Jagiellonian University Faculty of Biotechnology

XXXII Winter School of Faculty of Biotechnology

From Molecular Biology to Biotechnology

Zakopane,
March 3rd – 7th 2005

Organized by:
Faculty of Biotechnology, Jagiellonian University
Heme Oxygenase FanClub

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The XXXIII Winter School continues the tradition of the meetings of scientists and students in a relaxing atmosphere and beautiful surroundings of Tatra Mountains. This time we reconvene to discuss some novel aspects of the enormous progress in molecular biology and its applications in biotechnology.

"From molecular biology to biotechnology". The title of the present schoold reflects also our history, exemplified by the various names our institute possessed, as well as the current transition in our interests. Also because of the structure of our Faculty, the subject of our school is quite diverse. The other purpose of that variability is our willingness to provide the opportunity for all participants to learn some interesting facts during the few days. To better address those mentioned diversities we have invited several prominent scientists of various fields, both from Poland and abroad, who will present the current progress in several fields of molecular biology. We hope all this will create the atmosphere for an exchange of opinions, extensions or initiations of new collaborations, all of which will lead to the development of science at our faculty for the benefit of scientists, our present and future students.

Besides continuing the tradition the school is aimed also to introduce novel aspect for our discussions, which have not been yet so broadly represented during winter meetings. Therefore, the significant time will be devoted to the gene and cell therapy. Some scientific preferences of the organizers are also reflected in the programme. Additionally, we also want to discuss the reasons for so diverse perception of biotechnology by the society and to extend our knowledge on this subject which is so widely misinterpreted.

We are happy that so many colleagues from different departments and laboratories of our Faculty are taking part at the meeting. We welcome warmly all our guests from other Polish institutions and we do hope that our school will fulfill their expectations. We believe that lively atmosphere during the workshops will be also present at the ski-slopes, which we hope to be full of snow for yours and our fun!

Thank you for coming!

Heme Oxygenase FanClub
The Organizers

Venue

Dom Wypoczynkowy „Rzemieślnik”
ul. Makuszyńskiego 12
34-500 Zakopane
Poland
Phone: +48 18 20 66 416
http://www.rzemieslnik.zakopane.pl/

Time

From Thursday, March 3rd 2005
to Monday, March 7th 2005

Hotels

Kraków – invited speakers
Bursa im. St. Pigonia
31-131 Kraków, ul. Garbarska 7a
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Zakopane
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Language

English

Organization Committee

Józef Dulak
Alicja Józkowicz
Justyna Leja
Paulina Kucharzewska
Andrzej Rutkowski

PROGRAM OF THE SCHOOL

Thursday, March 3rd 2005

09:00	Invited speakers: minibus departure to III University Campus from hotel parking (Garbarska str. 7a)	
10:00	Bus departure to Zakopane	
13:00-14:00	Lunch	
14:15-14:30	**OPENING OF THE SCHOOL**	
14:30-16:15	***Session I***	***SIGNAL TRANSDUCTION AND TUMOR TARGETING***
	Chairpersons:	*Claudine Kieda and Bożena Kamińska*
14:30-15:00	**Bozena Kaminska**	TGFβ signalling and its role in tumor pathogenesis
15:00-15:30	**Grietje Molema**	Design of vascular endothelium specific drug targeting strategies for the treatment of cancer
15:30-15:45	**Aneta Kasza**	RhoA and MAPK pathways converge to regulate *SRF* gene expression
15:45-16:15	Discussion	
16:15-16:45	Coffee break and poster hang up	
16:45-18:45	***Session II***	***GENE EXPRESSION, GENE FUNCTIONS AND GENE POLYMORPHISM***
	Chairpersons:	*Ewa Bartnik and Artur Jarmołowski*
16:45-17:15	**Artur Jarmołowski**	New functions of the plant nuclear cap-binding protein complex
17:15-17:45	**Alicja Ziemienowicz**	Involvement of DNA ligase in *Agrobacterium*-mediated plant transformation
17:45-18:00	**Wojciech Strzałka**	Changes in PCNA gene expression during *Phaseolus vulgaris* growth and development
18:00-18:30	**Maria Kapiszewska**	The influence of genetic polymorphisms, nutrients and hormonal status on the DNA damage. The *in vivo* and *ex vivo* studies
18:30-18:45	Discussion	
19:00-20:00	Dinner and get-together party	
20:00	Discotheque, bar will be open	

Friday, March 4th 2005

08:00-09:00	Breakfast	
09:00-11:00	***Session III***	***GENE EXPRESSION AND THERAPY***
	Chairpersons:	*Grietje Molema and Maria Czyżyk-Krzeska*
09:00-09:30	**Maria Czyżyk-Krzeska**	Oxidative stress induces VHL- and prolyl hydroxylation-dependent ubiquitylation of Rpb1
09:30-10:00	**Claudine Kieda**	Glycobiotechnologies for cell therapy
10:00-10:15	**Agnieszka Łoboda**	Effect of atorvastatin on angiogenic gene expression in normoxic and hypoxic microvascular endothelial cells
10:15-10:30	**Paulina Węgrzyn**	Transcripts profile in HepG2 cells stimulated with proinflammatory cytokines
10:30-11:00	Coffee break and posters viewing	
11:00-13:00	***Session IV***	***MOLECULAR MODELING***
	Chairpersons	*Marta Pasenkiewicz-Gierula and Cezary Czaplewski*
11:00-11:30	**Bogdan Lesyng**	Overview of selected mesoscopic biomolecular modelling models and theories
11:30-12:00	**Cezary Czaplewski**	Multibody effects in hydrophobic interactions
12:00-12:30	**Jerzy Ciarkowski**	A hypothesis for GPCR activation
12:30-13:00	**Joanna Trylska**	Exploring global motions and electrostatic properties of the ribosome
13:00-14:00	Lunch	
14:00-14:30	Posters viewing and sponsor's announcement:	
	Renata Czachór (Merck, Poland)	Merck Biosciences – products and reagents for molecular biology

14:30-16:30	***Session V***	***HEME OXYGENASE IN HEALTH AND DISEASE***
	Chairpersons	*Anupam Agarwal and Józef Dulak*
14:30-15:00	**Anupam Agarwal**	Regulation of the human heme oxygenase-1 gene in disease
15:00-15:15	**Agnieszka Jaźwa**	Pro-angiogenic effect of heme and heme oxygenase-1 in human keratinocytes: regulation of VEGF and IL-8 synthesis in normoxia and hypoxia
15:15-15:30	**Jaroslaw Cisowski**	Biliverdin affects expression of angiogenic genes in human HaCaT keratinocytes
15:30-15:45	**Halina Waś**	Effect of heme oxygenase-1 on proliferation, viability and angiogenic potential of melanoma cells
15:45-16:00	**Dominika Nowis**	Heme oxygenase-1 protects tumor cells against PDT-induced toxicity
16:00-16:30	Discussion	
16:30-17:00	Coffee break and posters viewing	

17:00-19:30	***Session VI***	***STEM CELLS, BIOTECHNOLOGY AND SOCIETY***
	Chairpersons	*Alicja Ziemienowicz and Ewa Bartnik*
17:00-17:30	**Marcin Majka**	Stem cells – our key to longevity
17:30-18:00	**Edda Tobiasch**	Isolation and differentiation of human mesenchymal stem cells derived from adipose tissue
18:00-18:30	**Ewa Bartnik**	Frankenstein – the public perception of biotechnology
18:30-19:00	**Greg Becker**	Bram Stoker's Van Helsing or The private perception of science by science – and its duties to mankind
19:00-19:30	Discussion	
19:30-20.30	Dinner	
21:00	Discotheque, bar will be open	

Saturday, March 5th 2005

08:00-09:00	Breakfast	
09:00-11:15	***Session VII***	***GENE THERAPY AND BIOTECHNOLOGY***
	Chairpersons	*Alicja Józkowicz and Kazuhiro Oka*
09:00-09:30	**Kazuhiro Oka**	Inhibition and regression of atherosclerotic lesions
09:30-10:00	**Matthias Kapturczak**	AAV-mediated gene delivery to the vasculature and kidney
10:00-10:30	**Marcin Gruchała**	Therapeutic gene transfer in cardiovascular diseases
10:30-10:45	**Anna Zagórska**	RNA interference strategy for inhibition of HO-1 expression
10:45-11:00	**Tadeusz Pietrucha**	Biotech Consulting - commercialisation of research in the area biotechnology
11:00-11:15	Discussion	
11:15-11:45	Coffee break and posters viewing	
11:45-13:00	Free time, presentation of sponsors	
13:00-14:00	Lunch	
14:00-16:00	***Session VIII***	***MOLECULAR BACTERIOLOGY: MECHANISMS OF INFECTIONS AND METHODS OF DETECTION***
	Chairpersons	*Adam Dubin and Waleria Hryniewicz*
14:00-14:30	**Waleria Hryniewicz**	Human infections caused by *Staphylococcus* spp
14:30-14:45	**Artur Sabat**	Molecular methods for identification of clinically important species of the genus *Staphylococcus*
14:45-15:00	**Grzegorz Dubin**	Proteases of the *spl* operon of *Staphylococus aureus*
15:00-15:15	**Benedykt Władyka**	Study of the *stp* protease operon
15:15-15:30	**Malgorzata Rzychoń**	Probing structural basis for inhbitory specificity of staphostatins
15:30-16:00	Discussion	
16:00 -17:00	Coffee break and posters viewing	

17:00-18:00	***Session IX***	***SUNSET LECTURE***
	Leszek Kaczmarek	Cyclin D2 in adult neurogenesis

18:30-21:00 Special event – (sponsored by Sarstedt)

21:00 Discotheque, bar will be open

Sunday, March 6th 2005

08:00-09:00 Breakfast

09:00-13:00 Excursions, skiing
Free time
Company's presentations

13:00-14:00 Lunch

14:00-15:00 Posters viewing

15:00-16:30	***Session X***	***MOLECULAR BIOLOGY OF TUMOR AND IMMUNOTHERAPY***
	Chairpersons	*Grietje Molema and Jakub Gołąb*
15:00-15:30	**Jakub Gołąb**	Effective photoimmunotherapy of murine tumors induced by the combination of photodynamic therapy and dendritic cells
15:30-16:00	**Tomasz Stokłosa**	BCR/ABL recruits p53 tumor suppressor protein to induce drug resistance
16:00-16:15	**Joanna Cichy**	Plasmocytoid dendritic cells in host defense

16:15-16:30 Discussion

16:30-17:00 Coffee break and posters viewing

17:00-17:45	***Session XI***	***VARIA***
	Chairpersons	*Joanna Bereta and Tomasz Stokłosa*
17:00-17:15	**Renata Mężyk**	Elastase and cathepsin G augment the level of biologically active soluble form of TNFα
17:15-17:30	**Martyna Elas**	EPR oxygen images of tumors in living mice correlate with spatially and quantitatively with Oxyllite^{TM} oxygen measurements
17:30-17:45	**Dariusz Dziga**	The characteristics of cyanobacterial toxins and methods for their degradation
17:45-17:55	**Beata Bober**	Photodegradation of the microcystin-LR in the presence of natural plant products and chosen environmental factors
17:55-18:00	Discussion	
18:00-19:00	***FAREWELL LECTURE***	
	Kazuhiro Oka	Targeting site specific chromosome integration
19:00-20:00	Dinner and posters awarding	
20:00	Discotheque	

Monday, March 7th 2005

08:00-09:00	Breakfast
	Coming back

SESSION I
SIGNAL TRANSDUCTION AND TUMOR TARGETING

TGF-β signalling and its role in tumour pathogenesis

Bozena Kaminska, Aleksandra Wesolowska, Malgorzata Danilkiewicz

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Transforming growth factor beta (TGF-β) is a multifunctional cytokine involved in the regulation of cell proliferation, differentiation and survival/or apoptosis of many cells. Knock-out experiments for the three isoforms of TGF-β in mice have demonstrated their importance in regulating inflammation and tissue repair. TGF-β is implicated in the pathogenesis of human diseases, including tissue fibrosis and carcinogenesis. Malignant tumour cells often do not respond to TGF-β by growth inhibition, but retain responsiveness to cytokine in regulating extracellular matrix deposition, cell adhesion and migration. TGF-β receptors act through multiple intracellular pathways. Upon activation of TGF-β with its receptor, receptor-regulated Smad2/3 proteins become phosphorylated and associate with a common Smad4. Such complex translocates to the nucleus, where it binds to DNA and regulates transcription of specific genes. Negative regulation of TGF-β/Smad signalling may occur through inhibitory Smad6/7 that prevent activation of Smad2/3 or compete with Smad4 for complex formation with activated Smad2/3. Moreover, TGF-β-activated kinase-1 (TAK1) is a part of TGF-beta signalling and activates stress-activated kinases: p38 through MKK6 or MKK3 and c-Jun N-terminal kinases (JNKs) via MKK4. Previous studies demonstrated that TGF-β signal activates two independent pathways: the Smad- and MAPK-mediated pathways, however, recent data suggest a cross-talk between two signalling pathways.

In the brain, TGF-β, expressed at the very low level, increases dramatically after injury. Increased mRNA levels of the 3 TGF-β isoforms correlates with the degree of malignancy of human gliomas. TGF-β are secreted as latent precursors requiring activation into mature form. TGF-β inhibits proliferation of normal astrocytes but loses its growth-inhibitory potential towards gliomas due to alterations in the expression of cell cycle inhibitors. However, TGF-β has other effects such as local immunosuppression, induction of angiogenesis, and regulation of extracellular matrix unaffected. TGF β1,2 may stimulate production of vascular endothelial growth factor (VEGF) as well as plasminogen activator inhibitor (PAI-I) that are involved in vascular remodeling occurring during angiogenesis. Blockade of TGF-β action inhibited mammary tumour viability, migration, and metastases. Retroviral mediated introduction of a dominant negative TGFIIR to bone marrow cells led to generation of leukocytes capable of potent anti-tumor response and suppression of metastasis in melanoma and prostate cancer model. Reduction of TGF-β production and activity may be promising target of therapeutic strategies to control tumour growth.

Design of vascular endothelium specific drug targeting strategies for the treatment of cancer

Grietje Molema

Dept. Pathology & Laboratory Medicine, Medical Biology section, Endothelial Cell & Vascular Drug Targeting Research, Groningen University Institute for Drug Exploration (GUIDE), Netherlands; email: g.molema@med.rug.nl.
www.rug.nl/umcg/faculteit/disciplinegroepen/plg/medbiol/research/ec/index

Endothelial cells covering the blood vessel walls exert prominent functions to maintain body homeostasis. From the larger arteries down into the small microvascular arterioles and capillaries, and up via the postcapillary venules into the veins, they display strong heterogeneity in function. Angiogenesis or new blood vessel formation mainly takes place in capillary beds where endothelial cells and pericytes are the central cell types shaping the blood vessels. During tumour growth, chronic inflammatory processes, and hypoxic conditions, endothelial cells become activated while pericytes momentarily retract. Subsequent endothelial migration, proliferation, and maturation together with pericyte recruitment and maturation underlie the formation of new blood vessels. These cellular processes are characterized by specific molecular events that allow discrimination between tumour vascular and normal organ vascular endothelial cells. This fact forms the basis for the development of endothelial cell specific drug targeting strategies for the treatment of cancer and chronic inflammatory diseases.

