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Abstracts

Oral Presentations

Quantitative DNA-Analysis of Cholangiocellular Carcinoma: Predictive Value and Clinical Relevance

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Introduction: Patients suffering form cholangiocarcinoma (CC) are routinely transferred to surgery. But not all patients benefit from extended liver resection. So far, tumor stage, grade, metastases and complete tumor resection are used as prognostic factors to predict the outcome of CC-patients after liver resection. But their clinical value seems to be limited. Quantitative DNA-Analysis might be an alternative for prediction of survival probability. In this study, we investigated the predictive value of DNA-ploidy for outcome of patients resected due to CC.

Methods*:* This prospective study included 32 patients with CC within a period of 3 years. All patients received liver resection. Tissue specimens were taken from the tumor immediately after resection. The DNA-analysis was performed by means of image cytometry. The results of DNA-analyses were related to the histopathological tumor grade, stage and the clinical course. Patients were followed up for 1 to 3 years after liver resection.

Results: Tumor staging assigned 6% of patients to the tumor stage pT1, 8% to stage pT2, 35% to stage pT3 and 51% to stage pT4. Tumor grading showed mostly grade 2 (65%) and less frequent tumor grade 2 (22%) and grade 1 (13%). DNA-analysis classified tumors either as diploid (17%), polyploid (11%) or aneuploid (72%). Tumor-free margins of the liver resected parts were found in 57% (=R0-resection) of patients whereas R1-resection occurred in 5% and R2-resection was recorded in 38% of patients.

Survival was strongly related ploidy. Survival probability after 3-years for patients with diploid CC was 83% and for those with aneuploid tumors 7% (p=0.0006). All patients afflicted from metastasis at time of operation or thereafter suffered from aneuploid tumors indicating a poor prognosis.

Conclusion: DNA ploidy was the most accurate prognosis factor for resected CC patients. Patients suffering from diploid CC survived for a long time whereas aneuploid tumors indicated a poor prognosis with a rather short survival time.

We conclude that DNA-ploidy is a valuable diagnostic tool for identifying subgroups of patients that may be at higher risk for tumor progression and might not benefit from liver resection.

Cell Cycle Promoting Role of JunB through Cyclin A Activation

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JunB, an immediate early gene product and member of the AP-1 transcription factor is generally considered to be an attenuator of AP-1 action. In order to assess its function in cell proliferation fibroblasts were isolated from E9.5 *junB*$^{-/-}$ mouse embryos (1). Analysis of both primary and immortalized *junB*$^{-/-}$ fibroblasts showed normal cell proliferation but an altered cell cycle profile. Despite the large increase in the population of S-phase cells due to the loss of JunB repressing functions in the G1 to S transition (2, 3 and our own data) JunB-deficient fibroblasts did not exhibit an enhanced proliferation rate. A delay in the S- to G2/M- transition caused by impaired cyclin A-CDK2 and cyclin B-cdc2 kinase activity counteracts the accelerated S-phase entry. The kinetics of the cell-cycle dependent transcriptional activation of cyclin A is severely delayed in *junB*$^{-/-}$ fibroblasts. Here we report that JunB contributes to the activity of the CRE element in the cyclin A promoter. Upon reintroduction of a post-translationally inducible JunB-ER^{TM} expression vector the cell cycle distribution and the cell cycle associated cyclin A-CDK2 kinase activity could be restored. This work demonstrates that cyclin A is a direct transcriptional target of JunB and furthermore, identifies positive regulatory functions for the commonly known repressor JunB in cell proliferation.

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Upregulation of c-Myc and Ki-67 Proteins is Correlated with Level of Apoptosis in Tumour Cells after Cytokine Treatment.

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Human tumour cells Me-180, MCF-7, and TCC-Sup were treated with Tumor Necrosis Factor α (TNF), Interferon γ (IFN), and the combination of both (Ti). After 1 to 3 days of treatment increasing numbers of cells were released from the culture flask bottom, in particular after treatment with both cytokines (Ti). These floating cells turned out to be apoptotic, as shown by several apoptosis assays (Annexin-V-Fitc, mitochondria membrane depolarization (Mito-Track red), pre-G1 peak (PI), chromatin condensation (Acridin-Orange after acid denaturation), PARP-degradation). The cell lines Me-180 and MCF-7 were sensitive to apoptosis induction whereas TCC-Sup was more resistant.

The proliferation of the cells slowed down, however at the same time the c-Myc and Ki-67 expression levels were considerably elevated after TNF treatment, only slightly or not after IFN treatment, and the strongest increase was observed after Ti treatment. The level of c-Myc and of Ki-67 correlated well with apoptotis. When the levels of c-Myc and Ki-67 were high the percentage of remaining viable cells was low and this linear relationship was found for the sensitive as well as the resistant lines. Our data support the dual signal model (Evan, G, Cancer a matter of life and cell death, Int. J. Cancer 71, 709 (97). Elevated c-Myc induces proliferation and apoptosis pathways. When no survival signals rescue the cells to the proliferation pathway they become apoptotic. TNF is known to promote apoptotic rahter than proliferation pathways.

Replicative Senescence due to Telomere-Erosion in Hodgkin- and Reed-Sternberg Cells

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The malignant cell compartiment of Hodgkin's disease, i.e. Hodgkin and Reed-Sternberg (HRS) cells are clonal populations derived from the malignant transformation of a single B- or T-lymphocyte. They frequently display features of imminent cell death (mummification) and a markedly extended time to pass the cell cycle.In frozen sections, we identified the expression of senescence-associated beta-galactosidase by HRS cells. In order to test the hypothesis, that HRS cells are in replicative senescence due to telomere erosion, lymph node touch imprints of Hodgkin's disease were investigated by quantitative FISH (Q-FISH) for telomeric repeat sequences with peptide nucleic acid analogue (PNA) probes. The Q-FISH data were calibrated with telomere lengths in normal fibroblast cultures at different passage numbers. Non-Hodgkin lymphomas and touch imprints of invasive ductal adenocarcinomas of the breast were used as control groups. Our data suggest that the malignant cells of Hodgkin's disease frequently are in replicative senescence due to inadequate telomere maintenence. This phenomenon could explain the tumour kinetics and cytogenetic findings in Hodgkin's disease. This unexpected finding apparently sets Hodgkin's disease apart from other malignant neoplastic disorders.

Role of ERK MAP Kinase Signalling in Normal Cell Cycle Control and during Cell Transformation by V-Jun

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The *v-jun* oncogene accelerates G1/ S progression and enables cells to cycle in the absence of exogenous growth factors. Since v-Jun stimulates expression of heparin-binding epidermal growth factor (HB-EGF), we investigated whether autocrine signalling of HB-EGF via the ERK pathway might contribute to cell cycle deregulation by v-Jun. Unexpectedly, we found that in normal chick embryo fibroblasts (CEF) transient deactivation of ERK is sufficient for cell cycle exit induced by serum withdrawal and that active, dual phosphorylated ERK is regenerated and sustained at high levels in fully quiescent (G0) cells. Furthermore, transformation of CEF by v-Jun results not in an increase but a profound decrease in the basal level of active ERK, which also becomes refractory to stimulation by agonists such as serum, LPA and EGF. Biochemical analysis indicates that these defects are attributable to a combination of 1) inefficient signal propagation between Ras and Raf within the ERK pathway, and 2) increased tonic deactivation by MAP kinase phosphatases. The implications of these findings for normal cell cycle control and for the mechanism of cell transformation by v-Jun will be discussed.

Characterization of Poliferating B-Cll Cells

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CLL is an incurable B-cell malignacy characterized by the progressive accumulation of CD 5+ CD 19+ lymphocytes. This accumulation is attributed to the long survival of CLL cells, since the majority of circulating B-CLL cells are resting in the G0/ G1 phase of the cell cycle. Proliferation is minimal. The rates of cells in S/G2M range from 0,08 % to 0,6 %. (n=20).

We have characterized the proliferating cells in order to assess their malignant potential and their role in the biology of lymphocyte accumultion. We simultaneously analyzed DNA-content -quantified by the intravital dye Hoechst 33342- and surface antigens. Additionally we sorted DNA-stained cells for Western Blotting from the G2/M and S compartment.

Unlike their quiescent counterparts, proliferating peripheral cells displayed a high rate of CD86+ positivity and a slightly increased expression of PCNA and CD 21. However, other activation markers like CD 69 or CD 71 were not expressed on proliferating cells- or the expression did not positively correlate with DNA content, which was the case for CD 40 or the STAT cascade.

Comparative analysis of differentiation markers like bcr signaling components showed that proliferating cells were equally differentiated. We therfore conclude that the proliferating cells do not constitute a subpopulation of its own.

The proliferating cells were highly sensitive to the cytotoxic effect induced by the dye Hoechst 33342, as revealed by a high rate of proliferating cells among autofluorescent and PI positive cells.

The low proliferation rate, the limited activation status, the quiescent cell like phenotype and the low viability of circulating proliferating cells make the peripheral blood an unlikely compartment for CLL cell replication.

In the bone marrow, the overall proliferation rate exceeded that of peripheral blood cells by a factor of ten. However, this high proliferation rate was not due to CLL cells, since only about 0,5% of CLL cells were in S/G2M. Therefore lymphoid tissue must be taken into consideration as a source of proliferation as well.

Comparison of Scanning Fluorescent Microscope, Laser Scanning Cytometer and Flow Cytometer in the Field of Analytical Cytology

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In the last decades there has been a growing pretension to multi-parametric quantitative characterization of clinical and biological samples. With the highest versatility fluorescent assays are suggested for these purposes. Flow cytometry (FCM) and laser scanning cytometry (LSC) are used in the practice, but they need substantial primary investments that may be difficult to afford for small institutions and poorer countries. Computer aided scanning fluorescent microscope (SFM) with motorized object desk and digital camera could be a powerful competitor for them. This device is less cost intensive and needs only to be adapted to a present fluorescence microscope.

Aim of the study was to compare the analytic accuracy (finding of tumor and mononuclear cells in a dilution series) by an SFM program developed by the Semmelweis University running on a commercial microscope to that of the LSC and the FCM.

Materials and Methods: HT29 colon cancer cells stained by HEA 125 FITC antibody and lymphocytes stained with CD45 PE-TxR were mixed in different proportions (1:1, 1:2, 1:4, 1:8, 1:20, 1:50, 1:100, 1:500, 1:1000) in three replicates. Cell nuclei were counter-stained by Hoechst 33258 and TOTO-3. After drying smears were embedded in Pro-long mounting medium (Molecular Probes) in order to archive the fluorescent dyes for optimal analysis. 2,000-3,000 cells were analysed by SFM (Zeiss) and LSC (Compucyte), 10,000-15,000 cells by FCM (FACScan, BDIS).

Results By each machine the Hoechst and TOTO-3 staining, FITC or PE TxR labeled fluorescent cells were detected to an acceptable degree. Preset and measured frequencies were highly correlated ($r2>0.9$, $p<0.001$) irrespective of the device used for quantitation.

