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ABSTRACTS

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Andrea A. Robitzki
Annette G. Beck-Sickinger
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Svenne Eichler

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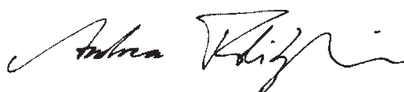
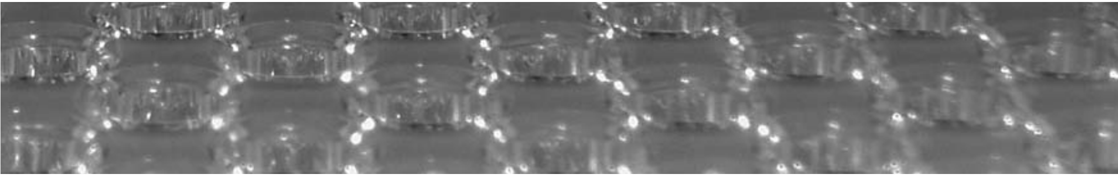
FOREWORD

The Center for Biotechnology and Biomedicine (BBZ) of the University of Leipzig is located in the BIO CITY LEIPZIG complex. Over the past few years it has established itself as partner for R&D in the fields of Protein Engineering & Bioanalytics, Molecular Medicine & Therapeutics, Biomedical & Cell Engineering as well as Nanobiotechnology & Nanoelectronics. The research center is structured in an inter- and transdisciplinal way and brings together molecular and cell biologists, pharmacologists, health professionals, (bio)chemists, (bio)physicists and engineers. At the crossways of, for example, biomedicine and biotechnology as well as micro- and nanotechnology, new modules for automation, parallelization and synchronization of analytical processes in Life Sciences are being developed. The focus is set on applied and industry-oriented bio-molecular research and development and on creating biomedical and biotechnological networks that can supply innovative approaches. The center cooperates with the most renowned national and international scientific and industrial research institutions in France and Great Britain (such as the Institute of Nanotechnology Cambridge, Nexus – European Company Network). Synergies, complementary R&D areas and expertise aiming at strengthening the European position can thus be identified and exploited: in fact, the BBZ offers services in areas such as bioanalytics, protein structure analysis as well as functional proteomics or metabolics for Pharmaceutics & Life Sciences, and it is also involved in R&D projects related to (Ultra)-High-Throughput-Screening.
The 4th Biotechnology Symposium demonstrates to which degree the alliance BBZ Leipzig, its industrial partners and research institutes are taking an active part in advancing international competition for innovative technologies.



Prof. Dr. Andrea A. Robitzki
Chair of the Center for Biotechnology and Biomedicine

1. PROTEIN ENGINEERING AND BIOANALYTICS



PRESENTATIONS

1.1 Creating novel proteins for therapeutic applications

Rainer Rudolph, Hauke Lilie, Ulrike Fiedler

Recombinant proteins are by now widely used for the treatment of various diseases. For some therapeutic applications (e.g. cancer treatment) natural proteins offer only limited strategies for therapeutic intervention. For these applications, we designed novel modular proteins. We used polyionic adaptor peptides containing cysteine residues as highly specific heterodimerization motifs. Fusion of these polyionic peptides to both a protein module recognizing specific cell surface epitopes, and another module inducing cell death resulted in disulfide-bonded immunotoxins, which possess a very high and specific cytotoxicity.

The polyionic adaptor motif was also used to specifically modify virus like particles. As modular delivery systems, composed of a specific recognition domain, *in vitro* assembled capsids, and the therapeutic cargo (e. g. DNA) specific cell transformation could be achieved.

Finally, novel binding molecules were created. For this purpose, two very stable human proteins, γ-crystallin from the eye lens, and ubiquitin were randomized in a solvent-exposed beta-sheet area. Applying suitable display and screening techniques specifically binding molecules can be isolated for both low molecular weight substances (e.g. estradiol) and globular proteins.

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1.2 Dark matter of the genome – The human genome contains thousands of non-coding RNAs

Peter F. Stadler

In contrast to the fairly reliable and complete annotation of the protein coding genes in the human genome, comparable information is lacking for non-coding RNAs. A computational screen of vertebrate genomes evaluating sequence conservation, secondary structure conservation, and thermodynamic stability of the putative RNA structures predicts more than 30,000 structured RNAs in the human genome, almost 1,000 of which are conserved across all vertebrates. Roughly a third is found in introns of known genes, a sixth are potential regulatory elements in untranslated regions, about half are located far away of any known gene. Only a small fraction of these sequences has been described previously. EST data demonstrate, however, that the majority of them is at least transcribed. We estimate, therefore, that the human genome contains at least as many RNA genes as protein coding genes.

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1.3 Functional annotation of high-throughput screening data with structural protein interactions and textmining

Michael Schröder

Currently high-throughput screening with gene expression and RNAi generate large amounts of data, which need to be annotated for interpretation. I will discuss two tools to support this task by identifying potential interaction partners based on structural domain-domain interactions and by textmining using hierarchical vocabularies such as the GeneOntology. The approaches will be illustrated with example screens identifying overexpressed genes during osteoclast differentiation.

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1.4 TRANSIL® technology – Immobilized proteins for innovative assays and for ADME prediction in drug discovery

Joachim Nöller

NIMBUS' TRANSIL® platform is a proprietary bead based assay technology allowing for the biofunctionalization of solid supports. Based on this platform, we are providing bead based assays for important stages in drug discovery.

About 40 % of late-stage failures are associated with poor pharmacokinetic properties leading to a huge loss in time and money. Early profiling of drug like properties provides important information to screen out insoluble, poor absorbing and toxic compounds.

As one contribution of NIMBUS to this bottleneck in drug discovery the TRANSIL® technology allows the functional immobilization of serum proteins (e.g. Human Serum Albumin) on solid supports. After surface passivation, protein molecules are covalently attached on the bead surface taking care that important pharmacologically binding sites are freely accessible and therefore suitable for the determination of the unspecific binding to serum proteins, as an important ADME related parameter. This parameter for a compound is used in combination with the membrane affinity and the molecular weight for the prediction ADME parameters like volume of distribution or plasma organ partitioning coefficients.

Furthermore technology enables also binding assays for transmembrane proteins which will be described for the measuring the binding of AGEs (Advanced Glycation Endproducts) to the plasma membrane of Caco-2 cells.

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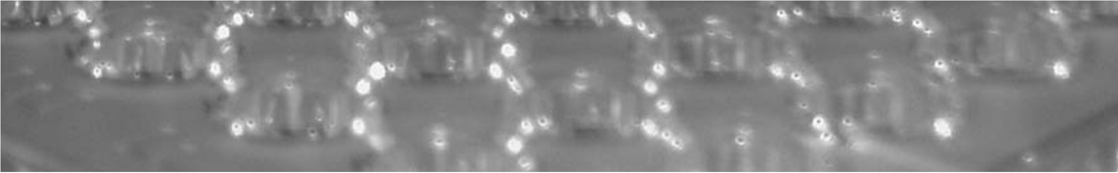
2. MOLECULAR MEDICINE AND THERAPEUTICS



PRESENTATIONS

2.1 Tumor targeting

Dario Neri

One avenue towards the development of more selective, better anti-cancer drugs consists in the targeted delivery of bioactive molecules (drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules (e.g. human antibodies) specific for tumor-associated markers.

Angiogenesis, i.e. the proliferation of new blood vessels from pre-existing ones, is an underlying process in many human diseases, including cancer, blinding ocular disorders and rheumatoid arthritis. The ability to selectively target and occlude neovasculature will be potentially useful in diagnosis and treatment of angiogenesis-related diseases.

A good-quality marker for both tumoural and non-tumoural neovasculature is the extra-domain B (ED-B) of fibronectin, a sequence of 91 aminoacids that can be inserted into the fibronectin molecule by a mechanism of alternative splicing. Anti-EDB antibodies stain vascular structures in tumour sections and selectively target tumour neovasculature, as shown in tumour-bearing mice using infrared fluorescence and radioactive techniques. The ability of the radiolabeled anti-EDB antibody L19 to target tumors in patients with cancer has recently been demonstrated using scintigraphic detection methods. A number of derivatives of the L19 antibody (fusions to cytokines, pro-coagulant factors, photosensitizers, drugs, radionuclides, etc.) have been studied in animal models. The results obtained are of therapeutic relevance, since the ED-B domain of fibronectin, a naturally-occurring marker of angiogenesis identical in mouse and man, is expressed in the majority of aggressive solid tumours, but is undetectable in normal vessels and tissues.

In the last part of my presentation, I will show how we are using the *in vivo* biotinylation of tumor bearing mice by terminal perfusion, followed by proteomic analysis of tumor specimens and normal organs, for the discovery of novel tumor-associated vascular targets.

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2.2 NAD$^+$-dependent regulatory processes on human monocytes/macrophages

Sunna Hauschildt

Human monocytes are capable of degrading extracellular NAD$^+$ to biologically active compounds and to use NAD$^+$ as a substrate for covalent protein modification. The reactions are catalyzed by CD38 and mono-ADP-ribosyltransferases (ARTs) resp. Furthermore NAD$^+$ causes a rapid elevation of $[Ca^{2+}]_i$ mediated by the influx of extracellular calcium. During differentiation of monocytes to macrophages by down-regulating CD38 expression the availability of NAD$^+$ to serve as a substrate for ARTs increases which in turn may account for macrophage specific functions. As membrane proteins oriented towards the extracellular compartment CD38 and ecto-ARTs represent good candidates for development of drugs which will be useful tools in understanding the action and biological functions of these proteins.

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2.3 Diabesity – Molecules and mechanisms

Michael Stumvoll

Regulation of glycaemia and body weight represents a fundamental biological principle, and failure of the regulation underlies type 2 diabetes and obesity. A myriad of molecules is likely involved in the complex aetiology of "diabesity" and dissecting the relevance of a single represents a challenge. Translation of burgeoning new basic science findings into a physiological and clinical context calls for novel and imaginative clinical experimental tools. For the purpose of this review four molecules (adiponectin [APM1], stearoyl desaturase 1 [SCD1], insulin receptor substrate-1 [IRS1], peroxisome proliferator-activated receptor γ [PPARG]) or visfatin (PBEF1), each with a plausible role in the disease process, have been selected to illustrate the use of such techniques in humans. These include procedures as diverse as isotope dilution for turnover studies (e.g. glycerol turnover as a proxy for lipolysis), conventional and modified clamp procedures, association studies of functionally relevant SNPs in candidate genes (e.g. IRS-1 and PPARγ), multivariate correlational analyses (as with plasma adiponectin), magnetic resonance spectroscopy to quantify intra-tissue lipid deposition and regional fat distribution, gas chromatography to determine fatty acid patterns in selected lipid fractions as proxy for intrahepatic enzyme activity and mRNA expression studies in selected tissues. A concerted effort by scientists from many disciplines (genetics and cell biology, physiology and epidemiology) will be required to bridge the growing gap between basic scientific concepts of biological modifiers of glycaemia and what is truly relevant for human obesity and type 2 diabetes.

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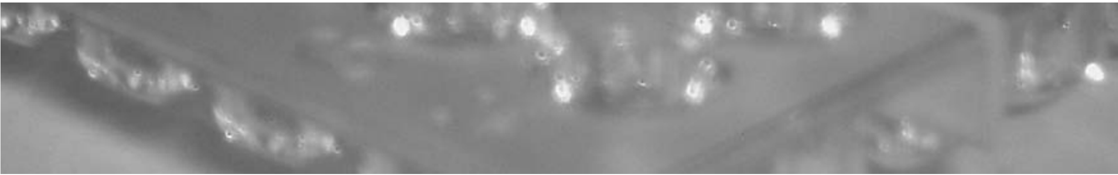
3. BIOMEDICAL AND CELL ENGINEERING



PRESENTATIONS

3.1 Modeling cardiac electric signals – From ion channels to the ECG

Olaf Dössel

The electrocardiogramm ECG is a well-known diagnostic tool. The electrical signals that can be measured on the surface of the body originate from electrophysiological processes in the heart. The “sources” of these electric signals are the ion channels embedded into the membrane of the myocytes that open and close according to basic rules. These rules have been described mathematically first by Hodgkin and Huxley. Today the complete chain from the ion channels (nanometer scale) over the myocytes (micrometer scale) to a description of the tissue (millimeter scale) and ending with the ECG on the body surface (meter scale) can be described explicitly with mathematical models.

A large set of membrane proteins is taken into account today in order to describe the electrophysiology of isolated myocytes. A very detailed description of the electrophysiology of the heart is necessary for realistic modeling of excitation propagation. Knowing the bioelectric source distribution in the heart the time course of the body surface potential map BSPM can be calculated.

In future better therapeutic measures can be designed using computer models of the human heart. It will be possible to predict the effect of new drugs on the rhythm of the whole heart by knowing the effect on the ion channels. Optimized strategies for RF ablation can be found. Maybe also new electrode configurations and pulses for electric stimulation like they are used in a heart pacemaker or a defibrillator can be investigated.

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3.2 Generation of reliable diagnostics and effective vaccines by recombinant DNA technologies

Hermann Müller

The focus of research at the Institute for Virology is on basic science, applied science and diagnostics in the field of animal viruses and viral infections of animals. Viruses of various avian species are in the centre of these investigations. Among these are infectious bursal disease virus (IBDV) affecting developping lymphoid B cells in chickens, avian polyomaviruses such as budgerigar fledgling disease virus (BFDV)and goose hemorrhagic polyomavirus (GHPV) as well as avian circoviruses, e. g., psittacine beak and feather disease virus (BFDV) and pigeon circovirus (PiCV). To fight against these virus infections, some of which are of considerable economic importance, diagnostic tools and virus vaccines are developed by the application of the techniques of molecular biology. Some examples will be presented.

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3.3 Microstructuring of surfaces for life science

Klaus Zimmer, Thomas Rudolph, David Ruthe, Rico Böhme, Joachim Zajadacz, Bernd Rauschenbach

High throughput analysis sensing on cell level and one-way life science products call for microstructures, functional surfaces and eventually for microsystems. Microsystems technologies are able to meet these requirements after some adaptations and allow the fabrication of surface microstructures with defined properties. For instance the handling of minute quantities of liquid samples for analysis and agent development requires microfluidic solutions. Combining nano- and microstructures and chemical modification provide highly functionalised microstructures. Standard techniques of microsystems technologies for microstructuring and physical/chemical surface modification as well as modern processing techniques such as laser and e-beam writing, plasma and UV-engineering and replication techniques have been exploited to achieve appropriate surfaces and microstructures for life science applications.

After touching the principles of microstructuring some examples of laser micromachining of surfaces are presented. Especially the direct laser processing for etching of glass and polymers to achieve micotopographies or for chemical modified surface areas for functionalisation are considered. Different examples of microstructured surfaces for morphological investigations of cells, for modification of low cost sensors, for microfluidic systems, and for application of microspotting techniques are presented and first results of practical applications are discussed.

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3.4 Dopaminergic neurons from midbrain-derived neural stem cells

Sigrid C. Schwarz

Parkinson's disease (PD) is a chronic, progressive neurodegenerative movement disorder characterized by a continuous and rather selective loss of dopaminergic neurons in the *substantia nigra pars compacta* with subsequent striatal dopamine depletion. Since dopaminergic neurons are a rather small and well defined population of neurons in the brain, restorative strategies may be especially effective in PD. The use of embryonic midbrain tissue is difficult secondary to ethical and logistical problems as well variable efficacy.

We transplanted neural precursor cells (NPCs) and neuron-restricted precursor cells (nrPCs). When long-term expanded human midbrain derived NPCs or nrPCs were differentiated *in vitro*, they expressed specific markers of dopaminergic neurons and displayed neuronal characteristics including sodium currents and action potentials. Following transplantation, there was improvement of amphetamine induced rotational behaviour and survival of tyrosine-hydroxylase immunoreactive (TH-IR) cells within the graft four months after transplantation. Animals with more than 2,000 TH-IR cells in the graft showed reversal of rotational asymmetry. Four of five animals grafted with nrPCs showed functional recovery as well as minimal immune responses. Only animals that received NPCs without immunosuppression showed mild activation of astrocytes, microglia and macrophages. Histological analyses did not reveal any sign of tumor formation.

Human midbrain derived NPCs (preferably nrPCs) represent a tissue source for transplantation of homogenous and non-immunogenic cells potentially capable of reconstituting dopaminergic neurons *in vivo.*

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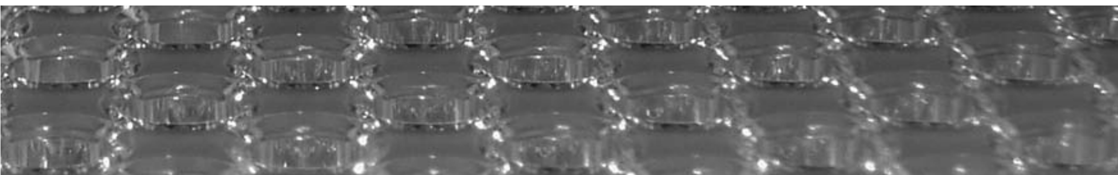
4. PROTEIN ENGINEERING AND BIOANALYTICS



POSTERS

4.1 Characterization of *B. burgdorferi* cyst formation

Samiya Al-Robaiy, Hassan Dihazi, Johannes Kacza, Johannes Seeger, Jürgen Schiller, Daniel Huster, Reinhard K. Straubinger

B. burgdorferi, the agent of Lyme borreliosis, has the ability to undergo morphological transformation from motile spirochetes to non-motile cystic forms in the presence of unfavourable conditions. However, little is known about cyst formation procedure.

We compared spirochetes and cysts induced under controlled conditions such as nutrient depletion or osmotic changes.

From the transmission electron microscopy it seems that cyst formation begins with membrane budding followed by spirochetal structures folding inside the pronounced budding membrane. The role of the periplasmic flagella involved in the motility and cell shape of *B. burgdorferi* was studied using a mutant lacking the major flagellar protein FlaB. Scanning electron microscopy showed that the induction of cyst formation was not affected in this mutant. Lipid analysis with MALDI demonstrated that the spirochetes and cysts contain the same lipid contents. Neither phosphatidylcholine (PC) nor phosphatidylglycerol (PG), the major phospholipids in *B. burgdorferi* which form the bilayer membrane of these cells were changed with structural transformation.

Protein profiling on western blots showed identical antigenic patterns for spirochetes and cysts with decreased expression intensities for cysts. However, an up-regulation of different low molecular proteins in the cysts was determined with SELDI.

Our results demonstrate that cyst formation is a process in which proteins as well as a physical rearrangement of the outer surface membrane are involved. Further analysis to identify the involved proteins is in progress.

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4.2 A different approach for cloning and purification of the 5S subunit of transcarboxylase multienzyme complex

Rakesh Kumar Bhat, Stefan Berger

Transcarboxylase (EC2.1.3.1) (TC) from *Propionibacterium shermanii* is a complex biotin-containing enzyme composed of 30 polypeptides of three different types. It is composed of six dimeric outer subunits associated with a central cylindrical hexameric subunit through 12 biotinyl subunits with three outer subunits on each face of the central hexamer. Each outer dimer is termed a 5S subunit, which is associated with two biotinyl subunits. The enzyme catalyzes a two-step reaction in which methylmalonyl-CoA and pyruvate form propionyl-CoA and oxalacetate, the 5S subunit specifically catalyzing one of these reactions. We report here our attempts for cloning, sequencing and expression of the monomer of the 5S subunit. The gene was identified by matching nucleotide sequences from the database available, isolated from authentic 5S peptides with the deduced sequence of an open reading frame present on a cloned *P. shermanii* genomic fragment. The cloned 5S gene encodes a protein of 519 amino acids, M, 57,793.

Initially we successfully cloned the gene into the pET 28a(+) vector. But there were problems while purification of the protein. After cleaving the His–Tag, the protein gets precipated and the cleavage was also not 100 %, which may give problems during NMR studies. Currently we tried to clone and purify the gene by a different strategy based on IMPACT-I system.

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4.3 Residue specific studies of NH exchange rates performed on ubiquitin

Torsten Brand, Garreth A. Morris, Eurico J. Cabrita,
Hans-Jörg Hofmann, Stefan Berger

The aim of the present study was to establish a new method to invesigate NH exchange rates in proteins. These measurements are based on residue specific diffusion experiments and were demonstrated on human ubiquitin since it is a small protein (9 kDa, 76 residues) for which resonance assignment as well as NMR structures are available.

For NMR studies a 1.8 mM solution of uniformly ^{13}C,^{15}N-labelled ubiquitin in 90 % H_2O, 10 % D_2O was used. Experiments were carried out at 300 K on a Bruker Avance 700 spectrometer equipped with a cryo probe. In order to resolve most signals of the protein ^{1}H,^{15}N-DOSY-HSQC spectra were recorded. Diffusion Ordered Spectroscopy was used to obtain an apparent diffusion coefficient D for each separated signal.

Analysis of the apparent diffusion coefficients showed several "fast moving" NH protons. Subsequently, the exchange rates were derived directly from the decay data of the diffusion experiment by applying a model deduced from the assumption of two-site exchange with water and the "pure" diffusion coefficients of water and protein.

Molecular modelling and MD studies have been performed in an attempt to predict exchange rates from structural or dynamical features of ubiquitin. Several parameters have been checked with respect to their utility for such a prediction, including static parameters like NH bond order, partial charge at the nitrogen and accessibility of the NH protons as well as the dynamics of hydrogen bond breaking. Whereas none of the parameters examined so far yielded a strong correlation, attempts to use a weighted combination of these parameters are topic of current research.

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4.4 Developmental pattern of tau isoforms in rat brain

Torsten Bullmann, Max Holzer, Wolfgang Härtig, Nicolas Sergeant, Andre Delacourte, Rohan de Silva, Hiroshi Mori, Thomas Arendt

Aggregates of hyperphosphorylated microtubule associated protein tau are found in Alzheimer's disease and other neurodegenerative diseases called tauopathies. These aggregates differ in their tau isoform composition, phosphorylation of specific isoforms and filament confirmation as well as in the affected cell types and regional distribution of lesions in brain. Some studies demonstrated a link between regional variation in tau isoform expression and susceptibility to neurodegeneration. Therefore distribution of tau protein isoforms in adult rat brain and during development was studied using recently avaiable specific antibodies, western blotting and RT-PCR. Single cell RT-PCR in combination with Laser microdissection may be a useful tool to study tau mRNA splicing in human brain to clearify whether a subset of neurons posses a specific tau protein isoform pattern that renders them highly vulnerable to degeneration.

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4.5 New structural and functional aspects of phosphofructo-1-kinase from *Pichia pastoris*

Anke Edelmann, Katrin Tanneberger, Jürgen Kirchberger, Jörg Bär, Torsten Schöneberg

The methylotrophic yeast *Pichia pastoris* is a highly efficient system for heterologous gene expression and of great interest in many biotechnological applications. Yeast growth and expression properties essentially depend on energy-providing pathways such as glycolysis. The phosphofructo-1-kinase (Pfk-1) is one of the key enzymes of glycolysis. By cloning the genes encoding the α- and β-subunits (about 100 kDa each), we could demonstrate that *P. pastoris* Pfk-1 (*Pp*Pfk-1) is a hetero-oligomer $\alpha_4\beta_4$). Surprisingly, a molecular weight of 975 kDa was determined for the native *Pp*Pfk-1. The unexpected higher molecular weight was due to a third protein component which was co-purified and partially sequenced by MALDI analyses. Based on these initial sequence data, the respective open reading frame (1,056 bp) and 2,070 bp of the 5’ non-coding region were isolated. The polypeptide chain contains 351 amino acid residues and has a predicted molecular mass of 40.8 kDa. To investigate the relevance of this protein as *Pp*Pfk-1 subunit, we examined the formation of *Pp*Pfk-1 in *pfk*-deficient yeast strains. These findings provide further evidence that *Pp*Pfk-1 contains a third type of subunits, which is probably necessary for enzyme assembly and catalytic activity.

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4.6 Recombinant expression and structure determination of phosphodiesterases

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PDEs catalyzing the hydrolysis of the 3'-5' phosphodiester bond of cyclic nucleotides (cAMP, cGMP) comprise a family of enzymes that modulate the immune response, inflammation, cell growth and many other functions. 11 families of PDEs have been described including multiple isoforms with varying selectivities for cAMP and/or cGMP. The isoforms are differentially expressed and regulated in different cell types, and they appear to modulate the location, substrate selectivity, kinetics, and response to activators or inhibitors of the enzyme. As essential regulators of cyclic nucleotide signalling, PDEs are recognized as important drug targets for the treatment of diseases such as heart failure, asthma and erectile dysfunction.

The aim of the current project is to develop expression constructs for the production of sufficient material for crystallization of full length PDEs and catalytic domains. The X-ray structures of these PDEs will allow for the rational design of specific inhibitors as therapeutics. 3D structures of the catalytic domains of PDE1, -3, -4, -5 and -9 are currently available.

For the expression of the catalytic domains we designed different truncations of PDEs to find good constructs for subsequent protein purification and crystallization. Expression in *E. coli* as well as in *P. pastoris* often yields insoluble products. To avoid the refolding processes of the proteins we also try to express the catalytic domains in an *E. coli* based cell-free *in vitro* system. This system allows for the simple modification of many expression parameters and hence it is maybe possible to directly produce soluble and active protein.

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4.7 Site-directed mutagenesis in the substrate binding region of R-3-hydroxybutyrate dehydrogenase (3-HBDH)

Claudia Feller, Marlis Grunow

3-HBDH belongs to the family of short-chain dehydrogenases/reductases (SDR). At present, about 4,000 primary structures and 40 3D structures of the SDR family are annotated in sequence databases. The enzymes included in the SDR family span several EC classes, from oxidoreductases and lyases to isomerases, with NAD- or NADP-dependend oxidoreductases forming the majority. In this class, many enzymes of different substrate specificities are found acting on steroids, prostaglandins, aliphatic alcohols and xenobiotics.

The aim of our work was to gain an insight into the substrate binding mechanism of 3-HBDH. How does 3-HBDH interact with its small and hydrophilic substrate, knowing that substrates as well as coenzymes are bound in a hydrophobic substrate binding cleft, as seen by SDR structures? Because a crystal structure is not currently available, a three-dimensional model of 3-HBDH was build using known structures of five oxidoreductases as templates. Aspects of the model concerned with 3-hydroxybutyrate binding have been tested by site-directed mutagenesis of residues Gln91, His141, Lys192 and Gln193 to Ala and Lys149 to Ala and Arg. Because of their position in the substrate binding cleft of the model and their positive charge, these residues were the main candidates concidered to play a crucial role in the binding of 3-hydroxybutyrate. Enzyme variants have been characterized in terms of kinetic constants. The results obtained with enzymes mutated at positions 91, 141, 193 and in particular at Lys149 confirm the assumed interactions of these residues with the carboxylate group of the substrate.

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4.8 Site-specific synthesis of Amadori-modified peptides on solid-phase

Andrej Frolov, David Singer, Ralf Hoffmann

Glycation of peptides and proteins is a slow chemical reaction of reducing sugars and amino groups resulting in formation of Amadori products undergoing further oxidation and degradation, leading to advanced glycation end-products - markers of ageing, diabetes mellitus and Alzheimer's disease. Here, we describe a solid-phase strategy for glycation of specific amino groups on partially protected resin bound peptides using a global post synthetic approach. The peptides were synthesized by standard Fmoc/tBu-chemistry using carbodiimide activation. The lysine to be modified was protected during the synthesis on the epsilon-amino group with the very acid labile methyltrityl group, which was cleaved after completion of the peptide synthesis selectively with 1 % TFA in dichloromethane. The partially deprotected peptide was glycated using 0.25 mol/L D-glucose in DMF at either 70 °C for 45 h or 110 °C for 25 min. The overall yields were about 35 % for the tested hexapeptides. Neighbored residues and their bulky protecting groups slightly reduced the yields to 30 %. Whereas the peptide eluted before the glycated peptide all other byproducts eluted much later on RP-HPLC allowing a simple purification even for medium-sized peptides. Thus, the general strategy presented here allows routine syntheses of Amadori peptides at reasonable yields and purities after purification by RP-HPLC using standard protocols established in most laboratories synthesizing peptides.

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4.9 Enantioselective high performance liquid chromatography of astaxanthin from microbial sources

Carola Griehl, Claudia Grewe, Sieglinde Menge

A method for determining the enantiomers of all-E-astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dion), an important colorant in the feed industry, was developed. Synthetically produced the carotenoid consists of a mixture of three optical isomers in the ratio 3S,3S' : 3R,3S' : 3R,3R' 1:2:1[1]. For the separation different stationary phases were tested on their ability to resolve the optical isomers of astaxanthin: β-cyclodextrin derivatives (Chiradex, Merck), dinitrobenzoylleucin derivatives (Pirkle L-leucine, R,R-Ulmo, Regis Technology) and a cellulose-tris-3,5-dimethyl-phenylcarbamate (Chiracel OD-RH, Daicel). A sufficient separation of astaxanthin stereoisomers was only achieved employing the Daicel OD-RH as stationary and a mixture of acetonitrile and phosphate buffer as mobile phase. Direct resolution of all occurring optical isomers was achieved. Separation conditions are described allowing the determination of the isomeric forms produced in microalgae (*Scenedesmus sp.*) and yeasts (*Xanthophyllomyces dendrorhous*) as well as the identification of synthetically derived astaxanthin for example in fish feed.

Notes

1 Bjerkeng B., Coral-Hinostroza G.N., Comparative Biochemistry and Physiology Part B. 133: 437-444 (2002).

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4.10 IZKF Leipzig Core Unit "Fluorescence Technology" – Confocal laser scanning microscopy

Jens Grosche, Andreas Reichenbach

The confocal laser scanning microscopy (cLSM) has become the accepted technology for obtaining high resolution images in light microscopic morphology. The technology is based on two essential features: the application of high-energy laser light to excite the fluorophores and the placing of a pinhole in the beam path which is conjugated to the focal plane of the microscopic lens. Thus it is guaranteed that only light from the focus level contributes to the final image, while out-of-focus information is greatly reduced. In this way it is possible to generate a 3-D reconstruction of a specimen by stacking optical sections collected in series, or to reread the distribution of labeled organelles within single cells.

The cLSM is particularly advantageous when using multiple markers, because the filter settings can be adapted and optimized for each fluorophore. Even strong overlapping emission spectra can be precisely seperated from each other or from autofluorescence of the tissue.

In addition to morphological investigations, it is also possible to analyse functional parameters by cLSM. Vital dyes which react sensitively to physiological parameters can be used to measure the change of the fluorescence intensity dependent on time. Furthermore recent improvements of the emission filter setups enable the study of molecular interactions between two protein partners in living tissue by fluorescence resonance energy transfer (FRET), as long as they are located within a close proximity of 1-10 nm.

The cLSM is becoming an increasingly valuable imaging tool for a variety of applications in biological and medical basic research.