Drug targeting constructs consists of homing ligands or moieties specifically interacting with a target epitope of choice on the angiogenic endothelium, carrier molecules containing the drug, and where appropriate, molecular entities improving construct behaviour in vitro and in vivo. Drugs studied for therapeutic intervention at the level of the tumour's blood supply include toxins, blood coagulation inducing factors, toxic chemicals, apoptosis inducing peptides, and RNAi, among others. The (extracellular or intracellular) location of the molecular target for the pharmacologic effector moiety determines which target epitope to choose on the cell membrane. It also governs the biological hurdles that need to be overcome to achieve effective drug delivery. Target epitopes on tumour endothelium are mainly transmembrane proteins expressed during angiogenesis, although also epitopes of basal membrane and extracellular matrix components unmasked during neovascularization are employed. Over 30 epitopes have been proposed as targets, only selections of them have been actually studied for drug targeting purposes.

In the last decade a wide range of tumour endothelial directed drug targeting strategies have been reported to exert significant therapeutic effectiveness in pre-clinical models. The challenge that lies ahead to develop them into clinical therapeutics includes unravelling of the spatiotemporal control of tumour endothelial heterogeneity as a result of which only a small fraction of endothelial cells are affected by single target strategies. Furthermore, improvement of chemical conjugation strategies leading to well defined drug targeting constructs is an essential prerequisite. Examples of attempts to address these issues will be discussed.

RhoA and MAPK pathways converge to regulate *SRF* gene expression

Kasza A.*, O'Donnell A., Zeef L., Gascoigne K., Hayes A. and Sharrocks A.D.

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**Present address: Faculty of Biotechnology, Jagiellonian University, Kraków*

Serum Response Factor (SRF) is a member of the MADS box family of transcription factors and is an important regulator of many genes associated with cell growth and differentiation. SRF can regulate gene expression via formation of ternary DNA-bound complex with another transcription factor, Elk-1. This interaction is mediated via DNA contacts and protein-protein interactions between the B-box of Elk-1 and the DNA binding domain of SRF. Elk-1 works in part through recruitment of the HATs CBP and p300. Using an EcR293 cell line that is stably transfected with an inducible dominant negative Elk-1 construct (Elk-1 fused to the repression domain from the *Drosophila* engrailed protein) we have identified *SRF* as a new Elk-1 target gene. Elk-1 regulates *SRF* expression directly via an ets-binding site in SRF promoter. Chromatin immunoprecipitation with an anti-Elk antibody confirms the binding of Elk-1 to the SRF promoter *in vivo*. Thus Elk-1 regulates the expression of its partner SRF. This regulation occurs in a B-box-dependent manner suggesting that it acts through ternary complex formation. Mutation of the Elk-1 B-box abolishes the inhibitory effect of dominant negative Elk-1 on SRF expression. The Elk B-box interacts with the same domain of SRF as MAL, myocardin-related SRF coactivator. Therefore we propose a new mechanism of regulation of *SRF* expression, via both the RhoA and MAPK pathways.

SESSION II
GENE EXPRESSION, GENE FUNCTION AND GENE POLYMORPHISM

New functions of the plant cap-binding protein complex

A. Jarmolowski

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Nuclear cap-binding protein complex (CBC) is involved in several aspects of RNA metabolism. The CBC binds to the cap structure of all polymerase II transcripts and promotes efficient splicing of pre-mRNA, nuclear export of U-rich small nuclear RNAs, mRNA 3'-end formation and also plays a role in nonsense-mediated mRNA decay (NMD). The cap-binding protein complex consists of two subunits called cap-binding proteins (CBP): CBP20 and CBP80. In our laboratory we have characterized CBC from *Arabidopsis thaliana* (AtCBC), which is composed of AtCBP20 (29,9 kDa) and AtCBP80 (96,5 kDa). AtCBP20 is highly conserved and shows 68% identity and 82% similarity with human CBP20. The most unique feature of AtCBP20 is a long C-terminal extension containing two nuclear localization signals (NLS) within it. The second subunit of AtCBC, AtCBP80, is less conserved and, unlike other CBP80 homologues, does not have any NLS. Due to the presence of two NLSs, AtCBP20 is actively transported into the nucleus, while AtCBP80 can reach the nucleus only in a complex with AtCBP20. Site-directed mutagenesis was performed in order to inactivate NLS1, NLS2 or both nuclear localization signals of AtCBP20. The GFP-AtCBP20 constructs were introduced into tobacco protoplasts and subjected to transient expression assay. Mutation in one of the two NLSs did not inhibit transport of AtCBP20 from the cytoplasm to the nucleus. These results indicate that NLS1 and NLS2 act independently and one of these two signals is sufficient for accumulation of AtCBP20 in the nucleus. Using co-transfection of tobacco protoplasts with GFP-AtCBP80 and AtCBP20 ΔNLS1, AtCBP20 ΔNLS2 or AtCBP20 ΔNLS1ΔNLS2 mutants, we have showed that each NLS can function independently in transport of the AtCBP20/AtCBP80 complex from the cytoplasm to the nucleus. Employing RNAi technology, we obtained *Arabidopsis thaliana* mutants with very low level of AtCBP20 or AtCBP80. These two mutants show identical phenotypes: slightly slowed growth and serrated leaves. Moreover, both mutants are abscisic acid (ABA) hypersensitive suggesting that CBC in plants is involved in ABA signaling.

Involvment of a DNA ligase in *Agrobacterium*-mediated plant transformation

[1]Wu Y.-Q., [2]Balwierz A., [1]Hohn B. and [2]Ziemienowicz A.

[1]*Friedrich Miescher Institute, Basel, Switzerland;* [2]*Faculty of Biotechnology, Jagiellonian University, Krakow, Poland.*

Agrobacterium tumefaciens, a plant pathogen, possesses a unique ability to transform plants by transferring a segment of DNA to plant cells. The transferred DNA (T-DNA) resides on a large Ti (Tumour inducing) plasmid, and the transfer of T-DNA is controlled by virulence (Vir) proteins of *Agrobacterium*.VirD2 protein cleaves the T-DNA at border regions and becomes covalently attached to the 5' end of the nicked DNA. The nicked DNA is then displaced from the plasmid producing single-stranded T-DNA. The T-DNA-VirD2 complex and the VirE2 protein are believed to be transferred to the plant with the help of a pillus-like structure containing VirB and VirD4 proteins. In the plant cell, T-DNA is coated with the single-stranded DNA-binding protein, VirE2. The T-DNA-protein complex (T-complex) is imported into the nucleus, where the T-DNA is integrated into the host genome. The VirD2 protein most likely is transferred into the nucleus in conjunction with the T-DNA; it may remain attached to it up to the integration step. Our recent results showed that plant factors are involved in T-DNA integration, although the nature and mechanism of function of these factors are still obscure.

To investigate the roles of VirD2 and plant proteins in T-complex ligation and integration, both *in vivo* and *in vitro* experiments were performed. By employing yeast two-hybrid system, affinity chromatography and immunoprecipitation we were able to isolate and to identify several plant proteins interacting with VirD2, including a DNA ligase. Next, we tested the enzyme for its potential involvement in T-DNA integration. *Arabidopsis thaliana* DNA ligase I was found to be able to perform T-complex ligation *in vitro*. Furthermore, VirD2 was shown to modify the activity of the DNA ligase by stimulating its adenylation and enzyme activity. Analyses performed with wild type and mutant VirD2 protein revealed that the C-terminal region of VirD2 encompassing a nuclear localisation signal is required for both the binding of VirD2 to the ligase and for efficient T-complex ligation, while for stimulation of the ligase adenylation, the full length VirD2 protein is required.

Identification of other plant proteins involved in T-DNA integration will not only widen our knowledge on this process, but also result in creation of plant lines that are more susceptible to T-DNA transfer.

Changes in PCNA gene expression during *Phaseolus vulgaris* growth and development

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During the life cycle all of the higher eukaryotes undergo different stages of development starting from the divisions of the zygote that leads at the late end to their death. Differentiation is not a random but very precisely programmed process executed according to the biological clock. There are many factors responsible for these changes and their cross-talks lead to the unique gene expression patterns.

In our project we focus on the proliferating cell nuclear antigen (PCNA) that is a homologue of DNA polymerase III β subunit of *E. coli*. PCNA is one of the most conserved proteins in higher eukaryotes. Up till now the best characterized function of this protein in mammalian cells is its role in the DNA replication process. PCNA, known as a sliding clamp, is a crucial factor for the main eukaryotic DNA replicating enzyme, DNA polymerase δ, that requires this protein for its activity.

In our studies we tested the protein profile for the presence of PCNA in embryos from different legumes species. In the preliminary studies we prepared total protein extracts from embryos dissected from dry seeds of *Cicer arietinum*, *Lens culunaris*, *Luinus luteus*, *Phaseolus vulgaris*, *Pisum sativum* and *Vicia faba*. Using the western blot technique we detected PCNA protein with anti-human PCNA monoclonal antibody. All of the investigated species except for *P. vulgaris* contained a protein with M.w. around 33 kDa, corresponding to PCNA. In *P. vulgaris* we detected additional cross-reacting protein with M.w. ~43 kDa. In a next step a time course experiment has been conducted that showed dissappearance of this protein after 24 hours of germination. Taking into account these results we employed the 3` and 5` RACE technique to identify cDNA encoding these two PCNA proteins. Recently, we have amplified and cloned the cDNA coding for the 33 kDa PCNA protein.

In the close future we will purify and characterize the 33 kDa form of PCNA. Our goal is also to find the PCNA interacting plant protein partners. Moreover, we want to elucidate whether the additional 43 kDa protein is a second form of PCNA or rather different cross-reacting protein that might be strictly related to the embryo formation process.

The influence of genetic polymorphisms, nutrients and hormonal status on the DNA damage. The *in vivo* and *ex vivo* studies

Maria Kapiszewska, Małgorzata Kalemba, Ewa Sołtys

Faculty of Biotechnology, Jagiellonian University, Kraków, Poland

Human health depends on life style, nutrition and, at almost the same degree, on genome, which is the information how the organism is built and how is functioning. The genetic code is almost identical for all humans but its more detailed analysis allows to find out population variations being the consequences of selections caused in turn by conditions of the environment existing in particular geographical area where they lived, often by hundreds of generations. These small alterations within genes, the single nucleotide polymorphism (SNP), in particular, allowed our ancestors very often not only to survive, but to better use the available natural resources as well. The same selective advantage, when this population change the life style and/or diet, may be life threatening. Since our genetic ancestors take 65% of their energy requirements from plant foods, it is likely that polyphenols were among nutrients necessary for survival. Thus, the demand for the polyphenols, which were abundant in such diet, has been much higher. Recently, a renewed interest in flavonoids, the most broadly studied group of polyphenols was fueled by the new research data showing that flavonoids, even at very low concentrations, are not only important antioxidant but also have a great impact on the regulation of gene expression, production of steroid hormones, as well as on the cell-mediated immunity. It is possible that drastically reduced amount of consumed fruits and vegetables, particularly in those population whose distant ancestors were exposed to the plant-origin diet, introduces the disturbances in some metabolic pathways, resulting in the so-called "civilisation diseases". The differences in the frequency of SNP in the genes responsible for the metabolism of flavonoids and compounds competing for the same enzymes, for example estrogens, among the people living in differed geographical locations and of various ethnic background, coincide with the different prevalence of cancers. This observation suggests that health benefits or harmful effects of high flavonoid diet strongly depend on inherited variation amongst individuals in their genetically defined ability to metabolize flavonoids. Therefore, the knowledge how diet and genetic background influence human health and how proper nutrition depends on the genetic make up. The relationships between the level of endogenous DNA damage involved in the cancer risk and polymorphism in *COMT* and MTHFR genes, dietary habits, particularly high-polyphenol diet, as well as the estrogen level in the female subjects are discussed in the paper.

SESSION III
GENE EXPRESSION AND THERAPY

Oxidative stress induces VHL- and prolyl hydroxylation-dependent ubiquitylation of Rpb1

Maria F. Czyzyk-Krzeska, Monika L. Ignacak, Svetlana Kolobanova, Justin Striet.

Department of Genome Science, GRI, University of Cincinnati, Cincinnati OH, 45237

VHL-associated E3 ubiquitin ligase complex binds and ubiquitylates the largest subunit (Rpb1) of RNA Polymerase II. This interaction requires hyperphosphorylation of Rpb1 on the C-terminal domain, and hydroxylation of proline 1465, within the LGQLAP motif, similar to that on HIFα (*PNAS, 2003, 100, 2706-2711).*

Here we show that oxidative stress induced by low concentrations (25-50 uM) of H_2O_2 or intermittent hypoxia (IH) causes VHL-dependent ubiquitylation of hyperphosphorylated Rpb1 in PC12 and RCC 786-0 cells expressing wild type VHL protein, but not in cells that do not express VHL. This ubiquitylation correlates in time with statistically significant 20% decrease in total mRNA transcription only in cells expressing VHL. Low doses of H_2O_2 do not affect distribution of hyperphosphorylated Rpb1 on the DNA, as measured by immunocytochemistry. In contrast, higher doses of H_2O_2 (0.25-1 mM) induce VHL-independent, immediate ubiquitylation of Rpb1 in PC12 and RCC cells with or without VHL, followed by delayed ubiquitylation only in VHL positive cells. This is accompanied by change in Rpb1 staining from the uniform to the peripheral in relation to the DNA.

Both, low or high doses of H2O2 increase prolyl-hydroxylation of Rpb1, measured using a specific antibody developed against Rpb1 peptide containing hydroxylated P1465. *In vitro* prolyl hydroxylation assays using 37 amino acid long Rpb1 peptide show that nuclear extracts from cells treated with H_2O_2 have augmented prolyl hydroxylase activity towards the Rpb1 but not HIFα peptide, as measured by [^{35}S]VHL binding. Transfections of cDNA for histidine–tagged Rpb1 wild type or carrying P1465A mutation demonstrate that only wild type Rpb1 co-immunoprecipitates with VHL and undergoes ubiquitylation in response to H_2O_2. The experiments are in progress to identify involved prolyl hydroxylases and to determine functional significance of VHL-dependent ubiquitylation of Rpb1.

Glycobiotechnologies for cell therapy

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Glycobiology has matured rapidly, and with it, the far reaching clinical implications are becoming understood as well as its potential to improve the practice of Medicine has been well recognised. This final frontier of science is being conquered.

Indeed, from embryogenesis to pathogenesis, glycosylation plays a pivotal role.

The pathogenesis of viral infections involves sugars at every turn. Consequently, immune mechanisms will be a major focus for clinical intervention involving modulation of both the innate and adaptive immune defence in which disease associations with sugar changes are plentiful, as for example the function of the T cell co-receptor CD8.

Sugar associations with cancer have been recognised for some time. There continues to be new data concerning cancer, but research is expanding into new areas such as the pathological mechanism associated with the GPI anchorage of the prion protein or genetic diseases which, as muscular dystrophy, are caused by genes that encode glycosyltransferases.

Largely documented autoimmune arthritis has been associated with the generation of remnant glyco-epitopes by gelatinase B/matrix metalloproteinase-9, which is an inflammatory mediator and effector. Determination of T-cell reactivity against the gelatinase B-cleaved fragments of collagen II indicates that, there are many glyco-epitopes, thus reinforcing the role of glycopeptide antigens in autoimmunity.

