg/ month over 5 months). Target parameters of the study were the tolerance of the therapy and the elimination of disseminated tumor cells in the bone marrow one month after immunotherapy.

Results: We did not find any complications during therapy. Immunotherapy was well tolerated. Four patients showed no disseminated tumor cells in the bone marrow after the therapy, in two patients there was no evidence for 17-1-A AG expression on the reduced tumor cells. Two patients showed evident decrease of the 17-1-A AG expression. Two patients refused controll. All patients are still alife after two years without any sign of relapse.

Conclusion: Immunotherapy with PANOREX® is a well tolerated adjuvant and alternative therapy in patients too ill to undergo chemotherapy. Further investigations are recommended.

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RFT5/Ki-4(scFv)-ETA, a new recombiant bispecific immunotoxin directed against CD25 and CD30 receptor for the treatment of Hodgkin's disease

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A novel strategy for the expression of functional rITs directed to the periplasmic space of *E.coli* has recently been developed. Recombinant immunotoxins were recovered by sonication of pellets from bacterial shaking cultures grown under osmotic stress in the presence of compatible solutes. Compatible solutes are low molecular weight osmolytes naturally occurring in halophilic bacteria known to protect proteins under high salt concentrations. Using these compounds for the cultivation of *E.coli* under osmotic-stress, not only allowed the bacteria to grow under these otherwise inhibitory conditions, but also produced a periplasmic microenvironment for the generation of high concentrations of correctly folded rITs. In this study, we used the periplasmic expression protocol to produce a functional bispecific immunotoxin. Anti-CD25 (RFT5) and anti-CD30 (Ki-4) IgG V-genes were linked together and genetically fused to a deletion mutant of *Pseudomonas* exotoxin (ETA') using the new pET-derived expression vector pBM1.1. The resulting RFT5/Ki-4(scFv)-ETA' protein was directed to the periplasmic space of *E.coli* and purified by IMAC and size exclusion chromatography. First in vitro data will be presented and its value as a new immunotherapeutic agent for the treatment of patients with Hodgkin's lymphoma will be discussed.

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The recombinant anti-CD25 immunotoxin RFT5(scFv)-ETA' exhibits highly specific cytotoxic activity against disseminated human Hodkgin lymphoma in SCID mice

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Since recombinant DNA technology permits an easy production of large amounts of recombinant protein, we previously constructed a new anti-CD25 immunotoxin by fusing the RFT5 single chain variable fragment to a deletion mutant of *Pseudomonas* exotoxin A (ETA'). The recombinant immunotoxin (rIT) was directed into the periplasmic space of *E.coli* using the bacterial expression vector pBM1.1 and our newly developed expression/purification protocol. Biologically active RFT5(scFv)-ETA' was isolated by sonication and subsequently purified by immobilized metal ion affinity and molecular size-chromatography. In vitro characteristics of the rIT were assessed by ELISA, FACS and cytotoxicity assays. RFT5(scFv)-ETA' was subsequently used for the treatment of disseminated human Hodgkin's lymphoma in a SCID mouse model. The mean survival time (MST) of L540rec challenged SCID mice was 38.1 days. A single i.v. injection of 40 µg rIT 1 day after tumor challenge resulted in 100% tumor-free mice, extending the MST to more than 220 days ($P<0.0001$). All animals were assessed for soluble interleukin-2 receptor α, which is directly correlated to tumor burden. Soluble CD25 was not detectable in mice treated with the rIT. Thus, RFT5(scFv)-ETA' might also be suitable for further evaluation against Hodgkin's lymphoma in man.