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4.11 Depletion of high-abundant proteins from human serum

Katja Hanisch, Tobias Langrock, Ralf Hoffmann

Alzheimer's disease (AD) is considered as being the most important neuronal degenerative disease and accounts for approximately 60 % of all senile dementias. Despite all progress in recent years a valid biomarker is still missing, as neither Aβ nor tau are specific for AD and most presumably disease-specific alterations were also found in age-matched controls.

Our work aims to identify novel biomarkers in serum or cerebrospinal fluid (CSF). Difficulties in this field arise from a few dominant proteins present in these body fluids, such as serum albumin and immunoglobulins, which prevent detection of low-abundant proteins. The concentration range of proteins in human biological fluids spans over nine to twelve orders of magnitude, with 85-90 % of the total protein content being represented only by six proteins, that is, serum albumin (HSA), IgG, IgA, haptoglobin, antitrypsin and transferrin. Thus, we have tested several chromatographic techniques to deplete abundant proteins in human serum. Removing HSA with a blue column was not very effective. More effective were two commercially available columns that used specific antibodies. Whereas the POROS® Affinity Depletion Cartridges (Applied Biosystems, Langen, Germany) quantitatively removed only HSA and IgG the multiple affinity removal system (MARS, Agilent Technologies GmbH, Germany) removed HSA, IgG, IgA, haptoglobin, antitrypsin and transferrin from human serum. The depleted samples were analyzed by two-dimensional gel electrophoresis using either a colloidal coomassie or a SyproRuby stain.

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4.12 Study of antibacterial and antitumoral peptides

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The increasing resistance of more and more bacteria strains against common antibiotics threatens the western societies, thus the development of new classes of antibiotics is a major focus of current pharmaceutical research. A promising approach relies on antibacterial peptides, since it appears likely that bacteria do not develop any resistance against these peptides.

Peptides with antibacterial activity prevent many organisms, especially insects but also mammals, from infections. For example, short proline rich peptides isolated from insect hemolymph show a broad spectrum of efficiency against many bacteria strains. The typical approach to identify such drug leads is to challenge insects with a bacterial infection, isolate the produced peptides and determine their structure. Based on the native sequence, more potent compounds are developed using solid-phase peptide synthesis by incorporating unnatural amino acid derivatives to enhance the antibacterial activity and to increase their protease resistance to circulate longer in the blood.

The aim of this study was to synthesize and modify native antibacterial peptides and to determine their antimicrobial activity as well as their serum stability. Furthermore, the antitumoral activity against glioblastoma and neuroblastoma cell lines was tested, for example for prophenin, which is a proline and phenylalanine rich 79mer peptide isolated from porcine lung. The bactericidal properties of the C-terminal 18 residues ("PR 18") are comparable to the full-length peptide. We will also present data on the antitumoral activity of "PR 18" and other prophenin derivatives.

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4.13 Lipophilicity and protein binding as key descriptors for adme behavior – Innovative high-throughput screening tools

Thorsten Hartmann, Daniel Nimptsch, Cornelia Röhring, Johannes Schmitt, Joachim Nöller

Recent developments in combinatorial chemistry and robotics provide increasing numbers of lead compounds. Picking out candidates with appropriate physicochemical properties in an early drug discovery phase calls for fast and fully automated procedures.

The binding of pharmaceutically relevant molecules to neutral or ionic lipid membranes (expressed as the lipid/water partition coefficient log $D_{Lipid/Water}$) is directly related to permeation and intestinal absorption, while the binding of compounds to serum proteins like human serum albumin (HSA) determines the bound fraction (f_b) in the blood, thus affecting compound distribution. The proprietary TRANSIL® technology, based on lipids and proteins on solid supports, allows a fully automated assessment of both parameters in conventional microtiter plates (96 or 384 well) enabling high-throughput measurements of drug candidates.

TRANSIL® beads are functionalized with either a well defined single lipid-bilayer, which is no covalently attached, or with HSA, immobilized on a passivated surface. Utilizing these beads, the assay is performed in a simple set-up without using specific laboratory equipment which is presented along with their use for ADME prediction.

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4.14 Selection of an aptamer against the tissue inhibitor of matrix metalloproteinases

Julia Hoßbach, Rolf Gebhardt

Matrix metalloproteinases (MMPs) are a group of enzymes that regulate the degradation of a variety of tissues. They play an important role in growth, development and tissue repair.

These Enzymes are regulated by their inhibitors, the TIMPs. This regulation is very sensitiv, if it fails, a diverstity of diseases may result. For example, an overproduction of TIMP may lead to fibrosis.

To interfere with the regulation of MMPs and TIMPs, we took a closer look at the possibilities to neutralize the effects of the TIMPs. One way was to select RNA species (aptamers) that may bind to the TIMPs and therefore decrease the efficiancy of the inhibiting effect of the TIMPs.

Several properties of aptamers make them attractiv agents. They act by folding a three dimensional structure, based on their nucleic acid sequence to bind their targets. The binding of aptamers is usually a very specific interaction, with the ability to discriminate between related proteins.

Systematic evolution of ligands by exponential enrichment (SELEX) was used to obtain binding species for TIMP1 out of a pool of RNA oligomers. During the selection process different methods of immobilisation of the target were tested, to avoid the problem of an enrichment of unspecific binding sequences. Using this technique three different sequences were enriched. Crosslinking studies reveal that two of the three sequences bind TIMP specifically. Further binding tests revealed a dependence of bound amounts of these sequences from the amount of TIMP present.

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4.15 Probing laminin self-interaction by chemical crosslinking and ESI-FTICR mass spectrometry

Stefan Kalkhof, Sebastian Haehn, Christian Ihling, Neil Smyth, Andrea Sinz

We employ chemical cross-linking in combination with high-resolution fourier transform ion cyclotron resonance (FTICR) mass spectrometry to obtain structural information about self-interacting regions between N-terminal domains of laminin (LN domains). The heterotrimeric glycoprotein laminin is the main non-collagenious component of basement membranes and controls cellular activities such as adhesion or migration, differentiation and polarity, proliferation or apoptosis. Laminin is essential for formation of basement membranes through interactions with itself and other components. LN domains were cross-linked with a number of homobifunctional, isotope-labeled, amine-reactive cross-linkers, such as sulfo-DSG, DSA and BS^3, bridging distances between 7 and 12 Å. The extent of cross-linking was monitored by one-dimensional SDS-PAGE of the cross-linking reaction mixtures. The bands of the created cross-linking products were cut out and digested *in gel*. For enzymatic digestion we applied a variety of proteases, such as trypsin, AspN, or LysC as well as combinations of these enzymes in order to obtain fragments of appropriate mass (*m/z* 500-4,000 Da) to facilitate detection of cross-linking products. The resulting mixtures of enzymatic peptides were separated by nano-HPLC which is directly coupled to the FTICR mass spectrometer equipped with nano-ESI source. The excellent resolution and mass accuracy of FTICR MS enhances confidence in the identification of cross-linking products in addition to using isotope-labeled cross-linkers. The obtained cross-linking products give information on interacting regions between LN domains.

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4.16 Recombinant expression and characterization of the ecto-nucleotidase CD39

Matthias Krause, Norbert Sträter

In contrast to their intracellular function as a chemical driving force, extracellular ATP and other nucleotides play an important role in autocrine and paracrine cell signalling. They are known to act as molecular cues in many physiological processes, such as thrombosis, inflammation and synaptic signalling. Cellular responses are induced by binding on specific G protein coupled receptors or ligand-gated ion channels.

Signalling effects of extracellular nucleotides are mainly controlled through the hydrolytic breakdown by extracellular nucleotidases. CD39 family members or eNTPDases (ecto-NTP-diphosphohydrolase) catalyze the sequential hydrolysis of β and γ phosphate of triphosphonucleosides. The eNTPDases are bound to the cell membrane by N- and C-terminal transmembrane helices, with their large catalytic domain facing the extracellular space.

These enzymes are now considered as potential new drug targets or drugs themselves. Soluble forms from mammalian cell culture are shown to inhibit platelet aggregation *in vivo.* This might be of high therapeutic use in treatment of coronary thrombosis and stroke. On the contrary, inhibition of eNTPDases may prolong the effect of nucleotides at their respective receptor.

We have established an *E. coli* expression system for insoluble production of the catalytic domain of CD39 and optimized the *in vitro* refolding. This allows for large scale production of active protein for further biochemical characterization and clinical application. X-ray structure determination of the refolded protein will provide valuable insights in the rational design of medically relevant inhibitors.

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4.17 Crystal structure of the 2-oxoglutarate dependent dioxygenase RdpA

Jörn Krauße, Anne Westendorf, E. Bartholomeus Küttner, Roland H. Müller, Norbert Sträter

The most widespread group of mononuclear non-heme iron enzymes is the family of 2-oxoglutarate (2-OG) dependent oxygenases. Its members are found in bacteria as well as eukaryotes and catalyze a large variety of reactions. The catalysis is always coupled with the decarboxylation of 2-OG and the consumption of molecular oxygen. A 2-OG dependent oxygenase was isolated from *Delftia acidovorans* MC1. It carries enantiospecific activity for the etherolytic cleavage of R-(2,4-dichlorophenoxy)propionate (Rdp) and is thus referred to as RdpA. Its substrate belongs to the group of chlorinated phenoxyalkanoates which were widely used herbicides in agriculture and remain as severe soil pollutants. The crystal structure of RdpA is of special interest to analyze its stereospecific substrate recognition in comparison to a homologous enzyme (SdpA) which catalyzes the decomposition of S-(2,4-dichlorophenoxy)propionate (Sdp) exclusively. Such knowledge would possibly allow the design of new specific catalysts.

Initial crystallization conditions for purified RdpA were found using the sparse matrix method. Further refinement of the conditions was carried out to obtain crystals suitable for X-ray diffraction experiments. Data collection was performed at BESSY, Berlin. Orthorhombic crystals of RdpA show anisotropic diffraction up to 2.5 Å resolution. Data scaling was limited to 2.9 Å. The structure was determined by molecular replacement using the homologous protein tauD from *E. coli* as phasing model. First crystallographic data show that RdpA is a tetramer.

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4.18 Application of photoactivatable GFP to investigate dynamics of human mitochondria

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Only 5 % of all mitochondrial proteins are encoded by the genome of these organelles (16,569 bp in *Homo sapiens*). The other 95 % are encoded by the nuclear genome and have to be transported to the mitochondria.

Fluorescent microscopy is a current method for studying cells and fusion proteins.

For tracing the route of the proteins through the cell and to investigate intracellular protein dynamics it would be very helpful to have the possibility to colour only a small region of the cell or only some mitochondria with fluorescent dyes.

An excellent tool for this approach is a photoactivatable GFP (PA-GFP). This is an engineered variant of the *Aequorea victoria* green fluorescent protein which was first published by Patterson and Lippincott-Schwartz[1]. After activation with 413-nm laser light it increases its natural fluorescence approximately by 100 fold when excited by 488-nm light.

By using PA-GFP as a tool for tracking photoactivated fusion proteins it is possible to reveal molecular movement in the cell by fluorescent microscopy with the focus on human mitochondrial proteins.

We engineered PA-GFP using the coding sequence of enhanced green fluorescent protein (EGFP) with the technique of site-directed mutagenesis. Using this approach we were able to convert GFP to the photoactivatable form which undergoes photoconversion upon illumination with ~400-nm light.

Our aim is to use the PA-GFP to generate different fusion proteins composed of PA-GFP and proteins that might play essential roles in mitochondrial biogenesis.

Notes

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4.19 Crystal structures of two thermophilic DNA nucleases as templates for protein engineering

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DNA-cleaving enzymes are a common tool in the molecular biology lab. The use of DNA applications at elevated temperatures, such as PCR, prompts the development of temperature-stable enzymes by protein engineering. Exonuclease III (ExoIII) from the mesophile bacterium *Escherichia coli* (*E. coli*) has the desired catalytical properties but lacks sufficient thermostability. In order to facilitate the design of more thermostable variants of ExoIII we determined the structures of two homologous enzymes from thermophilic archaeons. These enzymes are thermostable but lack the desired catalytical properties. The genes of exonucleases from *Methanothermobacter thermoautotrophicus* (ExoMt, 257 amino acids) and *Archeoglobus fulgidus* (ExoAf, 258 amino acids) which exhibit highest activity at 60-70 °C (ExoMt) and 70-80 °C (ExoAf) were cloned and expressed in *E. coli*. After purification both enzymes could be crystallised in space group $P2_1$ using a 10mer DNA oligonucleotide as an additive. Complete X-ray diffraction datasets were collected from cryo-cooled crystals (100 K) to a maximum resolution limit of 1.7 Å (ExoAf) or 1.8 Å (ExoMt). The three-dimensional structures were solved by the molecular replacement method using the structures of *E. coli* ExoIII (PDB id: 1AKO) [→ ExoAf] or human APE1 (PDB id: 1DEW) [ExoMt] as search models. The structural refinement of ExoAf and ExoMt with bound DNA oligonucleotides is in progress. It is hoped that the crystal structures will provide valuable insight into structural aspects of increased thermal stabilitiy in order to engineer thermostable *E. coli* ExoIII variants for applications in DNA technology.

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4.20 Strategies for the identification of hydroxyproline-isomers and hydroxylysine in collagen

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In addition to the common twenty proteinogenic amino acids several hundred co- and posttranslational modifications, mostly a result of sequence-specific enzymatic activity, are present in proteins. This study targets hydroxylation of proline and lysine in collagens. Typical for all collagen-types is their triple-helical structure formed by three collagen chains containing a high degree of glycine and proline, which is further stabilized by sequence-specific enzymatic hydroxylation. Even though collagen has been widely studied, the distribution of hydroxyproline (Hyp) and hydroxylysine (Hyl) within the sequence as well as the stereochemistry of the Hyp-residues are still not well understood.

The aim of this study was to analyze the Hyl and Hyp content of various collagens. Especially the distribution of the four Hyp isomers, i.e. cis-3-, cis-4-, trans-3- and trans-4-hydroxyproline exhibits an analytical challenge, as these four compounds have very similar physical and chemical properties and are isobaric with leucine and isoleucine. Therefore at least these six amino acids (aa) must be separated to identify them via MS-detection.

We describe two chromatographic approaches for the separation of most proteinogenic and hydroxylated AAs. A direct separation of unmodified aa relies on hydrophilic interaction chromatography (HILIC), while separation by RPC requires pre-column derivatization, in our case performed with an analogue of marfey's reagent, i.e., N^2-(5-fluoro-2,4-dinitrophenyl)-L-valine amide (L-FDVA).

In both cases, on-line coupling to an MS was essential for identification of coeluting compounds.

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4.21 The regulatory environment for the development and approval of biopharmaceuticals

Rüdiger Laub, Ullrich Pigla

The regulatory environment for the pre-clinical development of biopharmaceuticals, the setup of clinical trials, and the application for marketing authorization is a continuously growing jungle made up of regional, national, and international guidelines and legislation.

Yet academic research on a potential investigative new drug (IND) should consider regulations laid down in the guidelines for good laboratory practice (GLP). Only pre-clinical data established under GLP are acceptable for their later inclusion in study protocols, or application trials. The manufacture of the active pharmaceutical ingredient, and its galenic formulation must comply with the guidelines for good manufacture practice (GMP) and its current 19 annexes. Guidelines for good clinical practice (GCP) have to be followed in clinical study plans and in the execution of clinical trials. Although these guidelines provide basic requirements for single steps in the development of an IND, most details are hidden inside legislations which are not harmonized among local areas. Their underestimation may block the next stage of development, and jeopardizes young biotech companies with small finance ceilings.

We here present paradigmatically important regulations which have to considered in the development of a monoclonal antibody-derived biopharmaceutical.

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4.22 Discrimination of vaccinated and infected pigs by *Salmonella*-specific IgA antibodies

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Due to intensified stockbreeding the risk of Salmonella transmitted to man by consumption of animal food products increased. Vaccination with live attenuated vaccines is a tool for reduction of *Salmonella* burden of pig herds. Unfortunately, there exists no reliable assay to discriminate vaccinated from infected pigs so far. Ig isotype switching leads to generation of high affinity antibodies (Ab) during the immune response. This can be applied for differentiation of early and late infection stages. The aim of this study was to evaluate the applicability of a novel Ig-isotype specific *Salmonella Typhimurium* (STm) Ab ELISA for discrimination between vaccinated and infected pigs.

Serum IgM, IgG, and IgA Ab were captured by a whole cellular extract antigen of STm and detected by isotype-specific HRP-conjugated secondary Ab. Sera from vaccination/challenge experiments were used for evaluation of the test.

Using this ELISA, different patterns of specific IgM, IgG and IgA Ab were detected following immunisation with a STm live vaccine or experimental infection with a wild-type strain of STm. Interestingly, STm-specific IgA Ab represented an excellent tool for the recognition of acute infection in vaccinated pigs. Under SPF conditions, we were able to differentiate between naive, vaccinated, experimentally infected non-vaccinated and vaccinated animals. However, the highest specific IgA levels were detected in challenged vaccinated pigs. Results from field trials confirmed these findings.

This novel test offers a hopeful tool to promote the acceptance of immunisation of pig herds using live vaccines under diagnostic control.

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4.23 Selective inhibition of protein kinases by emodin-related compounds

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The enzymes CK-I, CK-II and GSK-III are ubiquitous serine/threonine protein kinases and essential for viability and multiple mechanisms of regulation in eukaryotic cells. The hydroxyanthraquinone emodin, extracted from the rhizome of *Rheum palmatum L.*, was recently described as potent, ATP-competitive inhibitor of cell cycle regulating proteins. In fact, derivatives of emodin seem to be attractive compounds for antiviral and anti-tumor chemotherapeutics.

Emodin and the derivatives rhein, emodinic acid and the emodinic acid ethyl ester are very effective (IC_{50}-values between 0.5 and 5 µM) and fairly specific (within 1:20 to 1:120 of IC_{50}-values against CK-I and GSK-III) inhibitors of protein kinase CK-II. This kinase is abnormally elevated in a wide variety of tumors. The results show, that the activity of CK-II can be highly selective inhibited.

Some of these compounds show a higher cytotoxic activity for the tumor cell line HepG2 in comparison to normal hepatocytes. By further modification, undiserable side-effects can be minimized and new therapeutically possibilities may arise.

With the help of computer-assisted analysis and molecular modelling we want to verify our results and give an outlook on new promising derivatives.

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4.24 Chip calorimeter as a control device for the monitoring of bioprocesses

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We demonstrated the suitability of a miniaturized calorimeter for incorporation into technical relevant bioprocesses as a monitoring device. The calorimeter is sensitive enough to detect metabolic heat production rates in microbial suspensions of few micro-litres. Due to the small time constants a fast on-line operation in connection with bioreactors is possible even for aerobic processes.

As example for the monitoring of aerobic and anaerobic processes the growth of *Escherichia coli* DH5α and *Halomonas halodentrificans* CCM 286T cultures, respectively, was calorimetrically determined. Periodically, small portions of bacterial suspension were taken out of a bioreactor and transferred into the calorimetric chip transducer of the system. The actual heat production rate of the bacterial culture was indicated with a time delay of less than five minutes. The time dependence of the heat production rate was sufficiently described by thermokinetic models.

In a second example the high flexibility of the chip calorimeter system was demonstrated by measuring the metabolic activity of a biofilm which was established inside the calorimetric chip transducer. For example, biofilms cause damages of technical tubing systems by clogging. Therefore, a great interest exists in methods for the non-destructive inspection of tubings with respect to the actual state of the contamination. As proof of principle the internal surface of several calorimetric transducers was externally loaded with *Escherichia coli* DH5α. Then, the metabolic activity of the bio-film was tested by injecting of carbon-substrate solution into the calorimetric chip transducer.

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4.25 Myeloperoxidase-induced effects on spermatozoa

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Inflammations in the male genital tract play a steadily increasing role in the reduced male fertility. Different leukocyte subpopulations including neutrophiles, monocytes/ macrophages and lymphocytes are invading into inflammatory loci. The heme-enzyme myeloperoxidase (MPO) is released from stimulated neutrophils and becomes attached to negatively charged cell membranes. Due to its extraordinary reactivity it contributes to the production of reactive oxygen species and causes halogenation of biological material. The binding of myeloperoxidase to the spermatozoa surface could be demonstrated in connection with myeloperoxidase antibodies by confocal fluorescence microscopy. The presence of albumin in the incubation mixture decreases the binding of myeloperoxidase to spermatozoa epitopes. MPO impairs the vitality of spermatozoa in dependence on the enzyme activation with hydrogen peroxide from 52 to 31 %. MPO-derived hypochlorous acid induces the externalisation of phosphatidylserine as detected flowcytometrically with annexin V, impairs the cell vitality and induces an enhanced formation of lysophospholipids. A significant increase of annexin V binding to 43 % can be noticed at 10^{-5} mol/l hypochlorous acid, while an increasing of lysophospholipids first occurs with 10^{-4} mol/l hypochlorous acid. Vitality lost can already be seen at 10^{-6} mol/l hypochlorous acid. These results indicate drastical changes in membrane structure caused by myeloperoxide-derived hypochlorous acid whereas phosphatidylserine-exposition precedes the lysophospholipid formation.

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4.26 IZKF Leipzig Core Unit "Fluorescence Technology" – Flow cytometry and cell sorting

Andreas Lösche, Viola Döbel

Flow cytometry is a local measuring technique to analyze and/or sort suspended individual cells (or particles). Optical signals (scatter, fluorescence) are sequentially generated by each single cell and detected and displayed for cell distribution analysis.

There are three analysers in our lab (FACScan, LSR and FACSCalibur from Becton Dickinson) and a high-speed-sorter FACSVantage SE (Becton Dickinson). Up to three independent laser beam spots (UV, 488 nm, and 633 nm) are used as light sources and up to six fluorescence signals can be collected for each cell.

By correlating the forward scatter signal (FSC) and the side scatter signal (SSC), subpopulations corresponding to lymphocytes, monocytes and granulocytes can be detected in peripheral blood samples, and unwanted signals such as cell debris ar cell aggregates can be excluded from analysis.

Staining cells with fluorochrome-conjugated antibodies or fluorochromes which interact directly with specific targets such as DNA, cell subpopulations can be characterised according to phenotype and/or cell cycle phase.

The sorter has the ability to selectively deposit cells from particular populations into tubes, or other collection vessels such as wells of microtiter plates in a sterile environment.These selected cells can be used afterwards for further experiments, culture, or staining with another dye/antibody and reanalysis.

Flow cytometric applications include:
- cell cycle analysis
- apoptosis analysis
- multicolor (phenotypic) analysis
- functional analysis
- stem cell analysis
- sterile cell sorting.

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4.27 IZKF Leipzig Core Unit "Fluorescence Technology" – Laser scanning cytometry

Andreas Lösche, Anja Mittag, Attila Tarnok

The laser scanning cytometer (LSC) combines the advantages of flow cytometry, image analysis, and automated fluorescence microscopy. It allows measurements of light scatter and fluorescence but also records the position of each measured object. Cells of interest can be re-located, visualised, re-stained, re-measured and photographed. In common with flow cytometers, the LSC measures multicolour fluorescence and light scatter on a single cell basis. This technology is of particular interest if cell numbers are low, small subpopulations are being examined or morphological information is required in addition to phenotypic information.

The LSC in our lab (CompuCyte) is equipped with two lasers (Argon ion 488 nm and HeNe 633 nm) and is designed around a standard Olympus BX50 fluorescence microscope. The motorised stage has a stepper motor moving in 0.5 µm increments. In this way a pixellated bit map on the slide is built up in 0.5 µm squares. For each pixel, the fluorescence value for each measured parameter is recorded. So a number of parameters can be measured, e.g. area (the number of pixels occupied by the cell), the integral value (the sum of the fluorescence values for each pixel in the contour) and the maximum pixel (the position and value of the brightest pixel within the contour). Additional factors can also be measured, e.g. cell perimeter and "texture".

Examples of applications:

- cell cycle analysis
- cell proliferation
- apoptosis
- immunophenotyping
- enzyme kinetics
- cytogenetics
- cell-cell interaction.

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4.28 Binding proteins for advanced glycation endproducts isolated from different rat tissues

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Non-enzymatic glycation of proteins by reducing sugars and subsequent metal-catalyzed oxidation leads to a formation of advanced glycation end products (AGEs). AGEs are involved in several diseases, such as Alzheimer's disease, diabetic retinopathy and nephropathy, atherosclerotic deletions of blood vessels, and inflammation reactions. The accumulation of AGEs in the body is suspected to lead to an inhibition of cellular functions and transport processes, and finally to cell dysfunction and death. AGEs are known to bind specifically to different cell surface receptors and other AGE-binding proteins. A number of AGE-binding proteins have been identified, including the receptor for advanced glycation end products (RAGE), oligosaccharyl transferase-48 (OST-48 or AGE-R1), 80K-H phosphoprotein (AGE-R2), and galectin-3 (AGE-R3). These proteins have been shown to be present in many different cell types, for example in monocytes, macrophages, endothelial cells and microglia. In the present study a method for purification of AGE-binding proteins is described. Organs from liver, lung and kidney of male Wistar rats were used. Membrane proteins were isolated by differential centrifugation and subsequent purification by an AGE-affinity chromatography. AGE-binding proteins were analyzed by 1D-SDS-PAGE and Western blot. The membrane fractions contained at least 10 proteins in all tissues interacting with AGEs. Our results indicate the existence of different binding proteins in rat tissues. This work suggests a suitable method to identify new binding proteins for AGEs

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4.29 Characterization of recombinant antibody fragments to human CD4 expressed in transgenic tobacco

Dirk Mischke, Sunna Hauschildt, Jörg Böhme, René Brecht, Frank Emmrich, Ullrich Pigla, Rüdiger Laub

The minimization of biopharmaceutical molecules is an important step to improve its performance and safety in therapeutic or *in vivo* diagnostic applications. Large proteins such as monoclonal antibodies may possess several disadvantages. They may have limited distribution, retarded clearance, and may exert other side effects simply due to their molecular size. Further, they may elicit severe immunological responses to repeated applications.

We have engineered single chain variable-fragment (scFv) and minibodies (mb) from the variable region of the heavy (VH) and light (VL) chain domains of a monoclonal antibody to human CD4, a typical surface molecule on helper T cells and monocytes. The coding sequences for the scFv and mb were cloned into a plasmid of Agrobacterium spec. which was used to transform tobacco plants.

We have established a panel of quality control analyses to test the purity of the transgenic fragments extracted and purified from plant tissues. The binding and specifity of the transgenic fragments were demonstrated by flow cytometric assays using peripheral mononuclear cells (PBMC) as indicator cells. Investigation of functional parameters revealed, that the mb but not the scFv is able to modulate the CD4 molecule on helper T cells like the parental anti-CD4 mAb does.

While important functional properties are retained, we expect from minimization better pharmacokinetic characteristics, and significantly reduced immunogenicity.

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4.30 Separation of phosphopeptide isomers using capillary-zone-electrophoresis

Marika V. Mützelburg, Peter Hoffmann, David Singer, Ralf Hoffmann

One of the most common posttranslational modifications of proteins is O-phosphorylation of seryl-, threonyl- and tyrosyl residues. Many proteins are phosphorylated at different sites. At the molecular level the phosphorylation pattern is often very complex and inhomogeneous. The separation of phosphopeptide isomers, which often exist in mixtures, by RP-HPLC or other techniques is very difficult or impossible due to the very similar properties. As phosphopeptide isomers have equal masses, high-resolution mass spectrometry fails too.

The tau protein, which plays an important role in the Alzheimer's disease, is a well investigated phosphoprotein. At least 26 different phosphorylation sites have been determined by immunological techniques and sequence analysis. The phosphorylation sites are mostly located in four regions of the tau protein, each region spanning about 20 amino acid residues. Singly to quadruply phosphorylated peptides are present in tryptic digests. The distribution of the phosphate groups on the molecular level, that is, the phosphopeptides isomer pattern, is still unknown.

The aim of this work was to separate singly, doubly and triply phosphorylated tau peptide isomers with capillary electrophoresis. Fused silica capillaries have been used to separate acidic and basic peptides. The impact of different buffer systems, with and without organic solvent, on the migration times and the resolution of the peptides was studied. By optimizing the separation conditions it was possible to establish a method to separate phosphopeptide isomers for both acidic and basic sequences.

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4.31 Mass spectrometric methods and the investigation of the kinetic properties of cellular phospholipases

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Phospholipases are important heterogeneous group of cellular enzymes. Their activation is an important step in the intra- and inter-cellular signalling processes. Phospholipases produce various second messengers that further influence the activity of other cellular enzymes - e.g. protein kinase C.

Various methods are nowadays established for the investigation of the activity either of isolated or the cellular enzymes. A serious drawback of these methods is that most of them use modified substrate. The use of natural substrate is preferred, since in this case the phospholipase activity might not be altered. With that respect, mass spectrometric (MS) methods are advantageous over other, well established methods for the investigation of the activity of phospholipases. In the assays employing MS in most cases lipids - a substrate or one of the products - are measured. Along with kinetic parameters important structural information are easily obtained by MS.

In spite all advantages offered by MS methods in this field, there are only a few examples on their application and they concern the activity of phospholipase A_2. These applications are presented in this poster, together with experiments to use matrix-assisted laser desorption and ionisation time-of-flight MS for the investigation of the kinetics of cellular phospholipase D and phospholipase C.

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4.32 Phenotyping of mutans streptococci by intact cell MALDI-TOF-MS after caries excavation

Stefan Rupf, Knut Merte, Knut Breitung, Susanne Kneist, Klaus Eschrich

The study aimed to analyze the heterogeneity of *Streptococcus mutans* strains from the cavity floor of deciduous molars by intact cell matrix-assisted-laser-desorption/ionization-time-of-flight mass spectrometry (ICM).

Hard dentin samples from 32 teeth of 20 children were taken after chemo-mechanical caries excavation (T_1) and after eight weeks of temporary filling (T_2). 261 strains of mutans streptococci (MS) were isolated. Strains were identified and classified by ICM and hierarchical clustering based on known MS reference strains. Results were confirmed by species specific PCRs and 16S rDNA sequencing.

258 of the 261 MS strains were identified as *S. mutans*, two as *Streptococcus downei* and one as *Streptococcus sobrinus*. *S. mutans* was found in 19 of the 32 investigated teeth at T_1 and in 13 of 21 teeth at T_2. Identical S. mutans strains were found in the same teeth at T_1 and T_2, as well as in different teeth of individual patients. Between one to seven different phenotypes of S. mutans were found per cavity. Overall 59 phenotypes occurred in the population of 258 *S. mutans* strains. The complexity of the isolated *S. mutans* population was similar at T_1 and T_2. The overall statistical comparison of the phenotypes revealed solutions on different similarity levels.

At a low infection level clonal heterogeneity of *S. mutans* remained in carious lesions after chemomechanical caries removal which persisted after a period of temporary filling. ICM was found to be appropriate to detect rare species in a uniform MS population. The species *S. downei* was identified in a human carious lesion for the first time.