Rheumatoid atrhritis is a common disorder where sensitive diagnostic is awaited from potential of IgG glycosylation.

Once disease mechanisms have been understood, the next step is to determine whether this information can be used to devise therapeutic options. An example is the use of polysaccharide to block skin inflammation. But, cellular glycoengineering for fully human and optimised glycosylation of proteins is therefore going to be important if these molecules are going to be fully and specifically active. Most pharmaceutical proteins are expressed in bacteria, yeast or mammalian cells resulting in proteins lacking glycosylation or carrying glycans which largely differ from human carbohydrate chains in various aspects including sialylation. However, relationships between the N-glycan and O-glycan structures and biological activities are now better understood. It is fundamental and apparent that novel glycoprotein expression technology is developed to address this problem and data is now available to demonstrate how this can be done. The new developments using cell therapy for delivery of replacement glycosyltransferases genes are illustrating the strategy.

Effect of atorvastatin on angiogenic gene expression in normoxic and hypoxic microvascular endothelial cells

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Background: Hypoxia (low oxygen tension) is a well known modulator of angiogenesis. It affects the expression of many genes by both up-regulating pro-angiogenic and down-regulating anti-angiogenic gene expression. Statins, which are inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and are used as a potential drugs lowering cholesterol level were also reported to affect angiogenesis. Interestingly, limited data are available concerning the effect of statins on angiogenic gene expression in hypoxia. Therefore, we investigated the effect of atorvastatin, a member of statins family both in normoxic and hypoxic human microvascular endothelial cells (HMEC-1).

Methods and results: Complex effect of hypoxia and atorvastatin on HMEC-1 was observed as both pro-angiogenic and anti-angiogenic genes were influenced. Macroarray analysis showed that hypoxia up-regulated the expression of pro-angiogenic VEGF-D, endoglin, inhibitor of DNA binding 3 (ID3) or transforming growth factor beta receptor (TGFBR1). On the other hand, thrombospondin-1 (TSP-1) and tissue inhibitor of matrix metalloproteinases-1 and 2 (TIMP-1 and TIMP-2) were down-regulated after hypoxia treatment. Interestingly a decrease in IL-8 mRNA and protein level in hypoxic conditions has been observed in HMEC-1. Dual effect of statins on VEGF-D and endoglin has been noted being stimulatory in normoxic and inhibitory in hypoxic conditions. Moreover, both VEGF-A and IL-8 were down-regulated after atorvastatin treatment as shown by RT-PCR real-time RT-PCR and ELISA.

Conclusions: Atorvastatin seems to exert anti-angiogenic activity in human microvascular endothelial cells. Interestingly, two pro-angiogenic factors VEGF-A and IL-8 are regulated in a completely different way by hypoxia in HMEC-1. Our results showed that pro-angiogenic effect of hypoxia is connected with the increase in VEGF-A but not in IL-8 synthesis. Changes in IL-8 synthesis both by hypoxia and atorvastatin might be connected with anti- inflammatory activity of statins.

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Transcripts profile in HepG2 cells stimulated with proinflammatory cytokines

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Different gene expression is essential for both normal development and pathological processes. Changes in expression level can occur in response of cells to different external stimuli, such as cytokines, growth factors, viruses or bacterias. The purpose of our study was the comprehensive analysis of transcripts profile in human hepatoma HepG2 cells stimulated with IL-1 and IL-6 and the mixture of IL-1/IL-6 during different time of exposure. Both cytokines are principal proinflammatory agents involved in inflammation process and regulation of expression of acute-phase proteins. Differential display method was applied to analyse differentially expressed genes. Observed changes in transcript level were confirmed by RT-PCR, northern blot analysis, or slot-blot analysis. All together, we identified 87 modulated cDNA sequences represented functionally known genes, and 41 cDNA fragments complementary to unknown genes or hypothetical proteins. According to the function of the protein products encoded by these genes they can be classified into 13 functional groups. Genes encoding proteins involved in metabolism, protein synthesis and signal transduction are broadly represented. Transcripts identified so far in HepG2 cells encode cytoplasmic and nuclear proteins but not secreted proteins. Cytokine stimulation revealed three types of gene response: early response, late response and continuous response. Several genes represent biphasic response. Analysis of gene expression reveals that response to proinflammatory cytokines is more complex and need to be investigated intensively to explain the mechanism of the acute phase response.

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SESSION IV
MOLECULAR MODELING

Overview of selected mesoscopic biomolecular modelling models and theories

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In order to study structures of large biomolecular systems and simulate their dynamics in a long-time scale one requires fast methods which allow to compute their free-energy function. An important contribution to the free energy function is a mean-field electrostatic contribution based on Poisson-Boltzmannn (PB) and/or Generalized Born (GB) models. The GB model is a mesoscopic, semi-analytical approach, capable to reproduce the mean-field PB electrostatic energy of a molecular system in continuous medium, characterized by the dielectric constant and the ionic strength.

Coupling of a fast quantum SCC-DFTB method and PB or GB models will be discussed. SCC-DFTB is applicable to large molecular systems. CM3 charges generated by the SCC DFT TB method, depending on conformation and reproducing precisely molecular dipoles are used in the GB model. Other faster, but less precise procedures, like a charge equilibration approach, could also be implemented. Coupling of SCC-DFTB and GB or PB models, allows to compute the mean-field electrostatic energy of molecular systems in molecular environments close to real ones. The PB and GB models give, in particular, electrostatic reaction field energies of molecular environments, which contribute to hydration energies. The ongoing research is focused on designing molecular mesoscopic interaction potentials, applicable to (bio)molecular systems and utilizing conformationally dependent atomic charges.

It is assumed that the solvation energy consists of the mean-field electrostatic and nonpolar (hydrophobic) energy contributions. The ongoing studies are based on parameterization of the interaction free energy, as function of *microscopic* atomic parameters, like partial charges, VdW radii and L.J. atomic parameters. In our approach the generalized Born (GB) model is used to describe the electrostatic part of the free energy, while the hydrophobic contribution is either a function of the molecular surface exposed to solvent, or approximated by a polynomial of reciprocal values of the Born radii. Also, following Levy and coworkers, a mean Van der Waals energy contribution is being accounted in this model.. The hydrophobic energy has already been fitted to reproduce experimental energies of solvation contained in the data-base provided us by Dr. D.Truhlar (University of Monnesota). In particular, the nonpolar energies are fitted to solvent surface accessible area (SASA), or represented by a polynomial series depending on reciprocal values of the Born radii. Presence of the mean VdW energy on the quality of the fits is also analyzed.

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Multibody effects in hydrophobic interactions

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The structuring of water molecules in the vicinity of nonpolar solutes is responsible for hydrophobic hydration and association thermodynamics in aqueous solution. We studied the potential of mean force (PMF) for the formation of a dimer, trimers and tetramers of methane molecules in specific configurations in explicit water to explain multibody effects in hydrophobic association on a molecular level. The main effect of the multibody contribution to the PMF is a reduction of the height of the barrier between the contact and solvent separated minima and a narrowing of the region of its maximum, while the region of the contact minimum is affected only weakly. The cooperative contributions to the PMF agree very well with those computed from the molecular surface of the systems under consideration, which further supports earlier observations that the molecular surface can be used with good accuracy to describe the energetics of hydrophobic association. We analyzed the packing and orientation of water molecules in the vicinity of the solute to explain the effect of ordering of the water around nonpolar solutes on many-body interactions. A simple mechanistic picture of hydrophobic association is drawn. The free energy of hydrophobic association depends primarily on the difference in the number of water molecules in the first solvation shell of a cluster and that in the monomers of a cluster; this can be approximated by the molecular surface area. However, there are unfavorable electrostatic interactions between the water molecules from different parts of the solvation shell of a trimer because of their increased orientation induced by the nonpolar solute. These electrostatic interactions make an anti-cooperative contribution to the PMF, which is clearly manifested for the linear trimer where the multibody contribution due to changes in the molecular surface area is equal to zero.

A hypothesis for GPCR activation

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Some 50% total and ~25% of top-selling 100 drugs act via transmembrane G protein-coupled receptors (GPCR). Their global sales exceed 30 billion USD per year A GPCR, usually activated by a specific extracellular molecular signal, stimulates in turn a specific G protein, hence receptor's name. G protein is a cytosolic peripheral membrane heterotrimer (Gαβγ), binding guanine nucleotide GDP/GTP. The receptor mediating visual perception, rhodopsin (RD), is unique for several reasons. It belongs to a GPCR subfamily termed opsins, characterized by a covalently bound retinal, taking part of antagonist in the 11-*cis* conformation, and of agonist in the *all-trans* conformation. RD is the only GPCR of resolved atomic structure so far (in inactive form). Hence, RD is commonly accepted as a structure template of an inactive (antagonist-bound) receptor of any CPCR Class A, a major GPCR family, to which RD belongs. Yet only the structure of Meta II (activated RD*, with the *all-trans* retinal), docked to an inactive protein Gtαβγ·GDP (transducin) could potentially make a template for a complex of a class A GPCR with an agonist. Excluding opsins, other receptors are activated by non-covalent interactions with an endogenous agonist. Despite numerous clues from biochemical experiments, details of activation and structure of an activated GPCR are unknown. Leading pharmaceutical companies, contributing in the GPCR-dependent drug market, work in their R&D labs on elucidation of GPCR activation and biological signal transduction. Growing evidence that rhodopsin (RD) and related G protein-coupled receptors form functional dimers/oligomers, followed by direct proof (using atomic force microscopy) that in the retina disc membranes RD associates into a paracristalline network of rows of dimers, need models on RD-transducin (Gt) complex that would envision an optimal RD dimer/oligomer amenable to satisfy all well documented interactions with Gt. Of the models proposed so far, only a few refer to RD dimers and only one of them proposes a complex of Gt with an RD oligomer (Filipek et al, 2004, *Photochem Photobiol Sci* 3, 628-638). This lecture puts forward a hypothesis on another arrangement of RD monomers into the reported network of rows of dimers. Arguments for compatibility of this set-up with interactions and activation of RD in the complex with Gt, in particular with the well documented as vital for RD activation movement of transmembrane helix 6 and cytosolic loop 3, are discussed.

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Exploring global motions and electrostatic properties of the ribosome

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Ribosomes are the ribonucleoprotein assemblies that translate the information contained in the mRNA sequence into polypeptides. Bacterial ribosomes are made up of two unequal subunits; the small subunit contains one ~1500 nucleotide long RNA chain and 21 proteins, and the large subunit contains two RNA chains (~2900 and 120 nucleotides long) surrounded by over 30 proteins. Translation is a highly dynamic, remarkably accurate and fast process, with the rate of protein elongation of 10-20 amino acids incorporated per second. All the stages of this process are controlled by various factors binding to the ribosome. Factor binding, peptide bond formation and global ribosome motions are interdependent processes, most probably driven by electrostatic interactions.

The electrostatic properties of this highly charged macromolecular machine were explored qualitatively based on the Poisson-Boltzmann implicit solvent model. Studies accounted for various conformations of the ribosome derived from the normal mode analysis and cryo-electron microscopy studies. To solve the Poisson-Boltzmann equation, effective parameters (charges and radii), applicable to a highly charged backbone model of the ribosome, were developed. Regions of positive potential were found at the binding site of the elongation factors G and Tu, as well as where the release factors bind. Large positive potential areas are especially pronounced around the L11 and L6 proteins. The region around the L1 protein is also positively charged, supporting the idea that L1 may interact with the E-site tRNA during its release from the ribosome after translocation. Functional rearrangement of the ribosome leads to electrostatic changes which may help the translocation of the tRNAs during the elongation stage. Therefore, ribosome motions modulate electrostatic properties of this system.

Global coupled motions and correlations among the movements of various ribosomal parts were additionally studied with half a microsecond of coarse-grained molecular dynamics. A low resolution anharmonic network model that allows for the evolution of tertiary structure and long-scale sampling, was developed and parameterized. Most importantly, we found that functionally important movements of L7/L12 and L1 lateral stalks are anti-correlated. Other principal directions of motions include widening of the tRNA cleft and the rotation of the small subunit which occurs as one block and is in phase with the movement of L1 stalk. The effect of the dynamical correlation pattern on the elongation process was analyzed. Small fluctuations of the 3' tRNA termini and anti-codon nucleotides show tight alignement of substrates for the reaction. The model provides an efficient and reliable way to study the dynamics of large biomolecular systems composed of both proteins and nucleic acids.

SESSION V
HEME OXYGENASE IN HEALTH AND DISEASE

Regulation of the human heme oxygenase-1 gene in disease

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Heme oxygenases catalyze the rate-limiting step in heme degradation resulting in the formation of iron, carbon monoxide and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Recent attention has focused on the biological effects of product(s) of this enzymatic reaction, which have important antioxidant, anti-inflammatory and cytoprotective functions. Two major isoforms of heme oxygenase (HO) enzyme have been described, an inducible isoform, HO-1, and a constitutively expressed isoform, HO-2. A third isoform, HO-3, closely related to HO-2 has also been described. Several stimuli including heme, nitric oxide, growth factors, angiotensin II and cytokines implicated in the pathogenesis of diseases such as atherosclerosis, kidney failure, vascular restenosis, organ transplant rejection and sepsis, induce HO-1. Induction of HO-1 occurs as an adaptive and beneficial response to these stimuli in animal models of these diseases. This presentation will focus on the molecular regulation of the HO-1 gene in tissue injury by heme and will highlight the interspecies differences, predominantly between the rodent and the human HO-1 genes.

Pro-angiogenic effect of heme and heme oxygenase-1 in human keratinocytes: regulation of VEGF and IL-8 synthesis in normoxia and hypoxia

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Background: Upon skin injury blood vessels are disrupted and large amounts of heme are released. Heme is a potent pro-oxidant which is a substrate for heme oxygenase-1 (HO-1), releasing carbon monoxide, iron and biliverdin, subsequently reduced to bilirubin. Recently the involvement of HO-1 in angiogenesis has been implicated, however, the role of heme and HO-1 in angiogenic events in wound healing has not been yet investigated.

Results: Hemin (30 μM, 6 hours) very strongly induced HO-1 expression in human HaCaT keratinocyte cell line, as determined by RT-PCR, Western blot and ELISA and enhanced HO activity, confirmed by elevated bilirubin production. Concomitantly, hemin enhanced the VEGF mRNA expression through upregulation of VEGF promoter and VEGF protein synthesis was enhanced. Involvement of HO-1 was confirmed by inhibition of VEGF expression by SnPPIX, a blocker of HO activity and by attenuation of HO-1 mRNA expression with specific siRNA. HaCaT cultured in medium containing low amounts of glucose (5.5 mM) produced more VEGF than cells growing in 25 mM glucose, but treatment with hemin were able to overcome the inhibitory effect of high glucose on VEGF synthesis. Interestingly, hypoxia (1% oxygen) augments VEGF expression in HaCaT, but induction of HO-1 expression is much weaker in comparison to treatment with hemin. In contrast to VEGF, the production of IL-8, another important angiogenic mediator is less reliant on HO-1 induction after heme treatment, and hypoxia did not enhance its production.

Conclusions: HO-1 is involved in induction of VEGF expression in human keratinocytes by hemin, but does not play a role in hypoxia-mediated VEGF expression. HO-1 overexpression may be beneficial in restoring the proper synthesis of VEGF disturbed in diabetic conditions.