Conclusion These results prove that the SFM software using a commercial microscope and camera provide the same quality of finding rare fluorescence labeled cells as the LSC or FCM. Due to lower investment costs SFM may prove an acceptable adaptation to conventional fluorescent microscopes in order to perform rare cell detection.

Apoptosis and Caspase Activity in Peripheral Blood

Lymphocytes of Patients with Lupus Erythematosus Detected by Flow Cytometry

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Cells undergoing apoptosis can be detected flow cytometrically be a variety of different methods including annexin V assay, disruption of mitochondrial membrane potential ($\Delta\Psi m$), TUNEL Assay, detection of poly(ADP)-ribose-polymerase fragmentation, and sub-G_1-peak for example. Sequential activation of initiator and effector-caspases (cysteine proteases with aspartic acid specificity) is a biochemical hallmark of cells undergoing apoptosis. Commonly used techniques for the detection of activated caspases in different cell systems are western blot analysis, colorimetric and fluorometric assays. These are time consuming methods and additionally have the disadvantage that a simultaneous labelling with specific mAbs is not possible. To overcome these problems in the present preliminary investigation we used covalently to rhodamine 110 [$(L\text{-}Asp)_2$-rhodamine 110] bound aspartyl compounds as pan-caspase substrate. The complete complex is colorless, but after caspase-induced cleavage the fluorochrome is released and can be detected either by flow cytometry, fluorescence microscopy or laser scanning microscopy (excitation 488 nm, emission 515 - 545 nm). Since patients with lupus erythematosus display an increased in vivo frequency of blood cells undergoing apoptosis, we analyzed peripheral lymphocytes of these patients. Freshly obtained whole blood samples were compared to density gradient centrifugation isolated PBMC. Additionally, apoptosis was measured by using the annexin V assay.

We could find caspase activity in peripheral lymphocytes of patients with LE as compared to healthy controls with this technique. $(L\text{-}Asp)_2$-rhodamine staining was possible in PBMC as well as in whole blood samples. Annexin V Labelling of membrane phosphatidylserine exposure did not correlate with $(L\text{-}Asp)_2$-rhodamine cleavage. Taken together, the obtained results show that $(L\text{-}Asp)_2$-rhodamine 110 is an interesting tool for the flow cytometrically based investigation of pan-caspase activity, because (i) it is a rapid method suitable to screen pan-caspase activity on single cell level, and (ii) it can be used with simultaneous mAb staining. Further investigations to confirm this observation and clarify it in more detail are currently underway.

Growth Factors and Herceptin Show Different Effects on c-erbB-Receptor Activation and Interaction and Cause Different Cell Cycle Kinetics in Breast Cancer Cells in spite of c-erbB2 Overexpression

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Aims: The c-erbB2 receptor targeted antibody therapy Herceptin has brought clinical benefit to reast cancer patients. Nevertheless, the clinical outcome after antibody treatment is difficult to predict. Beside overexpression of c-erbB2 its interaction to related receptors of the EGFR family is frequently disregarded. Here, we investigated the effect of Herceptin on receptor interaction and related cell cycle kinetics in c-erbB2 overexpressing cell lines SK-BR-3 and BT474. In addition we analyzed erbB-receptor activation by growth factors and Herceptin rreatment.

Methods: In order to analyze the coaggregation of receptors on the cell surface level we used the flow cytometric Foerster-Type-Fluorescence-Resonance-Energy-Transfer (FRET) technique. Phycoerythrin and

Cyanine-5 served as donor and acceptor fluorochrome pair. Receptor interaction were examined under specific growth factor treatment (EGF and HRG) both in the presence and absence of Herceptin.

Results: Specific growth factor application to SK-BR-3 and BT474 induced cell type specific but different c-erbB2 receptor activation and different homo- and heteromeric clusters of erbB receptors. Consequently the effect to cell proliferation was different. Herceptin reduced receptor interaction specifically and in a different amount and inhibited cell proliferation of SK- BR-3 but not of BT474 cells. The effect of Herceptin can partially be compensated by growth factor treatment.

Conclusions: The observed impact of growth factors and Herceptin on breast cancer cells depends on the coexpression of several members of the EGFR family rather than on c-erbB2 receptor overexpression alone. Lateral receptor communication, responsible for the initialization of signal transduction and specific cellular response, is affected in different amounts in c-rbB2 overexpressing cells. Therefore c-erbB2 appears an insufficient marker for cellular response and specific therapy. The improvement and specification of receptor targeted therapeutics requires to take into account the communication amongst receptors in more detail.

The First Phase of the Syngenic Fœtal Organ Implant Development : Crucial and Mysterious Process.

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As it is known from previous works, soon after syngenic implantation into a syngenic adult fœtal organs undergo « destructuration » phase before further « reconstruction ».

Aim: The aim of the present work is to try to understand what happens at this moment and its meaning for the implant as as well as for the host. **Material and methods**. 40 « Fischer » rats received into an ear subcutaneous pouch a piece of digestive organ or heart obtained from 15-20 days old fœti. At day 0 to 10, biopsies were taken from the implants for optic and electron microscopy, host serum was obtained for IGF-1 determination. **Results.** During the first p.o. days both apoptosis and necrosis were observed mainly among differentiated cells. At the 4-5th p.o. days, only isolated and altered enterocytes, acinar cells, cardiomyoblasts or neurons might be found among a mass of uneasily identifiable cells. At this moment, a significant increase of host seric IGF-1 was found. The vascularization of the implant took place in these days. The further « reconstitution » of the implanted organ seemed to follow ontogenetic pattern, though it was not always complete.

Discussion and conclusion. The first « destruction » phase seems to be specific for implants that will recover (it is absent in allogenic grafts without immunomodulation). Destruction seems to affect differentiated cells unable to survive with only diffusion nutrition. At the same time, cell death in the implanted tissues may be a trigger for increasing host IGF-1 production at the days 4-5 p.o. That may, in its turn, enhance the regeneration processes in the implants vascularized by host vessels at this very time. So the first phase after syngeneic implantation of different fœtal organs has common features, is determinant for the future of the implants and implies activity of both host and implant.

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Toluidine Blue Colour Test for Sperm Nucleus Status. Application of RGB Image Analysis

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Sperm nucleus status is an independent parameter of sperm quality and can be tested with structural staining probes. We have reported previously the similarity of results obtained by in situ DNA denaturation test using Acridine orange (AO) with the test using Toluidine blue (TB), elaborated in our laboratory (Erenpreiss et al., 2001), both revealing metachromasia in staining the sperm chromatin with impaired conformation. In the present work, image analysis on 37 samples of human sperm has been applied determining average OD for 300 cells in red, green, blue (RGB) colour filters. The data of image cytometry were verified by spectrophotometry using monochromator and by AO-test of the same samples. OD in green filter showed comparable data with the results of the AO test (r=0.7). We have preliminary determined in absolute OD units the threshold of unequivocally infertile sperm corresponding 30% of the orange-red stained sperm heads in AO test. In turn, the last value has been independently reported by several workers. The results displayed in spectrodensitograms were also analysed in relation to clinical sperm parameters (concentration, motility, morphology) and the cases of discrepancy between AO- and TB-test results. The initial analysis suggests that sperm cell heads displaying in TB staining the high OD value in red filter possess the particularly disordered chromatin structure, which may distort balance between metachromatic and orthochromatic staining in the AO-test.

Probing the Silica Surfaces by Red Blood Cells

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Highly dispersed silicas with modified surfaces can provide selective adsorption of various toxic metabolites that may persist in human organism ("sorption medicine"). However, differently modified silicas may have diverse effects on cell membranes. It is well known that silica of numerous polymorphic forms reacts with red blood cells (RBCs) causing hemolysis. Therefore, perspective samples of silicas to be modified should be tested for their membranotoxicity prior to the clinical use. In our study, the impact of thermally treated and chemically modified Aerosil (fumed SiO_2) on RBCs was examined by optical methods.

Washed human RBCs were used to interact with Aerosil A-300 and silicas prepared from the initial A-300 by dehydroxylation at various thermal conditions. Their light scatter (forward and side light scatter) in 0.01% silica colloidal dispersion was measured uninterruptedly within the first five minutes of the reaction by means of the flow cytometry ("flow erythrogram"). The hemolytic effect of SiO_2 particles was evaluated by photometric measurement of hemoglobin in the supernatant by 90 min of the reaction. The light scatter of affected RBCs in conjunction with the degree of hemolysis revealed that the silica particles with different surface properties had different influences on the RBCs. After thermal treatment, samples, in general, showed the tendency to increase, and then, to decrease their membranotoxic effect with the maximum for the sample heated at 600°C. Thus, the spatial organization of particle swarms and surface hydroxyls is likely to be a critical point in the adsorptive behaviour of silicas.

Finally, we examined the hemolytic properties of silicas modified by $-CH_3$, -RCOOH and $-RNH_2$ groups. The initial A-300, compare to modified silicas, was most hemolytic. The hemolytic activity of "aminoaerosil" was equal to 1/3 of initial A-300 activity. However, silicas modified by $-CH_3$ and -RCOOH groups actually did not cause RBC lysis due to changes in the electrostatic interaction between silica surfaces and cells.

Because of ready availability and high sensitivity to external factors, RBCs offer a convenient model for probing the surface properties of silica. The method of "flow erythrogram" allows one to analyze cell responses on the initial phase of silica-cell interaction.

Comparison of Inhibitory Concentrations IC50 Using the Cell Proliferation Assays MTT and the Flow Cytometric BrdU-Hoechst Technique Applying CDK-Inhibitors

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The analytical techniques applied for high throughput screening or hit verification of chemical compounds record single parameters only. These data give limited information on the real biological function of chemical molecules relevant for therapeutic applications since it does not reveal the complexity of kinetics and the specificity of action on subpopulations or even within the cell cycle. We investigated and compared the usefulness of two different cell proliferation methods: the frequently used MTT microtiter plate assay quantifying the number of of viable cells, and the rarely applied flow cytometric BrdU-Hoechst quenching technique. The proliferation inhibitory effects of the CDK inhibitors Kenpaullone, Alsterpaullone and Indirubin-3'-monoxime were evaluated on a variety of suspension and adherent tumor cell lines. With the flow cytometric analysis a simple linear calculation of the non-dividing versus the dividing fraction displays at any time point of analysis the quantitative inhibitory fraction of cells.

In summary, similar IC50 values were obtained for both the MTT assay and the BrdU-Hoechst flow cytometric analysis. In 22 out of 27 analyses comparable IC50 values for the MTT and flow cytometric BrdU/Hoechst technique were obtained for 9 cell lines and 3 CDK inhibitors tested. The advantages of the flow cytometric analysis, however, are twofold: a) it reveals the specificity of the compound action on the cell cycle compartments, and b) the quantitative and qualitative results are obtained after one day instead of five applying the MTT assay.