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4.33 Interaction of myeloperoxidase with human serum albumin

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Myeloperoxidase (MPO) - is a glycosylated heme-enzyme with molecular weight 140 kDa. It is present in neutrophils and in monocytes and released by activated phagocytes into the phagolysosome or into the extracellular space in responce to a variety of agonists. We investigated interaction of MPO with the plasma protein albumin. Albumin - is an important plasma protein with a molecular weight of 66 kDa. It is responsible for maintaining the transendothelial oncotic pressure gradient and regulating the transport of fatty acids, steroids, thyroxine and amino acids. Albumin also exists in the extavascular space.

The association of MPO and albumin was confirmed by ELISA technique and native gel electrophoresis. MPO (pI~10) exhibited a time-dependent binding to albumin (pI~4.7) coated microtiter plates in PBS solution by pH value 7.4 but not by pH value 5.0. This binding could also be blocked in the presence of 0.5 % Tween20 or 40 mg/ml albumin in PBS solution (pH 7.4). By FACS analysis, it was demonstrated that interaction of RITC-labelled MPO with albumin-coated silica particles can be reversed by incubation with albumin solution. We also used MALDI-TOF mass spectrometry to map the sites of MPO-mediated albumin modification. Especially modifications of methionine as well as halogenations of tyrosine residues of albumin were observed.

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4.34 Comparing the biophysical properties of sterols in lipid membranes – What is special about cholesterol?

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Cholesterol is a major constituent of mammalian cell membranes. Its biophysical properties are essential for several membrane functions. However, a recently described generation of knock out mice, entirely lacking cholesterol[1], showed surprisingly mild phenotype. Since the cell membranes of these mice contained desmosterol, the direct precursor of cholesterol, we compared the biophysical properties of membranes containing cholesterol, desmosterol and lanosterol (a more distant precursor).

Membrane packing properties were studied by ^{2}H NMR, fluorescence, and EPR spectroscopy. It was found that cholesterol and desmosterol showed a very similar behavior, while lanosterol produced significantly weaker effects.

We also investigated the effect of the sterols on the lateral diffusion of the lipids and of the sterols, respectively, by ^{1}H PFG MAS NMR spectroscopy. In the liquid-crystalline phase, the phospholipid diffusion coefficients are decreased in the presence of all three sterols. In the lo phase this decrease strongly depends on the cholesterol concentration. Cholesterol mirrors the lipid behavior, but exhibits a slightly faster diffusion. In the lanosterol and desmosterol containing membranes, the lipid diffusion rates are somewhat higher than those for cholesterol. While desmosterol diffusion rates are also only slightly higher than for the lipids, lanosterol diffusion rates significantly exceed lipid diffusion rates. This indicates weaker interactions between the lipids and lanosterol, which explains the weaker condensation effect of lanosterol.

Notes

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4.35 Studying calmodulin-adenylyl cyclase 8 interaction by chemical cross-linking and FTICR mass spectrometry

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Chemical cross-linking combined with mass spectrometry has emerged as an alternative strategy to obtain low-resolution structural data of protein interfaces from low quantities of proteins within a relatively short time[1].

In our present work, we study the interaction between the C-terminal region of adenylyl cyclase 8 (AC 8) and its binding partner calmodulin (CaM). Adenylyl cyclases are a critically important family of multiply regulated signaling molecules[2]. AC 8 is regulated by Ca^{2+}/CaM and possesses an N-terminal Ca^{2+}-dependent CaM-binding motif (amino acids 35-54), as well as a Ca2+-independent IQ-motif[3] located at the C-terminus (amino acids 1197-1210).

A 25-amino acid peptide comprising the IQ-motif was synthesized. The cross-linking reaction was performed by using different amine-reactive, isotope-labeled (d_0 and d_4) cross-linkers (BS^3 and BS^2G) with varying spacer length and a zero-length cross-linker (EDC). The cross-linked 1:1 complexes were investigated by MALDI-TOFMS to check the extent of chemical cross-linking. Separation of intramolecular and intermolecular cross-linking products was carried out by one-dimensional SDS-PAGE. After excision of the bands of the cross-linked complexes, *in gel* digestion was performed using trypsin. The resulting mixtures were analyzed by nano-HPLC/ nano-ESI-FTICRMS. We expect to gain further insight into the CaM/AC 8 interaction based on the identified intermolecular cross-linking products.

Notes

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4.36 *In vitro* evolution of a thermostabilized Exonuclease III

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Exonuclease III (ExoIII) of *Escherichia coli*, a DNA-modifying enzyme with four activities, is very frequently used in molecular biology[1]. To expand its applicability a thermostabilized variant of ExoIII could be useful for a method of recombining of homologous DNA sequences[2]. *In vitro* recombination methods have been successfully used to enhance enzyme thermostabilities[3]. Especially DNA family shuffling is a powerful tool for directed evolution combining sequences from individual genes by recombination[4]. In order to generate a library of chimaeric genes we successfully isolated, cloned and sequenced genes encoding the ExoIII gene products from four microbial species closely related to *E. coli*. DNA family shuffling was used to generate a library containing recombined sequences of these homologous genes[5]. Directed evolution experiments require selective screening of great libraries, hence we needed to be able to determine the ExoIII activity against any background activity of *E. coli* in a high throughput compatible way. We therefore established a reliable protein expression and purification protocol as well as an ExoIII activity assay using microtiter plate technology. The assay is based on contact mediated fluorescence quenching in a linear duplex DNA probe and could be shown to be qualified for sensitive screening of ExoIII activity.

Notes

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4.37 Purification and structural characterization of the annexin II / p11 tetramer from porcine small intestine

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The annexin II / p11 heterotetramer (A2t) is a multifunctional protein composed of two annexin II and two p11 (S100A10) subunits. Ca^{2+}-binding mediates its interaction with phospholipids in membranes. A2t plays an important role in membrane interactions, membrane trafficking, and signaling.The purified tetrameric complex was used for structural studies of the A2t interface.

A2t was purified from pig (*Sus scrofa domestica*) small intestine[1]. Briefly, mechanically disrupted mucosal scrapings were washed repeatedly with buffer and centrifuged, retaining the pellet with membrane-bound A2t in the presence of calcium. Addition of EGTA released the tetrameric complex into the supernatant. Further purification was achieved by ion exchange chromatography. The proteins were *in gel* digested and analyzed by nano-HPLC/nano-ESI-FTICRMS and MALDI-TOFMS. The cross-linking reactions were performed using the amine-reactive, isotope-labeled cross-linkers BS^3, BS^2G (d_0 and d_4 each) and DSA (d_0 and d_8).

Sequence coverages from peptide mass fingerprinting of annexin II and p11 were 100 % and 72 %, respectively. Our data on the sequence of porcine annexin II confirm the recently published amino acid sequence[2]. The distance constraints from chemical cross-linking are in good agreement with the X-ray structure published for monomeric annexin II.

The A2t complex will be further characterized with respect to its three-dimensional structure employing chemical cross-linking and FTICRMS.

Notes

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4.38 Protein-ligand interaction of S-adenosyl-L-methionine and the methyltransferase SU(VAR)3-9

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The recently developed Saturation transfer difference (STD) NMR technique[1] was used to investigate the interaction of S-adenosyl-L-methionine (AdoMet) with SU(VAR)3-9 from *Drosophila melanogaster*. SU(VAR)3-9 has a SET domain and plays an important role in methylation of lysine-9 of histone H3 which results in formation of heterochromatin and gives rise to gene silencing.

We were able to detect a binding interaction of SU(VAR)3-9 and AdoMet in solution and determined the binding epitope of AdoMet. Comparision of the data with a crystal structure of SET7/9 ([2], another SET protein) shows a good agreement for most of the protons.

We therefore conlude, that beside differences for certain ligand protons of AdoMet, the overall binding interaction is similar for the crystal state and SU(VAR)3-9 in solution.

The binding epitope will lead to further investigation of SU(VAR)3-9 mutants with different activity. We plan to combine STD measurements with the transfer NOE technique to test possible conformational changes of the ligand.

Notes

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4.39 Characterisation of antibodies recognizing phosphorylation dependent epitopes in the tau protein

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The microtubule-associated protein tau promotes the assembly and stabilisation of neuronal microtubules. In Alzheimer's disease, tau becomes hyperphosphorylated and self-associated to form paired helical filaments (PHF). Monoclonal antibodies (mAbs) are very useful tools to analyse the phosphorylation status of tau proteins and might be of diagnostic value, if a disease specific phosphorylation pattern is recognised. For that commercially-available polyclonal and monoclonal antibodies directed against several known phosphorylation sites, including Ser212 and Ser231, were tested for their specificity in both ELISA and Western blots. Thus, sequences corresponding to human tau-regions containing several phosphorylation sites, especially 207-219, 224-240 and 390-410 with phosphorylation sites at Ser212, Thr214, Ser217, Ser218, Ser231, Thr235, Ser396, Thr400, Ser403 and Ser404, were synthesised on solid-phase using the Fmoc/tBu-strategy. The peptides were phosphorylated on the solid-phase before deprotection by the global phosphorylation approach using phosphoamidite. After cleavage with trifluoroacetic acid, purification by RP-HPLC and characterisation by mass spectrometry, the phosphopeptides containing up to four phosphate groups were used to map the antibodies for their phosphate-specificity. Furthermore, the antibodies were tested against bovine tau by immunoblots after two-dimensional gel electrophoresis.

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4.40 Proteome analysis of *Escherichia coli* using high-performance liquid chromatography and fourier transform ion cyclotron resonance mass spectrometry

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The basic problem of complexity poses a significant challenge for proteomics studies. To date, 2-DE (two-dimensional gel electrophoresis) followed by enzymatic *in gel* digestion of the peptides, and subsequent identification by mass spectrometry is the most commonly used method to analyze complex protein mixtures. However, 2-DE is a slow and labor-intensive technique, which is not able to resolve all proteins of a proteome. To overcome these limitations gel-free approaches are developed based on high-performance liquid chromatography (HPLC) and Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS). The high resolution and excellent mass accuracy of FTICRMS provides a basis for simultaneous analysis of numerous compounds[1]. In the present study, a small-protein subfraction of an *E. coli* cell lysate was prepared by size-exclusion chromatography and proteins were analyzed using C4 reversed phase HPLC for pre-separation followed by C18 reversed phase nano-HPLC/nano-ESI-FTICRMS for analysis of the peptide mixtures after tryptic digestion of the protein fractions[2]. We identified 231 proteins and thus demonstrated that a combination of two reversed phase separation steps - one on the protein and one on the peptide level - in combination with high-resolution FTICR mass spectrometry has the potential to become a powerful method for global proteomics studies.

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4.41 Mapping protein interfaces by affinity cross-linking combined with MALDI-TOF and ESI-FTICR mass spectrometry

Andrea Sinz, Stefan Kalkhof, Christian Ihling

Chemical cross-linking of proteins in combination with mass spectrometric analysis of the reaction products allows a rapid mapping of protein interfaces, which is crucial for understanding protein/protein interactions. The identification of cross-linking products from the complex mixtures created after the cross-linking reaction however remains a daunting task.

In order to facilitate the identification of cross-linking products, we are exploring the use of the trifunctional cross-linker sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate), which possesses one amine-reactive and one photo-reactive site and additionally allows an affinity-based enrichment of cross-linker containing species via its biotin moiety. As model system, we chose the complex between calmodulin and its target peptide M13. The peptide M13 comprises 26 amino acids and represents a part of the C-terminal sequence of the skeletal muscle myosin light chain kinase.

After the cross-linking reaction, the reaction mixtures were subjected to tryptic *in solution* digestion. Biotinylated peptides were enriched on monomeric avidin beads after a number of washing steps had been performed. Peptide mixtures were analyzed by MALDI-TOF and nano-HPLC/nano-ESI-FTICR (electrospray ionization Fourier transform ion cyclotron resonance) mass spectrometry. We demonstrate that an enrichment of cross-linker containing species allows a more efficient identification of interacting amino acid sequences in protein complexes. This strategy is expected to be especially beneficial for investigating large protein assemblies.

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4.42 Localisation of the PHB-biosynthesis in recombinant yeasts

Claudia Stolz, Uta Breuer, Hauke Harms

The polyester polyhydroxybutyrate (PHB), a member of the family of the polyhydroxyalkanoates (PHA), possesses similar properties as polypropylene and polyethylene, which, however, are problematical wastes. PHAs are synthesized by many bacteria as an intracellular carbon and energy storage material with yields of up to 90 % of the biomass. The most interesting properties of these materials are the biodegradability and the biocompatibility. Recently, these materials became interesting for medical applications, e.g. in implants, scaffold in tissue engineering, or as drug carriers. For the production of recombinant PHB in yeasts, the three biosynthesis enzymes (Reductase, 3-Ketothiolase and PHB-Synthase) have been transformed in *Saccharomyces cerevisiae* INVSc1. Up to now the maximally obtained PHB-yield is 6.7 % per gram biomass. To optimize and increase the PHB-synthesis in yeasts the localisation of the terminal enzyme of the biosynthesis, the PHB-Synthase (PhbC) and the polymeric product is very important. The protein phasin (PhaP) is also important in this respect; it stabilizes the developing PHB granules thus leading to an increase of the PHB-synthesis. Probably, the recombinant PHB is accumulated in yeasts within the lipid bodies because of its lipophilic properties. Up to now no microscopic analyses involving staining could differentiate between lipids and PHB. To localize the recombinant PHB in yeasts, translational fusions of the PHB-Synthase and the phasin with the fluorescent reporter yEGFP (yeast enhanced green fluorescent protein) were constructed. First results are reported.

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4.43 *In vitro* selection of DNA aptamers binding ethanolamine

Beate Strehlitz, Dörthe Mann, Christine Reinemann, Regina Stoltenburg

Aptamers are ssDNA or RNA oligonucleotides with very high affinity to their target, developed by the SELEX-process. They bind to the target with high selectivity and specificity due to their secondary structure. Aptamers will present an attractive alternative for both *in vitro* and *in vivo* applications in the fields of clinical diagnostics and therapy.

We have identified novel aptamers binding to ethanolamine with high affinity. These specific aptamers were selected from an initial 96mer DNA-library in a FluMag-SELEX process. This process is characterized by the use of fluorescein-labeled ssDNA for quantification and magnetic beads as matrix for the immobilisation of the very small target molecule ethanolamine (M_r= 61,08). After this process the enriched aptamer pool was cloned to get individual aptamers, which were sequenced and characterized. The affinity constants (K_D) determined by binding curves range between 6-18 nM.

The aim of the development of ethanolamine specific aptamers is their use for the detection of this substance in clinical and environmental analysis. Ethanolamine is involved in the biosynthesis of glycerophospholipids and in the biosynthesis of acetylcholine as an important neurotransmitter. Disorders of this biosynthesis are connected with diseases like Schizophrenia or Alzheimer's disease as well as the storage disease Ethanolaminosis. The easy and fast determination of ethanolamine in blood or in plasma membranes should be helpful for the early diagnosis of these diseases. A biosensor based on ethanolamine aptamer receptor molecules could be a time and cost effective tool.

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4.44 The calcium-dependent regulation of TRPV4 involves the interaction with the carboxy-terminus of Calmodulin

Rainer Strotmann, Torsten Schöneberg

The calcium-permeable cation channel TRPV4 is activated by a number of stimuli, including hypotonic cell swelling, certain derivatives of arachidonic acid and phorbol esters.

A unique property of the TRPV4-mediated current is its calcium-dependent potentiation. In previous studies, we could demonstrate that the underlying molecular mechanism involves the binding of the cytosolic calcium sensor protein Calmodulin (CaM) to a C-terminal binding domain in TRPV4.

In the present study, we have used an *in vitro* proximity assay based on the AlphaScreen™ technology (Perkin Elmer) to map the TRPV4 binding site within the CaM molecule. CaM is a highly symmetrical molecule with two calcium-binding EF hand (EFH) motifs in the N- and C-terminus, respectively. Using truncated and partially calcium-binding deficient mutants, we could show that the interaction with TRPV4 occurs specifically with the C-terminus of CaM and involves the central helical and parts of the EFH3 domains. It could be shown that the AlphaScreen™ assay system can be customized to characterize protein-protein interactions on a nanomolar scale and in a high throughput fashion.

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4.45 The reverse transcriptase of HIV – Generation of a polymerase with reduced replication fidelity

Sascha Stumpp, Susanne Brakmann

Polymerases are vital for the processes of life. They maintain genetic information in every life-form in a characteristic manner. Isolated polymerases, for example, differ strongly in fidelity, processivity and substrate specificity. Our work focuses on the reverse transcriptase of HIV, which exhibits an average mutation rate of ~$5x10^{-5}$ per nucleotide per replication cycle[1]. As a result, viral offspring differs remarkably in its genotype. Genetic diversity of the virus makes medication very difficult. One possible approach is the further reduction of viral replication fidelity, driving viral progeny to complete loss of infectivity[2]. Using nucleoside analogs, extinction of HIV has been verified *in vitro*[3]. Our plan is to generate a novel reverse transcriptase with an increased error-rate. Currently we apply techniques like shuffling and error-prone PCR to generate mutant libraries and select for active variants with a genetic complementation system. In further studies, we will use a fluorescence-based assay for the identification of mutant reverse transcriptases with increased error-rates[4].

These newly generated polymerases could provide a basis for further studies of structure-function relationships and a future antiviral strategy.

Notes

1 Gao F. et al., J. Virol. 78:2426-2433 (2004).
2 Eigen M. et al., WO 94/01545, (2002).
3 Loeb L.A. et al., Proc. Natl. Acad. Sci. USA 96:1492-1497 (1999).
4 Marx A. et al., Ang. Chem. Int. Ed. 41:3620-3622 (2002).

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4.46 A phosphopeptide capturing Fe kit designed for selective isolation of phosphopeptides on the surface of superparamagnetic micro particles

Corinna Sykora, Peter Hoffmann, Thomas Elßner, Markus Kostrzewa, Ralf Hoffmann

Protein phosphorylation is one of the most important regulatory mechanisms in the cell. It regulates for example the activity, localisation or interaction with other proteins. Identification of phosphorylation sites is complicated due to low phosphorylation degrees, endogenous phosphatase activity, unspecific binding to metal surfaces and discrimination during the ionization process of mass spectrometrical analyses. The aim of the work presented here was to establish a general, robust method to enrich phosphopeptides from complex peptide mixtures based on immobilized metal affinity chromatography (IMAC).

The "Phosphopeptide Capturing Kit" (Bruker Daltonics, Bremen, Germany) is designed for selective enrichment of phosphopeptides, for example from protein digests. Here, purified synthetic phosphopeptides with different but well defined phosphorylation states (none, singly, doubly and triply phosphorylated) and digests of phosphoproteins as well as protein mixtures were enriched with IMAC. Thus, the enriched peptides were spotted on regular MALDI targets or Prespotted AnchorChip targets (Bruker Daltonics) and analysed using MALDI-TOF/TOF mass spectrometry.

This method worked for singly, doubly and triply phosphorylated peptides. The detection limit for singly phosphorylated peptides was approximately 500 fmol using a tryptic β-casein digest and 5 pmol for the quadruply phosphorylated peptide using a conventional AnchorChip. The "Prespotted AnchorChips" increased the sensitivity about 10 fold allowing identification of the singly phosphorylated at the 50 fmol and for the quadruply phosphorylated peptide at the 1 pmol level.

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4.47 FLOCKTYPE®recIBDV/recIBV/recNDV – The new generation of poultry virological ELISAs

Daniela Trepnau, Claudia Engemann, Katja Höschler, Roland Hackl, Michael Hess, Thorsten Arnold, Johannes-Joachim Arnold, Vikram N. Vakharia, Jörg Gabert, Jörg Lehmann

Several ELISAs, based on inactivated viral antigens, has been developed for serological diagnosis of poultry viral infections. The standardisation of those tests runs into some problems due to the non-homogenous distribution of single antigenic components within different lots of the native antigen.

Alternatively, recombinant proteins can be used as antigens. We have cloned and expressed three recombinant proteins derived from the infectious bursal disease virus (IBDV), the infectious bronchitis virus (IBV), or the Newcastle disease virus (NDV) and developed independent ELISAs by using the respective antigen. These novel tests were proved to be useful for the detection of virus-specific IgY antibodies induced by immunisation.

To prove that the new tests lead to comparable results obtained with conventional commercial test kits, we investigated reference sera obtained from vaccinated chickens with both kinds of tests. The diagnostic specificity and sensitivity were determined to be 100 %. Additionally, a field study with vaccinated chickens revealed that the antibody titre is variable for every flock. Some of them had high antibody levels, indicating that those flocks are well protected. However, other ones had a low antibody level, indicating the need of a booster vaccination. Taken together, all three recombinant protein-based tests are very sensitive, specific and reliable tools for quantification of the vaccination state of poultry flocks. The main advantage is the standardisation in antigen production, which yields high reproducibility and reliability of the new generation of poultry virological ELISAs.

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4.48 Novel protein microarrays for detection of posttranslational modification activities

Larissa Vasilets, Jörg Nerkamp, Carsten Voigt, Michael Braun

One of the most extensive groups of cellular protein activities is those which produce posttranslational modification. They are characteristic of all living cells and highly significant for proteome studies as well as for the development of pharmaceuticals. These activities trigger changes in the functional properties of modified proteins. BIOSCORA is a young biotechnology company, which uses proprietary lab-on-chip technologies to perform detection of posttranslational modification activities free of radioactive or dye labelling. Our technologies enable fast and effective real time detection of protein kinase, phosphatase and protease activities in multiplex. Biosensors for the detection of protein kinase activities are designed *in silico*, cloned in expression vectors, expressed in *E. coli* and purified as heterologous proteins. Assays can be performed in wellplate as well as in microchip scale, and will find applications in proteome studies, molecular medical diagnostics, studies of disease mechanisms, high-throughput screening and toxicological studies. Examples of the new generation of activity microarrays based on the label-free electrical detection and those based on optical detection methods will be presented and discussed.

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4.49 Lipid modifications of a Ras peptide lead to alteration of bilayer packing and softness as detected by 2H solid-state NMR

Alexander Vogel, Guido Reuther, Catherine Katzka, Herbert Waldmann, Klaus Arnold, Michael F. Brown, Daniel Huster

Many proteins involved in signal transduction are anchored to membranes by covalently attached lipid modifications. In this study, we investigated the insertion of the acyl chains of a doubly lipid-modified heptapeptide from the C-terminus of the human N-Ras protein into DMPC membranes using ^{2}H NMR. Information about the chain dynamics and membrane packing properties was obtained from powder-type samples of both deuterated DMPC with protiated ras peptide and protiated DMPC with deuterated ras peptide, at a peptide to lipid ratio of 1:15. De-Pakeing of the spectra yields a number of resolved quadrupolar splittings, which can be assigned to different CD_2 groups in the hydrocarbon chains of the peptide or the lipid, respectively. The order parameters determined for the ras chains are significantly lower than those of DMPC, meaning that the length of the alkyl groups of the peptide is adjusted to match the length of the phospholipid chains. Accordingly, the mean area per hydrocarbon chain is larger for the ras peptide than for the lipid, in good agreement with the assumption that the peptide backbone requires more space at the membrane interface than the lipid headgroups. Additionally, R_{1Z} relaxation rate measurements have been performed. While the square law plot for DMPC in the presence of ras peptide shows a straight line, the plot for ras peptide in a DMPC membrane is bent and is comparable to that of DMPC in the presence of a detergent like $C_{12}E_8$. Therefore, the hydrocarbon chains of the ras peptide are more flexible and softer than those of the surrounding lipid matrix.

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4.50 Characterization of the molecular distribution of bovine tau protein with respect to its posttranslational modifications

Daniela Volke, Peter Hoffmann, Ralf Hoffmann

The brain of Alzheimer's disease (AD) patients is characterized by senile plaques (SP) and neurofibrillary tangles (NFT). The major proteinaceous component of SP is the amyloid β-peptide, Aβ. The tangles represent dense accumulations of paired helical filaments (PHF), comprising excessively phosphorylated forms of the low molecular weight microtubule associated protein, tau. Functionally, tau binds to tubulin and PHF-tau practically does not caused by its hyperphosphorylation. In recent years, this hypothesis was extended to other posttranslational modifications, such as glycosylation and isomerization. However, this hypothesis is based mostly on separation of the tau-isoforms by SDS-PAGE and probing the western blots with different sets of antibodies, without separating the modified versions of each isoform.

Our work aims to separate all tau-isoforms including their posttranslationally modified versions using 2D gel electrophoresis followed either by mass spectrometrical identification of the spots or probing them with monoclonal antibodies (mAbs) of different specificity. Thus, we could show that more than 50 tau-versions are present in bovine brain. The image of these tau-versions after 2D gel electrophoresis was independent of the preparation protocols starting from fresh calf brain samples. This indicates that the obtained pattern resembles closely the molecular distribution *in vivo.* Furthermore, we could show that all tau-versions can be enriched by affinity-chromatography using mAb BT-2. The complex pattern of posttranslational modifications as well as a novel concept to explain the data will be presented.

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4.51 Monitoring of biotechnological processes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Patrick Wunderlich, Ina Schulze, Melanie Nickel, Katrin Emmerling, Dirk Mischke, Ullrich Pigla, Rüdiger Laub

The manufacture of biopharmaceuticals is a complex process of biotechnological methods such as fermentation and subsequent alteration and purification of active pharmaceutical ingredients (API) based on proteins. The analysis of discrete protein species is of pivotal significance to manage single production steps, as well as for the quality control of the final processed bulk itself.

We have established a GMP-conform process to produce a monoclonal antibody (mAb) in hollow-fiber bioreactors (upstream process, USP), which is subsequently proteolytically cleaved into Fab-fragments and chemically conditioned for radiolabeling (downstream process, DSP).

SDS-PAGE was validated according to the guidelines of the International Conference on Harmonization (ICH) and was used to demonstrate the presence and the identity of the target protein in the fermentation broth (USP). In DSP, the analytical method served as inprocess control to monitor (i) the capture of the target protein by affinity chromatography, (ii) the efficiency of papain-mediated cleavage, and (iii) the results of discrete purification steps.

Moreover, gels were prepared from probes of the final processed bulk to confirm the identity of the API according to the guideline ICH Q6B.

Taken together, SDS PAGE, in conjunction with other analytical methods, has been proven as a valuable tool to facilitate supervising and controlling of complex biomanufacturing processes.

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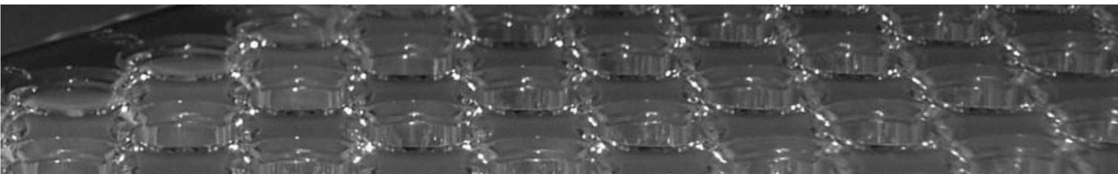
5. MOLECULAR MEDICINE AND THERAPEUTICS



POSTERS

5.1 A candidate gene association study in rheumatoid arthritis, strategies for gene and polymorphism selection and an application of the GENOLINK™ genotyping system

Peter Ahnert

Rheumatoid Arthritis (RA) is a common complex autoimmune disease of rather unclear etiology.

There is evidence that certain patterns of specific variants of specific genes distributed throughout the whole genome are implicated in the genetic risk of RA.

Our approach is to identify such genes in a candidate gene association study.

To select genes and polymorphisms, we aim to integrate current knowledge. So we develop network analysis strategies and apply public mining tools and mainly publicly available data sources. We select SNPs for their position, frequency, ethnicity, state of validation and reported or predicted function (e.g. interference with potential transcription factor binding sites, splice sites and exonic splice site enhancers, and peptide domain architecture). Our genotyping technology is the GENOLINK™; genotyping system (Bruker Daltonics). This comprises PCR, primer extension, and MALDI-TOF analysis. We develop mostly Java™ based software tools to speed up and improve snp selection, assay design and assay optimisation.

We also apply and try to improve current data analysis methods (e.g. decision trees, max. likelihood aproaches, parametric/nonparametric methods, pattern analyses, support vector machines...). In our current undergoing study we investigate 78 different genetic variations, most of them single nucleotide polymorphisms (SNPs) in 31 candidate genes in 450 individuals. Preliminary results based on TDT tests show the association of seven genes to rheumatoid arthritis with p values ranging between 0.01 and 0.001.

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5.2 Gabapentin-lactam failed to prevent rotenone-induced cell death in human neuroblastoma cells

Clemens Allgaier, Julia Kochen, Susana Garcia de Arriba, Ester Verdaguer

Mitochondrial complex-I inhibition by rotenone is used as a model for studying neurochemical alterations associated with Parkinson's disease[1]. As recently demonstrated, the neurotoxic effect of rotenone is counteracted by activation of ATP-sensitive potassium (K-ATP) channels. Gabapentin-lactam (GBP-L) has recently been showed to be neuroprotective and this effect has been related to its K-ATP mitochondrial channel opening properties. Therefore, we investigated in human neuroblastoma (SH-SY5Y) cells the possible neuroprotective effect against rotenone-induced cell damage. Rotenone (1-100 µM for 16 h) induced necrotic cell death in SH-SY5Y cells in a dose-dependent manner. The loss of plasma membrane integrity was assessed by flow cytometry using propidium iodide. In addition, it was demonstrated by staining of cell nuclei with the fluorescent dye Hoechst 33258, DNA laddering and cytochrome c release that apoptotic pathways contribute to rotenone-induced cell death. GBP-L (10-100 µM), pre- and co-applied with rotenone was not able to reduce cell death, to prevent DNA fragmentation or cytochrome c release related to rotenone application. Instead, GBP-L showed a certain level of toxicity that became evident at 100 µM by propidium iodide staining. In conclusion, in SH-SY5Y cells GBP-L did not prevent the rotenone-induced cell damage, but induced at higher concentrations some toxic effects by its own. Accordingly, the present data do not support the idea that K-ATP channel openers such as GBP-L may be useful in the treatment of neurodegenerative diseases related to mitochondrial dysfunction.

Notes

1 Betarbet R. et al., Nat.Neurosci. 3:1301-1306 (2000).

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5.3 The role of β-catenin mutations and expression of glutamine synthetase in the development of hepatocellular carcinomas

Madeleine Austinat, Frank Gaunitz, Andrea Tannapfel, Rolf Gebhardt

Hepatocellular carcinoma (HCC) is the most frequent cancer of the liver. Although activation of the Wnt signalling pathway as indicated by nuclear localization of β-catenin is frequently observed, the prognostic value is still a matter of debate. Recently, glutamine synthetase (GS) was shown to be a putative transcriptional target of β-catenin. Mouse tumors haboring activating β-catenin mutations are stained GS positive. To verify whether this is conferrable we analysed 43 patients suffer from HCC by immunohistochemistry and mutations in the β-catenin gene.