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Biliverdin affects expression of angiogenic genes in human HaCaT keratinocytes

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Background: Angiogenesis, a process of formation of new blood vessels from preexisting capillaries, is involved in many physiological as well as pathological processes. Vascular endothelial growth factor (VEGF) is one of the most ubiquitously expressed and the most potent angiogenic growth factor. Its expression is accelerated by numerous noxious stimuli including reactive oxygen species (ROS) and heme (1-3). Heme is also an inducer of the synthesis of heme oxygenase-1 (HO-1) and concomitantly acts as its substrate, which is decomposed to biliverdin (BV), Fe^{2+} and carbon monoxide. BV is subsequently converted to bilirubin (BR) by biliverdin reductase (BVR) . The aim of this study was to determine, whether BV and BR, products of HO-1 and BVR activity, can influence the expression of angiogenic genes in human HaCaT keratinocytes.

Methods and results: Hemin and BV induced VEGF mRNA and protein accumulation in HaCaT keratinocytes and activated a full length VEGF gene promoter. Preincubation of HaCaT with actinomycin D, an inhibitor of transcription, was able to completely reverse the effect of BV, which suggests that BV does not increase the stability of VEGF transcript. Instead, preincubation of HaCaT cells with mithramycin A, which inhibits binding of Sp-1 to its cognate DNA sequences, or with PD 98059, which blocks MEK p42/p44 pathway, down-regulated VEGF synthesis. BV was also a potent inducer of interleukin-8 (IL-8) expression in HaCaT cells. BR, at the same concentrations as BV, was unable to mimic its effect on VEGF synthesis. Moreover, HMEC-1 endothelial cells and primary human keratinocytes, in contrast to HaCaT, were resistant to stimulation with BV. It did not enhance VEGF protein synthesis in these cell types.

Conclusions: In HaCaT keratinocytes hemin elicits VEGF expression and BV, a product of heme degradation by HO-1, can be responsible for this activation, since exogenously added BV lead to a strong *de novo* expression of VEGF. Moreover, this effect seems to be cell type-specific since neither HMEC-1 nor human primary keratinocytes were able to respond to BV stimulation with VEGF synthesis.

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Effect of heme oxygenase-1 on proliferation, viability and angiogenic potential of melanoma cells

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Background: Heme oxygenase-1 (HO-1), an enzyme degrading heme to biliverdin, carbon monoxide and iron ions, may augment angiogenesis. Additionally, HO-1 can regulate cell cycle and apoptosis. Here we investigated the effect of HO-1 overexpression on the proliferation, viability and angiogenic potential of B16(F10) murine melanoma cell line *in vitro* and *in vivo*.

Methods and results: Establishing the HO-1 overexpressing cell line (B16(F10)-HO-1) was confirmed by PCR, RT-PCR and western blotting. Overexpression of HO-1 led to a significant increase in melanoma cell proliferation, as measured by BrdU incorporation. Moreover, B16(F10)-HO-1 cells were more resistant to oxidative stress. Four-hour exposure to 200 μM H_2O_2 resulted in massive death of wild-type melanomas, whereas almost 70% of B16(F10)-HO-1 cells were still viable, as assessed by trypan-blue exclusion. Accordingly, low concentration of H_2O_2 (0.78-3.12 μM, 18 h) induced apoptosis in B16(F10), but not in B16(F10)-HO-1, as measured by TUNEL assay. Finally, conditioned media harvested from B16(F10)-HO-1 more potently induced endothelial cell proliferation and formation of capillaries by endothelial spheroids than those collected from B16(F10). This effect was not modified by pre-treatment of media with antibodies blocking the vascular endothelial growth factor (VEGF). In accordance, HO-1 overexpression did not influence the generation of VEGF as checked at the promoter, mRNA and protein levels in melanoma.

To check the effect of HO-1 overexpression on tumor development 0.2 x 10^6 B16(F10) or B16(F10)-HO-1 cells were injected intracutaneously into the back of syngenic C57BL/6 mice. Overexpression of HO-1 in melanoma cells shortened survival time of B16(F10)-bearing mice. Surprisingly, there were no differences in tumor size between mice bearing B16(F10 and B(16)-HO-1 cells. Preliminary analyses suggest, however, that HO-1 overexpression was associated with reduced inflammatory edemas and necrotic areas in the tumors and with increased density of melanoma cells. In accordance, the levels of tumor necrosis factor (TNF), in serum and tumor lysates from mice injected with B16(F10)-HO-1 cells were lower than from those injected with the wild-type malanoma. Finally, immunohistochemical staining using anti-CD31 antibodies suggests also the stronger vascularization of HO-1 overexpressing tumors.

Conclusion: HO-1 increases viability, proliferation and angiogenic potential of murine melanoma cell line. Accordingly, overexpression of HO-1 in tumor cells leads to decrease in survival time of melanoma-bearing mice, inhibition of inflammatory reaction, and increase in tumor vascularization. It suggests that reduction of HO-1 activity might be beneficial in therapy of melanoma.

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Heme oxygenase-1 protects tumor cells against PDT-induced toxicity

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Photodynamic therapy (PDT), a promising therapeutic modality for the management of solid tumors, is a two-phase treatment consisting of a photosensitizer, oxygen and a visible light. Increasing evidence indicates that tumor cells in regions exposed to sublethal doses of PDT can respond by rescue responses that lead to insufficient cell death. Using a cDNA microarray analysis we identified gene products overexpressed in tumor cells exposed to PDT. One of these was heme oxygenase-1 (HO-1), a rate limiting enzyme involved in the degradation of heme into carbon monoxide, free iron and biliverdin. We decided to examine the role of HO-1 overexpression in the effectiveness of PDT and to investigate whether inhibition of HO-1 affects the antitumor activity of this treatment regimen. In the initial experiments we observed overexpression of HO-1 protein in cells exposed to PDT. Chemical induction of HO-1 with hemin resulted in a decreased sensitivity of the tumor cells to the cytotoxic effects of PDT. Inhibition of HO-1 activity using Zn(II) protoporphyrin IX – a competitive HO-1 inhibitor, led to potentiated antitumor effectiveness of PDT. We also established a tumor cell line stably transfected with HO-1 gene. These cells were more resistant to PDT than controls in *in vitro* as well as *in vivo* models. Leukocyte infiltration after PDT in the tumor sites composed of HO-1 transfectants was significantly delayed. The protective role of HO-1 in PDT-treated tumor cells is not dependent on the increased production of the antioxidant biliverdin or bilirubin. The mechanism of this interaction probably relays on the production of carbon monoxide and/or free iron. We conclude that inhibition of HO-1 activity is an effective treatment modality capable of potentiating the antitumor effectiveness of PDT.

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SESSION VI
STEM CELLS, BIOTECHNOLOGY AND SOCIETY

Stem cells – our key to longevity

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Computers and informatics dominated the XX century. They were the moving engines that push our civilization forward during last decades. But they are fading away and new disciplines are emerging as dominating forces. One of them is biotechnology. Progress in this area will dramatically change the quality of our lives.

Research on stem cells is an important part of biotechnology that will lead as predicted to the development of new therapeutic strategies. A lot of effort has been put to understand biology of the stem cells, to find genes that are responsible for their proliferation-self-renewal and differentiation. Many laboratories around the world are trying to discover new markers on their surface that can be employed for stem cell purification.

So far different cytokines and growth factors were used to expand stem cells, but no combination of these factors was identified, to effectively expand the most primitive hematopoietic stem cells. Recently, however, several new genes have been described as regulators of stem cells fate (Notch family) or stem cells self-renewal and differentiation (Hox family). Ligands for these receptors or these genes themselves are being already used for *ex vivo* expansion of stem cells and the first data are promising. This opens the new possibilities for clinical application of stem cells isolated from both hematopoietic and non-hematopoietic tissues.

New markers such as CXCR4 or CD133 has been discovered and shown to be present on a surface of hematopoietic stem cells. The same markers were recently also found to be expressed on neuronal-, hepatic- or skeletal muscle- stem cells. By employing these markers several laboratories try to isolate stem cells for potential clinical purposes.

New characteristics of stem cells have been also described. Several papers were published that showed that the committed of stem cells to a given tissue can be changed. For example, hematopoietic stem cells were described to be able to transdifferentiate into skeletal muscles- or neuronal- stem cells. This phenomenon was called "stem cells plasticity" and generated a lot of hope and excitement that adult somatic stem cells could be employed to regenerate tissue/organs. Unfortunately most of these results were not reproduced in subsequent independent studies. These obvious differences in reported results could be explained by a phenomenon of cell fusion or epigenetic changes in stem cells. However, the third possibility that was not taken carefully into consideration in previously published data, it is a presence of heterogenous population of stem cells in different organs/tissues. Recently our team has identified a population of tissue committed stem cells in the bone marrow. The number of these cells, however, is extremely low but if we would be able to expand them efficiently ex vivo, these cells perhaps could become our keys for regeneration/longevity.

Isolation and differentiation of human mesenchymal stem cells derived from adipose tissue

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Embryonal stem cells have the potential of long-term viability, self-renewal and multilineage differentiation. This makes them excellent candidates for tissue engineering and gene therapy applications. Nevertheless, adult stem cells are investigated extensively as another source of self-renewing cells mainly due to ethical and political issues.

Human mesenchymal stem cells (HMSCs) which are isolated from bone marrow stroma, peripheral blood, dermis, muscle and adipose tissue have the advantage of potential autologous transplantation ability. They can be differentiated in chondrogenic, osteogenic, adipogenic and myogenic lineages. Problems related with the more traditional extraction of HMSCs from bone marrow include low cell numbers, low isolated volumes, pain, and to some extend ethical concerns. Therefore, the isolation of HMSCs from human adipose tissue was recently identified as an alternative source, since these cells are easy to obtain in big cell numbers.

Adipose tissue is derived from embryonic mesoderm and it contains a heterogeneous stromal cell population. To achieve lineage-specific differentiation in the processed lipoaspirates (PLA) cells are cultured in media supplemented with the appropriate factors. To confirm, whether these cells represent a stem cell population, extended biochemical and molecular characterisations have been performed and published by various authors. Inductions of the cells into multiple mesenchymal lineages resulted in the expression of several lineage-specific genes, proteins and specific metabolic activity. Nevertheless, the single differentiation steps of the various lineages still have to be defined in more detail.

It is hypothesized that PLA may differentiate into a phenotype of other germ-layers such as neural cells derived from embryonic ectoderm as well. This characteristic would abolish the main disadvantage of adult stem cells if compared with embryonic derived cells.

In conclusion, the potential benefit of the multi-germline capacity of HMSCs seems to be a promising approach for allogenic cell therapy and human tissue engineering.

Frankenstein – the public perception of biotechnology

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We live in a world in which new products of the biotechnological industry are being introduced at a rapid rate. Some of them were accepted easily – such as HBV vaccines or human recombinant insulin. Others – especially plants produced using genetic engineering – arouse some fears. There is also a heated discussion concerning human cloning, especially in respect to embryonic stem cells.

This is not the first time that we encounter new and unexpected things due to human activity. The first experiments with recombinant DNA in the early 1970s led to a year-long moratorium to give time for scientists and society to reflect on the dangers posed by cloning (of genes) and how to prevent them. Introduction of the transplantation of human organs which could only be removed from persons who would not need them any more led to a change in the definition of death. In vitro fertilization led to the development of a new branch of medicine dealing with the problem of human infertility.

I will discuss the possibilities and perspectives of biotechnology and the hope, fear and objections which this field arouses.

Bram Stoker's van Helsing or The private perception of Science by Science – and its duties to mankind

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"(...) no good to her, to us, to science, to human knowledge, why do it?"
Bram Stoker. Dracula. Page 60

Bram Stoker's famous novel "*Dracula*" deals not only with a satanic and bloodthirsty Vampire but also draws an image of Science at the dawn of its next step of great progression. It is a story teaching us about important roots of today's Science and the nature of its duties.

Dracula is the symbol for the brute, wild motives and the deep ancient fears in man. He is the spirit of ancient times that always denies, following his instincts and will only. He stands for the evil disposition of man based on ignorance, beliefs and superstition. And so, he is the darkest myth of chaos that only can be defeated by knowledge as he plainly is the anti-civilising manifestation.

Dracula's arch-enemy is a colourful figure: ***Abraham van Helsing***.

He is a, physician, a researcher and erudite, a philosopher, meta-physicist, an ethicist, a cynic entertainer and man of world, but foremost he is a scientist. He represents Science that fought for its rational direction, but still was trapped in the struggle between the mystical search for wisdom and the will to work for truth and positive facts that speak for themselves only.

Van Helsing is not the first of the modern and thus rational scientists, but the last metaphysician of his profession in the past. According to Science, in one person *van Helsing* is THE LAST ACTION HERO, THE CLEANER and THE TERMINATOR, the one who enforces certainty against superstition, knowledge against beliefs and reason against fear. Second to none, for him responsibility for the good of mankind is the drive of Science and he dedicates all his knowledge to this purpose. This attitude provides an example for all Science, especially today.

We take the fictional and symbolic figure of *van Helsing* to give an outline on the self-conception of Modern Science against the background of its origin and its ethical duties to mankind.

We will show how current Science only has become possible by defeating superstition and ignorance.

We will explain why Science is more than to increase the level of knowledge and how it serves always the higher purpose to be responsible for the good of mankind.

Furthermore, we will talk about how this higher purpose has turned to a formal principle that has to be filled with life again. Additionally, we will portray the current involvement of Science in business and how this connection may cut Science from its goals.

We will describe how Science abandoned its place in society and why the current possibilities in Bio-Sciences – ironically – have created exactly the same horror than the defeated beliefs in unreal and satanic creatures in the past. Finally, we will discuss how the current self-image of Science has to be changed and what scientists have to do to win back the lost trust in the good of Science.

SESSION VII
GENE THERAPY AND BIOTECHNOLOGY

Inhibition and regression of atherosclerotic lesions

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The original view of atherosclerosis was that it was a slow, irreversible process. However, there is mounting evidence that it is both dynamic and reversible. Early studies of primates and hypercholesterolemic rabbits have shown that switching from a high to a low cholesterol diet results in regression of atherosclerotic lesions. Other models using thoracic aorta transplantation or transgenic mice carrying Mx1-Cre transgene have shown that long-term lipid normalization or stable expression of an anti-atherogenic protein induces regression of advanced lesions. Furthermore, these studies have found dramatic remodeling of atherosclerotic lesions into a more stable appearing phenotype, with less macrophage-derived foam cells, decreased smooth muscle cells in the blood vessel intima and media, and more collagen content. This translates into a lower probability of clinical events, such as atherosclerotic plaque rupture.

Somatic gene therapy is being explored as potential alternative treatments for patients who have elevated low density lipoprotein (LDL) cholesterol, but do not respond adequately to conventional LDL-lowering medical therapy. This approach has further evolved into use of gene therapy vectors as tools for the study of vascular biology in animal models of dyslipidemia. Previous studies of atherosclerosis regression in animals employed early generation adenoviral vectors and showed that overexpression of anti-atherogenic or lipid lowering proteins leads to regression of fatty streaks, or "early lesions." However, these studies have suffered from the methodological limitations including vector induced inflammation and transient nature of the transgene expression by these vectors. Our group has reported that helper-dependent adenoviral vector (HDAd)-mediated liver-directed gene transfer is useful for these studies. In apolipoprotein E (apoE)- or LDL receptor (LDLR)-deficient mice, apoE or LDLR gene transfer by HDAd vectors resulted in lifetime (greater than 2 years) phenotypic correction and protection against atherosclerotic lesion formation. We have used a HDAd vector to study regression of advanced atherosclerotic lesions. ApoE-deficient mice having advanced lesions analogous to American Heart Association type IV and V lesions in humans were treated by HDAd expressing human apoE3 (HDAd-gE3). Thirty-six weeks after treatment, the lesion was significantly reduced in mice treated with HDAd-gE3 compared to control mice. These results suggest that somatic gene therapy may be a prospective treatment for protecting against atherosclerosis progression, and may even result in inducing lesion regression.