Cell Cycle Regulation by the Ah Receptor

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Most, if not all, symptoms of dioxin toxicity and carcinogenicity involve alterations in cell proliferation and they are mediated by the Ah receptor. The Ah receptor is a transcription factor which under non-physiological conditions is activated by dioxin-like polyhalogenated aromatic hydrocarbon ligands (dioxins, e.g. TCDD). Most of the Ah receptor dependently regulated genes code for xenobiotica metabolising enzymes. Genes which could mediate dioxin toxicity are, however, largely unknown. We now used the previously defined model system of the continuously growing 5L rat hepatoma cell line to search for Ah receptor dependent pathways to the control of cell proliferation.

Proliferation is halted in the G1-phase of the cell cycle by TCDD exposure of 5L cells. Biochemical analysis indicates that TCDD-treated 5L cells accumulate at a stage where Rb is hypophosphorylated. Cyclin E dependent kinase activity is dramatically decreased although the protein levels of neither cyclins D/E nor the associated CDKs 2, 4, or 6 are reduced. Amongst the members of the Cip/Kip and Ink families of cyklin-dependent kinase inhibitors (CKIs) selectively p27Kip1 is induded by TCDD. A 4-5-fold increase in protein

levels is associated with a similar increase in steady state mRNA levels. The increase in mRNA levels is the cause for the induced protein levels since TCDD treatment did not alter the rate of protein translation or protein degradation. The increase in Kip1 mRNA appears to be a bona fide transcriptional induction since the mRNA synthesis rate was found increased in a nuclear run-off analysis. Furthermore, a reporter gene comprising 1610 bp upstream of the transcriptional start site in the murine Kip1 promoter suffices to confer TCDD inducibility to a luciferase reporter gene. Kip1 mediates TCDD effects on the 5L cell cycle because Kip1 antisense RNA expressing cells are resistant to TCDD. Induction of Kip1 strictly depends on the Ah receptor since it is not found in a receptor deficient subclone of the 5L cells ($BP8^{AhR-}$) but can be reconstituted by ectopic AhR expression in these cells ($BP8^{AhR+}$).

In cultures of fetal thymus glands (Fetal Thymus Organ Culture, FTOC) TCDD-exposure induces Kip1 and reduces proliferation rates of thymocytes by 30%. To test a role of Kip1 in this process FTOCs were derived from Kip wildtype and Kip-deficient (knockout) embryos. FTOCs from Kip1-deficient embryos were substantially less sensitive to TCDD though not completely resistant. Thus, induction of Kip1 upon activation of the Ah receptor by TCDD is likely to explain at least one aspect of dioxin toxicity, e.g. the thymus toxicicity. Other symptoms like the effect on spermatogenesis or the inhibition of liver regeneration may follow the same pathway but other toxic actions of dioxins like carcinogenicity are not easily explained by the induction of an inhibitor of proliferation.

A systematic search for additional target genes which could mediate carcinogenicity of TCDD identified N-myristoyl-transferase 2 (NMT-2) as TCDD-inducible gene. Metabolic labelling of 5L cells indicates that myristoylation of a subset of proteins is inducible by TCDD presumably as consequence of NMT-2 induction. NMT-2 expression is also induced in the livers of TCDD-treated mice. Since inappropriate protein myristoylation is associated with several forms of cancer it is tempting to speculate that ectopic NMT-2 expression mediates carcinogenicity of TCDD by induction of inappropriate protein myristoylation.

Quantification of Temporary and Permanent Stained Subpopulations of Bull Sperm by an Optimized Sybr-14 / Propidium Iodide Assay

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The quality of bull sperm is one key factor in the field of controlled reproduction. Viability is one important aspect of sperm quality, especially after cyropreservation. We investigated the commonly used Sybr-14 / propidium iodide assay to get additional information about the sperm - dye and dye -dye interactions. After optimizing filter settings, dye concentrations and incubation times we applied the relevant dyes interruption free during kinetic flow cytometric measurements to a mixture of viable and dead sperm. Our data analysis revealed the the following findings:

- there is an essential spectral overlap between Sybr-14 and propidium iodide between 590 and 700 nm.
- the difference of propidium iodide signals from viable and dead sperm is tenfold smaller than from leukocytes.
- Sybr-14 alone stains viable and dead sperm cells.

- the reduction of Sybr-14 fluorescence from dead cells is due to fluorescence quenching caused by propidium iodide induced energy transfer.

- an "intermediate population" betwen viable and dead cells appears within 10 min and disappears between 4 to 7 min later or shifts into the dead population. The extension of this "intermediate population" becomes greater after stress on the cell membrane induced by cryopreservation. Based on this knowledge, we conclude, that the Sybr-14 / propidium iodide assay is a relevant tool to assess the viability of bull sperm.

Low Temperature FISH of Chromosomes Analysed by Scanning Far-Field and Near-Field Optical Microscopy

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A non-enzymatic, low temperature Fluorescence in situ Hybridisation (FISH) procedure working at 37 °C was applied to metaphase spreads and interphase cell nuclei [1]. Denaturation of the chromosomal target DNA by heat treatment and chaotropic agents like formamide was completely omitted. Using the single stranded DNA probe pUC1.77, the staining quality (signal brightness, signal specificity, signal to background ratio etc.) was analysed for direct fluorochrome labelling and antibody labelling by means of confocal laser scanning microscopy (CLSM). An optimum in specificity and sensitivity was obtained for 15 hours hybridisation time. For these conditions, the chromosomal morphology was analysed by scanning near-field optical microscopy (SNOM) [2]. The results were compared to the morphology of chromosomes subjected to centromere labelling using the same specimen and probe preparation procedure but with heat and formamide treatment of the target. Furthermore, a comparison to centromere labelling according to a standard FISH protocol was done. The topographic (scanning force), light transmission and fluorescence near-field images obtained by SNOM simultaneously for each chromosome, differed substantially with the applied FISH procedure. The results indicate that for certain DNA probes specific chromosome labelling can be obtained without the usually applied heat procedure for denaturation of the DNA target resulting in a different chromatin morphology as visualised by SNOM.

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[2] Hausmann et al. (2001) Microsc. Anal. (Europ. Ed.) 5/2001: 13 - 15

Control of Cell Proliferation and Survival by the Transforming Proteins of Human Papilloma Viruses

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Human papillomaviruses (HPV) of the high-risk group, e.g. HPV-16, can immortalize and transform mammalian cells, including primary human cells, and thereby contribute to several proliferative disorders in humans, including cancer of the cervix. Apparently, the interaction of viral proteins with cellular growth-regulatory pathways leads to cell transformation. Our studies are focused on better understanding the role of the HPV-16 E7 gene in tumorigenesis..

It was shown that expression of E7 in mammalian cells overrides several cell cycle checkpoint controls, mainly at the G1/S boundary, and this activity depends on the physical interaction of the E7 protein with several regulators of the cell cycle. E7 also modulates cell survival by the interference with control elements in one or several apoptotic pathways; however, the detailed mechanisms of apoptosis control by E7 remain to be elucidated.

Using a yeast two-hybrid approach, we have isolated cDNA clones corresponding to the products of several human genes, including the gene encoding IGF-binding protein 3, a known regulator of apoptosis in mammalian tumor cells. We also identified several metabolic enzymes that are bound by E7, and obtained evidence that binding of E7 modulates the activity of M2 pyruvate kinase (M2-PK), a key enzyme of glycolysis. M2-PK activity is also modulated by other oncoproteins, including v-src and ras, suggesting that targeting of M2-PK by oncogene products may play a role in the control of cell proliferation.

Since the interaction of viral oncoproteins with cellular target proteins is critical for viral pathogenesis, a careful analysis of these interactions is required for a better understanding of virus-associated malignancies. We have used the peptide aptamer approach to design molecules that can antagonize certain aspects of E7 function; these experiments may lead to the development of new therapeutic approaches for HPV-associated diseases

Automated Micro-Axialtomography for Quantitative Analysis of the Genome Organisation

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To study the three dimensional organisation of the genome, it has been well established to analyse the topology of FISH labelling sites in intact cell nuclei by precise distance measurements using 3D confocal laser scanning microscopy. Unfortunately, this is time consuming for larger amounts of cell nuclei as

required for statistical reasons. In a more straight forward procedure 2D imaging can be applied in an epi-fluorescent microscopic setup. Under these conditions, however, the axial resolution does not allow for precise measurements.

Here, we present a new micro-mechanical device in the dimensions of an object slide miniaturised according to the principle published earlier [1, 2]. This new micro-axialtomograph allows computer controlled, micro-motor driven rotation of precise glass fibres perpendicular to the optical axis of a microscope lens. The cell nuclei are fixed on the fibre surface. A fully automated image acquisition programme registers a series of images during a 90 degree rotation of the cell nucleus in the field of view. From this image series high-resolution information can be obtained. For image analysis a software package based on FISH 2.0 [3, 4] has been developed which allows automatic segmentation of cell nuclei and labelling sites, determination of intensity bary centres and distance measurements between labelling sites and other nuclear coordinates. In addition an appropriate preparation protocol for cell nuclei and FISH on glass fibres has been developed and an advanced micro-preparation device has been constructed. The functionality of the complete setup has been proven for several examples.

[1] Bradl J et al. (1994) J. Microsc. 176: 211 – 221

[2] Bradl J et al. (1996) Microscopy & Analysis 44 (Europe) November 96: 9 – 11

[3] Kozubek M et al. (1999) Cytometry 36: 279 – 293

[4] Kozubek M et al. (2001) Cytometry 45: in press

APC Subsets Involved in Peptide Presentation during Induction of Tolerance versus Immunity

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Application of antigens to a naive animal may result in specifc T cell immunity or tolerance, depending on the mode of antigen application. We have developed a highly sensitive cytometric assay to directly assess peptide presentation ex vivo on the single cell level. To test the hypothesis that different APC are critically involved in the initiation of these differential reactions we have quantitated and phenotypically analyzed those APC which present physiologically relevant quantities (>100-200 peptides/cell) of a MHC class II restricted peptide following intravenous or subcutaneous/CFA injection of peptide. We can show that both ays of immunization result in antigen presentation by B cells and dendritic cells (DC). However, when comparing the kinetics and organ distribution of presentation and the phenotype of peptide presenting DC striking differences were observed. I.v. injection resulted in rapid but transient (6-12h) peptide presentation in spleen and all lymphnodes. Most of the peptide-presenting DC showed only weak expression of costimulatory molecules like B7.1/B7.2/CD40 but also high expressing cells were found to present the peptide. In contrast, peptide presentation following s.c. injection of antigen in CFA was almost completely restricted to the draining lymphnodes where the peptide-presenting DC had high costimulatory capacity: Additionally, s.c./CFA application resulted in a prolonged presentation up to 72 hours. These data suggest that tolerance is not induced by specialized APC. Instead, the total number of APC with low costimulatory capacity as well as the time of APC T cell interaction might be critical parameters.

Cell Cycle Dependent Variations in Transcription Factors Phosphorylation

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Mitosis is associated with chromosome condensation, transcription arrest and nuclear envelope breakdown in higher eucaryotes. These events certainly facilitate the correct segregation of chromosomes to daughter cells. This will be also the opportunity to yield two different daughter cells during asymmetric cell division. On the other hand identical daughter cells will have to reassemble the correct nucleoprotein structures on their chromatin to maintain cell memory. Our laboratory has followed the G2 to M transition in mouse and human cells to try to understand how the transcripional machinery is silenced during mitosis and reactivated in the next G1 phase.