Immunohistochemistry detected intracellular b-catenin in 22 samples. Twelve of these samples were also positively stained for GS. So far, sequence analysis of the ß-catenin gene was performed in 13 samples. In six out of seven samples showing co-localization mutations were identified within the TrCP recognition sequence, whereas in three out of six samples presenting only the β-catenin positive phenotype mutations were detected downstream of this site. Presumably, only specific activating β-catenin mutations are leading to the co-localized phenotype. Mutations in Axin1 or Axin2 may lead to positive nuclear staining of β-catenin due to deranged β-catenin degradation. This may be the case for the remaining samples where no mutations were found. Whether mutations in the Axin genes result only in an activation of β-catenin or may also lead to aberrant GS expression is currently investigated.

Only a subset of β-catenin mutations seems to lead to enhanced GS expression. This is of pathological importance, since it was shown that GS expression in HCCs is a negative prognostic marker.

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5.4 *In situ* cytokine profiling of UVB-exposed human skin by combination of dermal microdialysis and protein microarrays

Marco Averbeck, Simone Beilharz, Matthias Bauer, Carl Gebhardt, Friederike Kauer, Ursula Voith, Jonathan Sleeman, Jan C. Simon, Christian Termeer

Investigation of real time extracellular cytokine secretion *in situ* has been limited by low-cut off filtering membranes and sample volume size. Importantly, the evolving immune response is triggered by early release of a cytokine cascade into the extracellular space. Further, it becomes more and more obvious, that the simplification of the TH-1/TH-2 model cannot copes the *in vivo* situation. Therefore, we combined the methods of intradermal microdialysis and antibody protein arraying to profile the early cascade of multiple cytokines in a complex immune response exemplified by UVB-induced inflammation. Quantitative cytokine analysis of the microdialysis fluid revealed that UVB-irradiation results in a TH2 biased immune response (IL-4, IL10) after 24 h which evolved from a strong and rapid inflammatory response, mainly depending on the secretion of TNF-α, IL-6 and IL-8 in the first 8-16 h; but also included both, TH1 and TH2 associated cytokines by secretion of IL-2, IL-12, IL-4, IL-10, and TGF-β1. The combination of the intradermal microdialysis and protein microarray analysis enable us to get a more realistic picture of the complex and rapidly changing interstitial cytokine milieu in the acute response of human skin to UVB-irradiation.

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5.5 Large scale production of Taxol® from biosynthetical precursors by enzymatic semisynthesis

Martin Becker, Klaus Häupke, Jürgen Hensel, Daria Rybakova, Till Hornbogen, Rainer Zocher, Christian Pflieger, Doreen Liesicki, Dieter Frense, Gerald Lauckner

Taxol® or paclitaxel is a taxoid drug found in the bark and roots of several taxus species. Its unique way of action made paclitaxel one of the most successful therapeutics for treatment of different types of cancer discovered during the past decades.

From the discovery of its antitumor activities to the approval for wide spread clinical applications the supply of paclitaxel has been a constant problem. Highest concentrations of paclitaxel were found in the bark and roots of Pacific or European yews but destructing the endangered yew populations is no way to meet the growing pharmaceutical demand of the substance. Very soon the idea was born to transform biosynthetic precusors of paclitaxel from renewable parts of the yew into paclitaxel. Concentrations of paclitaxels precursor, baccatin, are small but its deacetylated precursor, 10-deacetylbaccatin, is found in the needles of the yew in concentrations up to hundred times higher than the concentration of baccatin or paclitaxel.

Since chemical acetylation of 10-DAB to baccatin is not economically feasible the aim of the presented work was to isolate an acetyltransferase from the roots of the European yew and develop a large scale semisynthetical process for the production of baccatin. Based on this enzyme a new approach for bac-catin production using an enzyme membrane reactor is presented as well as refined processes for the isolation of 10-DAB and baccatin. Semisynthetic production as large as 5 g baccatin per week was acchieved.

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5.6 Depot-specific differences in adipocyte biology and adipose tissue gene expression-parallel regulation in humans and mice

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Visceral and subcutaneous adipose tissue display important metabolic differences that underlie the association of visceral fat with insulin resistance, type 2 diabetes and the risk of cardiovascular disease. The functional and molecular differences contributing to this association are not completely understood. Murine and human visceral adipose tissue evidences a higher lipolytic activity and decreased insulin sensitivity in comparison to subcutaneous adipose tissue. Moreover, there is evidence for intrinsic differences in gene expression between visceral and subcutaneous adipose tissue in normal mice. Our aim was therefore to investigate whether gene expression differences detected by microarray analysis in adipose tissue of C57Bl6 mice could be confirmed in healthy humans using the same genomic approach. Gene expression analysis was performed using 10 µg of cRNA isolated from subcutaneous and visceral adipose tissue from C57Bl6 mice and hybridizd to Affymetrix oligonucleotide microarrays (MG U74A-v2 chips). In addition, RT-RCR was performed to confirm the most significant gene expression differences in mice and parallel in human. Of the 12,488 genes represented on the chip, 72 genes were identified as fat-depot-specific changed, including fatty acid synthase, lamin A, IL-6, β-adrenergic receptors, leptin, carnitine palmitoyl transferase 2,TGFβ, angiotensinogen, PKC β und δ. Our data suggest that instrinsic differences in gene expression between subcutaneous and visceral adipose tissue are evolutionary conserved and might therefore contibute to the link between visceral fat and insulin resistance.

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5.7 A novel approach to identify cellular target proteins of quercetin in human cells

Markus Böhl, Herwig O. Gutzeit

Despite the wealth of information concerning biological effects of flavonoids a systematic approach to analyse the molecular targets is still lacking and, for this reason, a rational evaluation of the risks or benefits of flavonoid- containing food or of possible pharmaceutical applications has not been possible. When the flavonoid quercetin binds to a target protein like BSA, the respective spectral properties of the protein and the ligand change. Upon excitation in UV the autofluorescence of the target protein is effectively quenched. When excited at longer wavelength a fluorescence signal is elicited in some protein/ligand combinations. This effect can be exploited to visualise quercetin target proteins in the fluorescence microscope that appear to be mainly present in the cell nucleus. For this reason we isolated nuclei of HL-60 cells and monitored the quercetin-dependent decrease of protein autofluorescence in protein fractions of nuclear extracts which were obtained by column chromatography. Those fractions containing quercetin target proteins were separated by SDS-PAGE and further analysed by MALDI-MS (collaboration with Prof. B. Hoflack, Biotec, TU Dresden). Amongst other proteins we identified actin as one of the target proteins. To verify the flavonoid/actin interaction we showed that actin autofluorescence decreases strongly after addition of quercetin. Using this approach, other target proteins of quercetin or other ligands can be identified systematically. This type of information will help to interpret the observed complex biological effects of flavonoids.

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5.8 DNAzymes designed to inhibit prothrombin gene expression are outperformed by an antisense oligodeoxynucleotide

Markus Böhl, Bernd Schwenzer

DNA with nucleolytic activity towards RNA like the extensively studied 10-23 DNAzymes, has been considered to be a potential agent useful in the treatment of various diseases because of the specific mode of action. Even tho many DNAzyme based agents are reported to cleave their substrate mRNA successfully in cell free activity test systems, the number of reported DNAzymes to be effective in cell culture is much smaller. The reasons for this are well known. DNAzymes have to overcome the same hurdles like any other nucleotide based agent when they should be active in living cells and there is the additional fact, that cell free test systems utilise optimal conditions for the cleavage reaction which can not be found in cells cytoplasm. Therefore DNAzymes for the use in living cells should not be selected by a cell free activity test.

We wanted to know if the inhibition of prothrombin gene expression which was observed by us for an antisense oligodeoxynucleotide in cell culture can be outperformed by DNAzymes directed against the same target region. This approach has the benefit over other reports dealing with this topic that we were able to direct the DNAzymes against a proven accessible site within the prothrombin mRNA. The substrate binding arms of the respective DNAzymes were designed in a way, that they do always cover the exact target sequence of the original antisense oligodeoxynucleotide. We found that the DNAzymes used in this study were outperformed in their inhibition efficiency by the antisense oligodeoxynucleotide directed against the same target region.

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5.9 Bioreactor "FiberReact" – The complete solution

Klaus Breese, Tanja Bloß

The FiberReact bioreactor is a complete cell cultivation system adaptable to the needs of all possible applications like production of cell mass or production of cell products like antibodies. Heart of the system are hollow fiber bundles embedded in plastic-cartridges. They are used as cultivation matrix for the cells (hydrophilic fibers) and for the oxygenation of the medium (hydrophobic fibers).

Developed for long term cell cultivation, the FiberReact system shows innovative features to make cell cultivation easier to handle, better reproducible and more cost-effective:

- online measurement and documentation of pH, pO_2 and temperature
- non-invasive, optical measurement of pH and pO_2
- use of disposables means less hands on time and less contamination risk
- modular construction system enables user specific solutions.

Additional benefit for the user can be reached by coating of the hollow fiber surface with cell specific molecules leading to better cell adhesion and higher quality of cells (e.g. specific differentiation). Appropriate technologies for the coating with bioactive molecules were developed, leading to new applications like cell specific therapies.

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5.10 Synthesis of novel 4-phenylhydrazinopyrazolo [3,4-*d*]pyrimidines as ligands for the human A_{2A} adenosine receptor

Detlef Briel, Richard Aurich, Klaus Unverferth, Ute Egerland

A prevention of dopamine receptor degeneration should lead to a synergistic action to the simultaneous administration of A_{2A}-antagonists and dopamine agonists or L-DOPA. These interactions could be the basis of new therapy conceptions of Morbus Parkinson[1-3].

Some pyrazolopyrimidines with specific substitution pattern are characterised by high receptor affinity and A_{2A}-selectivity[2].

For this reason our target was the synthesis of new 4-phenylhydrazino-pyrazolo[3,4-*d*]pyrimidine derivatives bearing a residue with low polarity at the pyrazole ring to obtain substances with A_{2A}-affinity.

Both imino-1,3-thiazines and pyrimidine carbaldehydes served as edducts. To synthesize pyrazolopyrimidines, different substituted hydrazines were used. Starting from imino-1,3-thiazines, 2-phenylsubstituted pyrazolopyrimidines were obtained. However, an alternative reaction path was favoured when pyrimidine carbaldehydes were used. Thereby the products were phenylsubstituted in the 1-position.

The affinity of the products towards the human A_{2A} adenosine receptor was determined in competitive radioligand-receptor binding experiments. Thereby the synthesized pyrazolopyrimidines proved out significant effects. A phenyl residue in the 1-position caused a higher affinity.

Notes

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5.11 Production and characterization of monoclonal antibodies against the nucleocapsid protein of Severe Acute Respiratory Syndrome-associated Coronavirus (SARS-CoV)

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In 2002, a new virus known as the Severe Acute Respiratory Syndrome-associated Coronavirus (SARS-CoV), caused an epidemic with atypical pneumonia in China, followed by fast worldwide spreading in 2003. One of its structural proteins, the nucleocapsid (N) protein, was found in serum and different body fluids of patients early after the onset of symptoms. The detection of this protein could be used for fast diagnosis and further studies of SARS-CoV infection.

The RNA of the N protein of SARS-CoV was reverse transcribed and cloned in a bacterial expression vector. A recombinant N protein (N-MBP) was expressed consisting of the fusion protein of SARS-CoV N protein and *E. coli* maltose-binding protein (MBP). Balb/c mice were immunized and monoclonal hybridoma cultures were generated from its spleen cells. Cell culture supernatants of the hybridoma cultures were screened for production of antibodies against the N protein by ELISA, Western blot and immunofluorescence. The obtained antibodies were characterized concerning isotype and bound epitope. In immunofluorescence assay the N protein was localized mainly perinuclear but also in core.

Ten independent monoclonal cultures could be established, all were of IgG type. Until now three linear and three conformational epitopes are identified.

Altogether the immunization of mice with N-MBP led to a strong immune reaction and B-lymphocytes that could be used to generate antibody-producing hybridomas. Antibodies obtained from these cell cultures may be useful in viral diagnostic tests and research on SARS virus pathogenesis.

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5.12 Gene silencing in acute and persistent measles virus infection

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RNA interference (RNAi) is induced by short interfering RNA (siRNA) which leads to specific mRNA degradation and therefore abrogates the specific protein expression. This mechanism is highly sequence specific. Even one mismatch can inhibit the RNAi effect. The aim of the experiment presented here was to analyse the antiviral usage of siRNA in cells lytically and persistenly infected with measles virus (MV), a nonsegmented negative-stranded RNA virus. Three siRNA Expression Cassettes (SEC) were constructed. The SECs are composed of the human H1 promotor, the coding regions for sense and antisense separated by a loop region and the terminator. According to the experimental protocol described by Castanotto et al (2002) two PCRs were established and optimised to produce the SECs. SECs were designed to hybridise with the mRNA of the Nucleocapsidgene (N) at the strongly conserved 5'-region. Two viral model systems were transfected with the SEC using lipofection: Lytically infected HeLa cells and persistently infected neuroblastoma cells (SH-SY5Y). The amount of N-specific RNA was measured. It was decreased up to 43 % for the lytical and 65 % for the persistent infection. The number of cells expressing N-protein was quantified by FACS analysis using monoclonal antibodies. A decrease for N-protein was seen in both, lytical and persistent infection. The results indicate that RNAi can be induced in lytically and persistently MV infected cells.

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5.13 Reduction of VEGF gene expression by antisense oligonucleotides in bladder and breast cancer cells

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Angiogenesis plays a key role in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is one of the major angiogenic factors. In the study we have evaluated the efficiency of antisense oligodeoxynucleotides (AS-ODN) against VEGF selected from computational prediction of VEGF mRNA structure. Twenty-five different AS-ODN in two different tumor cell lines were investigated. Treatment of cell line EJ28 by VEGF723 resulted in a 83.5 % suppression of VEGF protein when compared with control-ODN. Three further AS-ODN reduced VEGF protein more than 45 % in comparison to control-ODN. This was caused by an antisense-specific downregulation of the VEGF transcript determined by real-time PCR. Furthermore, antisense-mediated inhibition of VEGF was associated by a reduced cell viability. In MCF-7 cells VEGF protein was inhibited more than 45 % by two AS-ODNs. In conclusion, we found that computational prediction of potential single strand mRNA motifs is a well suitable method to elect effective AS-ODN.

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5.14 Antisense-mediated inhibition of tissue factor expression in different tumor cell lines

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Human tissue factor (TF) is the initiator of blood coagulation. Beside this function it is involved in tumor angiogenesis and metastasis. In the study we have evaluated the efficiency of antisense oligonucleotides (AS-ODNs) against TF selected from computational prediction of TF mRNA structure. Fourteen different AS-ODNs were tested in three cell lines of different origin with a high TF content. In cell line MCF-7 TF gene expression was inhibited up to 50 % by AS-7 in comparison to reference. To investigate the dependence of inhibition efficiency on the AS-ODN position inside a potential target motive we designed further AS-ODNs shifted 2-3 nt among AS-7. One AS-ODN was found that was more effective than AS-7. In cell line T508 we found moderate effects in inhibition of TF gene expression of 30 % by AS-4. In J82 cell TF protein was inhibited up to 68.6 % by two AS-ODNs. In conclusion, we compared inhibition of TF gene expression in different cancer cell lines and found that all effective AS-ODNs were located in the translated region of TF mRNA. Suitability of a target region of an AS-ODN is relativ independent on cell line. In contrast, optimal transfection conditions are dependent on cell line.

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5.15 Highway for light – Müller cells as living optical fibers

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Vision is one of our most important senses. The cells responsible for converting light into electrical impulses are the photoreceptor cells (PRs). However, due to the constraints of evolution, the vertebrate retina is the "wrong way round": in order for light to reach the PRs, it must first pass through several retinal layers of different types of cells. Particularly under low-light conditions additional structures are required which guarantee optimal utilization of the light. These structures ideally span the entire thickness of the retina and guide the light through all of the light-scattering layers to the PRs. Only the so-called Müller cells, the principal retinal glial cells, have these properties. Furthermore, these cells contact every single PR.

In the present work, the light guiding properties of individual Müller cells were studied. Cells were trapped between two counter-propagating, divergent infrared laser beams and additional visible light was sent through the cells. The light guiding efficiency of the cells for visible light was thereby measured.

These measurements unambiguously demonstrate the light guidance function of Müller cells. The observed intensity-dependent transmittance might serve as a protective mechanism of the inverted retina against photo damage of the PRs.

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5.16 Phospholipid metabolism detected by ^{31}P NMR spectroscopy and MALDI-TOF mass spectrometry

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Phospholipids (PL) and phospholipases A_2, C, and D play important roles in signal transduction. Alterations in PL and enzyme activities might affect membrane properties, which may have a direct impact on dysfunctions, certain pathologies or cell proliferation, differentiation and apoptosis. For successful regenerative therapy the knowledge about the details of the functional and dysfunctional PL metabolic changes are necessary.

Unfortunately, suitable methods of PL analysis are often time-consuming and tedious, since most of them include a variety of separation and derivatisation steps. Methods allowing lipid analysis in a single step would be highly useful. ^{31}P nuclear magnetic resonance (NMR) spectroscopy provides the quantitative detection of all relevant PL classes in a single step. The combination with matrix-assisted laser desorption and ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) is very useful to obtain further information about the acyl chain composition of the individual PL classes. Because of its very high sensitivity MALDI-TOF MS offers also the potential to perform measurements at low PL concentrations.

Thus, we are performing PL analyses by the combined application of ^{31}P NMR spectroscopy and MALDI-TOF MS to characterize the PL composition and metabolic changes in biological samples (tissues, cells, and body fluids). It will be shown that especially the content of lyso-phosphatidylcholine (lysoPC) represents a reliable measure of inflammation. The PC/lysoPC ratio may indicate the severity of inflammation, the success of therapy and regeneration, and may serve as an early diagnostic marker.

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5.17 Mechanisms of CD44 shedding in human melanoma cells – Relevance for tumor progression and target for therapeutic intervention

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Previous studies have shown that human melanoma cells (MM) can bind hyaluronic acid (HA), an extracellular matrix component, via the CD44 receptor. This interaction of HA with membrane-bound CD44 (mCD44) results in the release of autocrine growth factors from MM cells leading to proliferation and progressive tumor growth *in vivo.* On the other hand, certain MM are capable of shedding soluble CD44 (solCD44) from their surface. Interaction of solCD44 with HA abolishes proliferation of MM *in vitro* and *in vivo* by inducing apoptosis. Autocrine or drug induced secretion of solCD44 may thus exert potent anti-tumoral effects.

To elucidate the mechanism of CD44 shedding, a panel of well characterized MM cell lines differing in their capacity to shed solCD44 was analyzed. RNA analysis revealed that solCD44 is not the result of alternative splicing of CD44 mRNAs and the shedding activities are not linked with CD44 mRNA turnover. The different observed shedding activities are accompanied by similar expression of mCD44 on the cell surfaces suggesting an increased turnover of the protein. Moreover, CD44 shedding was reduced by serine protease or metalloprotease inhibitors. Further, mRNA levels of several MMPs were analyzed by realtime-RT-PCR. We found the low shedding cell line P2 lacking MMP1 and MMP3 mRNA while the high shedding cell line Bro expressed these MMPs. Addition of HA induced MMP1 and MMP3 expression in the low shedding cell line P2, indicating the regulatory role of the CD44 ligand HA. Since other authors suggested MMP14 as the main responsible protease we analysed MMP14 espression by FACS and ELISA.

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5.18 Tauopathies' relation to axonal transport

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A vital function in neurons is axonal transport (AT) along microtubules. A loss of synapses and later on a loss of neurons will be the result of disturbance or even disruption of the transport. Neurodegeneration in syndromes like Alzheimer's disease (AD) or Boxer's dementia (*dementia pugilistica*; DP) are very likely related to dysfunction of axonal transport. Additional evidence are the two main deposits found in AD and DP: A-β protein in amyloid plaques and tau protein in neurofibrillary tangles. The precursor protein of A-β is thought to be an adaptor protein between vesicles and molecular motors. And tau is a microtubule-binding and stabilizing protein, which may influence the functionality of the microtubule track. Tau's microtubule-binding is mainly controlled by its phosphorylation state.

Studying the axonal transport in primary neurons and neuroblastoma cells we focus on the interaction of transport, tau phosphorylation and mechanical stress.

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5.19 Glial cells from human retinas produce CXCL8 and express CXCR1 and CXCR2 receptors

Iwona Malgorzata Goczalik, Maik Raap, Michael Weick, Andreas Reichenbach, Mike Francke

Interleukin-8 (CXCL8) is a pro-inflammatory chemokine. Several diseases of the eye (e.g. PVR, uveitis) are associated with increased levels of CXCL8 in the vitreous. Various cell types are potential sources for the secreted CXCL8. The aim of our study was to evaluate whether retinal glial cells are able to produce and secrete CXCL8 and/or to express CXCL8 receptors. We established primary cultures of isolated glial cells from human donor retinas and used an immortalized human Müller cell line. The cultures were prepared for immunohistochemistry (IHC), Western blotting and RT-PCR. Ca^{2+} imaging was performed to test the activation of CXCL8 receptors. Additionally, we examined surgically removed PVR membranes and retinal cryosections from organ donors.

CXCL8 immunoreactivity was accompanied by GFAP immunoreactivity and was co-localized with other glial specific markers. CXCL8 mRNA could be detected in human Müller cell cultures. Immunoreactivity for CXCR1 and CXCR2 was observed in human primary cultures and in the Müller glial cell line. Western blot analysis revealed proteins with about 40 kDa. The RT-PCR method confirmed the expression of CXCR1 and CXCR2 in the human Müller cell line. Application of recombinant CXCL8 protein caused an increase in intracellular Ca^{2+} levels in subpopulations of Müller cells. We determined CXCR1 and CXCR2 expression in GFAP-positive cells in PVR membranes. It is concluded that Müller cells may participate in the inflammatory response of pathologically altered or injured eyes. Surprisingly, we also detected the expression of CXCR1 and CXCR2 in healthy retinas from organ donors by means of IHC.

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5.20 Morphometrical investigations on different regions of the newborn skin

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The aim of the investigations was the elucidation of differences in the morphology of different regions in the skin of newborns. Features were the thickness of the skin and epidermis, resp. Moreover the relative amounts of collagenous fibers of type I and III in the extracellulare matrix were determined. Further features involved the depth and the distance of papillae of dermis. The investigations were carried out on conventionally stained (HE,CROSSMON) and silvered (GOMORI) histological sections of the skin of deceased newborns (six female, five male). The relevant regions were forehead, neck, upper arm, forearm, palm of the hand, thoracic and abdominal wall, back side, femoral, lower leg, sole of the foot. The data of the features of the different regions were compared to each other. A relatively uniform skin thickness was observed in the different regions by using morphometrical methods of the light microscopy. In contrast to skin thickness the thickness of the epidermis showed significant differences between some regions. Highest thickness, depths and distance of papillae of dermis were detected for the sole of the foot and the palm of the hand, the lowest for the forehead. No correlation was found between the thickness of the skin and the epidermis, resp.

The areal arrangement and orientation of the bundles of collagenous fibers and the pattern of the collagen type I and III distributions suggest a proceeded mature process at the time of birth compared to adult skin. The results of the statistical tests give hints for possible transplantations of skin regions, which resembles each other corresponding to considered feature.

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5.21 Experimentally induced rat brain cholinergic deficiency as a suitable tool to evaluate new radioligands for the vesicular acetylcholine transporter (VAChT) – Immunohistochemical evidence of VAChT loss after immunolesion

Udo Großmann, Wolfgang Härtig, Dietlind Sorger, Elena Kouznetsova, Reinhard Schliebs, Matthias Scheunemann, Jörg Steinbach, Osama Sabri

The basal forebrain cholinergic system is known to be involved in normal brain cognitive function and cortical cholinergic deficits seem to be implicated in Alzheimer's disease and other demential disorders. The VAChT localized in the membrane of vesicles of acetylcholine storage and transport is a possible target molecule for novel vesamicol derived fluorine-18 labelled radioligands to *in vivo* image these deficits by Nuclearmedicine methods like SPET and PET.

It is the aim of this study to validate immunhistochemically (IHC) that a quantitative reduction of VAChT occurs following a single dose of intracerebroventricular applicated immunotoxin to degenerate cholinergic terminals in cholinoceptive target regions.

The IHC labelling of VAChT was performed with free-floating, coronal frozen sections from perfused paraformaldehyde-fixed rat brains 6 weeks after a unilateral ventricular stereotaxic injection of 2 µg 192 IgG-saporin. Controls received a saline injection. VAChT immunoreactivity was visualized both by immunoperoxidase and carbocyanine immunofluorescence staining, respectively, using commercially available antisera. Following immunolesion a drastic loss of VAChT-immunoreactivity in the magnocellular basal forebrain nuclei and in the vast majority of VAChT immunopositive fibres projecting to neocortex and hippocampus was observed. It was shown that the VAChT represents a potential target molecule to reflect cholinergic deficits in brain. The used animal model of cholinergic immunolesion is an appropriate tool to test novel radiolabelled analogues of vesamicol for their sensitivity to detect cholinergic reductions.

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5.22 Extended tropism of HIV vectors expressing two different envelope glycoproteins

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Background: Env protein of HIV-1 is assembled into stable trimer, and oligomerization is required for maintaince of viral infectivity. HIV isolates differ in use of CXCR4 and CCR5 as coreceptors for infection of target cells. Coreceptor usage depends on the particular viral glycoprotein. Simultaneous expression of genetically different glycoproteins on single virus may lead to different biological and immunological properties.

Methods: Two expression cassettes, each containing a different HIV-1 or HIV-2 glycoprotein gene were cloned into an eukaryotic expression plasmid and used for the generation of infectious replication-incompetent HIV vectors. Vector constructs were added CD4+, CXCR4 or CCR5-expressing cells in culture. Infection was monitored by determination of marker gene expression.

Results: Virus vectors with different glycoprotein have different degrees of viral infectivity. Expression of a second glycoprotein (of identical tropism) interfers with vector infectivity. HIV vectors produced with plasmids containing a CCR5-tropic HIV-1 and a CXCR4-tropic HIV-2 glycoprotein gene or the genes of two HIV-1 envelope glycoproteins of different tropism exhibited dual tropism.

Conclusion: HIV vectors generated with expression plasmids containing two different HIV glycoproteins have new biologic properties probably due to expression of a mosaic of different glycoprotein trimers on the surface of the vector particles. Different glycoprotein molecules may interact in a “dominant-recessiv” fashion.

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5.23 Expression of glutamatergic and muscarinic receptors in organotypic brain slice cultures of Munc13 knock-out mice

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Munc13 proteins are presynaptic phorbol ester receptors, which are essential for synaptic vesicle priming and glutamate release. Approximately 90 % of synapses are solely dependent on Munc13-1 but in a small number of synapses Munc13-1 and Munc13-2 isoforms are present. Thus, glutamate release is reduced by 90 % and 100 % in neurons of Munc13-1 knock-out mice and Munc13-1/2 double knock-out mice, respectively. The regulation of Munc13 proteins is partly mediated by binding of DAG to the Munc13 C1 domain. Several biological processes - including the proteolytical processing of the amyloid precursor protein - depend at least in part on Munc13 function. Phenotypic characteristics observed in Munc13 knock-outs are either directly due to the presynaptic functions of Munc13 proteins (i.e. vesicle fusion and glutamate release) or represent secondary consequences of impaired glutamate release (i.e. lack of stimulation of glutamate receptors). To distinguish between these scenarios, we established organotypic brains slice cultures from newborn Munc13 knock-out mice. These cultures were maintained for different periods of time and characterized with regard to the temporal expression of DAG-linked Type I metabotropic glutamate receptors (mGluR-I) and M1 muscarinic receptors (M1 mAChR) by RT-PCR, radioligand binding, and immunhistochemistry. Our results indicate that both, mGluR-I and M1 mAChR are expressed by neurons in brain slices cultured for ten to 18 days. Thus, these cultures are useful experimental tools to reveal mechansims of Munc13 function in pharmacological stimulation experiments.

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5.24 Phenotype-dependent differential vulnerability to oxidative stress in cholinergic SN56 cells

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Basal forebrain cholinergic dysfunction is a consistent feature of Alzheimer´s disease, which has been suggested to cause, at least partly, the cognitive deficits observed. This has raised the question of what causes the specific degeneration of basal forebrain cholinergic cells in this disease, and moreover, whether the cholinergic system is particularly sensitive to various pathophysiological conditions. The clonal hybrid cell line SN56 derived from the medial septal region of the mouse basal forebrain, expresses typical cholinergic neuronal characteristics such as choline acetyltransferase (ChAT) and acetylcholinesterase, and thus should represent an appropriate model to study the susceptibility of cholinergic cells to pathogenic insults. Differentiated and non-differentiated SN56 cells that demonstrate different levels of ChAT expression, were exposed to increasing concentrations of H_2O_2 to induce oxidative stress. Using MTT and LDH viability assays, SN56 cells differentiated with 1 mM dbcAMP/1µM retinoic acid were shown to be more susceptible to low dosis of oxidative stress as compared to nondifferentiated cells, an effect that could partly be prevented when cells were pre-exposed to 10 µM L-nicotine. The differential vulnerability suggests a distinct activation of the cellular anti-oxidant defence system. Gene expression analyses in phenotypically different cells following oxidative stress will reveal key signaling molecules that are involved in cell maintenance.

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5.25 Different vulnerability to oxidative stress in nondifferentiated and differentiated cholinergic SN56 cells

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A characteristic feature of Alzheimer's disease is the preferential loss of basal forebrain cholinergic neurons, which causes the progressive impairment of cognitive function. The mechanisms underlying the higher susceptibility of basal forebrain cholinergic neurons to pathogenic insults are still largely unknown. The disease-associated increase of oxidative stress may play an important role in the early degeneration of the cholinergic cells. The clonal hybrid cell line SN56 derived from the medial septal region of the mouse basal forebrain, expresses typical cholinergic neuronal characteristics such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), and thus should represent an appropriate model to study the susceptibility of cholinergic cells to pathogenic insults. Differentiated and nondifferentiated SN56 cells that demonstrate different levels of ChAT and AChE expression, were exposed to oxidative stress induced by H_2O_2. Using MTT and LDH viability assays, SN56 cells differentiated with 1 mM dbcAMP/1µM retinoic acid were shown to be more susceptible to low dosis oxidative stress as compared to nondifferentiated cells. This data suggest that the cellular anti-oxidant defence system is differentially expressed in the various cell types. The different responses to oxidative stress should be investigated by performing microgene arrays. A comparison of the stress mediated responses in the phenotypically different cells may explain their differential vulnerability.

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5.26 Pathological changes caused by copper overload in the livers of the Wilson disease gene knock-out mice

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Wilson disease (WD) is a severe genetic disorder associated with copper (Cu) accumulation in tissues, particularly in the liver. The affected gene, *ATP7B*, is well known; however, molecular mechanisms and cellular events leading to liver pathology remain poorly understood. We describe morphological, histochemical, and biochemical changes in the livers of *ATP7B*(-/-) knock-out mice at different time points.