AAV-mediated gene delivery to the vasculature and kidney

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Over the past decade significant advances in molecular genetics have led scientists to initiate gene therapy trials in humans and indeed, this modality has been attempted in several clinical situations, especially in the treatment of certain metabolic disorders, cancer, hypertension and sepsis. Gene therapy has the potential to provide a therapeutic strategy for numerous vascular diseases such as atherosclerosis, diabetes, hypertension and renal diseases such as polycystic kidney disease, Alport syndrome, and inherited renal tubular disorders as well as chronic transplant rejection and acute renal failure. However, gene therapy in the vasculature and kidney has not as yet been achieved with great success, due to many obstacles that have to be overcome. The selection of vectors, methods of delivery, choice of therapeutic genes and targeting to the specific compartment are critical issues for the success of gene therapy for vascular and kidney diseases and related entities. The purpose of this presentation is to provide a discussion of the available vector systems, particularly the recombinant adeno-associated virus vector (rAAV), for gene delivery to the vasculature and the kidney and the potential for gene therapy as a strategy for selected renal diseases. rAAV has become an attractive tool for gene therapy due to its ability to transduce both dividing and nondividing cells, the capacity for imparting long-term transgene expression with a limited immune response, and. Previous studies have utilized rAAV serotype 2 predominantly and found that transduction of vascular cells is relatively inefficient. Using alternate serotypes of rAAV, particularly AAV1 and AAV5, we have observed significantly better transduction of the otherwise resistant vasculature, particularly in endothelial cells. These results suggest the unique potential of rAAV1 and rAAV5-based vectors for vascular-targeted gene-based therapeutic strategies.

Therapeutic gene transfer in cardiovascular diseases

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Standard techniques for the treatment of common cardiovascular diseases are often limited by the by-pass graft failure or restenosis occurring at the site of therapeutic intervention. Gene therapy is a novel rapidly evolving field of medicine, which potentially offers new treatments for cardiovascular diseases. With the use of gene transfer methods it is possible to modify somatic cells in vascular wall or myocardium to overexpress or inhibit pathologically important proteins and achieve therapeutic effects. Prevention of restenosis after vascular interventions such as percutanous coronary angioplasty (PTCA), percutanous peripheral angioplasty (PTA) or stent implantation, prevention of venous grafts failure and therapeutic angiogenesis are the major aims of experimental studies and clinical gene therapy. The promise of gene therapy in the treatment of cardiovascular diseases remains high. Experimental studies have well established the proof of principle that gene transfer to cardiovascular system can achieve therapeutic effects. First human clinical trials provided the initial evidence of feasibility and safety of the novel therapy. There are also first successful reports on the prevention of neointimal hyperplasia and promotion of therapeutic angiogenesis in clinical trials. However, there are still important questions regarding utility, efficiency and safety of gene therapy in the treatment of diverse cardiovascular diseases.

RNA interference strategies for inhibition of heme oxygenase-1 expression

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Background: Heme oxygenase-1 is a stress-inducible enzyme that catalyzes the degradation of heme to carbon monoxide, iron, and biliverdin, subsequently converted to bilirubin. Recently, it has been shown that the products of HO-1 activity exert numerous antioxidant and cytoprotective effects. Increased HO-1 expression has been observed in various tumours where it could contribute to growth, insensibility to oxidative stress, and angiogenesis. The latter effect might be mediated via upregulation of vascular endothelial growth factor (VEGF) - a key regulator of angiogenesis, whose induction is claimed to constitute a trigger of the "angiogenic switch" during cancer growth. Therefore, the inhibition of HO-1 in tumours could augment effectiveness of various therapies. Chemical inhibitors of HO, zinc or tin protoporphyrins, were shown to act unspecifically (1, 2), thus RNA interference is the method of choice for downregulation of HO-1. The aims of this study were to:1) design siRNA sequence that would efficiently silence heme oxygenase-1 (HO-1) expression, 2) to check the effects of siRNA-mediated inhibition of HO-1, 3) to construct vectors enabling efficient intracellular expression of chosen siRNA, and 4) to construct adeno-associated virus vectors that could provide effective and safe tool for obtaining long-term expression of siRNA *in vivo*.

Methods and results: Two hypothetical sequences of siRNA against HO-1 were designed according to the Ambion Guidelines. siRNAs were obtained by in vitro transcription with T7 RNA polymerase and introduced to cells by lipid transfection. Only one of two designed siRNA sequences caused a decrease in HO-1 mRNA, protein, and function, confirmed by RT-PCR, Western Blot, and HO-1 activity measurement, respectively. The inhibition of HO-1 was followed by the reduction of VEGF mRNA and protein levels, measured by RT-PCR and ELISA. The efficiency of transfection strongly depended on cell line and transfection agent as verified by fluorescein-labeled chemically synthesized siRNA. Vectors enabling intracellular expression of siRNA were constructed by ligation of U6 RNA promoter (cloned from HMEC-1 cDNA library) with the siRNA template. The complete cassette was ligated into pAAV-MCS vector, which may be used for construction of AAV vectors, and into pcDNA3.1+, which permits stable transfection of cells.

Conclusions: siRNA could be successfully used for inhibition of HO-1. The transfection efficacy, however, constitutes the limiting factor for the usage of this technology. Therefore, vectors enabling endogenous expression of siRNA seem to become more convenient tool both for research and for therapeutic purposes.

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1. Jozkowicz and Dulak, *Acta Biochim Pol. 2003: 50:69-79*
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Biotech Consulting – commercialization of scientific research in the area of biotechnology

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In terms of money circulation, the relationships between basic (life) science and biotechnology is described by this simple relation: basic (life) science transforms money into knowledge, whereas biotechnology converts knowledge into money. If the money coming from the commercialization of scientific results is bigger then the money directed to financing the scientific research, than the relationship between science and economy is proper. Such relationship is crucial for the knowledge based economical development.

Commercialization process of scientific research can be divided into two stages:

- creation of market products
- selling products on the market

Creation of market products

To provide something more from research than scientific paper(s) the following aspects, among others, have to be taken into account:

- The commercialization aspect of commercialization should be included into the grant formulation project
- Scientific results with the commercialization potential should be patented before their publishing
- The research procedures should meet the Good Laboratory Practice procedure

Selling products on the market

One of the most popular way of selling scientific intellectual property is by licensing the results of scientific research. A license is a right that is given to a person or to the company to use our intellectual property, which otherwise is unauthorized and illegal.

Upon granting the license, the patent owner does not divest himself of ownership in the patent or the technology in question and continues to exercise all rights as an owner therein.

The other one is assignment. An assignment is a complete transfer of one's rights and interests in a patent or other intellectual property to someone else.

In both cases, the results of scientific research have market value and are evaluated and priced.

SESSION VIII

MOLECULAR BACTERIOLOGY: MECHANISMS OF INFECTIONS AND METHODS OF DETECTION

Human infections caused by *Staphylococcus* spp

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Staphylococci are responsible for a great variety of diseases both community and hospital acquired. Out of many staphylococcal species described the most important is coagulase-postitive *Staphylococcus aureus* being the main human pathogen. Coagulase- negative staphylococci (CNS) although represented by a big number of species produce mostly infections in patients with multiple risk factors as well as immunocompromised host. Prosthetic devices including heart valves, endovascular prosthesis, LVADs (left ventricular assist devices) or orthopedic devices constitute major target for coagulase negative CNS, in particular *S.epidermidis*.

Staphylococcal diseases have different clinical and epidemiological patterns in the general community, in newborns, in menstruating women and among hospitalized patients. They comprise both local and generalized infections which can be life-threatening. Out of the localized infections in the community the most common are those involving purulent changes of the skin and soft tissue whereas in hospital setting surgical site infections. However, the most severe picture is observed in systemic or organ specific infections such as bacteremia, sepsis, endocarditis and pneumonia. The last entity is not only prevalent as nosocomial infection but also post-influenza complication. In addition to organism invasiveness related infections *S. aureus* produces several toxin-mediated diseases such as staphylococcal scalded skin syndrome, diarrhea and the most severe staphylococcal toxic shock syndrome. Several virulence factors enabling entry to the host, adhesion to the host tissues , spread especially through hematogenous route and tissue injury render this microorganism so successful. In addition to significant pathogenic potential *S. aureus* elaborated several mechanisms of antibiotic resistance through mutation or acquisition of foreign resistant genes. The most important is resistance to methicillin (MRSA) rendering this bacteria resistant to all beta-lactam antibiotics. The epidemic character of the resistance was recognized and clonal spread of MRSA has taken a global dimension. Resistance to other drugs is common especially among hospital derived MRSA. Recently, the most annoying has been an acquisition by MRSA strains glycopeptide resistance, considered as drugs of last chance.Traditionally, MRSA was considered a typical nosocomial pathogen, however it has been recently shown that MRSA can cause life threatening infections in the community such as necrotizing pneumonia and sepsis related to the production of Penton-Valentin leukocidin.

Molecular methods for identification of clinically important species of the genus *Staphylococcus*

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Staphylococci are one of the most common pathogens in hospital-acquired infections. Rapid and accurate identification of Staphylococcus species is necessary in order to further evaluate

the virulence potential and clinical significance of these microorganisms. Biochemical methods exist,though their overall accuracy is low due to variable expression of certain genes. Although these methods are still widely used, there is an increasing number of molecular techniques, such as PCR and probe based, for detection and identification of staphylococcal species. Publicly accessible electronic database focusing on comparative DNA sequence analysis of the genes of conserved macromolecules was developed in order to facilitate and improve diagnostics. Recent information and the features of various molecular diagnostic methods for staphylococcal species will be discussed.

Proteases of the *spl* operon of *Staphylococcus aureus*

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Staphylococcus aureus is a gram-positive human pathogen which causes a wide variety of infections ranging from superficial skin infections to endocarditic, osteomyelitis, toxic shock syndrome and finally sepsis. Among a great arsenal of virulence factors, like secreted toxins, immune-modulators and adhesion molecules or signaling factors, there are serine, cysteine and metalloproteases.

In our study we analyze the unique operon *(spl)*, which was shown to be present in more than 60% of *S. aureus* strains, encoding six serine protease-like proteins (Spl) named SplA-F. Spl proteins share from 44 to 95% amino acid sequence identity with each other and 33-36% sequence identity to staphylococcal serine glutamylendopeptidase commonly referred to as V8 protease. Contrary to known *S. aureus* proteases, Spl proteins do not posses pro-sequence of any kind, but typically for exoproteins they have a putative N-terminal signal peptide. According to previous works, purified SplB and SplC possess proteolytic activity against casein as shown by zymographic analysis. The reports, however, do not describe detailed characterization of the proteins nor any data on remaining proteases.

The scope of our investigation was to obtain each of the six active proteases of the *spl* operon in recombinant model and their detailed characterization. By means of molecular biology methods *spl* gen were cloned into pGEX vectors and expressed. The preliminary data showed however, that the recombinant Spl does not manifest any enzymatic activity against casein. Further experiments revealed that artificial, additional two amino acids present on the N-terminal of the recombinant proteins were responsible for such inactivation. The newly developed expression system allows production of active recombinant Spl proteins identical with naturally occurring ones which are currently being characterized.

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Study of the *stp* protease operon

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Staphylococcus aureus is the well-known etiologic agent of various diseases in humans and domestic animals. This bacterium secretes three types of proteolytic enzymes; serine, cysteine and metalloprotease that are consider as virulence factors. *S. aureus* produces two extracellular cysteine proteases referred as staphopain A (ScpA) and staphopain B (SspB). Takeuchi and co-workers, however, have purified and cloned an additional thiol protease expressed by *S. aureus* isolated from a chicken with dermatitis (Takeuchi *et al.*, 1999, Veterinary Microbiology 67, 195-202; Takeuchi *et al.*, 2002, Veterinary Microbiology 89, 201-210).

Staphylococcal thiol protease (StpC, staphopain C) is over-expressed by the bacteria since it constitutes *c.a.* 80% of the total protein in the culture fluid. The enzyme was purified to homogeneity with yield over 15 mg/l of media using ion exchange chromatography. StpC is cross-inhibited by the specific staphopain inhibitors, staphostatins A from *S. aureus* and *S. epidermidis* but not by staphostatin B. Sequence encoding mature form of StpC was cloned into pETDuet-1 vector. Lack of the expression of the recombinant protein suggests toxicity of the enzyme for *Escherichia coli*.

Analysis of DNA sequence downstream of StpC gene has revealed existence of an open reading frame coding for 105 amino acid protein with high homology to staphostatins; 54.3% and 36.2% identity to staphostatin A from *S. epidemidis* and *S. auerus* respectively. Further investigations into the sequence suggest a new operon in which, similarly to other staphopains, after *stpC* is the gene (*stpD*) encoding a putative inhibitor of the enzyme, staphostatin C. StpD coding sequence was cloned into expression vectors to study a role of the protein and its interactions with staphopains.

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Probing structural basis for inhibitory specificity of staphostatins

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The development of antibiotic resistance in Staphylococci requires alternative antibacterial strategies, and secreted bacterial proteases are considered to be possible targets for therapy. Recently, a new class of cysteine proteinase inhibitors has been identified. They were named staphostatins because of high specificity towards staphopains, staphylococcal secreted papain-like cysteine proteases. Staphostatin A inhibits staphopain A and staphostatin B inhibits staphopain B but no cross-inhibition was observed. Staphostatins bind directly to the enzyme's active site cleft in a substrate-like mode, avoiding cleavage due to unusual conformation of the P1 residue.

The structural basis for the specificity of staphostatins has been investigated. The employed strategy included the inversion of inhibitory specificity through progressive replacement of staphostatin B fragments by corresponding segments of staphostatin A. The staphostatin B variants with substitutions in the region defined as the inhibitor binding loop (Lys-94-Ile-103) and in an additional region of contact corresponding to the His-50-Ile-78 segment of the inhibitor were produced by site-directed mutagenesis, *E. coli* expressed, purified and compared to wild-type staphostatins A and B with respect to their inhibitory activity and affinity for staphopains.

Mutagenesis studies revealed that point mutations within the inhibitor binding loop either do not influence the inhibitory activity of staphostatin B or reduce it, whereas substitution of the entire binding loop extinguishes inhibition. However, the protein saves its affinity for the target protease (staphopain B). The exchange of the additional region of contact introduces the affinity for staphopain A yet without giving it the ability to inhibit it. Moreover, the inhibitor is still capable of reducing the activity of staphopain B, its original target protease. The replacement of both the binding loop and the additional region of contact switches the inhibition profile. Likewise, substitution for the C-terminal half of the molecule reverses the inhibition profile and increases the inhibitory activity of the mutant. Nevertheless, in all cases its affinity for the protease is much lower than that of the wild-type inhibitor.