Studying the SWI/SNF chromatin remodelling machine we observed that the two alternative helicases of these complex Brm and Brg1 are phosphorylated late in G2 . Phosphorylation is associated with the dissociation of the complex from nuclear structures and from the chromatin. In addition phosphoylated Brm is degraded during this transition. The complex re-enters the nucleus after Brg1 dephosphorylation while Brm is resynthsized in the following G1 phase(Muchardt et al EMBO J. 15,3394,1996). Further studies by the group of R. Kingston have shown that the phosphorylated complex is enzymatically inactive (Sif et al Genes Dev 15,2842,1998). In addition to the phosphorylation dependent inactivation of a chromatin remodeling complex it was shown that several of the RNA polymerase II general transcription factors including TBP are phosphorylated during mitosis (Segil et al, Genes Dev ,10:2389,1996).

However mitotic related phosphorylation of transcription factors is in fact a much more frequent event. Studying the sub- units of the AP1 transcription complex composed of heterodimers between Jun, Fos and ATF proteins we have shown that all three Jun proteins:JunB, JunD and c-Jun are phosphorylated during the G2/M transition. At least for JunB we could reproduce the pattern of phosphorylation with pure Cdc2-CyclinB mitotic kinase. Phosphoylated JunB is triggered for degradation probably by the Ubiquitin-Proteasome pathway. Mutations of serine or threonine acceptor sites inhibit the phosphorylation and stabilise the protein. On the contrary to JunB, c-Jun is phosphoylated on serines 63 and 73 that do not change the stability of the protein but increase its transcriptional activity. The change in the activity and concentration of the different Jun proteins seems to play a role in driving the next cell cycle. In fact we could show that while c-Jun and even more phosphorylated c-Jun are strong activators of the Cyclin D1 promoter JunB represses its transcription. The change in the ratio between cJun and JunB in the beginning of G1 will generate a wave of Cyclin D1 transcription essential for G1 progression. These studies illustrate a novel inter- dependence between the cell cycle machinery and transcription factors (Bakiri et al EMBO J. 19,2056,2000).

Reflection Microscopy for Radioactive In Situ Hybridization on Hard Keratinized Structures"

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Commonly, the detection of radioactive In Situ Hybridization (ISH) signals are visualized by darkfield microscopy combined with bright field optics on a conventional microscope. This method, however, has serious drawbacks. Optical disturbances in the dark field are methodologically caused by the sensitivity on dust particles and, in particular, by breakage of light on structures tightly composed of proteins. By investigating the expression of hair keratins, we found that these disturbances are dramatically caused by the inherent structure of the hair fibres, which are composed of densely arranged hair cells (trichocytes) and filled with masses of interconnected filaments, as well as cells producing melanin. On those specimens ISH signals often could not be clearly correlated to their respective cellular structure, especially when only rather faint signals existed. To eliminate these optical disturbances, confocal laser scanning microscopy (LSM 510 UV, Carl Zeiss, Germany) was used. This instrument allows simultaneous visualization of reflection signals of radioactive ISH in epi-illumination and transmitted light in the bright field for hematoxyline staining. Both images were taken with a He-Ne laser operating at a wavelength of 633 nm. The transmission image was done in the green channel and electronically changed into black/white using the LSMib-software (Carl Zeiss), whereas the reflection image was done in the red channel (false colours). The two signal channels were combined with an overlay. On this base the pattern of gene expression (ISH on reflection) - even of rather weak signals - could be clearly seen and precisely correlated to its corresponding cellular compartments (HE staining on bright field). Moreover, a combination of ISH with immunofluorescence was also possible. In principal, this technique of reflection microscopy can also be done using a conventional microscope (epi-illumination of polarised light for reflection image) and transmitted illumination for hematoxyline staining, combined with a CCD recording system.

Detection of Allo– and Autoreactive Antibodies in Patients with Protein Losing Enteropathy (PLE) in Fontan Patients by Flow and Laser Scanning Cytometry (LSC)

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PLE is a feared complication 5-10yrs after Glenn/Fontan type of cardiac surgery with 5–15% of the patients exhibiting a substantial decrease of serum protein and an increased secretion of protein in the stool. The mortality among patients with a manifest PLE is >60% but its aetiology is yet completely unknown. In a follow up nine patients after Fontan surgery were immunologically analysed over a period of five years by flow cytometry (FCM) and serology. The immune sequel of one patient who developed PLE 9 months after surgery was compared to that of seven patients with a manifest PLE after Fontan and healthy controls.

There is a high correlation in the immune alterations after PLE, which includes the dramatic selective loss of >80% of the circulating T-helper (CD3+4+) cells. In our essays we tried to find the rational for the selective cell loss. With an FCM based assay we found in the serum of 25% of the PLE patients antibody binding to leukocytes, especially to T-helper cells. Serum of PLE free Fontan patients and of healthy controls was negative. We developed different assays for the LSC to quantify binding of autoantibodies to cells and tissues. In 25% of the patients with manifest PLE we found antibodies against myocardial structures. None of the PLE patients but one of the Fontan patients without PLE had antinuclear antibodies. Because of these results we hypothise that autoimmunity is at least participated in the aetiology of PLE.

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Combined in Vitro Chemosensitivity Testing and Subcellular Localisation Studies to Assess New Hematoporphyrin-Derived Platinum Conjugates

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Porphyrin-platinum conjugates are conceived as a new approach in cancer therapy by combining the cytostatic activity of cisplatin (one of the most widely used chemotherapeutics) and the photodynamic effect of hematoporphyrin derivatives (already established as photosensibilisators in photodynamic therapy) in the same molecule. Analysis of cytotoxicity combined with the investigation of subcellular localization should help to elucidate the mechanism of action of these new compounds.

Four platinum complexes differing in solubility and type of platinum fragment (derived from cisplatin and oxaliplatin) and the corresponding porphyrin ligands (leaving groups) were studied on low-differentiated J82 cells, a model of invasive bladder cancer. The anti-proliferative potential of the compounds was determined in a crystal violet-based chemosensitivity assay. Phototoxicity was assessed by irradiating the cells with an incoherent light source (λ = 600 – 730 nm) at a light dose of 24 J/cm^2. Intracellular localization was identified by fluorescence microscopy due to the intrinsic fluorescence of the leaving groups and costaining of cellular organelles.

Without irradiation the complexes (0.5 µM, 1 µM, 5 µM) showed anti-proliferative effects similar to cisplatin in the chemosensitivity assay, whereas the porphyrin ligands were inactive at a concentration of 0.5 µM and 1 µM. After irradiation the conjugates (0.5 µM) exhibited a synergistic effect compared to cisplatin and hematoporphyrin alone or a combination of the drugs.

Interestingly, only the water soluble porphyrin-platinum conjugates were diffusely distributed in the cytosol and associated with the cell membranes, in particular with those of the lysosomes.

In conclusions, porphyrin-platinum conjugates, especially the water soluble species, are promising candidates for a development of a novel type of selective photosensitizers with intrinsic cytotoxicity.

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The Scanning Image Cytometer MetaCyte: New Features and Applications

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The scanning image cytometer MetaCyte completely automates fluorescent cell analysis. It combines automatic slide scanning, automatic low light level image capture in up to six simultaneous color channels, focus stack imaging, multiple feature measurement, and quantitative image analysis. Due to its flexible design the system is easily adaptable to a variety of applications. In addition to high throughput interphase FISH analysis in cell suspensions and tissue sections we will present fully automated Comet (single cell gel electrophoresis) assay analysis. The principles of operation as well as results of case studies will be discussed.

Monitoring the InVivo Differentiation of Antigen-Specific T Cells Following DNA Vaccination

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Vaccination with DNA coding for the antigen of interest is a versatile tool to induce cellular and humoral immune responses. To optimize vaccination strategies and to analyze the modulation of the specific immune response by certain vectors or vector combinations we have established a transfer system for direct analysis of antigen-specific T cells *ex vivo* following vaccination with DNA or antigens in adjuvant. Purified naive OVA-TCR-transgenic T cells were labelled with CFDA and transferred into congenic Balb/c recipients. Following immunization reactive cells can be identified according to transgenic TCR expression and loss of CFDA fluorescence upon cell proliferation. These reactive cells were directly analysed for the expression of phenotypic markers or the production of cytokines and chemokines upon restimulation *in vitro*. This system will give direct information on the differentiation status and the effector function of specific T cells *in vivo* in response to different immunization protocols. Based on these direct read-out of antigen-specific cells it will be possible to modulate vaccination strategies to obtain optimized immune responses.

True Platelet Counts and Reticulated Platelets as a Measure of Thrombopoietic Activity

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Analysis of low platelet counts in thrombopenic disorders and the question of their reconstitution is a demanding clinical problem. Hematology analysers show problems esp. interferences in cases with helmet cells (e.g. hemolytic uremic syndrome), microcytosis or macrothrombocytosis. Microscopic counting with a hemocytometer also shows enormous variation because of low counts (10 platelets per chamber for 10,000 plt/µl) or morphological changes (e.g. destruction in ITP) and requires considerable experience. We therefore developed a flow cytometric method for immunological counting of platelets and a new stable method to analyse young platelets showing a high RNA content. These are called reticulated platelets in analogy to their red cell counterparts, the reticulocytes. For counting purposes thrombocytes are stained with CD41- FITC, then red blood cells are lysed and diluted by addition of 2ml lysing reagent. The test tube also contains fluorescent microbeads of known concentration to calibrate the measured sample volume. Reticulated platelets can be stained with RNA specific dyes like thiazole orange as described by RINDER et al., however there is a continuous increase of staining intensity over time because of unspecific dye uptake in the platelet granules. After degranulation and staining for 1.5 hours a stable end point is reached resulting in a narrow range of normal values (2 +/- 0.5%). Increased values can be obser- ved in cases of low platelet counts to differentiate immune thrombocytopenia from reduced megakaryopoiesis. In patients with elevated platelet counts there are also cases of normal and elevated reticulated platelets where the clinical relevance is not yet clear. Examples will be given of practical use of that new technology.

Flow Cytometric Analysis of Leukocyte Chemotaxis

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Flow cytometry has been successfully applied in the analysis of leukocyte function like phagocytosis, oxidative burst , cytokine secretion and apoptosis in basic and clinical research as well as for diagnostic purposes. However, chemotaxis was restricted to microscopy and manual counting due to the contruction of the classical boyden chamber. We dissected the process of leukocyte migration and found an adhesion molecule (L-selectin), shape change and transmigration to be critical steps. We used a combi- natorial approach to cover the chemotaxis phenomenon and found these steps to be independently affected in patients. Most frequently L-selectin becomes downregulated in septic patients in the blood vessel before emigration into the inflamed tissue. In healthy adults and children it seems to be a robust process. Test design, normal values, chemotactic agents and some case reports will be presented.