Hepatic Cu in *ATP7B*(-/-) livers rises quickly and reaches a maximum at 5-6 weeks, approximately 20-40 fold higher than in controls. Cu accumulates predominantly in the cytosol, bound to low-molecular-weight proteins. Among organelles, nuclei accumulate proportionally more Cu, resulting in a marked increase in nuclear size and nuclear damage. High Cu concentration remains constant up to 20 weeks of age, and then declines. Histological changes associated with Cu accumulation are very striking and depend on the stage of the disease. By 6 weeks 50 % of the mice show focal to diffuse but mild necro-inflammation. At 20 weeks the changes are universal and dramatic, with extreme degrees of hepatocellular injury and dysplasia, foci of nodular regeneration and bile duct proliferation. Over the next 6 months a remarkable degree of recovery of large portions of the liver is accompanied by the localized occurrence of adenocarcinoma arising from the proliferating bile ducts. Biochemical characterization of *ATP7B*(-/-) liver homogenates revealed significant up-regulation of glutathione-S-transferase isoform Pi on protein and mRNA levels. *ATP7B*(-/-) mice represent a valuable model for analysis of Cu toxicity and regeneration in the liver.

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5.27 Postnatal development of glial aquaporin and K^+ channel expression in the rat retina

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The osmotical and ion homeostasis of the inner retina is maintained by Müller glial cells. Müller cells express various subtypes of K^+ channels in their membranes that mediate fast transglial spatial buffering K^+ currents. Osmotically coupled to the transglial K^+ currents, Müller cells mediate water fluxes that are facilitated by aquaporin (AQP) water channels. The aim of the study was to compare the postnatal expression of AQPs and K^+ channels in the retina of the rat.

Retinal slices obtained from young rats at different postnatal days (P) were stained immunohistochemically against the glial K^+ channel subunit Kir4.1, AQP-1 and -4, and glutamine synthetase. Whole-cell K^+ currents were recorded in isolated Müller cells.

Glutamine synthetase as a marker of Müller cells was expressed in the rat retina at P11 and thereafter, and was not present at P5. In slices of adult retinas, immunoreactivities (IRs) for AQP-4 and Kir4.1 are co-expressed in Müller cell membrane domains that surround the vessels and abut the inner limiting membrane. Both IRs were expressed at P15 and thereafter. The IR for AQP-1 was expressed in the outer retina at P15 and thereafter. In Müller cells of adult retinas, the most prominent membrane K^+ conductance was found in the peri-somatic membrane domain. The K^+ currents of Müller cells increased during the second and third postnatal weeks.

The proteins for glial AQP and K^+ channels are simultaneously upregulated in their expression by Müller cells during the postnatal development. The expression of glutamine synthetase precedes the expression of AQP and K^+ channels in developing Müller cells.

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5.28 Detection of novel polyomaviruses by nested broad-spectrum PCR

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Mammalian polyomaviruses cause persistent subclinical infections which may develop to severe diseases after immunosuppression. Inoculation into non-permissive hosts often leads to tumor growth. Polyomaviruses of birds are primary agents of acute and chronic disease. Most of the polyomaviruses were identified by accident as contaminants of tissue cultures. In order to systematically screen for so far unknown polyomaviruses, degenerated PCR primer pairs were constructed on the basis of available polyomavirus genome sequences.

By establishing a nested PCR protocol amplifying part of the major capsid protein VP1 gene, the sensitive detection of nine different polyomavirus genomes was demonstrated. Screening of 100 field samples derived from different animal species revealed the presence of new polyomaviruses in the feces of a juvenile chimpanzee (*Pan troglodytes*) and in the inner organs of a bullfinch (*Pyrrhula pyrrhula*).

Phylogenetic analysis of the entire VP1-encoding region revealed that chimpanzee polyomavirus (ChPyV) is related to the monkey polyomavirus LPyV and the human polyomavirus JCPyV with 60.5 % and 61.0 % nucleotide sequence identity, respectively. The finch polyomavirus is most closely related to avian polyomavirus, with 50.4 % genome sequence identity. As this virus was detected in young birds died from acute systemic disease, an involvement in disease should be considered.

The results of our study indicate that polyomaviruses are widely distributed among different animal species. The application of the PCR protocol to larger sample numbers should help to assess their distribution and involvement in disease.

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5.29 Atrial natriuretic peptide (ANP) inhibits osmotic Müller cell swelling in the postischemic retina of the rat

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Retinal Müller glial cells are known to play an important role in the development of retinal edema, similar to the role of astrocytes in the pathomechanism of brain edema. One main cause for edema formation is glial cell swelling. Recently, we observed that Müller cell somata in slices from postischemic retinas of the rat swell upon hypotonic stress, which was not observed under control conditions. However, Müller cell swelling could be evoked by exposing slices of control retinas to Ba^{2+}. Under both experimental conditions the glial K^{+}-channels are downregulated or blocked, respectively. The aim of the present study was to investigate the effect of the atrial natriuretic peptide (ANP) on osmotic glial cell swelling. Acute application of ANP (100 nM) inhibited the hypotonic swelling of Müller cell somata. ANP may activate several types of natriuretic peptide receptors (NPRs), but acts particularly on the NPR-A. Agonists of the NPR-B (CNP) and NPR-C(C-ANP-[4-23]) mimicked the effect of ANP. Pharmacological experiments suggest that the ANP-effect is mediated by activation of neuronal NPRs, which stimulates phospholipase C and evokes an increase in intracellular Ca^{2+} and an activation of proteinkinase C. These signaling steps cause the synaptic release of glutamate which, subsequently, stimulates metabotropic glutamate receptors on Müller cells. This results in a release of purinergic agonists. Autocrine stimulation of A1 receptors by adenosine finally inhibits the osmotic swelling of Müller cells. Atrial natriuretic peptides are able to prevent glial cell swelling and might be beneficial to prevent and treat edema formation.

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5.30 *Borrelia burgdorferi* dissemination in the host – Comparison of the infection after intradermal and intravenous injection in mice

Jens Knauer, Reinhard K. Straubinger

Lyme disease caused by *B. burgdorferi* is characterized by disseminated inflammatory responses weeks after the infection. Most authors believe that these responses are induced by the spirochetes' presence at varying sites and in this context favour a blood-borne dissemination of the bacterium. Others, however, support a centrifugal spread of the spirochetes through the host's tissues based on clinical observations.

The aim of this work was to compare the infection pathways in mice, that received *B. burgdorferi* either intradermally (id) or intravenously (iv). Tissue and serum samples from 96 mice (6 per group) were collected in weakly intervals for six weeks and again after 26 weeks. Tissues were used for re-cultivation of spirochetes and serum samples were analyzed using ELISA and Western-Blots.

Fourteen id-injected mice and 3 iv-injected mice were positive in culture. Both experimental groups developed increasing antibody responses up to day 42. Thereafter, antibodies in iv injected mice that were mainly directed against outer surface proteins normally observed after vaccination had decreased while an increase of antibodies to a broad range of antigens was noted in the id injected group.

These data suggest, that iv injected spirochetes were unable to adapt to the host's haematogenous environment and where removed quickly form the blood stream, causing decreasing antibody titers. Id injected borrelia, however, adapted quickly and initiated a persistent infection with constant antigen exposure to the immune system. Therefore these results support the hypothesis of a centrifugal, tissue-based spreading of *B. burgdorferi*.

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5.31 Expression of ubiquitous phosphofructo-2-kinase/ fructose-2,6-bisphosphatase in glioma cell lines

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Many tumor cells maintain a high glycolytic rate even in the presence of oxygen (the "Warburg effect") to supply ATP and anabolic precursors essential for de novo nucleotide synthesis. High glycolytic flux correlates with tumor growth rate and malignancy.

Fructose-2,6-bisphosphate is the most powerful activator of 6-phosphofructo-1-kinase, the rate limiting enzyme of glycolysis. The synthesis and hydrolysis of fructose-2,6-bisphosphate is catalysed by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) which is expressed in four tissue-specific isoforms (*PFKFB1-4*).

The ubiquitous PFK2 is encoded by the inducible *PFKFB3* gene which is expressed constitutively in several transformed cell lines. The mRNA of ubiquitous PFK2 contains multiple copies of the AUUUA instability motif in the 3'-untranslated region. Therefore, ubiquitous PFK2 is the first enzyme of the intermediary metabolism with proto-oncogene features.

Alternative splicing generates six different isoforms of ubiquitous PFK2. Their expression pattern was determined in different astrocytoma cell lines on mRNA and protein level. The mRNAs of isoforms 5 and 6 were predominant throughout.

The *in vivo* interacting partners of ubiquitous PFK2 will be identified with the "tandem affinity purification" method (TAP), This method allows rapid purification of protein complexes under native conditions. As a first step the cDNAs of isoforms 5 und 6 cloned in the expression vector pcDNA3 were fused with the TAP-tag. The expression of the tagged PFK2 in different astocytoma cell lines was studied.

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5.32 Developmental and amyloid plaque-related changes in cerebral cortical capillaries in transgenic Tg2576 Alzheimer mice

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There is experimental evidence that cerebral perfusion is decreased during aging and in Alzheimer's disease. To characterize the temporal relationship between amyloid deposition, plaque size and cerebrovascular abnormalities, a semiquantitative immunohistochemical study was performed in transgenic Tg2576 mice that express the Swedish double mutation of human amyloid precursor protein and progressively develop Alzheimer-like beta-amyloid deposits. Cortical cryocut sections, obtained from mice at ages ranging between 10 and 18 months, were immunostained to label glucose transporter type 1 (GLUT1), a marker of vascular endothelial cells, and thioflavine-S to visualize plaques. Regardless of age and transgene, a laminar distribution of capillaries was observed being highest in cortical layers IV and lower layer V. In transgenic mice capillary density in all cortical layers decreased steadily with age and plaque load. Around large thioflavine-S-positive plaques the capillary density was low, while diffuse plaques demonstrated a close association of capillaries with no signs of any damage. The data suggest that amyloid plaque deposition differentially affects the cerebrovascular system in an age-and plaque type-related manner.

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5.33 Antisense-mediated telomerase inhibition enhances the cytotoxic effects of chemotherapeutic drugs in human bladder cancer cell lines

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Purpose: Responses of bladder cancer (BCa) to commonly used chemotherapeutical agents such as mitomycin C (MMC), cisplatin (CDDP) and gemcitabine (GEM) are often disappointing. Since hTERT is tumor-specifically expressed and contributes to immortality and malignancy of the majority of tumors, it is regarded as a suitable anti-tumor target. The aim of this study was to investigate whether combinations of hTERT antisense-oligonucleotides (AS-ODNs) with common chemotherapy (CT) schedules may improve the drug-mediated anti-tumor effects.

Material and Methods: An initial screening for the enhancement of the inhibitory effects of MMC, CDDP and GEM on viability by treatment with 2 different hTERT AS-ODNs prior to CT was performed in 4 BCa cell lines. Apoptosis was assessed by annexin V staining and detection of activated caspase-3. A nonsense (NS)-ODN was used as a control in all experiments.

Results: All cell lines responded to the anticancer agents tested. Treatment with AS+CT resulted in a significantly stronger inhibition of viability than the NS+CT control in the majority of the combinations, indicating an antisense-specific enhancement effect. For example, ASt2331+MMC reduced the viability to 17 % in contrast to NS+MMC (58 %) in EJ28 cells. All ASt2331+CT combinations specifically increased the rate of apoptosis in a range from 1.3-fold to 3.0-fold in comparison to NS+CT. Apoptosis induction was associated with caspase 3-activation.

Conclusion: The chemosensitization by hTERT AS-ODNs allows a dose reduction of the chemotherapeutics and confirms the suitability of hTERT as a target in a specific therapy approach.

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5.34 Proteomics reveals alterations in thyroglobulin expression and in the H_2O_2 detoxifying system in cold thyroid nodules

Kerstin Krause

Szintigraphically cold thyroid nodules (CTN) are frequent findings in regions with iodine deficiency. While the presence of a CTN raises the possibility of thyroid cancer probably less than 3 % of CTNs acutally represent malignancy. The molecular etiology of benign CTNs is still unresolved. We applied 2 D-gel electrophoresis in combination with MS analysis to define a protein fingerprint of CTNs as a pre-requiste to get further insights into their pathogenesis. 10 benign CTNs (colloid nodules and follicular adenoma) and corresponding normal thyroid tissue (ST) of the same patient were studied. The proteomics approach resulted in a protein resolution of ~1,500 spots/gel. Differential regulation of protein expression in CTN vs. ST was observed for 250 spots, which were subsequently identified by ESI-MS or FTICR. Proteins consistenly upregulated (>2 fold) in CTNs fell into three major categories: 1. Cellular proliferation (amyloid precursor protein), 2. Tg trafficking (cathepsin B, PDI, HSP90, calreticulin, Tg fragments) and 3. H_2O_2 detoxifying system (peroxiredoxin 2 and 6, Glutathione S-transferase π DJ-1). Western blot analysis for selective proteins e.g. APP, Cathepsin B and Tg was performed and verified the 2D-gel results.

In summary we report the first characterization of the proteome of benign CTNs. Our results suggest specific alterations in Tg expression and upregulation of the peroxide detoxification system in CTNs possibly due to a failure in the processing and iodination of thyroglobulin (Tg).

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5.35 Introducing IZKF Core facility DNA technologies

Knut Krohn, Petra Süptitz, Birgit Thiele, Kathleen Stein

The Interdisciplinary Centre for Clinical Research (IZKF) in Leipzig runs a core facility for DNA technologies to support a growing number of research groups. This well established service includes microarray technology and DNA sequencing, analysis of heterozygous DNA, fragment analysis and detection of genomic markers (e.g. SNPs, microsatellites).

As technologies rapidly change the face of modern biology and medicine, instrumentation needs a regular update. In this regard our newly available array scanner allows detection of gene expression for more than 46,000 genes on a single GeneChip as well as the analysis of more than 100,000 SNPs (single nucleotide polymorphisms) on two arrays or the re-sequencing of 30,000 Bases per chip. Moreover we extended our sequencing capacity by running a new ABI 3,100 capillary sequencer.

An the moment we serve more than 50 groups within the IZKF, the university hospital, and the university itself as well as external partners with about 15000 sequences and 300 microchip readings per year. A large part of this effort is directed to study genes (e.g. TSH receptor, thyroid peroxidase, TNF-α, Wilson gene) involved in human diseases (e.g. rheumatoid arthritis, diabetes, thyroid disorders, intestinal diseases, tumors) and to elucidate their possible molecular causes.

Moreover the core facility coordinates exchange of laboratory protocols and organizes workshops and laboratory courses for molecular topics (e.g. biocomputing, microarray technology, sequence analysis).

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5.36 Inhibition of C13orf19 mRNA expression by siRNA in prostate cancer cells

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Background: Human C13orf19 (NM 017569) was previously identified to be down-regulated in prostate cancer (PCa). Until now its function is unknown. We therefore inhibited the C13orf19 mRNA expression by siRNA transfection.

Material and methods: We compared the downregulation of the C13orf19 mRNA expression by different siRNA duplexes by DOTAP-mediated transfection of the PCa cell line PC-3 since these cells showed higher C13orf19 mRNA expression compared to other PCa cell lines. We optimized the transfection conditions and selected the most efficient siRNA. The mRNA expression of C13orf19 and the PBGD reference gene were measured by quantitative PCR. The relative expression values were normalized to the non-silencing siRNA-control. Cellular viability was analyzed by WST-1 viability assay and apoptosis by annexin V-propidium iodide staining. Also, cell cycle distribution and clonogenic survival were examined. In addition, the effects of C13orf19 down-regulation in combination with chemotherapy on overall cell survival were examined.

Results: The application of these siRNAs showed no obvious effects on doubling time and cellular morphology. After 24 h siRNA D5 inhibits the C13orf19 mRNA expression down to 16 %. After 72 h there is still an inhibition to 31 %. Cell cycle distribution, clonogenic survival, apoptosis and cell viability showed no alterations as compared to the controls.

Discussion: The C13orf19 mRNA inhibition by D5 has no effects on cellular growth properties. We suppose that the inhibition leads to reduced apoptosis in PCa cells. Therefore, studies on the potential chemoprotective effects of siRNA D5 are underway.

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5.37 Addition of rituximab to a CHOP-like regimen improves outcome of all patients with low risk diffuse large B-cell lymphoma (DLBCL)

Markus Löffler, Evelyn Kuhnt, Christina Rogalski, Michael Pfreundschuh

DLBCL is a chemosensitive disease usually treated with CHOP-like chemotherapy. Adding monoclonal antibodies against the CD20-epitope (Rituximab, ROCHE/GENENTECH) has been shown to improve outcome in elderly patients[1], but there is only limited data for young low-risk patients. In an intergroup study conducted in 18 countries, untreated patients (18-60 years) with low-risk $CD20^+$ DLBCL (IPI 0 or 1, stages II-IV and stage I with bulk) were randomized to receive 6 cycles of a CHOP-like regimen (CHEMO) or CHEMO plus 375 mg/m^2 of rituximab (R-CHEMO). Radiotherapy was planned for sites of initial bulk and/or extranodal involvement. The primary endpoint was time to treatment failure (TTF). Between 05/2000 and 10/2003, 824 patients were recruited. We present here the full-set analysis on 823 evaluable patients (410 CHEMO; 413 R-CHEMO) with data base status at Oct 31st, 2004.

Patient characteristics were: median age 47 years; IPI=1: 57 %; stages III/IV: 28 %; elevated LDH: 29 %; bulk: 49 %. Toxicity was not different in the two arms. After a median observation time of 22 months, R-CHEMO patients had a significantly longer TTF ($p<0.001$), with estimated 2-year TTF rates of 61 % (CHEMO) vs 80 % (R-CHEMO).Overall survival was significantly different ($p<0.001$), with 2-year survival rates of 86 % (CHEMO) and 95 % (R-CHEMO).

The combination of six cycles of a CHOP-like regimen with rituximab sets the new treatment standard for young low-risk patients with $CDD20^+$ DLBCL. The trial also demonstrates the ability of the Coordination Centre for Clinical Trials in Leipzig to organize a multicenter, multigroup registration trial.

Notes

1 Coiffier et al., NEJM (2002).

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5.38 Keratinocyte-derived dranulocyte-macrophage colony stimulating factor accelerates wound healing in a transgenic mouse model

Amrit Mann, Kerstin Niekisch, Manfred Blessing

Poorly healing and chronic wounds represent a major clinical challenge in diverse disciplines of modern medicine as these ailments greatly contribute to the morbidity and high cost of patient care. GM-CSF has been employed in clinical trials of wound healing with some success despite the complexity and scarcity of mechanistic understanding of its role in the wound healing. In skin, granulocyte-macrophage colony stimulating factor (GM-CSF) is secreted by keratinocytes shortly after injury and mediates epidermal cell proliferation in an autocrine fashion. Many cells involved in wound healing, e.g. macrophages, lymphocytes, fibroblasts, endothelial cells, and dendritic cells synthesize GM-CSF and/or are targets of this cytokine. In the present study, effects of both augmented and decreased GM-CSF activity in the skin on excisional wound healing were evaluated. Overexpression of GM-CSF lead to accelerated wound healing, whereas overexpression of a GM-CSF antagonist lead to delayed wound healing as observed by the rate of scab rejection and reepithelialization of the wounds. Also, the wounds in the GM-CSF transgenics healed with minimal scar formation as assessed at day 14 post wounding. We propose that besides the direct effects of GM-CSF on keratinocyte proliferation, granulation tissue formation and neovascularization, also the indirect processes via the induction of beneficial secondary cytokines contribute towards wound healing. We anticipate that these animal models may contribute towards a better understanding of benefits, perils and mechanisms of GM-CSF in normal and impaired wound healing.

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5.39 Cellular localisation of iron in the *substantia nigra* of an adult rat brain

Christoph Meinecke, Markus Morawski, Tilo Reinert, Thomas Arendt, Tilman Butz

Iron appears to be one of the main factors in the metal induced neurodegeneration. Quantitative information on cellular, sub-cellular and cell specific distributions of iron are important to assess the correlation between metal disorder and neurodegenerative diseases. Therefore, it is important to know where the iron is localised and what quantity of iron is present in the healthy brain tissue, especially in the *substantia nigra (SN).*

For our investigations we used an adult rat brain as a model for the healthy human brain tissue. We analysed 6 µm thick embedded, unstained brain sections containing the *substantia nigra*.

We used particle induced X-ray emission (µPIXE) measurements to determine the quantitative iron content on a cellular and sub-cellular level. Moreover, the measurements carried out with the focussed proton beam (beam-diameter below 1 µm) yielded the cellular localisation of iron quantitatively.

In an integral analysis a factor of two more iron was observed in the *SN pars reticulata* then in the *SN pars compacta*.

The analysis of the regional native iron distribution in the *SN* indicates a difference in the quantitative, elemental acquisition between different cell types (neurons and astrocytes). On the cellular level we conclude that iron is more abundant in astrocytes than in neurons.

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5.40 Quantitative assessment of the cerebral α4β2 nicotinic acetylcholine receptors (α4β2 nAChR) in Parkinson's disease (PD) – A PET study using 2-[18F]F-A-85380 (2-FA)

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Nicotine is assumed to have neuroprotective effects in PD mediated via the most abundant α4β2 nAChRs. By using 2-FA-PET we tested the hypothesis whether nAChR are altered in PD. Non-smoking PD (n=12) of different clinical severity and normals (n=8) of comparable age underwent brain PET following short time infusion of 370 MBq 2-FA for 7 hours. We calculated parametric images of distribution volumes DV (Logan plot after correction of the arterial input function for plasma protein binding and radioactive metabolites) and binding potential BP by MRI based ROI analysis. In parallel, dopamine transporters (DAT) in basal ganglia were assessed using [123I]FP-CIT-SPECT and ROI analysis. In comparison with controls, PD showed decreased BP in the caudate heads (caud) [paralleled by decreased target/background ratio T/B(FP-CIT) in the caud], in the frontal cortex (fctx), parietal ctx (pctx), amygdala, midbrain, pons (-39.0/-48.1 %; $p<0.05$). In both caud, put in PD, T/B was positively correlated with BP (r=0.62, 0.79, p=0.01, 0.04). Correlations of T/B in caud, put were also found with BP in fctx, temp ctx, pctx (r=0.63-0.83, p=0.003-0.04). Our data indicate alterations of α4β2 in PD not only in the striatum, but also in other (sub)cortical areas. The finding of striatal DAT state being correlated with striatal and cortical α4β2 availability might indicate a close functional interrelationship of dopaminergic and cholinergic neurotransmission in PD. Since PD patients studied here only showed mild cognitive impairment (MMSE), loss or downregulation of α4β2 nAChR might predict (later) conversion to dementia.

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5.41 Specific binding of 2-[18F]F-A-85380 (2-FA) on α4β2 nicotinic acetylcholine receptors (α4β2 nAChR) in the cerebral white matter (WM) in patients with Parkinson's disease (PD) and Alzheimer's disease (AD) – Demonstration by PET and camparison with diffusion tenso MSI (DTI)

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α4β2 on subcortical projection fibers (sPF) in WM had not been quantified *in vivo* in PD/AD. Aims of this study were to test whether 1) nAChR in WM can be quantified by 2-FA-PET, which binds on alpha4beta2, 2) nAChRs are altered in WM of PD/AD, 3) axonal integritiy is altered in WM of PD/AD studied with DTI. 25 non-smoking PD, AD or normals (N) were studied by 2-FA-PET upto 8h, a subgroup after smoking a cigarette (7h). DTI: Fractional anisotropy (fA) was assessed and normalized (rel-fA). 2-FA-PET: Distribution volumes were calculated. After co-registration with DTI-fA, binding potential (BP) and rel-fA were calculated in med./lat. periventricular WM (pvWM), ventr. capsula int. (civ), corp. callosum (cc). Smoking a cigarette resulted in displacement of 2-FA from thalamus, brainstem, pvWM. BP in PD/AD: decreased in pvWM; civ[$p<0.05$]. Rel-fA in PD/AD: increased in med.pvWM; decreased in lat.pvWM[$p<0.05$], cc, civ(AD). Correlations (PD/AD/N): (N)BP vs rel-f A in pvWM, civ ($r>0.68,p<0.02$). (PD) MMSE vs BP in lat.pvWM (r=0.59,p=0,05), (PD)MMSE vs rel-fA in cc(r=0.83,p=0.01), (AD)in civ (r=-0.87,p=0.03); (PD)MMSE vs fA in lat.pvWM(r=-0.61,p=0.08), (AD)in civ (R=0.90,p=0.02). Smoking a cigarette resulting in displacement of 2-FA by nicotine (upto 45%) is *in vivo* evidence for specific 2-FA binding on sPF in WM. In PD/AD, nAChR alterations and axonal damage in pvWM and civ show a differential regional pattern. The correlations between dementia severity, nAChR availability and axonal integrity in WM measured with 2-FA-PET/fA-DTI, indicate high potential of this combined imaging to assess WM integrity and cholinergic function.

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5.42 New targets for novel immunotherapeutic strategies based on natural regulatory T cells

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The induction of immunological tolerance is an elusive clinical goal to treat autoimmune disorders.

Regulatory T-helper with high expression levels for CD25 (Treg) have been shown to exert powerful suppressive activity.

By flow cytometry, we identified a characteristic pattern of adhesion molecules which is constitutively expressed on Treg in human circulation. As compared to $CD4^+$ T cells, Treg cells express significant higher levels for CD49e, CD49f, whereas CD49d and CD62L were diminished. Following activation via CD3/CD28, Treg cells upregulate CD62L. That expression pattern suggests profound differences in migratory pathways of Treg and other Th cells.

To investigate a possible regulatory role of cytokine release by Treg cells, their cytokine release was analyzed. Beside the lead-cytokine TGF-b, we found a characteristic cytokine pattern comprising some, but not all, of the Th2 cytokines IL-4, IL-5 and IL-13. Levels for IL-6 and IL-10 were below those of Th cells. Levels of characteristic Th-1 cytokines like TNF-α, INF-γ and IL-12p70 were negligible.

We demonstrate the regulatory properties of Treg cells in cocultures with responder CD4 and CD8 T cells. Treg cells suppressed the up-regulation of CD25, CD69 on responder T cells. In contrast, Treg cells stimulated CD30, CD95, CD152 on responder T cells.In the presence of Treg cells, activated responder cells failed in down-regulation of CD62L, which is a normal cellular response of stimulation.

Novel immunotherapeutic strategies could make use of cytokines identified as being important in natural immunosuppression or regulatory cells themselves after they have been expanded *ex vivo.*

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5.43 Parkinson's disease – A possible link between accumulation of iron and loss of perineuronal nets in the *substantia nigra*

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Oxidative stress generated by iron-induced processes (e.g. Fenton reaction) might be essentially involved in neuronal cell death in Parkinson's disease (PD). Perineuronal nets (PNs), a specialized form of extracellular matrix containing large aggregating chondroitin-sulfate-proteoglycans complexed with hyaluronan and tenascin, surround subpopulations of neurons in the *substantia nigra pars reticulate* (*snpr*). Due to their glycosaminoglycan components, these PNs form polyanionical charged structures in the direct microenvironment of neurons, and might, thus, have the properties to scavenge and bind redox-active iron, thereby reducing the local oxidative potential in the neuronal microenvironment. In a previous study, we showed that PN ensheathed neurons accumulate up to 4,6-fold more iron than any other ECM structure. Here, we differentially investigated the intra- and extraneuronal iron concentrations of the *substantia nigra pars compacta* (*sn pc*) versus snpr using particle induced X-ray emission. The amount of iron in the substantia nigra was found to be increased in PD compared to healthy controls. Moreover, an almost complete loss of PNs specifically labelled by anti CSPG antibody was found in PD *snpr*. It is suggested that high levels of iron in PD might affect snpr neurons, potentially through NMDA receptors, subsequentially leading to increased GABA release which in turn might result in an increased inhibition of target neurons.Taken together, degenerative process in PD might be a consequence of the *snpr* neurons loosing their PN's, associated by a reduction of iron binding capacity and elevated levels of free iron.

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5.44 LSC as powerful tool for the analysis of DNA content in neurons

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The laser scanning cytometer (LSC) is a microscope-based cytofluorometer closing the gap between flow cytometry and fluorescence microscopy, allowing a quantitative fluorescence analysis of tissue sections. Thus, the tissue architecture and morphological details remained conserved. Furthermore it is possible to measure many cells in a relative short time, to distinguish between different cell types by immunohistochemistry, and also to analyse the DNA content of cell populations via the propidiumiodide (PI) - fluorescence.

Alzheimer's disease (AD) is a dementia, histopathologically characterised by the formation of neuritic plaques and neurofibrillary tangles and a loss of synapses and neurons. The re-expression of cell cycle proteins in brains of AD patients leads to the assumption that a malfunction of mitogenic signaltransduction and cell cycle control triggers neurons to re-enter the cell cycle, a process that subsequently leads to cell death. Until today it is not clear, up to which point neurons progress through the cell cycle. To our knowledge, there is only one report indicating that neurons in AD brain enter the S-Phase and replicate their DNA[1]. In the present study, we developed a technique to measure the DNA content of cells in brain sections to ascertain if re-entrance into the cell cycle in neurons in AD brain is associated with partial or entire duplication of the genome.

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Notes

1 Yang et al. (2001).

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5.45 The role of antiinflammatory cytokines in cryptococcosis

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Meningoencephalitis caused by the pathogenic yeast *Cryptococcus neoformans* is an important threat for AIDS patients. As experiments of our and other groups indicated Th1 cytokines, e.g. IFN-γ and IL-12, are important stimulators of the immune system to control and eliminate this yeast. On the other hand it could be shown that Th2 cytokines can exacerbate the infection.

Cryptococcosis is an airborne infection therefore we have established a murine intranasal infection model to test the involvement of Th2 cytokines, especially IL-4 and IL-13, in pathogenesis. We could show that mice that lack IL-4, IL-13 or the shared receptor, are much more resistant to a *Cryptococcus neoformans*-infection than wild-type mice which can not control the yeast and therefore have larger cryptococcomas in the lung and the brain. These cryptococcomas cause severe problems as meningitis and pulmonary disorders. In contrast to mice that lack IL-13, mice that over express this cytokine show higher organ burdens than wild-type mice. These results clearly show the adverse role of these Th2 cytokines, for the first time for IL-13, in pathogenesis.

The future aim is to examine the cells that are influenced by these cytokines with the help of cell specific IL-4/IL-13 receptor deficient mice. These findings could help to establish new therapies against this opportunistic pathogen.

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5.46 Structural alterations of the bladder wall in idiopathic urge incontinence and interstitial cystitis

Jochen Neuhaus, Thilo Schwalenberg, Lars-Christian Horn, Jens-Uwe Stolzenburg

Introduction: The high prevalence (41 %) of urinary incontinence (UI) in German female population stresses its social and economical significance. The most severe form is the interstitial cystitis (IC), a chronic disease, of which the etiology is unknown to date. Up to 0.9 % of female population has been reported with IC in the past, whereas recent epidemiological studies state at least 10-20 % prevalence of IC. Early diagnosis and effective therapies suffer from lack of physiological and histopathological basis data. Therefore, we looked for alterations of the bladder wall in patients suspected for IC.