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BW704(scFv)-ETA', a new recombiant anti-GD2 immunotoxin for the treatment of neuroblastoma cells

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Since the disialoganglioside GD2 is abundantly present on the surface of neuroblasoma cells, we constructed a new recombinant anti-GD2 immunotoxin by fusing a recombinant antibody fragment to a potent bacterial toxin (*Pseudomonas* exotoxin A, ETA'). We synthesized a single chain variable fragment (scFv) from the murine anti-GD2 antibody BW704 by linking the heavy chain variable (VH) to the light chain variable (VL) domain via a flexible peptide sequence. To retrieve a functional high affinity scFv, we cloned the assembled VH- and VL- genes into a phagemid vector. Filamentous phages displaying scFvs on their surface and their genes within the phage particle were produced, purified, and selected for binding on the neuroblastoma cell line IMR5. Three rounds of selection on the neuroblastoma cells with scFv-phages derived from a mini-repertoire of $1x10^6$ different clones were necessary to retrieve a binding BW704 scFv clone. The clone was characterized by sequencing, ELISA experiments and FACS analysis. The recombinant antibody was genetically fused to a deletion mutant of *Pseudomonas* exotoxin (ETA') using a new pET-derived expression vector (pBM1.1). The resulting BW704(scFv)-ETA' protein was directed to the periplasmic space of *E.coli* and purified by IMAC and size exclusion chromatography. This recombinant immunotoxin will be further investigated *in vitro* for its value as a new immunotherapeutic agent for the treatment of patients with neuroblastoma.

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Characterization and Partial Sequencing of a Human Anti-Idiotypic Antibody against Anti-GD_2 Chimeric Antibody CH14.18 from a Patient Treated for Neuroblastoma

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Treatment of refractory neuroblastoma with anti-GD_2 antibodies, 14G2a and ch14.18, yielded complete clinical remissions. Most patients developed significant levels of antibodies against the injected therapeutic antibody, with specific anti-idiotype reactivity in case of the chimeric construct. Here we further characterize this immune response.

Human antibodies from a patient treated with ch14.18 were cloned by EBV transfection of his PBLs and consecutive fusion with the mouse-human heteromyeloma K6H6/B5 by somatic cell hybridization to produce stable hybridomas. Additionally, phage display technique was used to rescue individual clones.

One stable subclone (anti-Id) was further propagated. The subclass of this clone was determined in ELISA to be IgG_2 with a kappa light chain. The anti-Id demonstrated a strong reaction with ch14.18. Purified anti-Id competed with GD_2 for binding to ch14.18. To further stabilize and sequence the anti-Id, the phage display technique was used for cloning of its Fab fragment. Sequence analysis of the kappa light chain revealed the origin from DPK22 and JK1 germ-line genes without somatic mutations.

Further characterization and cloning by phage display will not only be helpful in evaluating the impact of the anti-idiotypic network in the therapeutic efficacy of the latter antibody, but also facilitate easy anti-tumor vaccine production, if the anti-Id proves to be suitable as such.

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Trioma cells for the therapy of human B-cell lymphoma

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The trioma approach is a new immunotherapeutic strategy for the treatment of malignant B-cell lymphomas. Lymphoma cells are fused to a hybridoma secreting an anti-Fc receptor antibody. The resulting trioma cells express a bispecific immunoglobulin that contains the lymphoma-specific idiotype assembled to the FcR-binding arm. The latter redirects the Id against antigen-presenting cells and leads to its processing and to presentation of tumor-derived peptides. We demonstrated in a mouse model that even established lymphomas can be efficiently eradicated by immunizing with trioma cells and that this is dependent on the presence of both CD4+ and CD8+ T-cells. Here we show that this approach is feasible for the treatment of human Non-Hodgkin lymphomas.

Lymph node cells from two patients suffering from centroblastic and centroblastic-centrocytic lymphoma, respectively, were fused to the mouse hybridoma 197 that is specific for human FcγRI. The resulting trioma cell clones were extensively tested by FACS and PCR analyses for expression of the human and the murine Ig arm as well as for the presence of several markers that were identified on the parental human lymphoma cells (CD5, CD19, CD20, HLA-A2). Selected trioma clones were used for stimulation of autologous PBMCs. Stimulation, as measured by TNF-α secretion in a cell proliferation assay, was considerably enhanced in the presence of the trioma cells as compared to the parental lymphoma or hybridoma cells. These data show that the trioma approach is a promising strategy for anticancer vaccination.

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Mode of action of monoclonal antibody IDEC-C2B8 against CD20 expressing lymphoma cell lines.