RINGO, a New Family of Cell Cycle Regulators that Directly Activate Cdk Protein Kinases

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We have cloned a novel protein that can trigger meiotic G2/M progression in *Xenopus* oocytes in the absence of progesterone stimulation. We named this protein RINGO for Rapid INducer of G2 /M progression in Oocytes. RINGO also induces pre-MPF activation and germinal vesicle breakdown, one of the markers of meiosis I entry, in cycloheximide-treated oocytes. Conversely, antisense-directed ablation of endogenous RINGO mRNA inhibits progesterone-induced maturation. Endogenous RINGO accumulates upon progesterone stimulation and peaks at about the time of meiosis I. RINGO can bind and activate the kinase activity of Cdc2 and Cdk2, but has no amino acid sequence homology with cyclins. Moreover, overexpression of a catalitically-inactive Cdc2 mutant can block RINGO-induced oocyte maturation. These results suggest that the activation of Cdc2 and/or Cdk2 by RINGO is important for the meiotic G2/M transition of oocytes. In contrast with the activation by cyclins, activation of both Cdc2 and Cdk2 by RINGO does not require the phosphorylation of the conserved threonine on the activation loop. RINGO-bound Cdc2 and Cdk2 are also more resistant to negative regulation by inhibitory phosphorylations and by association with CKIs. The ability of Cdk/RINGO complexes to be potentially active under conditions where cyclin-bound Cdks are inhibited, suggest that they can play different regulatory roles. We have also cloned a family of mammalian RINGO proteins, which can all bind and activate Cdk protein kinases.

Novel Fluorescent Beads and Novel Cytometric Immunoassays

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Fluorescent microspheres have been shown to be superior to radioactive beads in biological applications. We present novel fluorescent beads for laser excitation, novel fluorescent nanobeads, and a cytometric immunoassay based on such beads.

The beads are dyed with new lipophilic fluorophores that absorb and emit in the longwave part of the visible spectrum and are excitable with either lasers or diode lasers (Table 1). The dyes are fully incorporated into the particle, not only on its surface. Hence, the beads are very bright and this lowers the detection limit.

Table 1. Absorption and emission maxima (in nm) of the lipophilic dyes in ethanol solution

Dye	*?max (abs)*	*?max (em)*	*laser*
LR530	536	574	krypton ion (528 nm) frequency-doubled Nd:YAG (532 nm)
LP550	556	592	frequency-doubled Nd:YAG (532 nm) helium/neon (543 nm)
LB645	644	665	krypton ion (647 nm)
LB630	633	653	diode (635 nm)
LB635	631	647	helium/neon (633 nm)
LG670	671	692	diode (665 nm)

We have also performed a cytometric immunoassay based on beads. Beads (from Luminex, 5.4 µm) were loaded with HSA (via NHS coupling). Anti-HSA was labelled with Fluorescent Orange 548 (FO 548, a new reactive label) in bicarbonate buffer following a standard labelling protocol. The beads were incubated with the antigen solution, washed and submitted to flow cytometry. HSA is detectable in the 0 - 150 mg/L concentration range (Fig. 1).

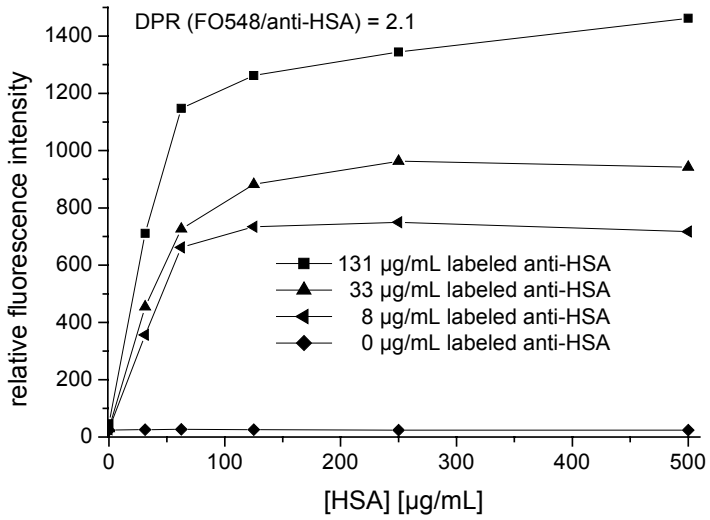

Fig. 1. Cytometric immunoassay on PS beads

We also have prepared new PAN nanobeads with a size of ~ 40 - 100 nm. These were applied to fluorescence resonance energy transfer (FRET) assays. Due to their extremely small size and high dye-loading they may be used for effective labelling of proteins, beads, and cells. A variety of longwave emitting and of long decaying beads were synthesized. They are not quenched by oxygen. The particles display high photostability and brightness. The large Stokes shift of some of the dyes allow measurements with minimal straylight from the light source. If gated detection is available, most of the background fluorescence can be eliminated, thus improving the signal-to-noise ratio.

Recruitment and Conformational Modulation of GPIIb/IIIa within Rafts on Platelets

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Disturbances in lipid metabolism which are associated with hypercholesterolemia are characterized by increased *in vivo* activation of thrombocytes. It is tempting to speculate that functional receptor co-operation within cholesterol and sphingolipid rich membrane domains (rafts) which has been described for several cell types (e.g. T-cells, monocytes) is a regulatory principle of platelet activation. In agreement with this hypothesis an association of Lyn protein tyrosin kinases with the high density lipoprotein (HDL) receptor CD36 has been shown. Furthermore receptors critically involved in platelet activation show oligomerization or co-association in complex 2-dimensional structures as for example the fibrinogen receptor complex GPIIb/IIIa, integrin associated proteins, receptor for van-Willebrand factor GPIb/IX/V or Fc?-receptor II (CD32). The regulation of receptor co-association, however, remains unclear. Finally, drug induced platelet activation or immunogenic neoepitopes have been accused to cause occasional thrombocytopenia.

We, therefore, analyzed the effects of the GPIIb/IIIa antagonist MK-383 (tirofiban) on platelet activation and GpIIb/IIIa conformation. At a concentration of 10^{-7} mol/L, we could confirm a complete inhibition of fibrinogen binding to in vitro stimulated platelets. Simultaneously, the GPIIb/IIIa expression density increased, to a similar extent as on activated platelets. However, no effect on P-selectin expression or the formation of platelet-leukocyte aggregates could be observed, indicating that MK-383 binding did not induce platelet activation. The conformation of the freshly recruited GPIIb/IIIa-complexes was further analyzed by fluorescence resonance energy transfer analysis between pairs of fluorochrome labeled anti-GPIIb/IIIa antibodies. As a result MK-383 induced a receptor conformation that differed from the resting as well as the activated receptor as induced by ADP or TRAP-6.

Furthermore we could identify an activation dependent composition of GPIIb/IIIa related receptor cluster. GPIIb/IIIa is associated with CD36 *ex vivo,* under activation, however, the HDL receptor CD36 is separated from the complex and the Fc-receptor CD32 is included in the cluster. Concomitantly under activation CD32 was associated to an integrin associated protein (CD9). This conformational modulation of GPIIb/IIIa related receptor complex presents an interesting mechanism which may be linked to affect mechanisms of the recruitment and conformational modulation of GPIIb/IIIa without inducing general platelet activation.

Laser-Rupture Induced Ca2+ Oscillations in Non-Excitable Connective Tissue Cells

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In addition to excitable but dormant myocytes non-excitable cells of the connective tissue significantly participate in the organization of cardiac scares which remain from myocardial ischemia and infarcts. Ventricular tissue scares are believed to constitute paroxysmic centers of automaticity by reentry along circular excitation pathways or by focal excitations. Focal excitations coincide with cellular hyperexcitability and an increased cytosolic calcium concentration.

To examine the function of non-excitable scare tissue cells in excitation paroxysms we equipped a confocal microscope with a laser microbeam: A TiSa-laser was coupled into the epi-fluorescence illumination beam path. This way we could rupture the plasma membrane of indivdual cells inside a confluent L929 fibroblast monolayer. The time course of the cytosolic calcium concentration was recorded with Calcium Green1.

Upon laser induced plasma membrane rupture the calcium diffusion along gap junctions in the syncytial monolayer could be examined.

Calcium diffusion measurements qualitatively agree with theoretical estimations of diffusion: The cytosolic concentration increases as faster as nearer located to the point of rupture. Beyond that the diffusion rate depends on monolayer density and on the size of the intercellular contact region. This shows gap junctions being the locations of membrane passage.

In addition to conventional diffusion fibroblasts closely neighbored to the ruptured cell developed calcium oscillations but not calcium spiking. The oscillations resemble the ones known from cardiac myocytes with regard to their shape. They differ in oscillation periods being in range of minutes. The oscillations stabilize the cytosolic calcium concentration near physiologic level.

The repetitive fibroblastic shuffling of calcium into the scare interstitium can induce calcium based hyperexcitability in the remaining cardiac myocytes and induce a paroxysmic transition from dormancy into focal excitations.

Optimization of Three and Four Color Multiparameter DNA Analysis in Lymphoma Specimens

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Aim: Proliferation activity of lymphoma cells is in correlation with survival, and response to therapy. Detection of aneuploidy helps to find the malignant cells without abnormal pattern of expressed antigens. Our goal was to establish a reliable and reproducible DNA content easurement on a benchtop flow cytometer in a well characterized population of lymphoma cells.

Methods: After immunostaining of fresh samples of peripheral blood, bone marrow and cell suspensions of lymph nodes, and an optimized post staining fixation procedure, several DNA dyes including propidium iodide, 7-AAD, To-Pro-3, and DRAQ5 were tested on FACS-Calibur and Coulter EPICS XL flow cytometers. In order to compare the different dyes an overall of 15 gently minced lymph nodes, 21 bone marrow samples, and 28 peripheral blood samples were used.

Results: 7-AAD and To-Pro-3 are not appropriate for clinical application due to either spectral characteristics or low quality of DNA histograms. Propidium iodide on a FACS-Calibur is limited to two color antigen staining. In parallel measurements with propidium iodide, DRAQ5 produced similarly good quality of DNA histograms. Because of excitation characteristics of DRAQ5 it is only applicable to flow cytometers equipped with an Argon laser source. DRAQ5 emits in the far red region, therefore just minimal compensation is needed in case of three as well as four color measurements.

Conclusions: DRAQ5 proved as the only dye to perform reliable multiparameter DNA measurements in combination with two or three color antigen staining. DRAQ5 enabled us to perform DNA measurements in as low as 4-5% lymphoma infiltration in bone marrow specimens without prior enrichment of B-cells. DRAQ5 is a promising new dye for multiparameter DNA measurements in the clinical routine.

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Magnetofluorescent Liposomes for High-Sensitivity Multicolor Immunofluorescence

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Magnetofluorescent liposomes conjugated to specific antibodies increase the signal-to-noise ratio of immunofluorescent labelling up to 1000fold compared to conventional methods. The high signal intensity allows the clear-cut detection of <200 molecules per cell. Furthermore, liposomes can be used as universal carriers for new hydrophilic as well as lipophilic dyes trapped either in the aequous liposome core or the lipid membrane. The high number of dye molecules included in a single liposome allows to use fluorochromes even at suboptimal excitation or emission wavelengths. We have generated liposomes containing Cy2, Cy3 and Cy5, which can be used as high-sensitivity reagents for multicolor analysis in standard flow-cytometers. The application of UV-excitable dyes for immunofluorescent applications is limited by the high cellular autofluorescence in this spectral area. We show here, that several UV-excitable dyes encapsulated into liposomes can be used as efficient reagents for multicolor immunofluorescent labelling uing UV-Laser excitation.