Method: incontinence group: n=7 (f); controls (tumor patients): n=5 (f), n=6 (m). Immunolabeling (IR): nerves: S100, endothelium: CD34, gap junction proteins: Cx40, Cx43, Cx45. Evaluation of the urothelium, lamina propria and the detrusor muscle.

Results: Despite of the severe clinical symptoms, none of the patients showed the histological picture of a full-blown IC. Cx43-IR was augmented in the incontinence group ($p<0.05$); Cx45-IR was unchanged and Cx40 was restricted to blood vessels. The suburothelial myofibroblasts were strongly labeled for Cx43, indicating extensive formation of functional syncytia, while Cx45-IR was slightly elevated in these cells. The innervation of the detrusor muscle was higher in 3/7 (=43 %) of the incontinent patients. Microvascularization, however, was significantly reduced ($p<0.05$).

Conclusions: We observed distinct structural changes within the bladder wall of urge incontinent patients, which might induce more effective diagnostics and new therapeutical approaches.

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5.47 Development of novel calcitonin derived carrier peptides with improved metabolic stability

Ines Neundorf, Robert Rennert, Annette G. Beck-Sickinger

The use of cell penetrating peptides is limited by their rapid clearance when in contact with cellular systems. Considering that a subsequent cleavage would prevent the successive translocation of the cargo one must not forget that the metabolic stability influences also the physiological degradation of CPP. This is an important factor when used for therapeutic purposes. Therefore, the metabolic fate of CPP after permeating the lipid bilayer is of great interest.

Recently, the selected C-treminal fragment hCT(9-32) was found to internalize into excised nasal epithelium while the receptor activating N-terminal part is lacking. This led to an increased interest in using hCT-derived sequences as carrier peptides.

In previous studies it was shown that an initial degradation of hCT(9-32) occurred in its N-terminal fragment through endopeptidase activity. Thus, we developed hCT(9-32) with position 12 and 16 replaced by D-Phe or N-methyl-Phe, respectively. We could demonstrate significant improvements on the metabolic stability in human blood as well as in cell culture supernatant. Furthermore, the uptake was not affected as proven by confocal laser scanning microscopy and all the new derivatives showed no toxicity. Accordingly, a novel generation of CPPs with significantly improved properties have been identified.

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5.48 N-methyl-D-aspartate (NMDA) receptor subunits in human and *Bennett* rat dorsal root ganglia – An immunohistochemical study

Vera Ogunlade, Ralf Schober, Stefan Grimm, Clemens Allgaier

NMDA receptors (NR) play a role in the transmission of pain signals to the central nervous system via the dorsal root ganglion (DRG) and may contribute to neuropathic pain. Therefore, information about NR subunits in DRG and spinal cord may help to understand the importance of the NR in processes related to chronic pain.

We investigated the distribution of NR subunits in the human DRG by immunohistochemistry (IHC). Possible changes in NR subunit expression in DRG were studied using the Bennett rat - a model for peripheral mononeuropathy (consisting in ligatures placed around the sciatic nerve). Lumbar human DRGs were fixed with formaldehyde. Rat DRGs were fixed by perfusion. Mayer's Hemalum counterstaining was used for IHC using anti-NR subunit-specific antibodies (Santa Cruz). NR immunoreactivity (IR) in the human DRG was found for all investigated subunits in neuronal cell bodies and axons, but also in other cell types. However, the expression pattern was found largely different among individual patients regarding the number of positive neurons and IR intensity. Mainly, the small neurons, which are supposed to be involved in nociception, were more intensively stained than the large neurons transmitting other sensory inputs. In neurons located in the ipsilateral (ligated) DRG of Bennett rats, the IR for NR2A and NR2B subunits was significantly increased as compared to the contralateral side. These alterations of NR expression were found in small and large neurons.

Our study demonstrates changes of functional significance in the expression of NR subunits presumably implicated in the development of neuropathic pain.

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5.49 Identification of P2Y receptor subtypes in human Müller glial cells

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Nucleotides act as signaling molecules via P2 receptors. Activation of metabotropic P2Y receptors results in an increase of the intracellular Ca^{2+} concentration $[Ca^{2+}]i$. The identification of P2Y receptors expressed in the retina may improve the understanding of retinal physiology. We investigated the expression of P2Y receptors in human retinal Müller glial cells. Since the access to human tissue is limited, we confined our study to the subtypes $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$. The use of the material was approved by the ethics committee of the University of Leipzig Medical School. Retinal tissue from patients with proliferative vitreoretinopathy was obtained during vitreoretinal surgery; Müller cells were enzymatically isolated. Because these cells express Ca^{2+}-dependent K^+ (BK) channels, an increase in $[Ca^{2+}]i$ could be recorded electrophysiologically. Application of different P2Y agonists resulted in BK current increases, pointing to the existence of different receptor subtypes in Müller cells. Subsequently to the current recording, the cytoplasm of the recorded cell was harvested into the recording electrode and expelled into a PCR tube. After reverse transcription, the cDNA from a single cell was split into two samples and separately used for a PCR reaction with $P2Y_1/P2Y_2$- (n=44) and $P2Y_4/P2Y_6$-specific (n=17) primers, respectively. The mRNA for all investigated P2Y receptor types could be found in human Müller cells, the incidence of $P2Y_2$ receptors was significantly lower than that of the other subtypes. In addition, we used human retinal tissue to demonstrate the existence of P2Y receptor subtypes immunohistochemically.

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5.50 Norchloro-fluorohomoepibatidin – PET evaluation of tracer properties in pigs

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2-[F-18]fluoro-A-85380 (2-FA) is currently used as radiopharmaceutical for positron emission tomography (PET) imaging of α4β2 neuronal nicotinic acetylcholine receptors in the brain. Comparison of the biodistribution of both enantiomers of F-18 labeled norchloro-fluorohomoepibatidine (NCFHEB) (6-β-(2-fluoro-5-pyridinyl)-8-azabicyclo[3.2.1]octane) with 2-[F-18]fluoro-A-85380 (2-FA) in mice revealed superior properties of the enantiomers of NCFHEB. We have started the comparison of these tracer compounds by PET studies in pigs.

Radiolabeling was performed by reacting a solution of Kryptofix222/potassium carbonate/[F-18]fluoride complex with 0.3 to 0.7 mg of enantiomerically pure precursor NCBrHEB (6-β-(2-bromo-5-pyridinyl)-8-azabicyclo[3.2.1]octane) in a microwave. The product was purified by HPLC (Multospher 120 RP 18 AQ, 10x125 mm, NaH2PO4 (10 mM) pH 4, 5 % ethanol). The product peak was collected and (-)-[F-18]NCFHEB injected into a pig (20 kg bodyweight).

Precursor and product were well separated by semipreparative HPLC. (-)-[F-18]NCFHEB showed a high uptake in the brain displaying the typical distribution of α4β2 nAChR subtypes in the brain with very high accumulation in the thalamus, high accumulation in the cortex and low activity concentration in the cerebellum. The time activity curve for the thalamus showed a maximum at 115 min.

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5.51 Is there an interaction of the adaptor proteins FE65 and Mint3 with MAP-tau?

Ellen Ramminger, Max Holzer, Thomas Arendt

The main characteristic of neurodegeneration in Alzheimer's disease (AD) is progredient neuronal death within brain regions of high plasticity caused by intraneuronal neurofibrillary tangles (NFTs) and extracellular β-amyloid plaques. NFTs consist of abnormally phosphorylated tau protein, which is polymerized into paired helical filaments or straight filaments.

The role of tau protein in neurodegeneration is emphasized by pathological tau fibrils occuring separately, without β-amyloid-depositions in several neurodegenerative diseases, summarized as tauopathies. The identity of factors facilitating tau aggregation remains elusive. One approach to identify such factors is to screen for proteins that interact with human microtubule associated protein tau (MAP-tau).

FE65 was identified to be a potential MAP-tau-interacting protein by co-immunoprecipitation[1]. Colocalisation of FE65 and tau-proteins within intracellular tangles has been observed[2].

Our aim was to investigate if the proteins FE65 and a related protein Mint3 interact with human MAP-tau and to define the interacting domains by means of Yeast-two-hybrid system and co-immunoprecipitation.

Surprisingly no interaction of MAP-tau with FE65 or with Mint3 was observed using Duplex-Yeast-two-hybrid system and co-immunoprecipitation. This might indicate a transiently occurring interaction not detectable by these methods, or may point to an absence of interaction.

This work was supported by the IZKF/TP C24 and the DFG (HO23681-1).

Notes

1 Barbato et al., Neurobiol. Dis. 18:399.

2 Delatour et al., Am. J. of Path. 158:1585.

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5.52 Behavioural effects of the antiaggregatory therapeutic compound clopidogrel (Plavix®; Iscover®)

Ralph Regenthal, Frank Baumann, Cornelius Donat, Christian Gachet, Peter Illes, Ute Krügel

Clopidogrel (Plavix®; Iscover®) is an inhibitor of ADP-induced platelet aggregation via $P2Y_{12}$ receptors worldwide used for long term prevention of atherothrombotic events. It was shown that $P2Y_{12}$ receptors are present in the central nervous system, modulating behavioural changes induced by extracellular ADP. Here, the influence of clopidogrel on explorative motor activity and anxiety was investigated and its distribution in liver and brain of rats. Clopidogrel dose-dependently (1 to 25 mg/kg p.o.) reduced the explorative activity in a novelty open field situation and affected the behaviour on the elevated plus maze, a standard test for anxiety, one hour after administration. The percent of entries into the open arms and of the time spent there were significantly reduced. These results indicate that clopidogrel mediates central effects on motivation-related locomotor activity at doses used for therapy in humans (loading dose 300 mg, maintenance dose 75 mg once daily).

Clopidogrel, is a prodrug, hepatic metabolised to a highly labil active intermediate. Clopidogrel was detected in liver and brain with a maximum of tissue concentration between half and one hour after administration, which confirms with the antiaggregatory response. The concentration in the brain was much lower than in the liver (0.043±0.027 vs. 0.25±0.19 µg/g tissue).

It is unclear, whether clopidogrel is metabolised in the brain, or whether the active metabolite acts on endothelial cells known to be endowed with $P2Y_{12}$ receptors or passes the blood-brain barrier.

However, clopidogrel induces central side effects relevant for motivation-related behavior.

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5.53 Brain prolyl endopeptidase expression in aging, APP transgenic mice and Alzheimer's disease

Steffen Roßner, Ingo Schulz, Ulrike Zeitschel,
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Prolyl endopeptidase (PEP) is believed to inactivate neuropeptides that are present in the extracellular space. However, the intracellular localization of PEP suggests additional, yet unidentified physiological functions for this enzyme. We have recently demonstrated that PEP is associated with the tubulin cytoskeleton and important for the proteolytical processing of the amyloid precursor protein (APP) and for β-amyloid secretion in neuronal cell lines. Here we studied the expression, enzymatic activity and subcellular localization of PEP in adult and aged mouse brain as well as in brains of age-matched APP transgenic Tg2576 mice and in brains of Alzheimer's disease patients. In mouse brain PEP was exclusively expressed by neurons and displayed region- and age-specific differences in expression levels, with the highest PEP activity being present in cerebellum and a significant increase in hippocampal but not cortical or cerebellar PEP activity in aged mouse brain. In brains of young APP transgenic Tg2576 mice, hippocampal PEP activity was increased compared to wild-type littermates in the pre-plaque phase but not in aged mice with β-amyloid plaque pathology. This "accelerated aging" with regard to hippocampal PEP expression in young APP transgenic mice might be one factor contributing to the observed cognitive deficits in these mice in the pre-plaque phase and could also explain in part the cognition-enhancing effects of PEP inhibitors in serveral experimental paradigms.

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5.54 Active cardiomyocytes on collagen gels for biohybrid systems

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Cardiomyocytes show inherent contractile activity. Under the influence of stimulating agents like noradrenalin or angiotensin-II the frequency of these contractions rises. Contraction frequency can be determined optically, electrophysiologically (using Multi-Electrode-Arrays) or by a force sensor. For the detection of contractility by a force sensor a monolayer of cardiomyocytes is cultivated on a collagen gel or on a coated silicone membrane that serves as bottom of the cultivation vessel. Contracting and relaxing cardiomyocytes cause changes in tension which are detected by recording the degree of sagging of the gel or the membrane. An extremely sensitive pressure sensor is able to detect even minute changes in sagging, providing an ideal technique to automate contraction frequency and contractility determination. The system can be used to examine the influence of drugs and their antagonists on cardiomyocytes. Collagen being a vital component of the extra-cellular matrix offers the advantage of resembling more natural conditions for cells if used as substrate while culturing cells directly on the membrane allows for more sensitive detection in changes of contactility.

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5.55 Molecular genetic diagnosis of susceptibility to malignant hyperthermia

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Malignant hyperthermia is a pharmacogenetic disorder of the skeletal muscles which may be triggered by inhalational anaesthetics and depolarizing muscle relaxants. Exposure to these agents can lead in pre-disposed individuals to a potentially life-threatening MH crisis caused by an uncontrolled intracellular calcium release from the SR into the myoplasm. A mutated ryanodine receptor subtype 1(RYR1) appears to play the outstanding role in the pathogenesis of MH. The disposition to MH is autosomal-dominantly inherited and the incidence of a MH event was estimated as 1 in 60,000 balanced anaesthesias.

The pre-clinical standard investigation to determine the MH disposition is the *in vitro* contracture test (IVCT). This test was introduced by the department of anaesthesiology and intensive care medicine in 1986. In the 90th the diagnostic approach was extended by the identification of a number of MH associated mutations in the RYR1-gene. Until 2005 23 RYR1 mutations were classified as being causative for MH by the European MH Group. From 1999-2005 we have genetically investigated 715 persons out of 135 MH families. Altogether, 231 RYR1 mutation carriers out of 62 families could be idenified. They all showed a clear pathological response in the *in vitro* contracture test, representing a strong genotype-phenotype correlation. 205 individuals did not bear the familial mutation, almost all were MH negative in the IVCT.

24 different RYR1 mutations were detected in the three hot spot regions of the gene: 9 MH causative mutations, 2 known MH associated mutations (without functional evidence) and 14 new mutations (incl. one causative).

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5.56 Variability of the mitochondrial genome in cancer

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ATP, cell's energy currency, derives from the partial oxidation of energy-rich biomolecules. The final steps in the complex reaction process are carried out in mitochondria. The mutation rate of the mitochondrial DNA has been found to be almost ten times higher than in nuclear DNA. This is not surprising when considering the genesis of reactive oxygen species (ROS) that are generated during the reactions of the respiratory chain as side products. In the last decade, a variety of devastating neuromuscular diseases have been associated with mutations of the mitochondrial genome. These comprise single base replacements in tRNA and rRNA genes as well as protein genes. Moreover, length variations - insertions and deletions - were reported for other patients as well.

The regulatory region of the genome, the so called D-loop (displacement loop) is a noncoding stretch of DNA of approximately 1,100 bp. Very recently, reports on mtDNA variations within the D-loop were published for several cancers, but their functional significance remains still unclear. Clusters of hypervariable regions were found where mutations occurred with much higher frequencies. As replication and the repair machinery act in part independent from cell cycle, a novel DNA variation that is created during malfunctioning DNA replication and/or repair can expand very rapidly.

The goal of this project is to characterize the variations of the mitochondrial genome during tumorigenesis. Upon finding a direct linkage between tumorigenesis and defects of the mitochondrial genome, we also want to develop a test system for simplified diagnostics.

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5.57 Interaction of interleukin-1β with muscarinic acetylcholine receptor-mediated signaling cascade in cholinergically differentiated SH-SY5Y cells

Reinhard Schliebs, Kristin Heidel, Jenny Apelt

Increased expression of interleukin (IL)-1 has been found in Alzheimer brain, raising the question whether plaque-associated up-regulation of IL-1 may contribute to neuronal loss as it is able to stimulate the amyloidogenic path of amyloid precursor protein cleavage. However, less is known on participation of IL-1 in specific cholinergic cell loss. To test for this hypothesis, the cholinergically differentiated cell line SH-SY5Y was exposed to IL-1β for various periods of time followed by stimulation of muscarinic acetylcholine receptors (mAChR) and detecting key signaling molecules. The activity of acetylcholinesterase (AChE) in SY5Y cells was dose-dependently increased following stimulation with carbachol being highest at 100 µM. Pre-exposure of SY5Y cells to various concentrations of IL-1β (ranging between 1 and 50 ng/ml) for one up to 24 hours prevented the carbachol-induced mAChR-mediated effects on AChE activity, already detectable following one hour of prexposure at 10ng/ml IL-1β. Carbachol stimulation dose-dependently stimulated the activation of the transcription factors NFkB and AP-1 as revealed by EMSA. Pre-exposure of cells with 1 ng/ml IL-1β for two hours did not affect carbachol-mediated increase in DNA binding capacity of NFkB or AP-1. However, exposure of cells with 1 ng/ml IL-1β for 24 hours suppressed the capability of SY5Y cells to respond upon mAChR stimulation with increased NFkB or AP-1 activation. The data suggest interactive mechanisms between IL-1β and mAChR signaling cascades which may contribute to the cholinergic deficits in Alzheimer's disease.

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5.58 Cell cycle regulatory proteins in the adult mouse neocortex

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The developmental structuring of brain is the result of a strictly coordinated process that involves controlled cell division, neuronal migration and terminal differentiation. Once differentiated, neurons are incabable of further division but retain the capability of structural and functional plasticity. In general, the cell cycle progression is regulated by the sequential expression and activation of regulatory proteins like cyclins, cyclin dependent kinases (cdk) or cdk inhibitors (cdki). In terminally differentiated neurons, cell cycle activity is arrested by enrichment of cdkis. During the development as well as during the whole lifespan neuronal elements undergo a high degree of structural and functional remodeling and establish a neuronal network. This plastic process is accompanied by dynamic rearrangements of the cytoskeleton, i.g. neurofilaments, microtubules and actin. In the present study we have examind the expression of cell cycle regulatory proteins in the adult neocortex using immunohistochemical methods. Several cyclins and cdks could be predominantly localised to pyramidal neurons of layer V. Staining was prominent both in neuronal perikarya and dendrites. In parallel to immunohistochemical studies, expression of cell cycle proteins was verified by Western blotting. Quantitative real-time PCR was used to determine mRNA expression of cyclins, cdks and cdkis in single pyramidal neurons. Findings suggest that the expression of cell cycle markers in terminal differentiated pyramidal neurons of neocortex is likely associated with additional physiological functions beyond cell cycle regulation.

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5.59 Advanced glycation endproducts activate mitogenic signal transduction pathways towards neuronal cell cycle re-entry

Angela Schmidt, Thomas Arendt, Gerald Münch

Advanced glycation endproducts (AGE), protein-bound oxidation products of sugars, have been shown to be involved in the pathophysiological processes of Alzheimer disease (AD). In addition, AGEs contribute to inflammation and oxidative stress occurring in AD. Neurodegeneration in AD is associated with activated mitogenic signaling and signs of re-entry into the cell cycle, indicated by the presence of cell cycle specific proteins, such as cyclins, cdk´s and their inhibitors. In the present study, we analysed the colocalization of AGEs and the cell cycle marker cyclin D1 in AD. Furthermore, it is supposed that RAGE, a receptor for advanced glycation endproducts, mediates its intracellular signaling via the MAPK pathway and causes the activation of the transcription factor NfkB. In order to show that the MAPK pathway is involved in RAGE signaling, we silenced this pathway by anti-RAGE antibody and by oligo-anti-sense-RAGE. Thereby we could obtain evidence for an involvement of RAGE in cell-cycle-activation. Further, we analysed the phosphorylation of CREB and Rb, known to be critical regulators of cell-cycle-activation. Our result indicated that AGEs can potentially activate mitogenic signaling which leads to cell cycle re-entry of neurons in AD, and thus drive the progression of dementia.

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5.60 Neuronal activation of Ras regulates synapse formation and synaptic vesicle docking

Gudrun Seeger, Ulrich Gärtner, Thomas Arendt

The small G protein Ras, which is a molecular switch in neurotrophic signal transduction, is implicated in synaptic plasticity and synapse development during ontogeny and in the adult nervous system. To characterise the involvement of Ras-dependent signaling in synaptogenesis, the cortical synapse-to-neuron ratio was investigated in synRas mice overexpressing Val12-Ha-Ras in postmitotic neurons[1]. The number of synapses and neurons were analysed in cortical layers II/III of the somatosensory cortex at different stages of postnatal development by stereological methods. The synapse-to-neuron ratio was still identical in wild-type and synRas mice at postnatal day 4 before the onset of transgene expression. At P12, P47 and in the adult, analyses revealed a significant increase in the synapse-to-neuron ratio in synRas mice which correlated with the strength of transgene expression. We also show that neuronal expression of Val 12-Ha-Ras enhances docking of synaptic vesicles to active zones in adult mice. The data presented here provide evidence that Ras activity might be profoundly involved in synaptogenesis by reinforcing the formation or maintenance of synapses during the development and in the adult. We conclude that neuronal Ras activity contributes to the regulation of of synaptic plasticity in adult mammalian brain at the presynaptic level.

Notes

1 Heumann et al., J. Cell. Biol. 151:1537-1548 (2000).

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5.61 Neuropeptide Y and the selective Y_1 receptor agonist [F7, P34] pNPY inhibit the glutamatergic synaptic transmission on cortical neurons

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Neuropeptide Y (NPY) has been shown to be one of the most abundant and widely distributed peptides in the central nervous system. In rat, NPY immunoreactivity is prevalent in the cortex and in the hippocampus. These areas are also enriched with NPY receptors (Y). Several receptor subtypes of the Y receptor superfamily have been identified in the central nervous system. The peptide agonists allow differentiation between various Y receptor subtypes in these areas. In the present study glutamatergic synaptic potentials (PSPs) induced by electrical field stimulation were measured intracellularly by microelectrodes in pyramidal cells of the rat cingulate cortex in brain slices. The type of the NPY receptor responsible for affecting the PSPs was assessed by examining the effects of different NPY agonists and the selective Y_1 receptor antagonist (R)-N2-(diphenylacetyl)-N-[(4-hydroxyphenyl)-methyl argininamide (BIBP3226). NPY had an inhibitory effect on the PSPs. It decreased concentration-dependendly the amplitude of the PSPs by a biphasic effect. The inhibitory effect induced by low concentration but not by high concentration was reversible during washout. The bath application of the selective Y_1 agonist [F7, P34] pNPY showed the same effect as NPY on PSPs. The selective Y_1 receptor antagonist BIBP3226 (50 nM) completely inhibited the effect of [F7, P34] pNPY. Accordingly, the present study demonstrates that the Y_1 receptor is involved in the inhibition of glutamatergic neurotransmission in cortical neurones of the cingulate cortex.

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5.62 Flow cytometry in Alzheimer's disease diagnostics

Jens Stieler, Markus Kiefer, Thomas Arendt

The search for a biomarker of Alzheimer's disease (AD) is one of the challenges in the investigation of this neurodegenerative disease. At present an early diagnosis of AD is, regardless the recent findings of molecular mechanisms and novel imaging techniques, still complex and unreliable. Several biomarkers were investigated to support or replace of the present tests. Numerous reports suggest an involvement of peripheral cell types in the pathology of AD. Isolated lymphocytes of AD patients showed an impaired response after mitogenic stimulation. The proliferative response can be analyzed by detecting the CD69 expression using flow cytometry. However, the protocol involves several elaborative and time consuming steps which make it difficult to introduce this technique into the analytical laboratory routine.

In order to reduce the methodical expenditure and enhance the reproducibility we tested the lymphocyte proliferation assay on whole blood on 22 samples (13 AD, 9 controls). Stimulation, staining and measuring of the sample can be performed in only one tube. Using this assay we could statistically significant differentiate between AD and non-AD samples.

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5.63 Osmotic glial cell swelling in the rat retina is inhibited by triamcinolone acetonide

Ortrud Uckermann, Franziska Kutzera, Antje Wolf, Ianors Iandiev, Thomas Pannicke, Andreas Reichenbach, Sebastian Wolf, Peter Wiedemann, Andreas Bringmann

Anti-inflammatory corticosteroids such as triamcinolone acetonide are commonly used to treat macular edema during ischemic and inflammatory eye diseases. In addition to the leakage of blood-retinal barriers which causes extracellular edema, swelling of Müller glial cells (cytotoxic edema) has been implicated in the formation of macular edema. Recently, we have shown that hypotonic stress (that resembles hypoxia-induced cytotoxic edema in the brain) induces Müller cell swelling under multiple experimental conditions: In the postischemic retina of the rat, in retinas of eyes with LPS-induced ocular inflammation, and in control retinas in the presence of Ba^{2+}, H_2O_2, arachidonic acid, or PGE_2. Acute application of triamcinolone dose-dependently inhibited the glial cell swelling in slices of postischemic retinas and from LPS-treated eyes, as well as in control retinas. The inhibiting effect of triamcinolone on Müller cell swelling was largely abrogated in the presence of inhibitors of A1 adenosine receptors, of adenylyl cyclase and of PKA, respectively. This suggests that the effect of triamcinolone involves endogenous release or formation of adenosine and subsequent A1 receptor stimulation that results in enhancement of the intracellular cAMP level and activation of PKA. Acute application of trimacinolone rapidly inhibits the osmotic glial cell swelling which is a characteristic feature of retinas of the rat during ischemia-reperfusion and inflammation. The inhibiting effect on the cytotoxic Müller cell swelling may contribute to the edema-resolving action of triamcinolone observed in human subjects.

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5.64 The influence of neuropeptide Y and Y_1 receptor antagonist BIBP3226 on MCF-7 tumor spheroids

Winnie Weigel, Andrée Rothermel, Markus Rüffer, Randy Kurz, Heinz-Georg Jahnke, Levin Böhlig, Annette G. Beck-Sickinger, Andrea A. Robitzki

Neuropeptide Y (NPY) and its receptors have been shown to be involved in human cancer. As revealed for breast cancer, 85 % of patients have an elevated and modified expression pattern of NPY receptors in their tumors. In normal breast tissue Y_2 receptor mRNA is predominant, whereas breast carcinomas preferentially express Y1 receptor mRNA. Tumor spheroids of the human mamma carcinoma cell line MCF-7 were incubated with NPY and Y_1 receptor antagonist (BIBP3226) to examine the effects on proliferation, apoptosis and tumor growth. In the presence of BIBP3226 the size of tumor spheroids was reduced, whereas NPY caused an increase in size compared to control spheroids. Moreover, we observed that the expression of Y_1 receptor mRNA was significantly increased by the addition of NPY. Interestingly, we could also detect an elevated mRNA expression of Y_1 receptors if tumor spheroids were treated with BIBP3226. Furthermore, an antagonist-depending reduction of apoptotic cells was shown in comparison to untreated spheroids. On the basis of these findings MCF-7 tumor spheroids can serve as a new model system to analyse the role of Y receptors in breast cancer and to develop new therapeutic strategies.

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5.65 Protective effects of prolyl endopeptidase inhibitors in astrocytoma cell lines and rat primary astrocytes

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Steffen Roßner, Hans-Ulrich Demuth

The neuroprotective effects of prolyl endopeptidase (PEP) inhibitors are well characterized in a number of experimental *in vitro* and *in vivo* models. However, several clinical conditions are associated with the death of astroyctes, whose functions are important for the maintenance of neurons. In order to demonstrate the effects of PEP inhibitors on the survival of astrocytes in stress paradigms, the astrocytoma cell lines U-343 and U-138MG as well as rat primary astrocytes were subjected to toxic stimuli by means of staurosporine-, LPS- or oncostatine M-treatment. These treatments resulted in the death of 30-70 % of the astrocytes within a period of 24 hours as demonstrated by LDH release into the culture medium. Enzymatic activity assays and immuncytochemistry using an antibody against active caspase-3 revealed the contribution of caspase3/7 in astrocytic cell death under these experimental conditions. To reveal whether or not PEP inhibitors are protective in these *in vitro* paradigms, co-incubation experiments with the stressors mentioned above and three different PEP inhibitors of distinct chemical classes were performed. We observed a dose-dependent protection from the cytotoxicity for all PEP inhibitors used but with a different potency and kinetics.

These data indicate that PEP inhibitors protect astrocytes from cell death induced by different insults. Based on the known functions of astrocytes in neuroprotection/repair it is tempting to speculate that the observed neuroprotective effects of PEP inhibitors in experimental lesion studies *in vivo* are contributed by enhanced astrocytic survival.

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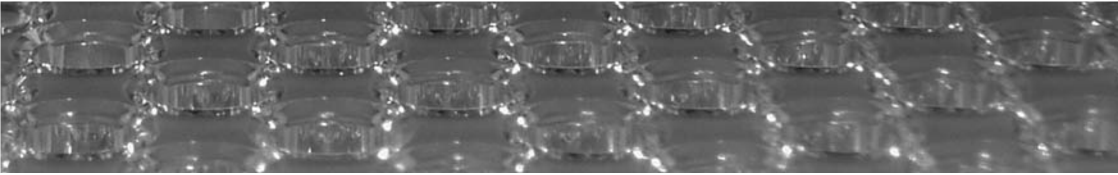
6. BIOMEDICAL AND CELL ENGINEERING



POSTERS

6.1 Optimisation of hepatocyte spheroid culture for application in bioartificial liver support systems

Matthias Funke, Doreen Beck, Christine Schulz,
Jan-Michael Heinrich, Augustinus Bader

In case of Hepatic Failure, the therapy options are today basically restricted to the use of biochemical and/or physical absorption and filtration process that are not suitable to substitute the complex synthetic and metabolic liver functions effectively. This task can only be fulfilled by cells of a healthy liver, namely the hepatocytes.

Our goal is the characterisation and further development of such biocomponents, especially hepatocyte spheroids, for bioartificial liver support systems. The 3D spheroid culture is characterized by the aggregation of single hepatic cells to a complex community, comparable to the native liver tissue, which promotes their viability and functionality.

The cells undergo different changes in the stages of formation and maturation of aggregates, caused by changes in mass transfer (supply with oxygen and nutrients) and by the establishment of extensive cellular contacts.

Regarding to the first point, we obsere the spheroid size and related morphological changes, especially the occurrence of necrotic or apoptotic cells inside the aggregates. At second we are focused on the differences at the formation of hepatocyte spheroids w/wo different kinds of nonparenchymal liver cells, and/or changes under the treatment with different combinations of growth factors. These experiments will result in the establishment of a continuous culture system for optimised cultivation and mass preparation of hepatocyte spheroids. The innovation of this culture system should be the production of spheroids with defined size by cutting of aggregates bigger than an upper limit, to prevent mass transfer limitations.

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6.2 Interaction of signaling pathways during capacitation of human spermatozoa

Sonja Grunewald, Uwe Paasch, Thomas Baumann, Christian Kriegel, Hans-Jürgen Glander

Introduction: Capacitation of sperm is a prerequisite for successful fertilization, although the exact molecular mechanism is not fully clarified. The calpain(CLP)-calmodulin(CM)-system is essential for membrane fusion during this process. Activation of caspases (CP) is a main feature of apoptotic cells. The objective of our study was to examine interactions of apoptosis signaling pathways and the CLP-CM-system during capacitation.