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The chimeric mouse human anti-CD20 monoclonal antibody IDEC-C2B8 is an effective treatment for patients suffering from non-Hodgkin's lymphoma. Since little information exists about the mechanism of its action, we examined cytotoxicity with a new flowcytometric cytotoxicity assay, which is also able to assess long term cytotoxicity. IDEC-C2B8 at concentrations above 100 ng/ml induced cytotoxic activity/ apoptosis against four out of eight CD20 expressing lymphoma cell lines (RAJI, DAUDI, JOK-1 and WT100). Moreover, it induced a moderate complement dependent cellular cytotoxicity (CDCC) after four hours with a pronounced increase of cytotoxicity after three days of culture. When peripheral mononuclear cells of normal donors were used as effector cells, IDEC-C2B8 induced a marked antibody dependent cellular cytotoxicity (ADCC) in seven out of eight lymphoma cell lines tested. Furthermore, we found that only the cytokine interleukin-2 moderately augmented ADCC against two out of eight examined lymphoma cell lines whereas the cytokines interleukin-12, interferon-α and GM-CSF were not effective at all. Interestingly, we could demonstrate a correlation between CD32 expression on lymphoma cell lines and IDEC-C2B8 induced apoptosis, indicating that crosslinking of CD20 with CD32 may be involved in the mechanism of cytotoxicity. We propose that simultaneous apoptosis, CDCC and ADCC results to the marked elimination of CD20 expressing tumor cells observed after treatment with IDEC-C2B8.

supported by Hoffmann-La Roche, Grenzach-Wyhlen and Megapharm, Sankt Augustin

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Zevalin™ Radioimmunotherapy of Relapsed or Refractory Non-Hodgkin's Lymphoma

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Zevalin™ (IDEC-Y2B8) is a murine IgG_1 kappa monoclonal antibody (ibritumomab) conjugated to the linker-chelator tiuxetan (MX-DTPA) which securely binds the pure beta emitting isotope ^{90}Yttrium (^{90}Y). The antibody targets the B lymphocyte antigen, CD20. A prior Phase I/II trial [Blood 1998 November;92(10):1722] determined the dose limiting toxicity to be hematologic without other significant organ toxicities. Severity of hematologic toxicity was best predicted by the clinical parameters of baseline platelet count (a surrogate for prior bone marrow damage from chemotherapy or external beam radiation) and percent bone marrow involvement with lymphoma and not by dosimetry. MTD was 0.4 mCi/kg; 0.3 mCi/kg in patients with pre-existing mild thrombocytopenia. Response rates were 67% in the combined low-/intermediate-grade and mantle-cell NHL population and 82% in the low-grade patients. Phase II and Phase III trials are now nearing completion. The Phase II trial is evaluating the safety and efficacy of 0.3 mCi/kg of ^{90}Y labelled Zevalin in 30 relapsed or chemotherapy refractory low-grade, follicular, or transformed NHL patients with mild thrombocytopenia (platelets: 100-149,000/ mm^3). A Phase III trial compares efficacy and safety of 0.4 mCi/kg of Zevalin with that of single-agent Rituximab (375mg/m^2 weekly x 4)in patients with low-grade, follicular, or transformed NHL. A separate Phase III trial is evaluating Zevalin in Rituximab refractory follicular NHL patients. Interim data will be discussed. Zevalin radioimmunotherapy is a safe and effective treatment for B-cell non-Hodgkin's lymphoma.