In summary, we show that magnetofluorescent liposomes can be used for high-sensitivity multicolor applications and that they can expand the spectral area and the number of fluorochromes available for immunofluorescent labelling approaches.

A Novel Single Tube Assay for the Simultaneous Quantification of Residual Leukocytes, Red Blood Cells, and Platelets in Fresh Frozen Plasma by Flow Cytometry

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European recommendations and German national guidelines define frequency of control and quality specifications of fresh frozen plasma (FFP). Counting of residual cells is performed before freezing the plasma. Cells should not exceed 6,000 red blood cells (rRBC), 20,000 platelets (rPLT) or 500 leukocytes (rWBC) per microliter (leukocyte depleted FFP: <1x106 rWBC/plasma unit).

Residual cells are stained in a TruCOUNT tube using thiazole orange as nucleic acid dye for rWBC. Monoclonal antibodies CD41a PerCP-Cy5.5 and anti-glycophorin-A FITC are used for labeling rPLT and rRBC. Fixation, permeabilizing or washing steps are not required.

Validation was done according to ICH guidelines and NCCLS EP10-T2. Cell free plasma was spiked with each cell type. Additionally, quality control samples from routine production were analyzed. Results were

compared with cell counts obtained by Nageotte (rWBC) and Fuchs-Rosenthal (rRBC) counting chambers or by BD LeucoCOUNT using a FACSCalibur flow cytometer.

Validation showed no carryover or drift. Unspecific background was <0.8 cells/µl for rWBC and rRBC, and <40 cells/µl for rPLT. Determinations of rWBC and rPLT counts were linear, imprecision ranged from 6-12%. Linearity and precision for rRBC diverged at concentrations >3,000 RBC/µl due to cross-linking by the divalent anti-glycophorin-A antibody, which can, alternatively, be replaced by monovalent Fab.

Conclusion: Residual cell counting in FFP by flow cytometry was successfully validated. This simple and reliable single tube assay can therefore replace time consuming and labor intensive manual microscopic counting.

CyFlow – Plant and Microbial Applications for an Ultracompact Multiparameter Flow Cytometer

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Within the last years the interest to use flow cytometry for determination of nuclear DNA amount in plants has permanently increased. In all these cases conventional flow cytometers suffer from being too expensive, service intensive for application like ploidy analysis, detection of plant hybrids, apomixis, effects of endoreduplication and the determination of genome size where only one or two fluorescence parameters are needed in addition to FCS and SSC parameters. The concept of the CyFlow offers here an alternative solution: Staining with propidium iodide and optimal excitation with the 532 nm line of the DPSS laser (CyFlow green) result in high resolution measurements of DNA with low CV values between 1,5 – 2,5 %.

Furthermore the performance for bacterial analysis and counting will be demonstrated on several examples.

Due to the progress in component development and the availability of powerful and robust solid state lasers the new compact flow cytometer, which has the size of a regular PC, only, was developed. Forward and side scatter analysis together with up to 3 fluorescence parameters can be measured with realtime data acquisition by using the Windows-based FCM software FloMax. The CyFlow allows to perform true absolute (volumentric) cell counting based on scatter signals after cell labelling with fluorescent dyes or antibodies and is therefore the perfect device for bacterial and cell counting in a size range of 0.2 µm to 50 µm. With this detection limits the scatter sensitivity of the CyFlow is as least as sensitive as conventional FCM devices run on large air or water cooled lasers.

It replaces the traditional cell counters because of higher specifity and accuracy as well as the full absence of clogging problems, the major limiting factors while counting small particles, platelets and bacteria using impedance counting principles.

Four different excitation wavelengths: a red laser diode (635 nm), DPSS lasers green (532 nm) and blue or a violett laser diodes (395 - 410 nm) will allow to perform all those measurements, too, which have been performed with large air cooled lasers up to now. The CyFlow runs on 12 V DC power (e.g. on a car battery) and therefore does not depend on a stabile regular power supply which might be important for remote places in field sudies, scientific expeditions or places which are close to fermentation processes.

Enzymatic Amplification Staining for Flow Cytometry

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Flow cytometric analysis of surface membrane molecules is a well-accepted technology. A major limitation of flow cytometric analysis has been the poor sensitivity of the technique. Dr. Kaplan has developed an enzymatic amplification procedure for the analysis of cell surface molecules by flow cytometry. In order to enhance the sensitivity of flow cytometric analysis of cell surface molecules on live cells, Dr. Kaplan adapted a catalyzed reporter deposition procedure. This technology has the potential to amplify the signal with enzymes that catalyze the deposition of labeled molecules on to the cell surface. Although the label binds covalently to any cell surface protein, the deposition is specific because it is proximity controlled by specific antibody-dependent binding to a targeted cell surface molecule. The EAS technology increased the fluorescence signal between 10 and 100-fold for all surface molecules tested. Conclusions: Enzymatic amplification staining produces a significant enhancement in the resolving power of flow cytometric analysis of cell surface molecules.

Towards Automated Mulicolor Immunohistology

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Multicolor tissue cytometry based on the KS400 image analysis environment, which we named Automated Fluorescence – Confocal Laser Scanning Microscopy [AF-CLSM, JIM 2000, 237:39-50] was developed. The triple staining results obtained show a highly significant correlation to flow cytometry and visual analysis. However, using AF-CLSM for

automated recognition of individual CD45+ leukocytes in renal tissue the standard identification strategy (SIS) meets its limitation with the identification of extremely clustered cell populations. In other words manual counting identified twice as many CD45+ and CD3+ cells as did counting with AF-CLSM/SIS. Therefore, new advanced identification strategies (AIS) for automated recognition of individual cells were developed and tested. Using novel AIS the automated recognition rate of CD45+ cells in renal tissue could be increased by 65% when compared to SIS and a mean of 68.1% of manually counted cells were identified. When DNA staining was included the mean recognition rate reached 95.2% of visually identifiable cells. Furthermore, depending on the antibody combination used this method allows an automated simultaneous analysis of the nuclear, cytoplasmatic and membranous staining intensities of identified cells. In summary, AF-CLSM allows automated multicolor analysis of cells in tissue and the generated information is comparable with that of flow cytometry except that cytometry adds the information about the location within the tissue and the cell as well.

Methods for Four-Colour Immunophenotyping of Tissue Sections by Laser Scanning Cytometry (LSC)

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In lymphatic organs the quantitative analysis of the spatial distribution of leukocytes would give relevant information about alterations during diseases (leukemia, HIV, AIDS) and their therapeutic regimen. Analysis of them in solid tissues is difficult to perform but would yield important data in a variety of clinical and experimental settings. We have developed an automated analysis method for LSC suitable for archived or fresh biopsy material of human lymph nodes and tonsils. Sections are stained with PI for DNA and up to three antigens using direct or indirect immunofluorescence staining. Measurement is triggered on DNA-fluorescence (Argon Laser). Due to the heterogeneity in cell density measurements are repeatedly performed at different threshold levels (low threshold: regions of low cellular density, germinal centers; high threshold: dense regions, mantle zone). Data are acquired by single- (Ar) or dual-laser excitation (Ar-HeNe) in order to determine from single- (FITC), up to triple-staining (FITC/PE-Cy5/APC). Percentage and cellular density of cell-subsets is quantified in different s tructural regions of the specimen. Comparison with manual analysis of identical specimens were highly correlated ($p<0.01$). With LSC a semi-automated operator-independent rapid and immunophenotyping of lymphatic tissues with simultaneously up to four antibodies is possible. This technique should yield new insight into processes during diseases and should help to quantify the success of therapeutic interventions.

Potential of Cytomics for Complication Prediction in Allogenic and Autologous Stem Cell Transplantation (SCT)

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Goal: The early recognition of upcoming complications like recurrent cytomegalovirus infections (CMVI) or chronic graft versus host disease(GvHD) after bone marrow SCT is of high importance for successful overall therapy. Cytomics as multiparameter cytometric analysis of cellular heterogeneity are particularly useful in this context.

Methods: Peripheral blood leukocyte 3-color immunophenotypes (FITC/PE/CY5, lyse no wash) CD3/16+56/45, TCRab/CD8/45, TCRgd/CD4/45, CD57/HLA-DR/TCRab, CD8/95/TCRab, CD45RO/27L/4, CD45RO/62L/4, then CD8/69/TCRab following mAb CD2&2R stimulation for CD69 induction, as well as intracellular (ICx) IFN-g, IL-2, IL-4, TNF-a, IL-13, CD69 determined as TCRab/ICx/CD8 following phorbol ester (PMA)+ionomycin stimulation were analyzed by flow cytometry in 80 patients (43 allogeneic/37 autologous) at 2,3,4,6,9,12,18,24 months post SCT including 117 healthy donors as controls. Cell frequency as well as mean intensity, intensity ratios and average packing density of antigens were calculated by quadrant analysis from FSC/SSC autogated lympho-, mono-, and granulocyte FITC/PE, FITC/CY5 and

PE/CY5 histograms containing altogether >95% of the collected cells of each measurement. The resulting 3.330 data colums per patient were classified (CLASSIF1, http://www.biochem.mpg.de/valet/classif1.html) for predictive information with the results at 2months post SCT serving as reference for the prediction of subsequent clinical complications.

Results: Complication free recovery (CFR), recurrent CMVI, chronic GvHD and survival were correctly predicted in >95% of the cases. Recurrent CMVI was predicted by changes of CD3/8/27/45/95 and chronic GvHD changes of CD4/8/45RO/62L/IL-2/TCRab and FSC. It was furthermore possible to simultaneously predict CFR, CMVI, GvHD, CMVI+GvHD.

Complication indicators were similarly increased or decreased for all complications while complication discriminators were differently affected. Complication indicators were e.g. increased CD4 and CD8 expression at decreased TCRab and TCRgd positive cell populations. Complication discriminators were CD3/8/16+56/69/TCRab/HLA-DR/IFN-g/FSC. Ultimate non survivors showed early on increased FSC levels e.g. on IFN-g+, IL-2+, IL-4+, TCRab+, CD69- or HLA-DR- lymphocytes.

Conclusion: The exhaustive analysis of the antigenic and light scatter information of peripheral lympho-, mono- and granulocytes provides standardized classifiers for the early predictive identification of SCT risk patients. The predictive parameter patterns are of interest for understanding the cellular pathogenesis of the various clinical complications.