Material and Methods: Semen samples of donors (n=14) were washed by density gradient centrifugation. Four aliquots were incubated in BWW at 37 °C 5 % CO_2 for 3h with: no additive (control); 3 % BSA (capacitation); 10 µM CLP-inhibitor III or 20 µM CM-inhibitor (Ophiobolin A). Capacitation was monitored by hyperactivation (CASA), CTC-assay and tyrosine phosphorylation (Western blot). FACS analyses were performed to evaluate CP1,-9 and -3 activation and the integrity of transmembrane mitochondrial potential (iTMP).

Results: Capacitation (proved by CTC-assay, increased levels of tyrosine phosphorylation and hyperactivation) resulted in inactivation of CP1:-9.9±8.2 %, CP9:-9.8±6.3 % and CP3: -4.8±8.3 % and improved intact TMP:+17.5±7.7 %. Inhibition of CLP during capacitation had no further impact on CP and iTMP, but diminished the capability to capacitate. In contrast, inhibition of CM resulted in both: blocking of physiological changes seen during capacitation and apoptosis (CP1:+64.0±27.2 %, CP9:+76.9±25.7 %, CP3:+78.9±25.2 %, iTMP:-68.3±20.5 %).

Conclusion: Interaction of both signaling systems seems to enable the capacitation process by prevention of apoptosis.

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6.3 Tissue engineered scaffolds for cartilage repair

Marcus Haberhauer, Andrea Deiwick, Augustinus Bader

Biological restoration of cartilaginous defects of the knee joint caused by trauma remains a challenging and controversial clinical problem and a major challenge for orthopaedic surgery since these defects do not heal spontaneously because of the limited regenerative capability of the articular cartilage.

Tissue engineering offers the possibility for the construction of autologous chondrogenic grafts for reconstructive surgery and represents a promising method for the treatment of large cartilage defects.

The intent of our project is the assortment of several biocompatible materials that could be considered as a three-dimensional carrier for autologous chondrocytes.

In our work, freshly isolated porcine chondrocytes were embedded in two biological degradable matrices and cultured either in static culture system for seven days and after that transferred to a roller bottle culture system for 30 days or cultured in roller bottle culture system for 37 days right after seeding.

Immunohistochemical and biochemical analysis, confocal laser scanning microscopy and histomorphological analysis were performed at defined time points to evaluate the phenotypic changes of the embedded chondrocytes, and the quality of the newly formed cartilaginous matrix.

Our experiments show that the varying treatments combined with the different matrices used in our experiments support the proliferation and the development of a stable and cartilage-like extracellular matrix with the opportunity to create a transplant for cartilage restoration.

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6.4 Effects of surface coatings using bone sialoprotein, collagen, and fibronectin on cells derived from human maxillar bone

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Stefan Thalhammer, Stefan Strasser, Franz Paul Armbruster

The interaction between the implant material and the surrounding tissues is believed to be one of the factors determining implant success. Bone sialoprotein (BSP) is both one of the major noncollagenous protein in the extracellular matrix of bone and a marker for osteoblastic differentiation. We compared BSP-, collagen-, fibronectin- and non-coated implant material in supporting the development of adult human maxillar bone at day 3, 5, 10, 15, 20 and 25 *in vitro*. The time course of the expression of BSP and fibroblasts was visualized immunohistochemically. The distribution patterns of cells were determined on the surface of TICER and glimmer. TICER consists of titanium with hydroxy-apatite ceramic with rough surface. Glimmer has a total smooth surface.

Cell countings revealed that most of survived cells are settled on the surface of the BSP-coated implants. Significantly different values were found at the 3. and the 5. Div, exclusively. The White-test using rankings of the median values gave evidence for BSP-coatings at rank 1 followed by collagen.

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6.5 Human retinal pigment epithelial cells produce and respond to placenta growth factor

Margrit Hollborn, Solveig Tenckhoff, Peter Wiedemann, Leon Kohen, Andreas Bringmann

Placental growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family and shares some biological functions of this molecule. PlGF seems to be implicated in the formation of fibrovascular tissue during proliferative diabetic retinopathy (PDR), since the vitreous level of PlGF is elevated during development of PDR and PlGF immunoreactivity is expressed in fibrovascular membranes. However, it is not known whether RPE cells produce PlGF. Therefore we determine whether human retinal pigment epithelial (RPE) cells express and respond to PlGF.

Human RPE cells express mRNAs for various members of the VEGF family of growth factors and for their receptors such as for VEGF-A, -B, -C, -D, PlGF, Flt-1, KDR, and neuropilins-1 and -2. The expression levels of the mRNAs for neuropilins-1 and -2 were higher when compared to the mRNAs for Flt-1 and KDR. Exogenous PlGF enhanced the expression of neuropilin-1. The Members of the transforming growth factor (TGF)-β superfamily, BMP-4, TGF-β1 and TGF-β2 are strong inducers of the PlGF gene expression, and evoke secretion of PlGF protein by RPE cells. Exogenous PlGF-2 induces chemotaxis in RPE cells and reduces slightly the cell proliferation at high concentrations.

The finding that RPE cells secrete and respond to PlGF suggests that the factor exerts an autocrine/paracrine action on these cells. Increased retinal expression of TGF-β-related growth factors during proliferative retinopathies may cause facilitation of PlGF expression by RPE cells that may contribute to the stimulation of cell migration as a critical component of the progression of epiretinal membranes.

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6.6 Investigation of RGD-oligonucleotide conjugates with respect to their hybridization with anodically entrapped oligonucleotides (ON) on titanium alloys and their binding ability to integrines on osteoblasts

Ina Israel, Jan Michael, René Beutner, Ute Hempel, Hartmut Worch, Dieter Scharnweber, Bernd Schwenzer

Titanium and its alloys are mainly used for implants because of their excellent biocompatibility. However, in the case of systemic diseases (e.g. diabetes) or bad local bone quality further improvement is necessary. A promising approach to stimulate the natural healing process is surface coating with components of the extracellular matrix (ECM) or peptides mimicking ECM functions. For the presented modular system different ON-conjugates were hybridized with ON which had been partially entrapped in an anodically grown oxide layer on the titanium alloy surface.

In this study complementary and non-complementary ON (with respect to the entrapped ON) were conjugated to the hexapeptide GRGDSP containing RGD as an integrine recognition sequence. Covalent linkage was carried out with suberic acid bis(N-hydroxysuccinimide ester). Characterization was accomplished by HPLC and MALDI TOF MS.

Immobilization and hybridization efficiencies were determined radioanalytically, using 32P-labeled ON. Hybridization efficiencies of the RGD-ON-conjugate and the corresponding ON were in a similar range. Thus, the bound hexapeptide hardly affects hybridization.

Successful conjugate binding to integrines on osteoblasts proved the preservation of biological activity of the GRGDSP hexapeptide.

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6.7 Destruction of endogenous mtDNA – Establishing a novel method utilising a restriction endonuclease

Alexandra Kneissl, Christian Kukat, Ingo Schäfer,
Peter Seibel

A variety of human disorders are based on defects of the mitochondrial genome. Among those diseases are cases of maternally transmitted epilepsy, muscle disorders, blindness, refractory anemia or diabetes. The broad spectra of symptoms already pinpoints toward the physiological problem of these disorders: the limitation of oxidative energy supply creates tissue and developmentally critical bottlenecks in different organs that are caused by genetic defects of the oxidative phosphorylation system. Very little is known about the overall molecular mechanisms underlying these diseases especially the interaction of nuclear and mitochondrial genomes.

Therefore we developed a novel system for establishing cells devoid of any endogenous mitochondrial DNA without using mutagenic substances. This system is based on a mitochondrially targeted restriction enzyme. Upon reaching the mitochondrial matrix the mtDNA is cleaved at specific sites and hence the mitochondrial genome can undergo degradation by endogenous mitochondrial nucleases.

The cell lines established so far with this method revealed a ρ^0-state by relying on supplementation of the growth media with pyruvate and uridine. The genetic loss of the mitochondrial genome was also demonstrated by PCR analysis.

Further investigations of the interactions between the mitochondrial and nuclear genome can now be envisioned. Moreover our research interest aims for the generation of an animal model that enables the study of mitochondrial biogenesis and oxidative energy supply *in vivo*, and to shed light on the molecular pathogenesis of these diseases.

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6.8 Evidence for abnormal iodination of thyroglobulin in cold thyroid nodules

Kerstin Krause, Stefan Karger, Marie-Christine Many, Sylvie Poncin, Ralf Paschke, Dagmar Führer

Using a proteomic approach we previously observed upregulation of proteins involved in the reduction of peroxide in cold thyroid nodules. These results point to either (I) a defect in the integrity of Tg which might prevent the organification and coupling of iodide (I-) to from thyroid hormones or (II) to a principal defect in the uptake and transport of I- in CTN. To investigate the functional protein expression characteristics in CTNs in more detail, immunohistochemical analysis was performed in a series of 12 CTNs and corresponding normal thyroid tissues. The following proteins were studied: (1) proteins involved in intrathyroidal iodine metabolism (Pendrin, ThOX 1/ 2 and TPO, Tg, T4-Tg); (2) proteins involved in the antioxidative system (PDRX 5, GST π) and (III) proteins involved in Tg-processing (cathepsin B). We found (I) complete absence of T4-rich iodinated Tg in CTNs while overall Tg expression was not altered, (II) higher expression of ThOXs and TPO in CTNs compared to ST, (III) upregulation of the antioxidant enzyme PDRX 5 in CTNs and (VI) expression of pendrin depending on follicular size (upregulation only in small follicles in normal thyroid tissues). Taken together, this study confirms the data obtained by proteomics and further reinforce the hypothesis of an altered Tg function with most likely compensatory yet ineffective upregulation of the thyroid hormone synthesis apparatus in CTNs. Moreover, it is tempting to speculate that increased proliferation in combination with deranged abundant peroxide generation may contribute to somatic mutations leading to clonal CTNs.

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6.9 Cardiomyocytes beat for microelectrode arrays: the use of electrogenic properties for functional monitoring of autoimmune antibodies in sera of preeclamptic patients

Randy Kurz, Andrée Rothermel, Markus Rüffer, Winnie Weigel, Heinz-Georg Jahnke, Holger Stephan, Andrea A. Robitzki

The aim of the study was the establishment of a high-sensitive screening system that enables the electronic detection of drugs and tissue-secreted factors involved in AT_1 receptor-mediated cardiovascular diseases. Spontaneously beating neonatal rat cardiomyocytes were cultured on multielectrode arrays and used as biological sensors. After evaluation of culture parameters, computer-based electronic measurement systems have been developed for counting contractions by recording the field potential of cardiomyocytes. Using the biosensor, angiotensin II, the predominant ligand of the AT_1 receptor, was detected at very low concentrations of 10^{-11} M via altered contractions of cardiomyocytes. Moreover, we demonstrated that cardiomyocyte coupled microarrays allow the detection of blood-derived low concentrated anti-AT_1 receptor autoantibodies of pregnant women suffering from preeclampsia. This study demonstrates the first well-suited electrophysiological recording of cardiomyocytes on multielectrode arrays as a benefit for functional biomonitoring for the detection of AT_1 receptor/ligand interactions and other marker proteins in sera directed to diseases.

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6.10 Comparison of entrapped strategies of hepatocytes culture

Aldo Leal-Egana, Jan-Michael Heinrich, Augustinus Bader

One of the principal difficulties to develop an Artificial Liver Support is the survival and the maintains of the metabolic characteristics of Hepatocytes cultured *in vitro.*

In spite of the multiples culture strategies developed, obtaining the optimal bioreactor design still remains as an unsolved problem. Nevertheless, current scientific publications show that the hepatocytes cultured in a three dimensions systems (3D) presents comparative advantages respect to the traditional culture in 2 dimensions (with adherent cells), basically due to the contact between the cells themselves.

Two techniques which allow the cell culture in a 3D system, besides aggregation culture, are the cell immobilization and the cell encapsulation, which presented other advantages, such as avoiding the shear and mechanical stress, and the possibility to freezing, maintaining a stable activity after defrost. Nevertheless, there are just a few reports which compare both systems using hepatocytes.

With the purpose to analyze the differences between both culture strategies, Mouse hepatocytes were immobilized in 500 µm diameter Alginate beads (app. 70 cells per capsule). At the same time, the same cell number was encapsulated in Poly-L-Lysine beads of the same diameter. In both cases the study was carried out without, with low and with high agitation.

The results show a clear difference in the analyzed parameters (albumin, glucose and lactate production, urea degradation, days of hepatocyte surviving) between the experiences carried out without and with low agitation, against the experiment with strong agitation, indicating that improving the mass transference could provide a potential enhancement on the survive capacity of the hepatocytes cultured *in vitro.*

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6.11 Interaction of nucleic acids with titanium alloy surfaces for improved immobilization and hybridization

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A modular system for bio-surface engineering of titanium implant materials based on anodic entrapment of single-stranded oligonucleotides (ON) and their subsequent hybridization with different ON-biomolecule conjugates was subject to further optimization.

Investigated parameters influencing interaction were
- terminal ON modification,
- pH of electrolyte,
- divalent metal ions (Mg^{2+}),
- buffer system,
- ON base composition,
- sample wettability.

Terminal phosphorylation is crucial for the extent, stability and regiospecificity of adsorption and entrapment.

Strength and quantity of immobilization depend on the pH-value of the electrolyte that influences electrostatic interaction between surface and ON. Above pH=8.0 repulsion between the - both negatively charged - titanium surface and ON is distinctly diminishing adsorption. Between pH-values of 3.0 and 8.0 Mg^{2+} acts as mediator by decreasing repulsion. Adsorption at pH=7.5 was better in TRIS buffer compared to borate buffer indicating an influence of the buffer system.

The influence of diminished surface wettability on ON adsorption due to progressive hydrocarbon contamination under atmospheric conditions was investigated comparing the interaction of water-stored (hydrophilic) and octane-treated (hydrophobic) surfaces with various ON. Their adsorption behaviour could not clearly be traced back to surface treatment or hydrophobic interactions at higher purine content. Unexpectedly, the sequences of the ON seem to influence the adsorption behaviour.

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6.12 Engineering of three dimensional mammalian retinal spheres by rotation cultures

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The aim of this study was the development of a reliable three-dimensional culture system of the mammalian retina that allows the analysis of retinal development and stratification as well as cellular differentiation processes *in vitro*. For the cultivation of retinal spheres, dissociated retinal cells of neonatal rats were maintained in culture dishes on a self-made orbital shaker. Based on well-defined rotation conditions, dissociated free floating cells re-aggregate in the centre of the culture dish to form multicellular cluster. Subsequently, cells begin to proliferate, whereby they form sphere-like retinal tissues with a diameter of 180-210 µm. The characterisation of mature retinal spheres by immunocytochemistry revealed the presence of ganglion cells, amacrine cells, Müller cells, bipolar cells, and rod photoreceptors which are arranged in different retina-like layers. Although a small number of cells undergo apoptosis, retinal spheres remain viable for at least 35 days in culture as shown by fluorescein diacetate and TUNEL staining. Since proliferation, differentiation, apoptosis, and survival are also detectable in retinal spheres this novel mammalian 3D culture system is well-suited to investigate aspects of retinogenesis *in vitro* under tissue-like conditions.

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6.13 Bioreactor technologies for culturing of skin substitutes

Thomas Rupf, Katrin Lorenz, Jürgen Salvetter, Augustinus Bader

Wound coverage of extensive burn injuries and other large deep skin defects represents a major problem in dermatology. The upper limit regarding surface area is set by the availability of autologous material to cover the site of injury provided by the patient. For this reason it is necessary to develop skin equivalents for transplantation consisting of dermal and epidermal components to support the regeneration of skin.

With the aim to produce skin equivalents under controlled conditions in perfusion bioreactors, the precultivation of keratinocytes on membraneous supports becomes an important technical requirement. On the other hand, to achieve bilayered skin substitutes, preformed keratinocyte sheets have to be combined in a second step with dermal equivalents.

Meaningful key parameters for quality assessment and process control are to be characterized in order to achieve standardized protocols. Special emphasis will be put on the role of the extracellular matrix (ECM) natively synthesized in the dermal equivalents for epidermal regeneration. To prove the concept of generation of functional dermal equivalents by fibroblasts in a three-dimensional configuration we made use of a fibrous biodegradable scaffold.

Fibroblasts were inoculated into the material and cultured under conditions optimized for ECM-production. When these constructs had accomplished a smooth coherent surface at the top, they were ready for the application of keratinocytes and organotypic cocultivation in perfusion bioreactors.

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6.14 Peptide mediated DNA import into mitochondria

Ingo Schäfer, Alexandra Kneissl, Christian Kukat,
Martina Seibel

Energy of eukaryotic cells is generated by the oxidative phosphorylation system located in the mitochondria. The compounds of this system are encoded either in the nuclear or the mitochondrial genome. Therefore genetic defects within the mitochondrial DNA can cause disorders in the cell's energy production and ultimately lead to neuromuscular dysfunctions. Mitochondrial DNA variations can range from single point mutations to deletions of large DNA fragments encoding for several genes.

Import of DNA from the cytoplasm into the mitochondrial matrix is an obligatory step for a site directed mutagenesis or gene therapy approach on mitochondrial DNA diseases. Up to now, no endogenous system mediating this transfer is known in mammalian cells. To close this gap we developed peptide conjugated DNA vectors that are capable of delivering nucleic acids to the mitochondrial matrix. The vector is made up of the mitochondrial signal peptide of the ornithine transcarbamylase (OTC, a nuclear encoded protein finally located in mitochondria) which is chemically cross linked to a nucleic acid component harboring the desired DNA molecule to be located in the mitochondrial matrix[1]. Due to the unique physical structure of the attached DNA, induction of regulated self-replication is emphasized upon reaching the final cellular compartment.

At the moment we focus on the composition of the DNA to achieve correct expression of the gene of interest (EGFP as marker) in the mitochondrial matrix.

Notes

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6.15 *In vitro* regeneration of liver cells

Christine Schulz, Jan-Michael Heinrich, Augustinus Bader

A special interest of regenerative medicine is the self renewing of the liver. The possibility of expanding hepatocytes *in vitro* would be an advantage for hepatocyte transplantations, liver support systems, drug screenings and especially for experimental use. Functional behaviors and growth activity of hepatocytes isolated from liver and cultured *in vitro* are incredible less in comparison to *in vivo*. The process of cell regeneration has to be divided into three phase: initiation, proliferation and termination, which are considerably controlled by different growth factors and cytokines[1,2].

This survey analyses the effect of several cytokines, growth factors and combinations on the regenerative potential of hepatocytes *in vitro*. A first the metabolic activity of cells was proved with ALAMAR-BlueTM to identify effective combinations. In addition we measured special hepatocyte behaviors i.e. albumin production rate, gluconeogenesis and cytochrome P 450 activity.

Main aim is to study intracellular signaling after treating hepatocytes with selected combinations of growth factors and cytokines. To get a global overview through changes in gene expression we used complete genome chips. After that we designed a custom made array containing approximate 500 genes which have significant roles in the regeneration process.

In summarize we can show differences in gene expression pattern after treatment with cytokines and growth factors.

Notes

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6.16 Solid-state NMR spectroscopy on bone implants

Jessica Schulz, Ina Khalaf, Mathias Pretzsch, Göran Zernia, Andrea Deiwick, Alexander Wild, Augustinus Bader, Daniel Huster

Solid-state NMR techniques are applied to quantitatively investigate the formation of organic and inorganic bone material in animals with osteoinductive bone substitutes. To this end, mesenchymal stem cells were seeded into porous β-tricalcium phosphate (β-TCP) cylinders and subsequently implanted into the femoral condyle of rabbits. The stem cells differentiate into osteoblasts and, while new bone material is produced by these cells, the β-TCP is resorbed by the animal's organism.

The formation of inorganic bone material was investigated by ^{31}P MAS NMR spectroscopy. The implants were removed from the rabbits after 2, 3, or 4 months and the formation of hydroxyapatite determined quantitatively. The ^{31}P NMR spectra are characterized by a superposition of β-TCP and calcium-hydroxyapatite, the major inorganic component of bone. From the peak integrals, the amounts of newly formed hydroxyapatite were determined.

The main organic component of bone, collagen type I, was investigated by ^{13}C solid-state NMR spectroscopy. ^{13}C CP MAS NMR spectra exhibit the characteristic signatures of collagen type I with good resolution for all major amino acids in collagen. Comparing the intensities of the collagen peaks with the peak intensity of a test substance, we have quantified the amount of collagen produced by the osteoblasts.

Information on the dynamics of collagen segments is obtained from motionally averaged ^{13}C-1H dipolar couplings. Thus, a quantitative comparison of the dynamical properties of collagen in bone and in the implants has been carried out.

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6.17 Novel bioreactor for simultaneous stimulation and cultivation of 3-dimensional cell constructs

Ronny Schulz, Andrea Deiwick, Nico Wüstneck, Christian Müller, Augustinus Bader

The novel bioreactor combine perfusion cultivation and mechanical stimulation of GMP-adequately produced three-dimensional, vital and mechanically resistant cartilage cell constructs in one bioreactor. The cultivated transplants can be used as substitute tissue material for the treatment of e.g. defects of the connecting and supporting tissue and degenerative articular illnesses. The main feature of the bioreactor is the transplant situated in a closed reactor area and multiply provided with *in vivo* adapted stimuli. One of these stimuli is the perfusion of the spatial cell construct with medium, which on the one hand leads to a production of organotypical shear forces at the cell membranes and on the other hand admits an increased metabolic exchange. In the closed bioreactor a piston, whose function is the application of force on the cell culture, is located above the transplant. This pressure plate is led through the bioreactor area and dynamic or static pressure stimulation is applied on the tissue transplant. Due to its degree of automation the apparatus minimizes the number of processes and thereby reduces the risk of infection for the cell culture. Furthermore, the automatic cultivation and stimulation of the transplants guarantee defined and reproducible processes. The parameters for bioreactor control include biochemical signals and the biomechanical parameters like compression, force and stiffness. Due to the constructional features of the bioreactor a closed circle is guaranteed and therefore a strictly autologous cultivation and stimulation of substitute tissue material adequate to the GMP guidelines is possible.

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6.18 Automation of the 3D *in vitro* pannus-model for rheumatoid arthritis with the CyBi-Disk workstation

Anja Skodda, Simone Fuchs, Jochen Ringe, Mehdi Yahyawi, Ingrid Schmid, Peter Götz, Michael Sittinger

The pannus-model for rheumatoid arthritis (RA) consists of a chondrocyte pellet culture interacting with RA synovial fibroblasts and was developed as a standardized screening test to analyse the effects of drugs and biological substances. Porcine chondrocytes were isolated from articular cartilage and cultured in media containing FBS. 3D cultivation was done in 96-well plates with $0{,}6x10^6$ chondrocytes per well. Automated media exchange was done with the liquid handling system CyBi®-Disk (CyBio AG Jena). A control software program has been generated with the aim of minimal shear forces for the pellet culture. After two weeks the chondrocyte pellets were covered with synovial fibroblasts to complete the pannus-model. The process was compared to manual handling and effects due to automation were analysed by scans, histochemistry and real-time PCR. Histochemistry comparison of both automated and manually operated pellets showed a formation of hyaline-like cartilage matrix. Components of the extracellular matrix like type II collagen were determined by immunohistochemistry, indicating a uniform distribution. Scans demonstrated a destruction of the manually operated pellet cultures, due to unsteady pipetting resulting in intense shear forces. Pellets operated by CyBi®-Disk were homogenous within the whole 96-well plate. Additionally a great advantage of the automation is the reduction in operation time per plate, 7 min 45 sec including tip washing compared to 19 min by manual process. Successful automation of the pannus-model allows for high throughput RA drug screening experiments. The transfer in human pellet cultures is in process.

Successful automation of the pannus-model allows for high throughput RA drug screening experiments. The transfer in human pellet cultures is in process.

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6.19 Aquaporin expression in the retina of the rat

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The development of retinal edema is a common complication of various ischemic and inflammatory ocular diseases. Fluid movements through the retinal tissue are facilitated by aquaporin (AQP) water channels expressed in plasma membranes of glial and neuronal cells. It is known that the retina of the rat express AQP4 and AQP1 water channel proteins while a possible expression of other members of the aquaporin protein family is unknown.

Transient retinal ischemia was induced in one eye of adult rats by elevating the intraocular pressure for 60 minutes. Total RNA was isolated and analyzed by RT-PCR. Retinal slices were immunostained against various aquaporin proteins.

The retina of the rat express mRNA for a diversity of aquaporins, including AQPs0,1,3,4,5,6,7,8,9 and 11. The mRNA for AQP2 remained under the detection level of the method used. AQP1 immunoreactivity is expressed by the outer retina and by single amacrine cells. AQP2 immunoreactivity was found at low level in horizontal cells. AQP4 immunoreactivity is strongly expressed in perivascular membranes and at the inner limiting membrane as well as in the plexiform layers. AQP5 immunoreactivity was found diffusely distributed above the whole retinal tissue. AQP9 immunoreactivity is expressed by single amacrine cells.

The retina of the rat express mRNAs for a diversity of different water channel proteins suggesting that the retinal water homeostasis is mediated by the cooperation of different aquaporins. The functional significance of the various aquaporin proteins for neuronal activity and in pathophysiological conditions remains to be determined.

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6.20 Effect of oxygen stress on the hepatocytes metabolic functions *in vitro*

Susanne Trettner, Jan-Michael Heinrich, Augustinus Bader

With the purpose recreate in a possible closer way the function of a Liver Support System (LSS), it is necessary to determinate some specific conditions of hepatocytes culture, as the adequate supply with oxygen.

According to the physiological oxygenation conditions (Liver blood flow represents a mixture of 75-80 % of oxygen depleted venous blood and which is only 20-25 % of fully oxygenated arterial blood[1], we decide to study the effect of oxygen stress, in hypo-, norm- and hyperoxygen conditions, in a system with mouse hepatocytes cultured *in vitro*, analyzing the changes in the metabolic activity of them.

For our study we cultured adult mouse hepatocytes on a flat membrane bioreactor designed and published recently by our group[2] under controlled oxygen conditions (2,5 %, 10 %, 20 %, 30 %) coated with collagen type I.

The effect of the different oxygen concentration was analyzed by measuring hepatic specific metabolic functions, for example proliferation rate, albumin synthesis rate, LDH- as well as cytochrome P450 activity.

In conclusion, we could show differences in metabolic activity, influenced by the availability of oxygen to the hepatocytes that are important in the design and control of future bioreactors.

Notes

1 Thews G., Anatomie, Physiologie, Pathophysiologie des Menschen, Stuttgart: Wiss. Verl.-Ges. (1999).

2 Langsch A., Bader A., Longterm stability of phase I and phase II enzymes of porcine liver cells in flat membrane bioreactors., Biotechnol. Bioeng. 2001 Sep;76(2):115-25.

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6.21 Behaviour of cells cultured on microstructured titanium surfaces

Hartwig Wolburg, Claus Burkhardt, Dieter Kern,
Jochen Seebach, Friederike Pfeiffer

Implantology is concerned with the cell-surface interaction. Fibroblasts seeded on microstructured epoxy resin surfaces which were sputtered with a ~50nm thick titanium layer, grow oriented according to the geometrical texture. EM analysis and immunogold labelling showed protrusions extending into the grooves and positive for vinculin. We combined the focused ion beam (FIB) technology for cutting and SEM for imaging in a CrossBeam instrument allowing site specific preparation of the internal interface at nm level. We observed tiny but numerous basal extensions of the cells where they contacted the Ti surface. This prompted us to ask for the expression of ERM-proteins (proteins connected to membrane formations such as microvilli or lamellipodia). As shown by CLSM, we observed an increased immunoreactivity against radixin on Ti versus non-Ti substrates. Then we tested the affinity of the cells on Ti versus non-Ti by means of shear-stress experiments using shear forces up to 100 dyn/cm^2. We found the attachment stronger on Ti than on non-Ti and the microstructured Ti not more effective than smooth Ti suggesting that Ti itself is more important for attachment strength than its surface geometry.

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6.22 Investigation of the dynamics of the macromolecules in articular and tissue engineered cartilage by NMR spectroscopy

Göran Zernia, Stephanie Höhle, Ronny Schulz, Matthias Zscharnack, Augustinus Bader, Jürgen Schiller, Klaus Arnold, Daniel Huster

To understand the viscoelastic properties of articular cartilage and for the development of tissue engineered cartilage, we have studied the physicochemical properties of the tissue using ^{13}C NMR methods. Cartilage is a complicated gel with extremely well adapted shock absorbing properties. The major macromolecular components of cartilage, glycosaminoglycans and collagen, can be studied individually by high resolution NMR and solid-state NMR methods, respectively. From relaxation time measurements, the motional parameters of the glycosaminoglycans are derived. These polysaccharides undergo almost isotropic motions and fast reorientations with large amplitudes. In contrast, the collagen component of cartilage tissue is rather rigid. Only small amplitude fluctuations on the nanosecond timescale are detected using solid-state NMR methods. Considering the high water content of cartilage and the almost isotropic mobility of the glycosaminoglycan molecules it is remarkable how little this affects the collagen dynamics. Therefore, the dynamics of cartilage macromolecules is broadly distributed from almost completely rigid to highly mobile, which lends cartilage its mechanical strength and shock absorbing properties. We have also applied the NMR technology to chondrocyte cell cultures and tissue engineered cartilage. In the cell culture, ^{13}C labeling of the extracellular matrix is carried out to increase the sensitivity of the experiments. Thus, NMR methods are proposed to carry out a quality control/quality assurance analysis of tissue engineered materials.

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7. NANOBIOTECHNOLOGY AND NANOELECTRONICS

POSTERS

7.1 Protrusion forces driving rapidly translocating cells

Claudia Brunner, Michael Gögler, Allen Ehrlicher, Bernd Kohlstrunk, Detlef Knebel, Josef A. Käs

Cell motility is a fundamental process of many phenomena in nature, such as immune response, wound healing, and metastasis. Mechanisms of force generation for cell migration have been described in various hypotheses1-5 requiring actin polymerization and/or molecular motors, but quantitative force measurements to date have focused on traction forces. Here we present a direct measurement of the forward force generated at the leading edge of the lamellipodium and at the cell body of a translocating fish keratocyte. To elucidate the sub-cellular force generation machinery, we additionally determined the forward force of locomoting lamellar fragments6, which lack their nuclei but remain motile. We positioned an elastic spring, the cantilever of a scanning force microscope (SFM), in front of a moving cell, which pushes this spring out of the way. The forward force was calculated using the detected vertical deflection of the cantilever in an "elastic wedge model", which considers cellular deformation. Our measurements of the propulsive forces, which are in the lower nN range and agree with expectations, will provide quantitative insight into how a polymeric network of active and passive molecular components act in concert as an active locomoting machine.