Thus, we conclude that selectivity of staphostatins is dependent on the inhibitor's interaction with the enzymatic body along quite a substantial surface area including regions outside the active site cleft.

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Cyclin D2 in adult neurogenesis

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Adult neurogenesis, *i.e.*, proliferation and differentiation of neuronal precursors in the adult brain, is responsible for adding new neurons in the dentate gyrus of the hippocampus and in the olfactory bulb. We have recently described that adult mice mutated in the cell cycle regulatory gene *Ccnd2*, encoding cyclin D2, lack newly born neurons in both of these brain structures. In contrast, genetic ablation of cyclin D1 does not affect adult neurogenesis. Furthermore, we have shown that cyclin D2 is the only D-type cyclin (out of D1, D2, D3) expressed in dividing cells derived from neuronal precursors present in the adult hippocampus. In contrast, all three cyclin D mRNAs are present in the cultures derived from 5-day old hippocampi, when developmental neurogenesis in the dentate gyrus takes place. Thus, our results revealed the existence of molecular mechanisms discriminating adult *vs.* developmental neurogenesis.

Kowalczyk A., Filipkowski RK, Rylski M., Wilczynski G.M., Konopacki F.A., Jaworski J., Ciemerych M.A., Sicinski P., Kaczmarek L. The critical role of cyclin D2 in adult neurogenesis. *J. Cell Biol.,* 167: 209-213, 2004.

SESSION X
MOLECULAR BIOLOGY AND TUMOR IMMUNOTHERAPY

Effective photoimmunotherapy of murine tumors induced by the combination of photodynamic therapy and dendritic cells

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Photodynamic therapy (PDT) is an effective therapeutic modality used for the management of solid tumors. The unique mechanism of tumor destruction resulting from apoptotic and necrotic killing of tumor cells accompanied by local inflammatory reaction and induction of heat shock proteins (HSPs) prompted us to investigate the antitumor effectiveness of the combination of PDT with administration of immature dendritic cells (DCs). The PDT treatment protocol used in these studies resulted in a mixture of apoptotic and necrotic cells. PDT resulted in slight induction of HSP70 and HSP90 and redistribution of HSP60 from cytosol to plasma membranes of C-26 cells. Immature DCs co-cultured with PDT-treated C-26 cells efficiently engulfed tumor cell remnants and acquired functional features of maturation. DCs produced substantial amounts of IL-12. Inoculation of immature DCs into the PDT-treated tumors resulted in effective homing to local and distant lymph nodes and stimulation of cytotoxic activity of T and NK cells. The combination treatment produced superior antitumor activity not only against the PDT-treated tumor but also against tumor cells inoculated into a distant site. The feasibility and antitumor effectiveness demonstrated in these studies suggest that treatment protocols involving administration of immature DCs in combination with PDT may have clinical potential.

BCR/ABL recruits p53 tumor suppressor protein to induce drug resistance

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Tumors expressing the ABL oncoproteins (BCR/ABL, TEL/ABL, v-ABL) can avoid apoptosis triggered by DNA damaging agents. The tumor suppressor protein p53 is an important activator of apoptosis in normal cells; conversely its functional loss may cause drug resistance. The ABL oncoprotein - p53 paradigm represents the relationship between an oncogenic tyrosine kinase and a tumor suppressor gene. Here we show that BCR/ABL oncoproteins employ p53 to induce resistance to DNA damage in myeloid leukemia cells. Cells transformed by the ABL oncoproteins displayed accumulation of p53 upon DNA damage. In contrast, only a modest increase of p53 expression followed by activation of caspase-3 were detected in normal cells expressing endogenous c-ABL. Phosphatidylinositol-3 kinase-like protein kinases (ATR and also ATM) -dependent phosphorylation of p53-Ser15 residue was associated with the accumulation of p53, and stimulation of p21Waf-1 and GADD45, resulting in G2/M delay in BCR/ABL cells after genotoxic treatment. Inhibition of p53 by siRNA or by the temperature-sensitive mutation reduced G2/M accumulation and drug resistance of BCR/ABL cells. In conclusion, accumulation of the p53 protein contributed to prolonged G2/M checkpoint activation and drug resistance in myeloid cells expressing the BCR/ABL oncoproteins.

Plasmacytoid dendritic cells in host defense

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Due to their key role in switching between immunity and tolerance, dendritic cells (DC) are among the most promising cellular targets for therapy against immune diseases. Myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) are the two principal subpopulations of dendritic cells. PDC are morphologically similar to plasma cells and are distinguished functionally from mDC by their ability to produce massive amounts of type I interferons in response to viral and microbial stimuli. Although, both mDC and pDC have an ability to stimulate T-cell responses, there is increasing evidence that mDC and pDC can be differentially recruited to promote variable types of immunologic reactions. Since the generation and maintenance of immune responses largely depend on the migratory behavior of immune cells, PDC trafficking pattern will be a major focus of the talk.

SESSION XI
VARIA

Elastase and cathepsin G augment the level of biologically active soluble form of TNFα

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Elastase and cathepsin G, the proteolitycal enzymes of neutrophiles (PMN) localized in the azurophil granules, are involved in PMN responses to various stimuli. In the sites of inflammation they participate in the degradation of extracellular matrix proteins but they also may cleave the proteins involved in development of inflammation such as cytokines and cytokine receptors.

In the previous *in vitro* study elastese and cathepsin G were shown to degrade soluble TNFα. However, since membrane TNFα shows strong biological activity, especially in local inflammatory lesions, it was worth investigating whether neutrophil enzymes might also destroy membrane TNFα and limit its biological activities. To avoid a possible influence of neutrophil proteases on ADAM17, the secretase of TNFα, the majority of experiments were performed using ADAM17$^{-/-}$ fibroblasts stably transfected with cDNA of human proTNFα ADAM17$^{-/-}$TNF^{+} cells). Elastase and cathepsin G strongly diminished the level of TNFα on the cell surface as measured by flow cytometry and this process was accompanied by the accumulation of soluble TNFα in the culture medium. After cathepsin G treatment of the TNFα-positive cells the augmentation of the level of soluble TNFα correlated with increased biological activity of the cytokin. This was manifested by the increase of both cytotoxic activity of TNFα and its ability to serve as a co-stimulator in the induction of iNOS. Unlike cathepsin G, elastase increased the amount of biologically active TNFα only at low concentration of the protease. At high concentrations elastase was able to degrade released TNFα. We confirmed that elastase but not cathepsin G was able to degrade soluble form of TNFα and identified the C-terminal of Val 93 and Val 117 as the cleavage sites for elastase in the soluble TNFα molecule.

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EPR oxygen images of tumors in living mice correlate with spatially and quantitatively with Oxylite™ oxygen measurements

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Tumor oxygenation predicts cancer radio- and chemo-therapy response and malignant phenotype. Several oximetric techniques, including polarographic electrode, fluorescence, NMR and EPR are employed to study tissue oxygenation in patients and in animal models. Comparison of different oxymetries is crucial for the validation and understanding of these techniques. Electron Paramagnetic Resonance (EPR) Imaging is a novel technique for providing quantitative high resolution images of tumor and tissue oxygenation. Data presented here compare tumor pO2 values from EPR oxygen images with sequences of oxygen measurements made along a track with an Oxylite™ fluorescent oxygen probe.

EPR oxygen images used spectroscopic imaging techniques to measure in each image voxel the width of the spectral line from an injected water soluble trityl spin probe (OX063, Amersham Health R&D). A simple calibration allows direct, quantitative translation of each line width to an oxygen concentration. These 4D EPR images, obtained in 45 minutes from FSa fibrosarcomas grown in the legs of C3H mice, have the spatial resolution of ~1 mm and oxygen resolution of ~3 torr. The position of the Oxylite™ track was measured within a few millimeters accuracy using a custom stereotactic positioning device.

A total of 9 images that involve 17 tracks were obtained. Of these, 14 showed good correlation between the Oxylite™ measured pO_2 and a track located in the tumor within the uncertainties of the Oxylite™ localizability. The correlation was good both in terms of spatial distribution pattern and pO2 magnitude. The strong correlation of the two modalities corroborates EPR imaging (EPRI) as a useful tool for the study of tumor oxygenation.

The characteristic of cyanobacterial toxins and methods for their degradation

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The incidence of cyanobacterial blooms in waters, including drinking water reservoirs, has increased over the past few decades due to rising nutrient levels. Some species of freshwater blue-green algae like Microcystis, Oscillatoria, Nodularia, Anabaena and Nostoc can produce many types of hepatotoxins or neurotoxins that can be harmful to humans as well as animals. Most common - microcystins and nodularins, are chemically very stable cyclic peptide and conventional processes have only limited efficacy in removing them from potable water. Several promising chemical and physical methods of hepatotoxins degradation being under test are here presented. Furthermore, biodegradation of microcystins and the allelopathic antialgal activity of plant-producing compounds are discussed.

Photodegradation of the microcystin-LR in the presence of natural plants' products and chosen environmental factors

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The increasing eutrophication of water by human activity has influenced the increasing of frequently occurrence and intensity the cyanobacterial blooms, which can be source of the toxins. These toxins have been recognised to cause accute or chronic toxicity and might be lethal to wildlife, domestic livestock and even to humans. One of the most common bioactive substances produced by cyanobacteria are hepatotoxic microcystins, which are strong liver tumour promoters. Their occurrence has been reported all over the world and can be especially dangerous for the drinking water reservoirs, due to difficult degradation because of their stability. The microcystins might be degraded or transformed by using natural environmental factors like: cyanobacteria pigments, humic substances or iron salts, but the mechanisms these reactions and optimalisation of the conditions are still studied. In this work mainly is reported promising method of degradation of microcystin-LR-photosensitized decomposition in the presence of phycocyanin using UV radiation. Microcystin-LR decomposed limitedly by exposure with UV radiation alone, but the addition of the phycocyanin extracted from cyanobacteria accelerated this degradation. The results suggest that using phycocyanin may be complementary method for the removal microcystin-LR in the drinking water treatment procedures.

Targeting site specific chromosome integration

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Helper-dependent adenovirus (HDAd) has improved the safety profile and duration of transgene expression *in vivo*. However, HDAd vectors are non-integrating viral vectors, and might be eventually eliminated from the host by cell division or cell turnover. This is a potential drawback in treating genetic and acquired disorders that require long-term expression of therapeutic genes. As an alternative approach, we have previously reported the success of re-administration of HDAd vectors with an alternate serotype. In theory, with relatively prolonged transgene expression following periodic vector treatments, one can maintain a therapeutic level of transgene expression almost indefinitely. Transplantation of genetically modified bone marrow cells is an important technique for gene therapy as well as for studying the roles of immune cells in atherosclerosis. HDAd vector has no role in stably modifying these replicating cells. Therefore, vectors derived from retrovirus and lentivirus are used. The problem associated with these RNA viral vectors is their propensity to integrate their genes into active host genes in an apparently random fashion, which may contribute to uncontrolled cell division. There are two known systems which mediate site-specific integration into host chromosome. They are adeno-associated virus (AAV) and phage integrases.

In order to circumvent the limitations of currently used gene therapy vectors for replicating cells, we have developed HDAd/AAV and HDAd/ΦC31 integrase hybrid vectors. The genome of wild type AAV is integrated within human chromosome 19 in the latent state. However, recombinant AAV vectors lack this ability due to elimination of Rep gene from the vector. We employed a multiple vector system to provide Rep protein in *trans* by mifepriston-inducible gene expression. Hep3B cells were infected with these vectors containing βgeo (a fusion protein of LacZ and neo selectable marker) as a reporter/selectable gene, and selected in the medium containing G418. Six out of seven randomly selected clones had site-specific transgene integration detected by nested PCR. With the HDAd/ΦC31 integrase hybrid vectors, we used two vectors which contain βgeo + attB (integrase recognition sequence) or ΦC31 integrase. Approximately 20 clones were isolated after G418 selection and the confirmation for site-specific integration is underway. These hybrid vector systems have taken advantage of the high infectivity and large cloning capacity of HDAd vector. Further optimization and *in vivo* applications are warranted.

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The analysis of the effects of nitric oxide on TACE expression and activity

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Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) in large quantities by macrophages and other cell types during inflammation exerts numerous biological activities. NO acts as a potent cytotoxic agent against pathogenes but also plays numerous immunoregulatory roles.

We investigated a potential role of NO in the regulation of the xpression and activity of ADAM17/TACE (tumor necrosis factor alpha converting enzyme), the secretase responsible for the shedding of TNFα as well as numerous other molecules involved in inflammation, such as both TNFα receptors, L-selectin, TRANCE, IL1-RII among others.

Our experimental model included two cell types, murine microvascular endothelial cells (MBE) and murine monocyte/macrophage-like cell line (P388D1). Both cell types produce large quantities of iNOS-generated nitric oxide upon their stimulation with cytokines and/or bacterial LPS. The activity of TACE in these cells was determined by the measurement of the levels of released TNFα receptor I (p55) from the surface of MBE cells and that of TNFα from P388D1 cells. The effect of endogenous NO was evaluated by a comparison of the effects of cytokines/LPS in the presence or absence of L-NMMA, the competitive inhibitor of NOS. The effect of exogenous NO released from NO-donor, SNAP (S-Nitroso-*N* -acetyl-D,L-penicillamine) was also examined.

We found that neither endogenous nor exogenous NO affected the levels of TACE mRNA, thus nitric oxide does not disturb the process of TACE transcription or TNFα-mediated stimulation of *TACE* expression. Endogenous NO had no effect on PMA-stimulated release of TNFα and TNFRI from the cell membrane what indicates that the intracellularly produced compound does not influence TACE activity.

However, exogenous NO, without affecting cell viability, inhibited TACE activity as judged by a significant decrease of the amount of TNFα and TNFRI released from cells. These observations are in contrast to the previously published data suggesting that NO may play a role of TACE activator (1).

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Dopamine D3 receptor gene promoter as a model for studying the effects of antidepressant drugs

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Dopaminergic system determines a lot of crucial processes to human body for example it controls endocrine function, motor activity, emotions and memory.

Dysfunction of dopamine, its receptors and agents may result in psychic disturbances (schizophrenia, depressions) and neural disorders (Alzheimer's disease, Parkinson's disease or Huntington's disease) which may prevent patients from live an independent and active life.

The aim of the study is explanation of the mechanism of antidepressant drugs action on biosynthesis of dopaminergic receptor D_3. The effect of different pharmaceuticals on the activity of both promoters is analyzed by estimation of the level of reporter gene's product (luciferase). New pharmaceuticals which not cause serious side effects are still desired. Neuroblastoma cells transfected with plasmid containing promoter of dopaminergic receptor D_3 and luciferase gene acts as a model for preclinical tests.

Construction of bicistronic vectors for overexpression of VEGF-A and FGF-4

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Introduction: Cardiovascular disorders constitute the leading medical problems in the developed world. Some of them, for example ischemic heart disease, may result from impaired angiogenesis, particularly in response to hypoxia. Therefore, therapeutic angiogenesis represents an attractive treatment option for a large number of patients who are not candidates for traditional revascularization procedures. Consequently, the delivery of angiogenic growth factors, most commonly vascular endothelial growth factor (VEGF), is currently being investigated in numerous animal models and early clinical trials. Although such angiogenic treatments were followed by an improvement in perfusion and other cardiac functions, it has appeared that VEGF alone is unable to restore the complete vascularisation. Therefore, we have chosen to combine VEGF with fibroblast growth factor-4 (FGF-4), which has previously been shown to give promising results in cardiac gene therapies. To enable the simultaneous expression of both therapeutic genes, plasmids containing internal ribosome entry site (IRES) were constructed.