The Mobility of Biomolecules in the Cell Nucleus

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During the interphase of the cell cycle numerous biological processes take place in the nucleus: transcription of active genes, splicing and processing of the synthesized RNA molecules, or the replication of the whole genetic material. All these processes require an efficient transport of molecules of various sizes within, into, and out of the nucleus. However, the three-dimensional arrangement of chromatin, functional and structural components is still quite unknown. Does the nuclear architecture play a regulatory role in such a way that a geometrical rearrangement promotes or impedes the mobility of molecules of a certain size? In addition recent studies have shown that a lot of apparently static cellular structures show a very dynamic balance of the constituents between free and bound states.

In order to investigate molecular mobilities and affinities to immobilized structures with a diffraction-limited resolution we have developed a confocal laser scanning and positioning system, the fluorescence fluctuation microscope (FFM), that allows the acqusition of conventional confocal fluorescence images on the one hand and the positioning of the laser in the sample with an accuracy below the optical resolution on the other hand. Using this setup, we can carry out fluorescence correlation spectroscopy (FCS) and fluorescence loss in photobleaching (FLIP) measurements at well defined positions in living cells which can be selected in confocal images. FCS makes accessible the diffusional behaviour and the concentrations (and in addition intramolecular processes, e.g. triplet state kinetics) of fluorescently labelled particles on the single-molecule level. In a rather complementary approach, with FLIP, the parameters of a binding equilibrium like the free and the bound fraction and the rate coefficients can be obtained. One major advantage of this setup is the use of the same optical path both for confocal imaging and for FCS/FLIP. Therefore the image signal can be used directly for concentration mapping.

Brownian motion in a living cell is vitally influenced by membranes, organelles, or statistically organized polymer systems like chromatin. Therefore we have adapted the theoretical basis of FCS and FLIP to the intracellular environment, taking into account geometrical restrictions as well as binding to the structures mentioned above.

In a first set of experiments the diffusional behaviour of inert molecules of different sizes as a function of the position in living cells has been investigated with FCS. Showing a faint variation with cell line and molecule size, the "microviscosity" has been found to be approx. 4 times larger than in aqueous solution everywhere in the cell. Especially in interphase nuclei we have observed a remarkable deviation from free Brownian motion that we have quantified in the framework of anomalous diffusion. With cells expressing Histone-AFP (autofluorescent proteins) chimera we have been able to map the diffusion obstruction as a function of chromatin density. This provides a first evidence that the influence of chromatin on the molecular mobility depends on the molecular size.

And we have investigated the mobility of functional proteins exemplified by the transcription termination factor TTF-I. In fluorescence images TTF-I-EGFP fusion proteins have been nearly exclusively localized in the nucleoli of interphase cells, but FLIP and FCS have revealed a fast diffusion controlled exchange of molecules between the nucleoli. The mean life time of the bound state in the nucleoli lies between 20 and 30 sec. In the remainder of the nucleus the proteins are freely mobile except from geometrical constraints.

Ultrastructural Changes and Activation Differences in Platelet Concentrates Stored in Various Media

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Substitution of platelets are important for treatment of patients after bone marrow and stem cell transplantation as well as patients with thrombocytopenia occurring after chemotherapy. Our study should demonstrate how the ultrastructural morphology of platelets stored in different media correlates with the expression of particular activation markers on their cell surface.

Buffy coat derived platelet concentrates were stored for 5 days in autologuous plasma or in platelet additive solution (PAS). Platelet activation was demonstrated by measuring the cell surface expression of activation markers CD62P, CD63 and the binding of thrombospondin using flow cytometry as well as by morphometry of ultrastructural changes of cell shape (form factor). Further vasoactive cytokines such as VEGF and RANTES were determined by ELISA. The activation markers were expressed to a higher extent in PAS compared with plasma and reached statistical significance on day 3 (66.37±2.44 vs. 37.83±2,03, $p<0.001$; 42.11±3.29 vs. 34.84±2.04, $p<0.05$ and 18.84±3.9 vs. 13.98±3,87, $p<0.001$, respectively). The form factor correlated highly significant with the cell surface expression of CD62P ($r=0.96$, $p<0.001$) and significantly with CD63 ($r=0.72$, $p<0.05$) and thrombospondin binding ($r=0.8$, $p<0.05$). VEGF and RANTES were increased in the PAS group.

With exeption of a baseline activation probably due to minimal handling procedures platelets remain relatively unaltered and more stable in plasma in comparison to storage in additive solution.

Poster Presentations

Quantitative DNA-Analysis of Cholangiocellular Carcinoma: Predictive Value and Clinical Relevance

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Introduction: Patients suffering form cholangiocarcinoma (CC) are routinely transferred to surgery. But not all patients benefit from extended liver resection. So far, tumor stage, grade, metastases and complete tumor resection are used as prognostic factors to predict the outcome of CC-patients after liver resection. But their clinical value seems to be limited. Quantitative DNA-Analysis might be an alternative for prediction of survival probability. In this study, we investigated the predictive value of DNA-ploidy for outcome of patients resected due to CC.

Methods: This prospective study included 32 patients with CC within a period of 3 years. All patients received liver resection. Tissue specimens were taken from the tumor immediately after resection. The DNA-analysis was performed by means of image cytometry. The results of DNA-analyses were related to the histopathological tumor grade, stage and the clinical course. Patients were followed up for 1 to 3 years after liver resection.

Results: Tumor staging assigned 6% of patients to the tumor stage pT1, 8% to stage pT2, 35% to stage pT3 and 51% to stage pT4. Tumor grading showed mostly grade 2 (65%) and less frequent tumor grade 2 (22%) and grade 1 (13%). DNA-analysis classified tumors either as diploid (17%), polyploid (11%) or aneuploid (72%). Tumor-free margins of the liver resected parts were found in 57% (=R0-resection) of patients whereas R1-resection occurred in 5% and R2-resection was recorded in 38% of patients.

Survival was strongly related ploidy. Survival probability after 3-years for patients with diploid CC was 83% and for those with aneuploid tumors 7% (p=0.0006). All patients afflicted from metastasis at time of operation or thereafter suffered from aneuploid tumors indicating a poor prognosis.

Conclusion: DNA ploidy was the most accurate prognosis factor for resected CC patients. Patients suffering from diploid CC survived for a long time whereas aneuploid tumors indicated a poor prognosis with a rather short survival time.

We conclude that DNA-ploidy is a valuable diagnostic tool for identifying subgroups of patients that may be at higher risk for tumor progression and might not benefit from liver resection.

Forward Scatter: Its Role in Functional Genomics

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In some functional genomic assays the FSC parameter plays an important role when using flow cytometry to analyse the effect of gene expression on cell size and on DNA content. Due to the necessity to analyse sub-populations of such samples using multiple lasers it is imperative that stream-in-air multi-laser systems are capable of giving the resolution necessary to achieve precise and accurate results in the analysis of FSC.

However, it is also generally accepted that the best resolution for this parameter is obtained on systems which use flow cuvettes. On stream-in-air systems the signal is said to be compromised due to noise emanating from the stream.

Presented will be data which shows a good comparison of FSC signals taken from the Facscan analyser(BD) - which incorporates a flow cuvette, and the MoFlo cell sorter(Cytomation) - a stream-in-air-system; plus data showing the effect of certain genes on cell size and DNA content.

Endopolyploidy in Higher Plants is rather Related to Taxonomic Classification than to Genome Size

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Endopolyploidy has been described for several plant species. It has been shown to be systemic and different between various tissues. It seems to be a feature especially of plant species with small genomes (e.g. Arabidopsis thaliana) so that a negative correlation between genome size and endopolyploidy was assumed. We studied 53 species within 16 families of higher plants by flow cytometry of which the genome size ranged from about 0.4 pg to about 118 pg. Our data revealed only a weak but significant negative correlation between genome size and endopolyploidization. However, there are strong and highly significant differences between different plant families: In some families (e.g. Lamiaceae, Asteraceae) nearly no endopolyploidy could be detected whereas in other families (e.g. Cucurbitaceae, Brassicaceae) all investigated members showed it in a high amount. Furthermore the previous observation was confirmed that the degree of endopolyploidization varies clearly within one species between different organs, e.g. cotyledones and leaf stalks usually showed occurence of a much higher level of endopolyploidy than leaves.

Protective Activity of Tea Phenol (-)-Epigallocatechin-3-Gallate (EGCG) against Oxidative Damage to DNA as Determined by the COMET Assay

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Phenolic phytochemicals such as (-)-epigallocatechin-3-gallate (EGCG) show cancer chemopreventive properties. EGCG is the key polyphenolic antioxidant in green tea and greatly contributes the overall protective properties of the beverage. In this study we addressed the question wheiter EGCG may also have inhibitory effects on oxidative DNA damage in human cells, since these basic mechanisms may protect from dietary associated cancers.

We investigated the ability of the polyphenol EGCG and a polyphenol extract from green tea to reduce oxidative damage to cellular DNA. For this we first preincubated human peripheral blood lymphocytes with the polyphenols and we induced DNA damage using a range of 0 to 300 µM hydrogen peroxide (H_2O_2). DNA double-strand breaks were then determined using the neutral version of the COMET assay (single cell gel electrophoresis), which separates the cellular DNA according to fragmentation. A long migration indicates a large number of DNA scissions.

EGCG showed an inhibitory effect against H_2O_2-mediated DNA double-strand breaks in human peripheral blood lymphocytes. The pre-incubation with physiologically relevant concentrations of2 µM EGCG reduced the length of migration. The combination of 2 µM EGCG with other polyphenols in the tea extract prevented oxidative damage in cellular DNA in an even more pronounced manner.

These data show that tea polyphenols, are not only antioxidative in the

classical sense,(e.g. as commonly demonstrated in non-cellular in vitro systems), but also effective in preventing the intracellular effects leading to oxidative DNA damage. Therefore, the phenols are also potentially protective during cancer initiation.

Study of the Glycogen Content during Refeeding in Hepatocytes of Rats with Liver Cirrhosis by Cytofluorimetric Method

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Using cytofluorimetric method (Kudryavtseva et al.,1974), the glycogen content were determined in hepatocytes of normal and cirrhotic liver of rats after a 48 hours starvation and during refeeding. The liver cirrhosis in animals wsa produced by inhalation of the hepatotropic poison CCl4. The animals were opisoned for 6 months in a special closed chamber three times weekly. The material were obtained from starved animals (48 hours without food, a water ab libitum) and after 10, 20, 30, 45, 60, 75, 90, 120 minutes after the oral administration of 30% solution of glucose (4 g/kg body weight). The content of total glycogen was measured cytofluorimetrically in the smears stained with a fluorescent PAS-reaction. Our study shown that after 48-hours starvation glycogen levels in cirrhotic liver hepatocytes were 1.2 times lower than control rats. After glucose intake to starved control animals the liver glycogen content gradually increases and reach maximal means after 75! minutes. The glycogen content in cirrhotic rat liver during 60 minutes of its resynthesis was significant lower as compared with thw control group. Besides, after 120 minutes refeeding glycogen levels were higher 1.6 times than in norm. In all, the glycogen content in hepatocytes increases during reffeding almost linearly both in norm and in liver cirrhosis. Thus, the our data show significant changes of the glycogen metabolism in hepatocytes of cirrhotical rat liver.