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7.2 Lipophilic nucleic acids as building blocks for highly functional membrane surfaces

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The functionalization of biological surfaces has attracted much attention in the field of nanobiotechnology. We have designed lipophilic nucleosides and oligonucleotides to combine the molecular recognition mechanism of nucleic acids and the self-assembly characteristics of lipids in planar membranes. The intention of this project is to develop versatile molecular compounds that can be used to specifically bind enzymes, probes, drugs, and so on to biological surfaces. To this end, we have synthesized molecular building blocks that consist of a single adanine or uracil nucleobase attached to ribose or desoxyribose. This hydrophobic headgroup is covalently bound to various lipophilic groups such as alkynyl chains and sterodial substituents to provide membrane anchoring. Alternatively, we have synthesized lipophilic oligonucleotides that consist of a 25-mer single stranded DNA with two covalently attached hydrocarbon chains. The membrane insertion and localization of these molecules was investigated by solid-state NMR spectroscopy. All molecules can be incorporated into phospholipid membranes at high concentration without destroying the bilayer structure or the formation of non-lamellar phases as shown by ^{31}P NMR and ^{2}H NMR of deuterated phospholipids. The hydrophobic headgroup of the lipophilic nucleosides and oligonucleotides was found to be localized in the lipid water interface investigated by ^{1}H nuclear Overhauser enhancement spectroscopy under magic angle spinning. Therefore, these molecules may be useful building blocks to obtain functionalized membrane surfaces.

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7.3 Binding specificity of peptides on semiconductor surfaces

Karsten Goede, Michael Bachmann, Wolfhard Janke, Marius Grundmann

Hybrid systems of anorganic semiconductors and small bio-molecules constitute on the one hand an easily accessible model system for studying the fundamentally important process of molecular self-assembly. On the other hand they provide us with a multitude of possible future applications which might range from biological sensoring to nano-bio electronics. We have studied the adhesion coefficient of peptide clusters with different amino-acid sequences on various classic semiconductor materials. The adhesion crucially depends both on the electronegativity of the involved surface atoms and their spatial arrangement and on the kind and succession of side chains in the amino acids of the peptide[1]. Low adhesion comes along with the appearance of eminently large and soft clusters. Starting from these empirical findings we present first simulations of relevant peptide convolutions and phase transitions and discuss the way towards a theoretical modelling of peptide adhesion on lattice surfaces.

Notes

1 Goede K., Busch P., Grundmann, M., Nano Lett. 4(11):2115-2120 (2004).

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7.4 Nano- and microstructured surfaces for selective and directed positioning of cells on electronic chips for laser-based microdissection and manipulation

Heinz-Georg Jahnke, Andrée Rothermel, Randy Kurz, Markus Rüffer, Winnie Weigel, Maik Schmidt, Andrea A. Robitzki

Positioning of single cells on well-defined surfaces and their directed growth is a precondition in modern biotechnology, nanotechnology and cell biology. For the reliable application of living cells on biochips and biosensors it is necessary to develop specific attracting or repelling surface topographies. Especially in neurobiology it is a great advantage if neuronal cells can be positioned in a defined neural network. Thereby, the influence of cell-cell and cell-matrix contacts or soluble factors on axon guidance or cellular growth in general can be investigated. For this purpose technical glasses or silicon-based chips were laminated with different silanes to screen their repulsive properties. Subsequently, the desired adhesive surface topography was generated easily by ablation of the silane coating via laser processing techniques. In our studies we investigated the growth of dendrites and the interactions between oligodendrocytes and nerve cells as well as the influence of chemical attractant or repellent factors like netrin. Netrin-1 is a prevalent laminin-like protein that showed both chemoattractant and repellent properties on neuronal cells. To analyse axonal growth OLN93 cells were cultured and transfected on nano- and microstructured glass chips with either sense or antisense netrin DNA constructs. Also first experiments with a lasermanipulation system were carried out because laserlight can induce and guide the growth direction of neuronal growth cones. These experiments are the basis for developing a electronic biosensor coupled with a laser-micromanipulation system.

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7.5 Chemosensitization of bladder cancer cells by VEGF antisense pre-treatment

Sabine Krause, Yvonne Förster, Kai Krämer, Susanne Füssel, Matthias Kotzsch, Uta Schmidt, Manfred P. Wirth, Axel Meye, Bernd Schwenzer

Due to the unsatisfactory success in treatment of bladder cancer and the low response rates to commonly used chemotherapeutics (CT) alternative and additive approaches have to be found. The function of vascular endothelial growth factor (VEGF) in neoangiogenesis and therefore in solid tumors makes it a promising target for a specific anti-tumor therapy. This study investigated the possibility to sensitize transitional bladder cancer cell lines (TCC) to CT by pre-treatment with VEGF antisense oligodeoxynucleotides (AS-ODNs).

The human bladder cancer cell lines EJ28 and 5637 were transiently transfected with three different anti-VEGF AS-ODNs followed by incubation with three different doses of the CT mitomycin C (MMC), gemcitabine (GEM) or cisplatin (CDDP). Both cell lines responded in a dose dependent manner to all CT. Combined treatment with VEGF-AS-ODNs and CT resulted in a reduced viability (WST-1 assay) compared to the isolated CT treatment. VEGF857 plus CT treatment could significantly reduce viability of both cell lines compared with NS-ODN plus CT treatment for all three CT ($p<0.007$). This detected chemosensitization was based on AS-mediated increase of apoptosis (annexin V staining).

One of the three AS-ODNs tested (VEGF857) significantly sensitizes human TCC cells to CT. We suggest VEGF as an additional putative target for an enhancement of therapeutical benefit for example for MMC and GEM instillation treatment schedules.

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7.6 Proton microscopy – Quantitative trace element analysis with sub-micron spatial resolution

Tilo Reinert, Daniel Spemann, Markus Morawski, Thomas Arendt, Tilman Butz

In recent years many nuclear microprobes around the world have developed to sophisticated tools for quantitative elemental analysis with high resolutions down to one micron. The principle is similar to an electron probe microanalysis. A focussed proton beam is scanned over the sample and the characteristic X-rays are recorded (microPIXE). The main advantage is the very low background and the precise knowledge of the principles of X-ray production and detection. Therefore, nuclear microprobes are widely used in trace element analysis mainly in the field of biological and medical research. Numerous successful studies on microscopic scale structures, e.g. cells, lead to the demand for higher spatial resolution or lower detection limits. Therefore, several labs started new efforts for sub-micron resolutions.

The Leipzig microprobe laboratory LIPSION, experienced in analysing brain tissue on the cellular level, has recently improved its analytical capabilities. We are now able to perform quantitative trace element analysis with sub-micron spatial resolution and μg/g (<100 μmol/l) detection limits for most of the physiological important elements. The smallest beam diameter with analytical capabilities for elemental analysis, we achieved thus far, was about 300 nm in diameter. It enables an outstanding microPIXE resolution. This contribution shows the applicability to biological samples. We perform sub-micron trace elemental analysis on neuromelanin, an intracellular protein agglomeration with high affinity to iron.

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7.7 Investigation of DNA binding proteins from the hyperthermophile *Sulfolobus acidocaldarius*

Mathias Salomo, Kati Kegler, Marc Struhalla, Jörg Reinmuth, Wiktor Skokov, Friedrich Kremer

The proteins Sac7d and Sac7e from the hyperthermophile archeon *Sulfolobus acidocaldari us* are reported to bind strongly to the minor groove of DNA and to introduce a sharp kink leading to a shortening of the DNA. Both proteins are very stable against heat, acid and chemical agents.

Our projects have the aim to investigate these proteins with optical tweezers, X-ray crystal structure analysis and evolutionary protein design.

With the help of optical tweezers we were able to establish a setup that allows to mechanically stretch and relax single DNA molecules with lengths between 1,000 bp and 6,000 bp that are immobilized between two microparticles. We can use this system to investigate changes in the elastic properties of DNA accompanying protein binding.

In the literature Sac7d is reported to bind DNA with lower affinity than Sac7e. We have crystallized Sac7e in order to solve its X-ray structure to compare it with the already known structure of Sac7d. This shall give rise to differences in the structure explaining the higher affinity of Sac7e to DNA.

Some other closely related proteins, like Sso7d isolated out of *Sulfolobus solfataricus*, own the ability to cut nucleic acids. By using evolutionary protein design we want to change the DNA binding protein Sac7d into a DNA cleaving enzyme. Therefore we randomize defined areas of Sac7d as well as the whole protein sequence generating large enzyme libraries which we will screen for the desired enzymatic activity.

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7.8 Investigation of the elastic properties of single DNA molecules

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Optical tweezers are commonly used to manipulate microscopic particles, with applications in cell manipulation, colloid research, manipulation of micromachines and studies of the properties of light beams. With their extraordinary resolution in space (~2 nm) and force (~1 pN) they became a irreplaceable tool for such purposes.

In our project we want to use them to study the elastic properties of single DNA molecules. Therefore we developed an easy and reproducible procedure for the immobilization of single double stranded DNA molecules 1,000 to 6,000 bp in length obtained by PCR between two micro particles supplying a general approach to address this problem.

A fully reversible elastic behaviour is found for the force-extension dependence of ds-DNA for relative elongations up to 50 % as described in the corresponding literature.

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7.9 Release of thioflavins from nanoparticles targeting fibrillar β-amyloid in transgenic mice

Thomas Siegemund, Bernd-R. Paulke, Herbert Schmiedel, Jens Grosche, Anke Hoffmann, Tibor Harkany, Heikki Tanila, Johannes Kacza, Wolfgang Härtig

Alzheimer's disease and age-dependent β-amyloidoses in transgenic mice are characterized by the occurrence of fibrillar amyloid (Aβ). β-sheet structures are selectively bound by fluorescent thioflavins and styrylbenzenes both in fixed tissues and after intravenous (iv.) infusion. In contrast, hydrophilic compounds such as thioflavin-T (ThT) are unable to pass the blood-brain-barrier. The modelling of brain functions with drugs only after their encapsulation with polymers suggests the targeting of Aβ using degradable, thioflavin-filled nanoparticles.

This study is based on different types of nanoparticles containing ThT or thioflavin-S. Core-shell particles were prepared by the polymerization of butyl-cyanoacrylate onto polystyrene cores containing either thioflavine or rhodamine. Purified nanoparticles had diameters between 40 and 150 nm as revealed by light scattering. The bioerosion of nanoparticles *in vivo* was mimicked by their enzymatic degradation with non-specific esterase *in vitro*. Next, aged double transgenic APP/PS1 mice displaying a high Aβ load received intrahippocampal or iv. injections of thioflavin-containing nanoparticles. Three hours or three days after injection, animals were perfused and sectioned. Thioflavin-staining of senile plaques was shown by Aβ-immunofluorescence labelling and subsequent confocal laser-scanning microscopy. For electron microscopic analyses of ThT released from nanoparticles, the fluorescent model drug was photoconverted into an electron-dense adduct and mainly found in plaques, but also in neurons and microglia. Our data suggest that drugs delivered by nanoparticles can target Aβ.

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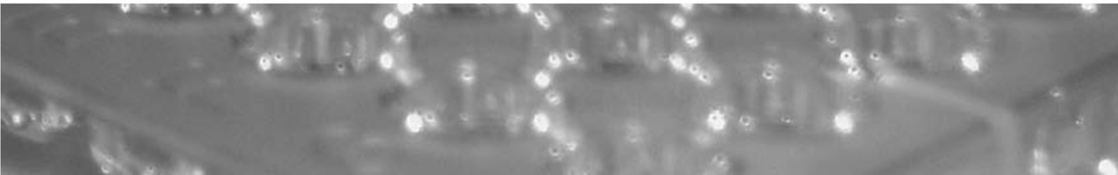
8. BIOINFORMATICS



POSTERS

8.1 Multiple Genome Rearrangement with conserved intervals

Matthias Bernt, Daniel Merkle, Martin Middendorf

The Multiple Genome Rearrangement problem is to find for species that are represented by gene orders a phylogenetic tree. The phylogenetic tree should describe the most "plausible" rearrangement scenario for the gene orders with respect to some distance measure. A gene order is described by a signed permutation over a set of integers where each integer denotes a gene and the sign denotes its orientation. The rearrangement operations that are considered in our work are reversals, which reverse the order and the sign of a subsequence of neighbored genes. We have studied the special case of three given gene orders, called Median problem.

In contrast to other studies of the Median problem we consider the additional constraint that possible gene groups should not be destroyed. The concept of conserved intervals for signed permutations is employed to describe such gene groups. Our algorithm has some similarities to the well known MGR algorithm but uses a different selection process for the applied reversals. It has been shown experimentally for different types of test problems that our algorithm produces better results than MGR for the standard Median problem and has the additional property that it tries not to destroy conserved intervals. The reversal selection procedure has also been integrated into the MGR and GRAPPA algorithms. This new versions achieve a significant speedup while obtaining solutions of the same quality on the test problems.

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8.2 Bio-Imaging platform LabImaging – A tool to develop software for biological, pharmaceutical and medical research and clinical diagnostics

Olaf Brenn

Kapelan Bio-Imaging Solutions is an ambitious German company founded in the year 2000. We are developing customer specific software for biological, pharmaceutical and medical research as well as clinical diagnostics based on our own bio-imaging platform. We are mainly focused on laboratory automation and image analysis.

Supported by our own bio-imaging platform we are able to create a wide range of applications within a short development process - from prototyping up to ready-to-use applications. Our applications provide faster time to market.

The platform provides a functional library with image analysing algorithms, e.g. separation and classification of objects, which can be combined to an analysis chain. Furthermore the bio-imaging platform features a complete graphically designed interface, online and offline updates, license management and workflow orientation.

Our know-how was already implemented in a software called LabImage for analysing 1D electrophoresis gel. An earlier version of this software has been worldwide successfully distributed by Kapelan for the last four years. Also we provide OEM development.

Further applications based on the bio-imaging platform are being developed for micro blot and micro array analysis as well as the analysis of cancer cells.

The bio-imaging platform is based on Eclipse. Eclipse is a Java-based open source universal tool platform, which is pushed and influenced by IBM.

We cooperate with research and university partners.

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8.3 Identifying targets of flavonoids by virtual docking studies

Robert Günther, Rolf Gebhardt, Hans-Jörg Hofmann

In the quest for new therapeutics, virtual screening methods have become a useful alternative to the expensive high-throughput screening (HTS) methods. Both methods have in common the testing of a great number of small compounds for interaction with one particular target to identify a lead structure. Thus, large compound libraries are needed. However, in academia and small companies often only a limited number of compounds is available, typically synthesized with special know-how. Thus, it might be of interest to identify targets interacting with these compounds.

One ansatz is the application of reverse docking methods. In contrast to common virtual screening methods, a panel of target proteins (or other macromolecules, whose 3D-structure is available) is screened for interaction with a limited number of small compounds in this approach. Beside the identification of a potential target, it is of value to know at an early stage of drug development, whether the projected therapeutic also interacts with other proteins possibly leading to undesired side effects. Furthermore, a reverse docking approach might help to shed some light on the function of hypothetical proteins.

Here, we present a preliminary reverse docking study of the screening for interaction partners of flavonoids. Flavonoids represent a vast number of compounds in higher plants. Thus, these ubiquitous plant constituents enter the food chain and might interact with various physiologically and possibly pathologically relevant proteins inside the human body.

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8.4 On the potential role of biomechanics in the growth of tumors *in vitro*

Stefan Höhme, Dirk Drasdo

Recently we have shown by computer simulations with a biophysical model that the growth kinetics of tumor spheroids and of expanding monolayers can largely be explained by a biomechanical form of contact inhibition in which growth stop can be triggered by the deformation of the cell.

Within this model each cell is represented as an individual object and parameterized by cell-biophysical and cell-kinetic parameters that can be experimentally determined.

Now we explore the consequences of this mechanism.

(1) We predict the growth kinetics in monolayers depends significantly on whether a growth stop is triggered either by a critical degree of cell deformation, or by a critical force exerted on the cell.

(2) We show, that the expansion of a tumor spheroid can largely be explained by a biomechanical form of contact inhibition while glucose (or oxygen) mainly determines the size of the necrotic core.

(3) We illustrate, that the relation between tumor diameter and tumor cell population size, and the cell size distribution in tumor spheroids is largely determined by the mechanism that controls the cell growth and propose mechanisms that are compatible with experimental observations.

(4) Based on our computations we propose a phenomenological growth law that we show covers the growth behavior of both, compact monolayers and multi-cellular spheroids.

Finally we give examples for properties of the cell environment that may significantly modify the growth kinetics of multi-cellular aggregates.

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8.5 Progressive multiple sequence alignments from triplets

Matthias Kruspe, Peter F. Stadler

The quality of progressive sequence alignments strongly depends on the performance of the pairwise alignment steps which are necessary to obtain the final alignment. The correct identification of insertions or deletions that occurred during the evolutionary history of the taxa considered is crucial. This becomes fundamental particularly for rather divergent nucleotide sequences where the information content within the sequences is scarce. The pairwise examination of the sequences only is in many cases not satisfactory.

We want to present a novel multiple alignment tool which simultaneously aligns three sequences in every step and so increases the information transfer to the alignment. This extra information is used to reliably derive the underlying phylogeny of the sequences under consideration. We use a natural gap cost model which deals with all different variations of gap openings or extensions for three sequences. Gap penalties are adjusted to global and local sequence properties. The phylogenetic history of the sequences is represented by a network which also determines the order of the three-way alignments. To speed up computation time and limit memory requirements a divide-and-conquer approach is used. We find the obtained alignments more reliable compared to alignments built with other alignment tools such as ClustalW. Because of the lesser information content compared to amino acid sequences, the benefit of our tool applies especially to RNA sequences. Furthermore we figure out that the natural gap costs model has a benefit compared to the quasi-natural gaps costs used in other algorithms.

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8.6 Non-coding RNAs in Ciona intestinalis

Kristin Missal, Dominic Rose, Peter F. Stadler

Motivation: The analysis of animal genomes showed that only a minute part of their DNA codes for proteins. Recent experimental results agree, however, that a large fraction of these genomes is transcribed and hence is probably functional at the RNA level. A computational survey of vertebrate genomes has predicted thousands of previously unknown ncRNAs with evolutionary conserved secondary structures. An extension of these comparative studies beyond vertebrates is difficult, however, since most ncRNAs evolve relatively fast at the sequence level while conserving their characteristic secondary structures.

Results: Here we report on a computational screen of structured ncRNAs in the urochordate lineage based on a comparison of the genomic data from Ciona intestinalis, Ciona savignyi, and Oikopleura dioica. We predict more than 1,000 ncRNAs with an evolutionarily conserved RNA secondary structure. Of these, about a quarter is located in introns of known protein coding sequences. Only a small fraction of the RNA motifs can be identified as known RNAs, including about 300 tRNAs, some 100 snRNA genes, and a few microRNAs and snoRNAs.

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8.7 From phylogenetic footprinting to the evolution of regulatory elements

Sonja J. Prohaska, Claudia Fried, Burkhard Morgenstern, Gunter P. Wagner, Peter F. Stadler

Regulatory elements are often conserved in non-coding DNA. A method to detect them by comparative genomics is called phylogenetic footprinting. We have developed our own method, the tracker approach (Prohaska et al.) which can handle a large set (a few tens) of genomic sequence fragments (of some hundred kb) and does not rely on a pre-supposed phylogeny among the input sequences.

Common footprinting programs based on alignments just yield high scoring alignments of conserved fragments that are in the same order and orientation. Since regulatory elements are rather insensitive to shuffling of single modules, we account for this fact in two ways. First, our method returns not only one set of consistent footprint clusters but all significant clusters, consistent and inconsistent. Second, we look for rearranged footprint clusters based on a decomposition of the detected footprint clusters into single footprints. Therefore, we dissect the clusters of conserved DNA into individual putative binding sites. Starting from this set of motifs we then look for the maximal set of footprints within a cluster of defined length that occurs in all sequences from syntenic regions independent from the order and orientation of the motifs.

The list of conserved footprint clusters or the conserved non-coding nucleotides can be passed on to statistical analyses on footprint loss and acquisition. We propose a method to test for rate differences in the modification of conserved non-coding nucleotides, as well as a method to estimate their rate of modification.

Reference

Prohaska S.J. et al., Mol. Evol. Phylog. 31:581-604 (2004).

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8.8 Functional adaptations of dendrites of pyramidal neurons in p21H-rasVal12 transgenic mice

Andreas Schierwagen, Alexander Schubert, Alán Alpár, Ulrich Gärtner, Thomas Arendt

If compared with the wildtype, in p21H-RasVal12 transgenic mice cerebral cortex volume is increased by about 20 %, due to enlarged pyramidal cells[1]. Pyramidal neurons in layer II/III show a significant growth and establish a more complex dendritic tree[2].

In this study we used compartmental modeling to explore the design principles and functional consequences of the enlarged transgenic mice pyramids. Two sets of pyramidal neurons (28 wildtype and 26 transgenic neurons) were reconstructed and digitized using NeurolucidaTM.

Using the simulation package NEURON, we calculated the morphoelectrotonic transform (MET) of each neuron. The MET maps the neuron from anatomical space into electrotonic space, using the logarithm of voltage attenuation as the distance metric. Since attenuation depends on signal frequency and propagation direction, we calculated METs at several different frequecies and for somatopetal and somatofugal inputs, respectively.

The statistical analysis of the sample METs computed with NEURON revealed global conformity, i.e. the electrotonic architecture of the transgenic neurons scales in such a way that signal propagation in the neuron models is scarcely affected.

In conclusion, our results suggest only a minor impact of p21Ras on dendritic electroanatomy, with negligible changes of the inner part of the corresponding neuromorphic transforms.

In a next step of analysis, active membrane currents will be added to investigate further the scaling problem in these neurons.

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Notes

1 Heumann et al., J. Cell. Biol. 151:1537 (2000).

2 Alpár A. et al., J. Comp. Neurol. 467:119 (2003).

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8.9 Fuzzy dynamic labeling in learning vector quantization for automatic classification of mass spectrometric data in clinical research

Frank-Michael Schleif, Barbara Hammer, Jens Decker, Ute Clauss, Thomas Villmann

Proteomic profiling based on mass spectrometry is an important tool for studying cancer at the protein and peptide level.

The underlying classification algorithms are one crucial point to obtain valid and reliable results. We propose a new prototype based approach for dynamic fuzzy labeling in learning vector quantization. Thereby we allow dynamically adapted fuzzy labels to indicate the responsibility of prototypes for a class. The new learning algorithm performs a gradient descent on a cost function adapted from Soft Nearest Prototype Classification (SNPC)[1].

Thereby, to each prototype a fuzzy class information is assigned indicating the probabilities for class responsibility.

We compare this Fuzzy Dynamic Labeling SNPC with a special variant of a Genetic Algorithm (GA). It incorporates k-Nearest Neighbor- and Centroid-Clustering into an advanced GA-scheme. The different paradigms are described and their performances on data taken from a clinical prostate cancer study is demonstrated.

We show that this new method can be successfully applied to the analysis of proteomic data and used for bio marker research.

Notes

1 Seo S., Obermayer K., Soft learning vector quantization. Neural Comp. 15:1580-1604 (2003).

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8.10 Multiple alignments of partially coding sequences

Roman R. Stocsits, Ivo L. Hofacker, Claudia Fried,
Peter F. Stadler

High quality sequence alignments of RNA and DNA sequences are an important prerequisite for the comparative analysis of genomic sequence data. Nucleic acid sequences, however, exhibit a much larger sequence heterogeneity compared to their encoded protein sequences due to the redundancy of the genetic code. It is desirable, therefore, to make use of the amino acid sequence when aligning coding nucleic acid sequences. In many cases, only a part of the sequence of interest is translated. On the other hand, overlapping reading frames may encode multiple alternative proteins, possibly with intermittent non-coding parts. Examples are, in particular, RNA virus genomes.

Therefore, the standard scoring scheme for nucleic acid alignments can be extended to incorporate simultaneously information on translation products in one or more reading frames. In our contribution, we present a multiple alignment tool, codaln, that implements a combined nucleic acid plus amino acid scoring model for pairwise and progressive multiple alignments that allows arbitrary weighting for almost all scoring parameters. Resource requirements of codaln are comparable with those of standard tools such as ClustalW.

We demonstrated the applicability of codaln to various biologically relevant types of sequences (bacteriophage Levivirus and Vertebrate Hox clusters) and showed that the combination of nucleic acid and amino acid sequence information leads to improved alignments. These, in turn, increase the performance of analysis tools that depend strictly on good input alignments such as methods for detecting conserved RNA secondary structure elements.

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8.11 Coarse-grained polymer models – On-Lattice vs. Off-Lattice

Thomas Vogel, Michael Bachmann, Wolfhard Janke

We investigated coarse-grained polymer models such as the HP on-lattice and the AB off-lattice formulations for heteropolymers ("proteins"). The poster will focus on the results of two problems.

Firstly, we will give results of investigations concerning designing sequences in the HP model on lattices. To this end we perform exact enumerations of the whole sequence-conformation space of HP proteins on the fcc lattice up to a certain chain length and compare with results from the simple cubic lattice.

Secondly we try to show how "far" lattice models are from "reality", or from similar off-lattice models, respectively. Therefore we simulate off-lattice AB model-like proteins with different potentials and compare putative ground states with those from on-lattice simulations.

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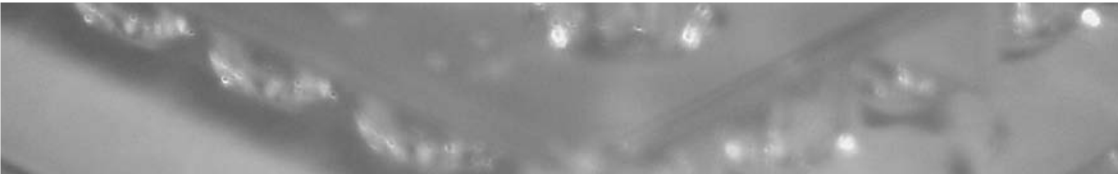

Center for Biotechnology and Biomedicine (BBZ) – An overview

In February 2003 the Center for Biotechnology and Biomedicine (BBZ) was established at the University of Leipzig. That paved the way for the BBZ to fullfill tasks set by the BBZ institutionally; which means to promote R&D in biotechnology, biomedicine and related disciplines, to initiate new degree courses and new types of in-service and further training and to facilitate the transfer of findings into economic activities.

With the framework program "The Saxon Biotechnology Offensive" in 2000 Saxony was given the pre-requisites to create such a center. The funding was granted for a period of five years (2001-2005) and was realized by the University Scientific Program and the European Regional Development Fund. The BBZ operates under the roof of BIO CITY LEIPZIG, where the city and the university combine scientific and economic activities in the fields of Biotechnology and Biomedicine and where synergies are set free for the progress of the location for science and economy in Leipzig.

The aim of the BBZ is to create an inventive atmosphere for science and development as well as for education, science and continuing education together with external research institutions, which consequently results in a transregional interest in this field by biotechnological and biomedical companies.

The concept of biotechnological biomedical focus in Leipzig is primarily being implemented by the faculty of biosciences, pharmacy and psychology, the faculty of chemistry and mineralogy, the faculty of medicine, the faculty of veterinary medicine, the faculty of mathematics and computer science as well as by the faculty of physics and earth science. These research fields all have become internationally renowned and competitive centers of research and teaching in molecular biotechnology and biomedicine in recent years.

The BBZ is an internationally competitive center which promotes research and development as well as teaching and advanced training in the areas Protein Engineering and Bioanalytics, Molecular Medicine and Therapeutics as well as Biomedical and Cell Engineering. Platform technologies and methods in biotechnology, biomedicine and nanotechnology are offered as services for academic institutions and industry. Here, the BBZ strengthens and supports collaborations between institutional and university research teams but also forms a unique interface between academia and industry in Europe. With expertise and competence, innovations are transferred to industrial utilization.

With a focus on red biotechnology and following the motive "From the molecule to the patient" the BBZ developes and offers chemical compounds, proteins and other biomolecules, cells as well as tissues as instruments and products for a wide variety of biotechnological and biomedical applications. To this end, novel analytical methods and preparative procedures are developed.

The center collaborates closely with the University Hospital and the Interdisciplinary Center for Bioinformatics (IZBI) at the University of Leipzig. A central office for management and acquisition supports the networking between science and business. In the BIO CITY LEIPZIG six academic research groups from the chemical, biological and medical sciences work under one roof with innovative biotechnological companies including many startups. In addition, 32 life science research groups in the Leipzig area are currently members of the BBZ.

The following heads of research groups are members of the Center for Biotechnology and Biomedicine:

From the Faculty of Biosciences, Pharmacy and Psychology:
- Prof. Annette Beck-Sickinger, Biochemistry / Bioorganic Chemistry
- Dr. Susanne Brakmann, Applied Molecular Evolution Research, Junior Research Group of BBZ (managing committee)
- Prof. Kurt Eger, Pharmaceutical Chemistry
- Prof. Sunna Hauschildt, Immunbiology
- Prof. Karen Nieber, Pharmacology for Natural Scientists (managing committee)
- Prof. Andrea Robitzki, Molecular Biological-Biochemical Process Technology (Chair)
- Prof. Martin Schlegel, Molecular Evolution and Animal Systematics with focus on Molecular Phylogeny (advisory)
- Dr. Thomas Greiner-Stöffele, Protein Engineering, Junior Research Group of BBZ
- Prof. Christian Wilhelm, Plant Physiology

From the Faculty of Chemistry and Mineralogy:
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- Prof. Athanassios Giannis, Organic Chemistry / Natural Products Chemistry
- Prof. Evamarie Hey-Hawkins, Inorganic Chemistry
- Prof. Ralf Hoffmann, Bioanalytics, Professorship of BBZ (managing committee)
- Prof. Helmut Papp, Technical Chemistry / Heterogeneous Catalysis
- Prof. Norbert Sträter, Structural Analysis of Biopolymers, Professorship on BBZ (managing committee)
- Dr. Andrea Sinz, Protein-Ligand Interaction by Ion Cyclotron Resonance Mass Spectrometry, Junior Research Group of BBZ

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- Prof. Klaus Arnold, Medical Physics and Biophysics
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- Prof. Gottfried Alber, Immunology
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- Prof. Josef Alfons Käs, Experimental Physics / Soft Matter Physics with focus on Cellbiophysics

The Center for Biotechnology and Biomedicine is interested in further research divisions dealing with molecular Biotechnology and Biomedicine. Please contact the Chief Executive Officer for further information. Applications can be filed to the Chair of the Center.

Prof. Dr. Andrea Robitzki (Chair)
Dr. Svenne Eichler (Chief Executive Officer)

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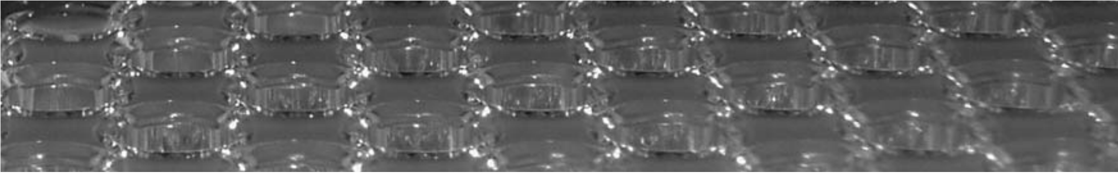

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