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HIGHLY EFFICIENT TREATMENT OF DISSEMINATED HODGKIN LYMPHOMA IN SCID MICE WITH THE NEW RECOMBINANT ANTI-CD30 IMMUNOTOXIN KI-4(SCFV)-ETA'

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The human lymphocyte activation marker CD30 highly overexpressed on Hodgkin/Reed-Sternberg cells is an excellent target for selective immunotherapy. We previously selected binding Ki-4 scFv on CD30-expressing L540rec cells by phage display technology. The antibody fragment was inserted into the pET-derived expression vector pBM1.1 and thus fused to a deletion mutant of *Pseudomonas* exotoxin A (ETA'). Functional Ki-4(scFv)-ETA' was directed into the periplasmic space of *E.coli* and subsequently purified by a combination of metal ion affinity and molecular size-chromatography. The characteristics of the recombinant immunotoxin (rIT) were verified by ELISA, FACS and cytotoxicity assays. To document in vivo antitumor activity, disseminated human Hodgkin's lymphoma in SCID mice were treated with the immunotoxin. The mean survival time (MST) of L540rec challenged SCID mice was 38.1 days. A single i.v. injection of 40 µg rIT 1 day after tumor inoculation resulted in 90% tumor-free mice, extending the MST to more than 200 days (P<0.001). Soluble CD30 antigen, which is directly correlated to tumor burden was not detectable in mice treated with the rIT. This new recombinant immunotoxin thus represents a promising candidate for further clinical evaluation in patients with Hodgkin's lymphoma or other CD30$^+$ malignancies.

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A bispecific single-chain diabody for the retargeting of cytotoxic T-lymphocytes to tumour cells

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Various trials with bispecific antibodies have demonstrated that the retargeting of cytotoxic T lymphocytes (CTL) to tumour cells can lead to an anti-tumour response. The initial trials were performed with bispecific IgG or $F(ab')_2$ molecules generated by chemical cross-linking or the hybrid-hybridoma technology. However, these bispecific antibodies are difficult to make and produce also a substantial number of non-functional molecules. Genetic engineering has led to the generation of various novel recombinant bispecific antibody formats with improved properties. We generated a bispecific single-chain diabody directed against the tumour-associated antigen carcinoembryonic antigen (CEA) and the T cell coreceptor CD3 (scDb CEACD3). ScDb CEACD3 expressed either in the VH-VL or the VL-VH configuration bound CEA in ELISA with identical efficacy. ScDb CEACD3 recognised also CEA in tissue sections of colon adenocarcinoma and bound specifically to CD3-positive Jurkat cells and T cells. As shown by rosetting experiments, scDb CEACD3 was able to retarget Jurkat cells to CEA-expressing tumour cells *in vitro* demonstrating correct assembly of both antigen binding sites. Furthermore, scDb CEACD3 mediated specific lysis of various CEA-positive tumour cell lines *in vitro* by activated CTLs in a concentration dependent manner. This scDb might be useful as immunotherapeutic reagent for treatment of CEA-expressing carcinoma.

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BISPECIFIC SINGLE-CHAIN DIABODIES

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Diabodies (Db) are small bivalent or bispecific dimeric antibody fragments generated by crossover pairing of two VH-VL fragments expressed in the same cell. We have generated a novel, monomeric single-chain diabody format (scDb) by joining the two VH-VL fragments by an additional linker. Bispecific single-chain diabodies are expressed in bacteria and as well as secreted by mammalian cells in active form and exhibit an improved *in vitro* stability in serum at 37°C. ScDb also possess antigen-binding activity when expressed intracellularly in the secretory pathway (intrabodies) or when displayed on the surface of mammalian cells opening additional therapeutic applications of scDb. The functional affinity of bispecific scDb could be increased by fusion with the Fc or CH3 region of human immunoglobulin γ1 generating bispecific IgG-like molecules with two binding sites for each antigen. A scDb directed against carcinoembryonic antigen and ß-galactosidase (scDb CEAGal) secreted from mammalian HEK293 cells specifically recruited the enzyme to CEA-expressing LoVo cells in cocultivation experiments and mediated tumor cell killing through conversion of a daunomycin-prodrug to the toxic drug. Another scDb (scDb CEACD3) directed against the same tumour marker and the T cell coreceptor CD3 was applied *in vitro* for the retargeting of CTL to tumour cells. ScDb molecules produced in mammalian producer cells *in vivo* may find applications in gene-directed antibody therapy combining antibody-mediated effector recruitment with a gene-therapeutic approach.