Prognostic Value of DNA Image Cytometry in Colorectal Cancer

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Objectives: In colorectal cancers, parameters which predict tumor behavior beyond the well characterized TNM classification could help to identify high risk patients and modify and improve treatment strategies.

Methods: The prognostic significance of DNA ploidy in fresh frozen tumor specimen from 75 patients with colorectal carcinoma was examined. Follow up could be determined in 27 patients (mean 27 months, range 1 to 61). Ploidy was evaluated by DNA-image-cytometry (ICM) and correlated with tumor stage, grading, survival and recurrence rate.

Results: Of the 75 carcinomas examined, 64 (85%) were classified as DNA aneuploid and 11 (15%) as diploid. Of the aneuploid tumors, Dukes stage was as follows: Dukes D: 19 (29,7%), C: 19 (29,7%), B: 19 (29,7%), A 7 (11,9%) whereas in the diploid patients Dukes stage was either A (54,5%) or B (45,5%). In the follow-up group 1/5 year survival was 61/52%. In five out of 27 patients (19%) which all had an aneuploid

primary tumor metachronic metastatic spread was reported (Dukes A: 1, Dukes B: 2, Dukes C: 2 patients). All patients with DNA diploid tumors (4/27) (either Dukes stage A or B) had a recurrence free 5 year survival.

Conclusions: Patients with low tumor stage and DNA aneuploidy might have a higher risk for metastatic spread than patients with diploid findings. Although a larger patient population with a long term follow-up is necessary, our results suggest that ICM is a useful, additional tool to predict clinical outcome and may serve as a prognostic factor in colorectal cancer.

Quantification of Temporary and Permanent Stained Subpopulations of Bull Sperm by an Optimized Sybr-14 / Propidium Iodide Assay

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The quality of bull sperm is one key factor in the field of controlled reproduction. Viability one important aspect of sperm quality, especially after cyropreservation. We investigated the commonly used Sybr-14 / propidium iodide assay to get additional information about the sperm - dye and dye -dye interactions. After optimizing filter settings, dye concentrations and incubation times we applied the relevant dyes interruption free during kinetic flow cytometric measurements to a mixture of viable and dead sperm. Our data analysis revealed the the following findings:

- there is an essential spectral overlap between Sybr-14 ad propidium iodide between 590 and 700 nm. - the difference of propidium iodide signals from viable and dead sperm is tenfold smaller than from leukocytes.
- Sybr-14 alone stains viable and dead sperm cells.
- the reduction of Sybr-14 fluorescence from dead cells is due to fluorescence quenching caused by propidium iodide induced energy transfer.
- an "intermediate population" betwen viable and dead cells appears within 10 min and disappears between 4 to 7 min later or shifts into the dead population. The extension of this "intermediate population" becomes greater after stress on the cell membrane induced by cryopreservation. Based on this knowledge, we conclude, that the Sybr-14 / propidium iodide assay is a relevant tool to assess the viability of bull sperm.

Flow Cytometric Examinations on the Effect of New Antibiotics of the Leptomycin Family on Tumor Cells

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As flow cytometry is a rapid method to analyse the state/ condition of cell cultures it can be used for in vitro studies. In vitro studies on cell- or tissue cultures are well known in pharmaceutical research. It is the first step to determine the effect of a new substance on a living organism. Only after finishing these studies the active substance is tested on animals and finally on human.

Ratjadon is one of these new substances with promising effect against tumor cells. It belongs to the well examined Leptomycin family. All known Leptomycins (Leptomycin A, B, Kazusamycin A, B, Dilactonmycin, Delactonmycin) inhibit the regulatory gene Rev translocation at nanomolar concentrations.

We focused on the basic investigations, dose-effect connection relationship. The permanent T-lymphocyt celline Jurkat and the permanent glioblastoma celline U87-MG are used to measure the vitality and cell number at different ratjadon concentrations. Furtheron we examine the cell cycle phase at which ratjadon causes arrest. Also determinations of pHi and membrane potential are carried out.

In the future several ratjadon derivatives will be examined towards their structure-activity relationship.

APC Subsets Involved in Peptide Presentation during Induction of Tolerance versus Immunity

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Application of antigens to a naive animal may result in specifc T cell immunity or tolerance, depending on the mode of antigen application. We have developed a highly sensitive cytometric assay to directly assess peptide presentation ex vivo on the single cell level. To test the hypothesis that different APC are critically involved in the initiation of these differential reactions we have quantitated and phenotypically analyzed those APC which present physiologically relevant quantities (>100-200 peptides/cell) of a MHC class II restricted peptide following intravenous or subcutaneous/CFA injection of peptide. We can show that both ways of immunization result in antigen presentation by B cells and dendritic cells (DC). However, when comparing the kinetics and organ distribution of presentation and the phenotype of peptide presenting DC striking differences were observed. I.v. injection resulted in rapid but transient (6-12h) peptide presentation in spleen and all lymph nodes. Most of the peptide-presenting DC showed only weak expression of costimulatory molecules like B7.1/B7.2/CD40 but also high expressing cells were found to present the peptide. In contrast, peptide presentation following s.c. injection of antigen in CFA was almost completely restricted to the draining lymph nodes where the peptide-presenting DC had high costimulatory capacity: Additionally, s.c./CFA application resulted in a prolonged presentation up to 72 hours. These data suggest that tolerance is not induced by specialized APC. Instead, the total number of APC with low costimulatory capacity as well as the time of APC T cell interaction might be critical parameters.

Dynamics of a Mixed Bacterial Population during Degradation of Xenobiotics

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There is increasing interest in degrading xenobiotics in contaminated building rubble using mixed bacterial populations. In order to obtain insight into microbial interactions in these degradation processes, a bacterial community was investigated that is able to degrade 2,4-DB. The gram-positive *Rhodococcus erythropolis* K2-3 is capable of starting this catabolic process by cleaving 2,4-DB etherolytically to 2,4-dichlorophenol (2,4-DCP). The process can then be continued by the action of the gram-negative bacterium *Ochrobactrum anthropi* K2-14. However, details of the mutual profit derived from the joint catabolism of 2,4-DB are unknown.

Investigations on a single cell basis are likely to provide the most thorough understanding of the subpopulations' roles within the mixed community, and to be most helpful in developing control strategies for optimising decontamination processes. Here flow cytometry was used as a powerful technique for analysing rapidly a variety of parameters of the individual bacterial cells within the mixed population.

To get information about the physiological states of two strains in the mixed bacterial population, at least two different but specific parameter like forward light scatter (FSC) and DNA fluorescence should be known. DNA was measured in the blue range of light. To differentiate between the two strains and to estimate the concentration of each of them (the number of cells in a special volume) a method exploiting fluorescently labelled lectin probes was developed. Since Concanavalin A (Con A) binds specifically to *R. erythropolis* K2-3, it was selected and linked to the fluorescent dye Bodipy 630/650, which has an excitation maximum in the red part of the visible light spectrum. The three parameters were conveniently monitored by dual and triple excitation flow cytometry in conjunction with double fluorescent staining techniques.

Laser-Rupture Induced Ca2+ Oscillations in Non-Excitable Connective Tissue Cells

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In addition to excitable but dormant myocytes non-excitable cells of the connective tissue significantly participate in the organization of cardiac scares which remain from myocardial ischemia and infarcts. Ventricular tissue scares are believed to constitute paroxysmic centers of automaticity by reentry along circular excitation pathways or by focal excitations. Focal excitations coincide with cellular hyperexcitability and an increased cytosolic calcium concentration.

To examine the function of non-excitable scare tissue cells in excitation paroxysms we equipped a confocal microscope with a laser microbeam: A TiSa-laser was coupled into the epi-fluorescence illumination beam

path. This way we could rupture the plasma membrane of indivdual cells inside a confluent L929 fibroblast monolayer. The time course of the cytosolic calcium concentration was recorded with Calcium Green1.

Upon laser induced plasma membrane rupture the calcium diffusion along gap junctions in the syncytial monolayer could be examined.

Calcium diffusion measurements qualitatively agree with theoretical estimations of diffusion: The cytosolic concentration increases as faster as nearer located to the point of rupture. Beyond that the diffusion rate depends on monolayer density and on the size of the intercellular contact region. This shows gap junctions being the locations of membrane passage.

In addition to conventional diffusion fibroblasts closely neighbored to the ruptured cell developed calcium oscillations but not calcium spiking. The oscillations resemble the ones known from cardiac myocytes with regard to their shape. They differ in oscillation periods being in range of minutes. The oscillations stabilize the cytosolic calcium concentration near physiologic level.

The repetitive fibroblastic shuffling of calcium into the scare interstitium can induce calcium based hyperexcitability in the remaining cardiac myocytes and induce a paroxysmic transition from dormancy into focal excitations.

Flow Cytometric Characterisation of Lactic Acid Bacteria

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In order to improve the quality of dairy products it is important to assess the bacterial erformances of the inoculated lactic acid bacteria. By using flow cytometric techniques it is possible to get information about the physiological activity of each single cell.

Here we represent first results of the flow cytometric investigation of two model organisms Lactobacillus delbrueckii DSM 20081T and Lactococcus lactis MG 1363.

Different staining procedures were established for both strains which were grown as pure cultures. The two strains were investigated separately as batch and as transient state cultures. Different glucose concentrations were tested in the chemostat model system to obtain information about the growth parameters (μmax- and Ks- values) of the cells depending on the carbon concentration. Further, lactic acid and acetic acid production were measured in correlation to the given carbon- and energy source. In order to relate the metabolic activity of the cells to the state of the cell cycle their DNA content was determined with the aid of 4',6-diamidino-2'-phenylindole (DAPI).

Ultrastructural Changes and Activation Differences in Platelet Concentrates Stored in Various Media

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Substitution of platelets are important for treatment of patients after bone marrow and stem cell transplantation as well as patients with thrombocytopenia occurring after chemotherapy. Our study should demonstrate how the ultrastructural morphology of platelets stored in different media correlates with the expression of particular activation markers on their cell surface.

Buffy coat derived platelet concentrates were stored for 5 days in autologuous plasma or in platelet additive solution (PAS). Platelet activation was demonstrated by measuring the cell surface expression of activation markers CD62P, CD63 and the binding of thrombospondin using flow cytometry as well as by morphometry of ultrastructural changes of cell shape (form factor). Further vasoactive cytokines such as VEGF and RANTES were determined by ELISA. The activation markers were expressed to a higher extent in PAS compared with plasma and reached statistical significance on day 3 (66.37±2.44 vs. 37.83±2,03, $p<0.001$; 42.11±3.29 vs. 34.84±2.04, $p<0.05$ and 18.84±3.9 vs. 13.98±3,87, $p<0.001$, respectively). The form factor correlated highly significant with the cell surface expression of CD62P ($r=0.96$, $p<0.001$) and significantly with CD63 ($r=0.72$, $p<0.05$) and thrombospondin binding ($r=0.8$, $p<0.05$). VEGF and RANTES were increased in the PAS group.

With exeption of a baseline activation probably due to minimal handling procedures platelets remain relatively unaltered and more stable in plasma in comparison to storage in additive solution.