Methods and results: VEGF and FGF-4 cDNA sequences were excised from plasmids pcDNA3.1(+)VEGF165 and pCAGGS-HST, respectively. Required restriction sites were added via PCR reaction with specially designed primers. The inserts were subsequently digested and ligated into bicistronic pTR vectors to create following constructs: pTR-VEGF-IRES-GFP, pTR-FGF4-IRES-GFP, pTR-VEGF-IRES-FGF4, and pTR-FGF4-IRES-VEGF. The plasmids were obtained by the standard cloning techniques and verified by restriction analysis and sequencing. Thereafter, those vectors where used to transfect human microvascular endothelial cells (HMEC-1). The efficiency of transfection varied between 5 and 10%, as measured by fluorescent microscopy. The level of VEGF and FGF4 in culture medium was measured by ELISA after different time following the transfection. In case of plasmids pTR-VEGF-IRES-GFP and pTR-FGF4-IRES-GFP the amount of VEGF protein was always several-fold higher than the amount of FGF4. Furthermore, transfection with plasmids containing both genes of interest revealed that IRES-dependent translation resulted in severely impaired level of protein in comparison to the preceding gene. The influence of incorporating the Woodchuck hepatitis post-transcriptional regulatory element (WPRE) was also checked. Although WPRE significantly augmented the level of proteins, it was not capable of restoring the equal amounts of both of them. Subsequently, condition media from transfected HMECs are going to be used in HUVECs cultures in order to check their possible effect on HUVEC's proliferation, viability, and angiogenic potential.

Conclusions: Bicistronic vectors do not allow the equal expression of both genes. Therefore, to obtain similar levels of FGF4 and VEGF we suggest either using the plasmid pTR-FGF4-IRES-VEGF rather than pTR-VEGF-IRES-FGF4 or introducing therapeutic genes on separate vectors. Moreover, due to the fact that VEGF mRNA/protein stability is far higher than this of FGF4, it is crucial to establish the optimal levels of both therapeutic proteins. Finally, as the high level of therapeutic gene may have negative effects on well-vascularised tissues, we are going to introduce hypoxia-dependent regulation of expression. This would enable us to do hypoxia/ischemia targeted gene therapy of cardiovascular disorders.

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Construction, propagation and optimalization of transduction of AAV vectors to different cell types

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Background: Adeno-associated viruses (AAVs) are non-pathogenic replication-deficient human parvoviruses and AAV vectors are considered as promising and safe tools for gene therapy.

Methods and results: AAV vectors were produced using Stratagene's AAV Helper-Free System. Vectors were obtained by triple co-transfection of HEK293 cells (stably expressing the adenovirus E1 gene) with the following plasmids: *pRC* (encoding the viral *rep* and *cap* genes), *pHelper* (encoding adenoviral *E2A, E4* and *VA* proteins required for packaging of viral DNA to capsids) and the plasmids containing therapeutic or reporter (*LacZ*) gene of interest. The following plasmids have been obtained: pAAV-CMV-HO-1 (with the heme oxygenase-1 cDNA), pAAV-HRE-HO-1 (containing HO-1 cDNA under the control of Hypoxia Responsible element - HRE) and pAAV-CMV-BVR (encoding biliverdin reductase). All these plasmids were digested with restriction enzymes and sequenced in order to verify the correctness of cloning.

HEK293 cells were subjected to three rounds of freeze/thaw. For transduction of various cell lines both crude extracts and vectors purified on the heparin-agarose column have been used. The quantity of the viral capsids was measured with the AAV ELISA Assay from *Progen*. Amount of infectious particles was assessed by a serial dilution titration protocol. Additionally, the method of dot-blot titration of the AAV genomic DNA is being developed.

Efficacy of transduction of various cell types has been determined with AAV-LacZ vector. Effectiveness depends on the three factors: type of cells, vector concentration and a presence of topoisomerase I inhibitor – camptothecin. Preliminary results show that the most susceptible to transduction with AAV vectors are the HEK293 cells (37% even in the absence of the camptothecin, when $2*10^{10}$ capsids were used), B16 melanoma cells (36% with $4*10^{10}$ capsids and 1 μg/ml camptothecin), human microvascular endothelial cells (HMEC-1) (20%, with $1{,}5*10^{10}$ capsids in the absence of the camptothecin), HeLa cells (13%, with $2*10^{10}$ capsids and 1 μg/ml camptothecin), HaCaT keratinocytes (4%, with $2*10^{10}$ capsids and 2 μg/ml camptothecin). In contrast, C26 adenocarcinoma cells are ineffectively transduced (below 1% with 10^{10} capsids and 1 μg/ml camptothecin).

Conclusions: AAV vectors can be produced in HEK293 cells using Stratagene Helper Free System in quantities sufficient to transduce various cell types in vitro. Transduction efficacy varied between different cells and could be improved in certain cases by camptothecin.

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Cell-specific targeting of adeno-associated virus vector

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Gene therapy presents a vivid perspective for curing many diseases. Though delivery of naked DNA to cells *in vivo* often appears to be very difficult, scientists developed viral vectors, which on one hand possess natural property to deliver DNA into cells, and on the other can be packed with desired therapeutic gene. Adeno-associated virus (AAV) drew much attention with regard to viral-based gene delivery systems, as they present lack of immunogenicity, ability to infect non-dividing cells, are not associated with any known disease.

Their cell entry comprises two steps. First, they attach to cell's surface via heparan sulfate proteoglycan (HSPG), and then they are bound by fibroblast growth factor receptor-1 (FGFR-1) and undergo receptor-dependent endocytosis. Thus, they are able to transduce only cells which present on their surface these two molecules. However it was shown that abundance of secreted HSPG may function as a trap for AAV and impair infectivity. Therefore in order to transduce cells of interest, two goals must be achieved:

- deprive the vector of the ability to bind HSPG,
- assure it's potency to bind antigens presented by cells of interest.

The poster briefs the ideas of targeting AAV vectors by various means, from chemical conjugation of viral capsid with biotin, to insertion of specifically targeting peptides into virus coat protein.

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Effect of heme oxygenase-1 on keratinocyte proliferation and migration

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Background: Keratinocytes are the prevalent cell type of human skin. Their migration and proliferation is required for the process of reepithelialization after tissue injury. In the wound, keratinocytes are the major source of vascular endothelial growth factor (VEGF), whose expression was shown to be upregulated by heme oxygenase-1 (HO-1), an inducible enzyme catalyzing the cleavage of heme, to biliverdin, carbon monoxide (CO) and iron ions. The aim of this study was to determine the influence of HO-1 on keratinocyte proliferation, migration and production of VEGF.

Methods and results: All experiments were performed using human immortalized keratinocyte cell line (HaCaT). Expression of HO-1 protein was undetectable in resting, unstimulated cells. It was, however, potently and concentration-dependently induced by 1 h or 6 h treatment with heme chloride (hemin; 10-100 μM), followed by a 23 h or 18 h incubation in fresh culture media, as demonstrated by RT-PCR and western-blotting.

Bromodeoxyuridine incorporation assay revealed that the high doses of hemin (30 and 100 μM) decreased the proliferation of keratinocytes (48 ± 27 % of the control level). This effect was not associated with any cytotoxicity, as assessed by the measurement of lactate dehydrogenase release and 3-(4,5-cimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide/MTT colorimetric assay). In accordance, incubation of keratinocytes in the presence of exogenous CO (500 and 1500 ppm) also results in inhibition of cell proliferation in a concentration-dependent manner.

Differently than in the case of proliferation, we were not able to demonstrate any significant influence of hemin on the migration of keratinocytes in a scratch assay, an in vitro model of re-epithelialization during wound healing. However, the HO inhibitor, tin protoporphyrin (SnPPIX), significantly delayed scratch closure. Importantly, effect of copper protoporphyrin (CuPPIX), which is not an HO-1 inhibitor, was much less pronounced. This suggests that the effect of SnPPIX might be dependent on HO-1 inhibition. Further analyses are necessary to confirm this supposition.

Conclusions: Compounds modulating HO-1 activity may influence the proliferation and migration of keratinocytes. It suggest that HO-1 pathway might play a role in regulation of wound healing.

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Angiogenic transcriptome in human keratinocytes and microvascular endothelial cells: macroarray and real-time PCR analysis

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Background: Decreased oxygen tension is a crucial mediator of the formation of new capillaries from preexisting vascular structures. This process, termed angiogenesis, is a hallmark in the pathology of many diseases and is also prerequisite for a proper wound healing. So far, however, limited data are available on the global analysis of angiogenic gene expression in human keratinocytes and endothelial cells which are both involved in wound healing process.

Results: Macroarray analysis has shown that hypoxia enhanced or decreased the expression of several genes involved in angiogenesis in HaCaT and HMEC-1 cells. In HaCaT keratinocytes hypoxia up-regulates very strongly (> 5 times) the expression of vascular endothelial growth factor-A (VEGF-A), the major angiogenic mediator. This effect was confirmed by RT-PCR for VEGF mRNA and ELISA for VEGF protein. Also the expression of integrin-α-5 and endoglin was enhanced more than 10 times and the expression of their ligands, fibronectin and TGFβ1, respectively, was increased. In HMEC-1 hypoxia strongly induced expression of VEGF-A and additionally augmented fibronectin, endoglin, TGFBR1, VEGF-D and prothrombin expression. In both cell lines the expression of maspin, anti-angiogenic mediator was decreased by hypoxia but real-time analysis showed different level of mRNA for this factor in HMEC-1 and HaCaT cells. Ct value (threshold cycle) for maspin in HaCaT keratinocytes was ~ 21 whereas in HMEC-1 it was ~ 34 indicating that maspin plays crucial role in HaCaT but not in HMEC-1 cells. Moreover, thrombospondin-1 (TSP-1) expression was down-regulated in both cell lines while tissue inhibitor of matrix metalloproteinases-1 and 2 (TIMP-1 and TIMP-2) were down-regulated only in HMEC-1. Interestingly, macroarray hybridization demonstrated that hypoxia augmented the expression of placenta growth factor (PlGF) in HaCaT cells and real-time PCR also shown ~15 times induction of PlGF. In contrast we did not observe such an effect in HMEC-1 .

Conclusions: Hypoxia significantly influences expression of several angiogenic mediators in human keratinocytes and endothelial cells. The pattern of genes influenced by decreased oxygen tension is different in these two cell lines what indicates their specificity and controlled mechanism of action. Present data points to potential targets for treatment of cutaneous diseases which are related to enhancement (eg. psoriasis) or impairment (eg. chronic diabetic wounds) of angiogenesis.

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The relationship between DNA damage and the reactive oxygen species generated during the oxidative burst in neutrophils from healthy donors treated by quercetin and etoposide *ex vivo*

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One of the most important questions in chemotherapy regards the possibility of prevention of DNA damage in healthy bone marrow cells during the etoposide treatment without lowering of the antitumor potency of the drug. Our recently published results provide the evidence that quercetin when applied both orally and/ subcutaneously protect the bone marrow cells in rats against genotoxicity of etoposide treatment due to an enhancement of antioxidant protection. At this study we attempted to elucidate can such results be related to the modulation of amount of reactive oxygen species released during the oxidative burst in neutrophils, investigated by chemiluminescence's assay. Moreover, the investigated effect was related to the extent of DNA damage in neutrophils evaluated by alkaline version of comet assay. Etoposide treatment enhanced the superoxide production in unstimulated neutrophils in dose-dependent manner (25 - 150 μM) and drastically induced the DNA damage. Quercetin, when used at the same concentrations, significantly decreases the amount of superoxide formation and the amount of DNA released to the comet tail is 10 fold lower as compared with etoposide treatment. However, both compounds inhibited the superoxide production in dose-related manner in neutrophiles activated by latex confirming their antioxidant ability. The 50 times lower concentration of quercetin is needed to give the maximal inhibition (by 30%) of superoxide production as compare to etoposide. The 10 μM quercetin entirely inhibited the latex stimulated production of superoxide. When, both compounds were applied, 1 μM quercetin enhanced the inhibitory activity of etoposide up to 50% what was also reflected in twice lower level of the DNA damage comparing with etoposide used alone. The study provides the evidence that DNA damaging activity of etoposide is strictly related to the superoxide production and the strong antioxidant activity of quercetin can protect the cells treated by etoposide due to scavenging the reactive oxygen species.

The oxidative DNA damage in lymphocytes is associated with catechol-O-methyltransferase polymorphism as well as folate and the 17β- estradiol concentrations in plasma of pregnant blood donors

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The pre-long exposure on the higher level of estrogens, one of the factors affecting genomic stability, can create the ground for further mutation. One of the reasons for that is the production of strongly reactive metabolites of the 17β-estradiol, catechol estrogens which if not detoxified can undergo the metabolic redox-cycling causing the oxidation of DNA bases. This reaction can be blocked via several detoxication pathways, including o-methylation of catechol estrogens by catechol-O-metyl transferase (COMT). COMT is polymorphic in human population, which results in differences in enzyme activity and causes different detoxication of catechol estrogens The aim of our study was to find out whether there is an association between the 17β-estradiol concentration in serum, the catechol-O-methyltransferase polymorphism folate concentration in plasma and the oxidative DNA damage in women's lymphocytes during the first trimester of pregnancy. The level of oxidative DNA damage was evaluated by the comet assay (using the specific DNA repair enzyme, endonuclease III) in lymphocytes isolated from the blood of 43 healthy women between the 6th to 12th week of pregnancy. The extend of oxidative DNA damage was only weakly associated with the 17β-estradiol concentration ($p=0,11$) but significantly depended on the catechol-O-methyltransferase polymorphism ($p=0,04$). More damage was observed in subjects with the combined *COMT* (HL+LL) genotype as compared to the *COMT* HH donors. In turn, the significant correlation was found between the folate serum concentration and the extend of the endogenous oxidative DNA damage ($p=0,03$). This indicates that the efficiency of the catechol estrogens removal from the circulation, which depends on the COMT activity as well as on the sufficient amount of folate, strongly influences the genomic stability, as measured by the extend of the oxidative DNA damage.

The antiinflammatory effect of acetylene compound from *Tanacetum parthenium. In vitro* study in HepG2 and HaCaT cell lines

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It was found previously (1) that in astrocytoma cell line the diacetylene compound (TP) clearly reversed some proinflammatory effects of IL-1 and oncostatin M (OSM), such as activation of transcription factors and modulation of antioxidant enzyme gene expression.The induction of a basal expression of antioxidant enzymes (catalase - CAT and glutathione synthetase - GSC) by TP, may play a protective role against reactive oxygen species in this cell model. The antiinflammatory effect of TP was restricted, however, to the low doses of this compound (5-10 μM).

The aim of this study was to compare the antiinflammatory activity of TP in other cell lines – HepG2 and HaCaT. Similarly to the astrocytoma cell line, in HepG2 and HaCaT cells the lower doses of TP (5 μM) exerted the strongest inhibitory activity towards IL-1-induced NFκB and AP-1 activation, while the higher concentrations of the drug were less active. The activation of STAT induced by IL-6 (but not by IFNγ) was downregulated by TP also in dose-dependent manner (2). The protective role of TP against H_2O_2 - caused leakage of LDH was observed in HaCaT cells, demonstrating its antioxidative activity. In both analysed cell lines TP was potent to modulate mRNA expression of some proinflammatory and antioxidant enzymes genes.