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Effective cytotoxicity mediated by a bispecific single-chain antibody directed against EpCAM/CD3 and cytokine-induced killer cells (CIK). Dimitri Flieger, Peter Kufer*, Imke Beier, Tilman Sauerbruch, Ingo Schmidt-Wolf. Medizinische Klinik & Poliklinik I der Universität Bonn, 53105 Bonn and *Institut für Immunologie der LMU-Universität München, 80336 München

Cytokine-induced killer cells (CIK) generated in vitro from peripheral blood mononuclear cells (PBM) by addition of interferon-γ, IL-2, IL-1 and a monoclonal antibody (mAb) against CD3 are highly efficient cytotoxic effector cells (1). In this study, we evaluated whether the cytotoxicity of CIK against the colorectal tumor cell line HT29 can be enhanced by the addition of a bispecific single-chain antibody (bs-Ab) directed against EpCAM/CD3 (2). For determination of antibody dependent cellular cytotoxicity (ADCC) we used a new flowcytometric assay, which directly counts viable tumor cells and can assess long term cytotoxicity (3). We found that the bs-Ab induced distinct ADCC at a concentration above 100 ng/ml with both PBM and CIK at an effector to target cell ratio as low as 1:1. CIK cells revealed higher ADCC than PBM, which appeared already after 24 hours whereas PBM showed the highest ADCC after 72 hours. The addition of the cytokine IL-2 at 20 ng/ml markedly enhanced ADCC of both PBM and CIK. When the bs-AK was combined with mAb BR55-2, which recognizes the $Lewis^y$-antigen, the ADCC was partly augmented, whereas mAb 17-1A, which binds to EpCAM as well, slightly suppressed ADCC. We conclude that in vitro or in vivo generated CIK combined with bs-Ab and IL-2 should be evaluated for the treatment of EpCAM expressing tumors.

1) Schmidt-Wolf I. et al, 1991, J. Exp. Med., 174:139. 2) Mack M. et al, 1995, PNAS, 18:7021. 3) Flieger D. et al, 1995, J. Immunol. Methods, 180:1.

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A Recombinant Bispecific CD19xCD3 Antibody induces rapid Lymphoma directed Cytotoxicity in unstimulated peripheral T-cells

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Bispecific antibodies activating T cells against malignant lymphoma have been shown to be effective in lysing lymphoma cells, their clinical use however has been hampered by the difficulty to produce clinical grade material in sufficient amounts. To overcome this problem we have generated a small recombinant lymphoma directed bispecific antibody by functional expression in CHO cells, that consists of two different single-chain Fv fragments joined through a Gly-Ser linker. One specificity is directed against the CD3 antigen of T cells, the other antigen binding site is directed against the pan B cell marker CD19. The latter antigen is uniformly expressed on the vast majority of B cell malignancies. FACS analysis demonstrated specific binding to CD19 and CD3. Surprisingly, unstimulated primary human T cells derived from peripheral blood became cytotoxic against CD19-positive lymphoma when incubated with the bscCD19xCD3 antibody at rather low concentrations of 10-100 pg/ml. Cytotoxicity was induced at Effector cell : Target cell ratios as low as 2,5:1. Remarkably, T cells displayed cytotoxicity after 4 hours without any apparent pre- or costimulation. In contrast, a conventional intact bispecific CD19xCD3 antibody, generated by the hybrid-hybridoma technique did not exert cytotoxicity with unstimulated human T cells even at concentrations up to 3000 ng/ml. Thus, the novel recombinant bispecific CD19xCD3 antibody appears to be unique in so far as it does not depend on preactivated T cells. Therefore clinical application of this novel agent appears to be warranted in patients with Non-Hodgkin Lymphoma.

Angiogenesis

Invited Speakers

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Oncogenes and the Control of Tumor Angiogenesis: Antibodies to the EGF Receptor or Her-2 as Possible Anti-Angiogenic Drugs in Vivo.

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Oncogenes may influence the growth and development of solid tumors indirectly by contributing to the angiogenic switch in addition to their direct effects on promoting aberrant cancer cell proliferation and survival (Kerbel et al, *Molecular Medicine, 4*: 286, 1998). For example, mutant *ras* oncogenes can result in a significant transcriptional upregulation of genes which encode various paracrine pro-angiogenic growth factors such as vascular endothelial cell growth factor (VEGF) (Rak, J. et al, *Cancer Res., 55:* 4575, 1995; Grugel et al, *J. Biol. Chem., 270:* 25915, 1995). The same appears the case for activation of receptor tyrosine kinase related (proto)oncogenes such as the EGF receptor (EGFR) or Her-2 (Viloria-Petit et al, *Am. J. Path., 151*: 1523, 1997). Consequently, it follows that treatment with drugs which target *Ras* (or Ras-related targets), or monoclonal neutralizing antibodies to Her-2 or EGFR, may inhibit tumor growth *in vivo,* indirectly, by suppression of angiogenesis, in addition to direct-acting growth inhibitory mechanisms. This would lead to a possible therapeutic benefit *in vivo*--where angiogenesis is required for progressive tumor growth-- but not in cell culture, where angiogenesis is irrelevant. Evidence for this will be summarized utilizing a variety of anti-human-EGF receptor (chimeric or humanized) monoclonal antibodies. The issue of acquired resistance to such signal transduction inhibitory agents arising during the course of long term therapy *in vivo* will also be discussed.

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Tumor factors and Dendritic cell differentiation.

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A major reason for the lack of host responses to tumor antigens is a tumor-associated defect in dendritic cells. We have discovered that vascular endothelial growth factor (VEGF), a molecule produced by most tumors and responsible for the induction of tumor neovasculature, also has a selective inhibitory effect on the ability of bone marrow precursors to differentiate into functional dendritic cells.

VEGF significantly inhibits NF-κB-dependent activation of reporter gene transcription and significantly decreased specific DNA binding by NF-κB. Blockade of NF-κB activity in HPC with a dominant IκB inhibitor of NF-κB reproduced the pattern of effects observed with VEGF. Blockade of NF-κB activation in HPCs by tumor-derived factors may therefore be a mechanism by which tumor cells inhibit effective antitumor immune responses.

In animal model experiments, we have shown that dendritic cell numbers and function can be improved in tumor-bearing animals after treatment with anti-VEGF antibodies, and that this results in improved efficacy for antigen-specific immunotherapy. We have studied dendritic cell function in cancer patients and found a correlation with stage, tumor site, and serum VEGF levels, and have shown improvement of these defects with treatment of the tumor and in preliminary experiments, following treatment of the patients with anti-VEGF antibodies. This knowledge suggests that the combination of anti-angiogenic blockade of VEGF and immunotherapy could be synergistic, and results in animal model systems suggest that this is the case.

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Apoptin® gene therapy

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Apoptin, derived from an avian virus, induces apoptosis in various human transformed and/or tumorigenic cell lines, but not in normal primary cells (Danen et al., PNAS 94, 5843-5847). Apoptin-induced apoptosis is independent of p53, is not inhibited by BAG-1 or Bcr-Abl, and is even accelerated by Bcl-2, indicating that Apoptin will be active when many (chemo)-therapeutic agents are known to fail.

To explore the effect of Apoptin in vivo, we have constructed an adenovirus vector expressing the Apoptin gene (Ad-Apoptin). By means of TUNEL assays, Ad-Apoptin was shown to induce apoptosis specifically in tumor cells. Experiments in rats show that Ad-Apoptin has no severe adverse effects. A single intra-tumoral injection of Ad-Apoptin into a xenogeneic hepatoma in nude mice resulted in a significant reduction of tumor growth. Histological examination of AdMLPvp3-treated tumors revealed relatively hypovascularized tumor tissue (Pietersen et al., Gene Ther. 6, 882-892).

The tumor-cell specificity of Apoptin and its unique apoptotic pathway make Apoptin a potential anti-tumor agent.

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Tumor Dormancy by Angiogenesis Inhibition

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By studying the phenomenon of the inhibition of tumor growth by tumor mass, we discovered angiostatin and endostatin, two potent and specific angiogenesis inhibitors. Angiostatin is a fragment of plasminogen and endostatin is a fragment of collagen XVIII. In vivo, systemic therapy with either agent induces a virtual complete blockade of angiogenesis and potently inhibits tumor growth. Using these agents, we have defined a type of tumor dormancy in which a high rate of tumor cell proliferation is balanced by apoptosis and death with blocked angiogenesis. In a series of experiments designed to determine if continued angiogenesis suppression would induce tumor dormancy, mice with several different types of cancer were treated with cycled endostatin therapy. Mice were treated for several months without any evidence of resistance to therapy. After several cycles of therapy, tumor dormancy persisted indefinitely off therapy. Prolonged continuous therapy with high dose endostatin can induce the same pattern of tumor dormancy. Recently, while studying the inhibition of tumor growth by tumor mass in human small cell lung cancer, we discovered that the cleaved or latent conformation of antithrombin III has potent antiangiogenic and anti-tumor activity. Taken together with the discovery of angiostatin these data suggest that the clotting and fibrinolytic pathways may have a direct role in the regulation of angiogenesis.

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The development of oligosaccharides as anti-angiogenic agents

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Basic FGF and VEGF express a mandatory dependence on heparan sulphate (HS) for the ability to activate their signal transducing receptors. The mechanism of activation probably involves receptor dimerisation, conformational change and tri-molecular complex formation (bFGF-FGFR-HS). Two classes of oligosaccharide have been identified that inhibit the biological activity of bFGF in vitro. These are heparin octasaccharides that are too short to activate the cytokine yet retain high affinity for bFGF (K_d 20nM). The IC_{50} for inhibition of bFGF activity is 10μg/ml, a clinically achievable and tolerable concentration. A second family of inhibitory oligosaccharides are the 6-O-sulphate deficient polysaccharides which bind bFGF yet prevent activation of the FGFr, possibly through failure of the bFGF-HS complex to engage the signaling receptor. 6-O-sulphate deficient sequences inhibit the biological activity of bFGF in vitro with an IC_{50} of 5 μM. In vivo evaluation of the activity of the compounds is ongoing.

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First preclinical and clinical results with the antiangiogenetic substance SU 5416 in malignancies. Paul Scigalla, Alison Hannah, Peter Langecker, Laura Shawver, Jeromy McMahon, Peter Hirth, SUGEN Inc., San Francisco, CA (USA). SU5416 is a potent small molecule which acts by directly blocking vascular endothelial growth factor (VEGF)-mediated receptor signaling. It inhibits *in vitro* proliferation of endothelial cells (IC50=40uM) and leads to a significant inhibition of a variety of tumors in *in vivo* models. ***Phase I*** studies with SU5416 in humans were designed to examine the toxicity of escalating doses of i.v. SU5416 administered twice weekly to patients with advanced malignancies. 69 patients: 34M/35F, median KPS 80 (60 – 100), median age 59 (23 – 77) were enrolled. Patients were treated at 13 dose levels between 4,4 – 190 mg/m2. Dose limiting toxicities were observed at the highest dose, consisting of projectile vomiting, headache, and nausea, reversible over 24 – 48 hours. Patients with a variety of advanced tumors had stable disease treated for greater than 6 months, including NSC lung (n=2), colorectal, renal cell, adenoid cystic and basal cell carcinoma. Of the 15 patients with NSC lung, 10 remained on SU 5416 for a longer period of time compared to during their most recent cytotoxic chemotherapy regimen. Encouraging results could be observed in patients with ***AIDS-related Kaposi's Sarcoma(KS)***.VEGF is very important regulator of the edema and angiogenesis seen in KS. To date, 20 HIV-+ patients with therapy refractory KS, ages from 32 – 52, (m=37 yrs.), with KPS of 70 – 100 have received SU5416.Among the first 18 patients in whom the outcome could be assessed, 11 have evidence of biological activity(flattening, shrinkage or dissolution of lesions; reduction or dissolution of edema), four patients had stable disease and three patients had progressive disease. Individual patients showing response reported pain reduction and increased mobility.