Conclusions: Antiinflammatory and antioxidative effects of diacetylene compound isolated from *T. parthenium* is not tissue-type specific as the results obtained in brain-derived cells (U373) were confirmed in hepatoma and keratinocyte cell lines.

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2. Stalińska K., Górnicka A., Malicki S., Sroczyńska P., Kisiel W., Guzdek A. A new diacetylene compound from *Tanacetum parthenium* modulates *in vitro* activation of transcription factors.13th International Symposium of Polish Network of Molecular and Cellular Biology, Kraków, 3-4 06, 2004

Comparison of anti-inflammatory properties of sesquiterpene lactone and its glycoside isolated from *Taraxacum obovatum*

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Several species of Asteraceae family contain a second metabolites - sesquiterpene lactones (SLs). These compounds are known to possess a wide range of biological and pharmacological activities including anti-inflammatory, antitumor and antimicrobial effects. A terpenoid C-15 sceleton and a γ-lactone moiety are common structural features of this class of compounds, whereas other structural elements can vary, influencing their biological activity. The sesquiterpene lactone and its glycosidic derivative were isolated from Taraxacum obovatum by Kisiel et.al. and the anti-inflammatory potential were evaluated in astrocytoma cell culture. This was acomplished by evaluation of the binding activity of transcription factors NFκB and AP-1 (known to be involved in the activation of several genes in inflammation). Moreover, the expression of several proinflammatory genes including antioxidant enzymes, IL-6 and antichymotrypsin genes was analysed.

After reaching the subconfluent stage, astrocytoma U373 cells were treated with proinflammatory cytokine – IL-1 (10 ng/ml) and/or sesquiterpene lactones (5, 50 μM/ml) for 60 min for nuclear proteins isolation and for 8 h for RNA isolation. EMSA was used for evaluation of NFκB and AP-1 binding and Northern blot for mRNA abundance.

Results: In U373 cell line sesquiterpene lactone in the concentration of 5 and 50 μM/ml exerted dose-dependent inhibitory activity towards IL-1-induced NFκB and AP-1 binding, while the effect of its glycoside was minor. In astrocytoma cell line IL-1 up-regulated expression of mRNA of manganese superoxide dismutase, IL-6 and antichymotrypsin, while down-redulated the expression of catalase, glutathione peroxidase and glutathione syntethase. Sesquiterpene lactone (5, 50 μM/ml) dose-dependently reversed these IL-1 elicited changes. Its glycosidic derivative was inefective or only weak effect of higher concentration of this compoud was observed.

Conclusions: Sesquiterpene lactone isolated from T. obovatum is able to inhibit IL-1-induced activation of NFκB and AP-1, and reversed the effects caused by IL-1 towards expression of several pro-inflammatory genes. Glucose moiety bound to sesquiterpene lactone substantialy abrogate the benefitial, anti-inflammatory effects.

The relationship between the polyphenol content and the protective ability against oxidative DNA damage of the Mediterranean plant extracts

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The protective ability of plant extracts was reported in many recently published studies. We studied the relation between the protective ability of 103 plant extracts from the Mediterranean region against oxidative DNA damage induced by hydrogen peroxide (25 μM, 5 min. at 4^0C) in lymphocytes isolated from male donors, and their polyphenol content. Fifty-five percent of investigated plant extracts at the concentration 10 μg/ml provided a significant antioxidant protection evaluated by a single cell micro-gel electrophoresis. This ability was, however, not related to their polyphenol content. In further studies six plant extracts were chosen to analyze the dose-response effect and the changes in total radical oxygen species level: three of them (*Amaranthus sp., Scolymus hispanicus, Thymus piperella*) having over 20% anti-oxidant protection , and three (*Crepis vesicaria, Origanum heracleoticum, Scandix australis*) with under 20% capability. Each extract, except *Scandix australis*, showed the dose-dependence protection within 0.1 to 10.0 μg/ml concentrations. The 100 μg/ml concentration of each plant extracts occurred much less protective, independently on the polyphenols content. When plant extracts were grouped according to the mean of polyphenol content, for the concentrations of plant extracts below 100 μg/ml, the protective ability increased with the dose up to 0.3 μg/ml of polyphenols but decreased when concentrations exerted 1 μg/ml of polyphenols. These six plant extracts did not influence the capacity of the repair process while being present for 2 h period after the hydrogen peroxide removal. The radical oxygen species scavenging activity of plant extracts in relation to polyphenols content and the DNA protective ability was also investigated and are discussed separately for each chosen extracts.

In conclusion, the protective ability of the plant extracts, when investigated in the lymphocyte model system, showed that a more complex mechanism is responsible for protection than only the antioxidant ability. Furthermore, it looks like that when the plant extracts exhibit the dose-dependent DNA protection, the density of polyphenols in these extracts plays more important role in their protection than the composition of polyphenols itself.

The influence of low density lipoproteins on peripheral blood monocytes function

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The infiltration of blood monocytes into the subendothelial tissue is a key event during atherogenesis. These cells differentiate into tissue macrophages that ingest modified lipoproteins and transform into lipid-loaded foam cells. In contrast to the extensive studies of foam cells formation and function, little is known about the reactivity of peripheral blood monocytes to modified lipoproteins. Low levels of oxidized low density lipoproteins (OxLDL) are present in human plasma and are known to increase in certain pathological conditions including acute myocardial infarction and carotid artery atherosclerosis. This suggests that circulating monocytes are in contact with increased, non physiological levels of modified plasma lipoproteins and prompted us to study the influence of LDL on monocyte function. Elutriation purified human blood monocytes and low density lipoproteins isolated from healthy plasma donors were used either unmodified - native LDL (nLDL) or oxidated with Cu^{2+} (OxLDL). Both were applied at the range of concentrations that were not cytotoxic for monocytes and the production of TNFαand IL-10 was measured after LPS stimulation. We demonstrate that the lipopolysaccharide (LPS)-induced production of IL-10 is strongly reduced in the presence of OxLDL in the concentration dependent manner, while production of pro-inflammatory cytokine TNFα is either not affected or even up-regulated. These data indicate that depending on oxidation status lipoproteins may influence the balance of pro- and anti-inflammatory cytokines produced by monocytes infiltrating arterial wall.

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Interaction of human peripheral blood monocytes with apoptotic cells and bacteria

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Human monocyte-derived macrophages efficiently phagocytose apoptotic neutrophils. In contrast to macrophages peripheral blood monocytes (MO) are not considered as cells able to engulf apoptotic cells. They are recruited into inflammatory sites and are believed to contribute to the immune responses by both as professional phagocytes and as antigen presenting cells but their interaction with apoptotic cells was not studied. Recently we observed that MO while in contact with apoptotic cells produce more anti-inflammatory cytokine IL-10 while the production of pro-inflammatory cytokines is rather reduced. Furthermore using flow cytometry, we provided some preliminary data suggesting that MO bind and/or engulf apoptotic cells. These data prompted us to ask whether: a) MO can phagocytose apoptotic neutrophils, b) monocytes stimulation with pathogens has any impact on recognition of apoptotic cells, and c) phagocytosis and killing of microorganisms by MO in contact with apoptotic is compromised. To answer these questions we used purified MO and neutrophills (PMN) of which some were induced to apoptosis by overnight culture without stimulation. As pathogen serum opsonized *S.aureus* was used. In some experiments monocytes and/or PMN were labelled with vital dyes PKH26 or PKH67 and *S.aureus* was labelled with FITC. The data obtained using confocal microscopy clearly indicate that MO engulf apoptotic but not freshly isolated neutrophils. Activation of MO with patogens or their products slightly reduced the ability of MO to phagocytose apoptotic neutrophils however, contact of MO with apoptotic cells had no influence on their ability to phagocytose and kill bacteria. These data suggest that MO that arrive into inflammatory site although contribute to resolution of inflammation are capable to effective elimination of pathogens.

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The cytokine production and phenotype of peripheral blood monocytes in patients with atherosclerosis

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Endothelial dysfunction, characterized by the loss of nitric oxide bioavailability, has been shown to precede atherosclerotic lesion development. In parallel to the loss of anti-atherosclerotic properties of endothelium, a robust activation of platelets and monocytes is observed. This prompted us to analyse the phenotype and function of peripheral blood monocytes in atherosclerotic patients in order to select parameters to be focused on during *in vitro* studies of monocytes interaction with dysfunctional endothelial cells. We present our preliminary data regarding CD16-positive monocytes with different surface expression of CD14. We looked at this population as we recently observed that $CD14^{++}CD16^{+}$ monocytes are main IL-10 producers in contrast to $CD14^{+}CD16^{+}$ monocytes that produce TNF. The proportion of monocyte subsets and cytokine production was measured in mononuclear cells isolated from patients and controls. In addition cytokine production was measured in cultures supplemented with apoptotic neutrophils as monocytes produce more IL-10 when activated in contact with apoptotic cells. We found the significantly higher proportion of $CD14^{++}CD16^{+}$ monocytes in patients than in controls. Furthermore, within patients group the proportion of $CD14^{++}CD16^{+}$ monocytes was significantly higher in patients with diabetes, high cholesterol level and those with myocardial infarct or stroke in anamnesis. The proportion of $CD14^{++}CD16^{+}$ monocytes correlated with increased IL-10 production and augmentation of IL-10 secretion in the presence of apoptotic cells. The increased production of IL-10 in the presence of apoptotic cells was not correlated to the reduced TNFα production. Finally, the activation of monocytes as evidenced by the increased spontaneous superoxide formation was in a group of patients with cardiac infarct or stroke in anamnesis. In summary our preliminary data strongly suggest that the increased proportion and activity of $CD14^{++}CD16^{+}$ monocytes is characteristic for atherosclerotic patients.

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Proteolytic activation of chemerin, a novel chemoattractant protein

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Chemerin is a chemoattractant protein, which acts through ChemR23 (chemerinR), a receptor expressed in immature plasmacytoid dendritic cells (PDC). Chemerin is secreted as an inactive precursor that undergoes proteolitic activation into highly specific agonist of ChemR23. Proteolytic activity responsible for chemerin activation remains unknown. Plasmacytoid dendritic cells are professional antigen-presenting cells (APC) that play a key role in both innate and adaptive immunity. In the present work we examined proteases synthesized by *Staphylococcus aureus* and human neutrophils as potential activators of chemerin. We demonstrate that cysteine protease SspB of *S. aureus* as well as proteases present in neutrophil granules convert full length chemerin into its active form via proteolitic cleavage. These findings suggest that indicated proteases contribute to a selective PDC recruitment to inflammatory sites.

Hyaluronan binding to tissue-specific endothelial cells

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Tissue-specific heterogeneity of endothelial cells, both structural and functional, plays a crucial role in physiologic as well as pathologic processes, including inflammation, autoimmune diseases and tumour dissemination. Recognition of vascular endothelium by blood-borne circulating cells and their subsequent migration through the vessel wall is mediated by multistep process that primarily involves; i/ selectins and their carbohydrate ligands, ii/ chemoatractants and their receptors, and iii/ integrins interacting with members of the immunoglobulin gene superfamily. Nonetheless, other primary (rolling) interactions have been described that involve hyaluronic acid (HA) present on endothelial cells, and its main receptor CD44 expressed on circulating leukocytes. The interactions between HA and CD44 also contribute to tumor metastasis. While the regulation of the CD44 expression and function on either leukocytes or tumor cells has been well characterised, much less is known about the ability of endothelial cells to expose HA on their surface. Therefore, in these studies we analysed HA surface levels in tissue-specific endothelial cells. Our results indicate that HA is retained on the surface of majority of endothelial cells examined through CD44-independent mechanism.

Comparison of gene expression profiles and signal transduction pathways induced by PDGFRα and PDGFRβ homodimers

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Platelet-derived growth factor (PDGF) is major *in vivo* mitogen and it exerts its stimulatory effects on cells by binding to two related protein tyrosine kinase receptors, PDGFα receptor (PDGFRα) and PDGFβ receptor (PDGFRβ) with different affinities. The different isoforms of PDGF give slightly different cellular effects which may be explained by their different interaction with the α and β receptors. Thus the expression of the two receptor types will determine the responsiveness of a cell to the different PDGF isoforms. The two closely related PDGFR isoforms share many signaling pathways. However, studies in tissue culture have indicated differences in the signal transduction pathways induced by the two PDGFR isoforms, i.e. Crk is only recruited to the PDGFRα and RasGAP only to the PDGFRβ. In this project we decided to focus our work on identifying differences in gene induction by the two PDGFR isoforms. Two PAE cell lines with PDGFR isoforms served as a system for investigation of the differences in the gene profile. These cell lines were expressing only one type of receptor. Using cDNA microarray technique we demonstrated that the majority of the genes induced by the two PDGFR isoforms were common for both isoforms. However, we were able to find genes which expression was significantly changed by one receptor isoforms but not the other. The microarray screen revealed 24 genes uniquely upregulated by PDGFRβ and 9 by PDGFRα. Three genes of interest that participate in MAP kinase pathway suppression were chosen for further investigation, i.e. MKP1, MKP2 and MKP3. The choice was based on the ability of the corresponding gene products to dephosphorylate MAP kinases in a specific manner. MKP1 selectively associates with p38, JNK and ERK1/2. MKP2 shows preference towards ERK1/2 and JNK whereas MKP3 selectively dephosphorylate ERK2. Using real-time PCR we were able to confirm the microarray results obtained for those genes. Furthermore, the phosphorylation of ERK and p38 was investigated by the Western Blot analysis in both PAEα and PAEβ cell lines. In PAEβ cells the ERK phosphorylation was sustained, whereas it was strongly decreased within 1 hour in PAEα cells. Moreover, p38 phosphorylation was decreased in PAEβ. These results correspond to the data obtained from cDNA microarrays.

Can we use proteasomal inhibitors in cancer therapy?

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This year Nobel Prize in Chemistry went to Aaron Ciechanover, Avram Hershko and Irvin Rose for the discovery of the ubiquitin mediated proteolysis degradation. That's why it's not surprised that a lot of research projects concern this process. It's clear now that degradation of cellular proteins is a highly complex and temporally controlled process that plays a major role in the cell. Degradation of proteins via proteasome includes two steps:

1) conjugation of multiple ubiquitin moieties to the substrate;

2) degradation of a tagged protein by 26S proteasome complex.

The activation of transcription factor NFκB depends also on proteasome degradation. NFκB is sequestered in the cytoplasm and rendered inactive by its inhibitor proteins IκB. After stimulation (LPS, TNFα, IL-1, IL-2, UV) IκB is phosphorylated and dissociate from NFκB, which translocates to nucleus and regulate expression of a wide range of genes, including those involved in cell survival, cell adhesion and antiapoptotic genes (Bcl-2, cIAP).

Proteasome is excellent target for cancer therapy because of its critical metabolic function. Proteasome inhibition enhances the sensitivity of cancer cells to traditional anticancer agents in both in vitro and in vivo preclinical studies. It has been noted that actively dividing cells are more susceptible to proteasome inhibition than are quiescent or differentiated cells. There is a lot of known proteasome inhibitors: natural (lactacystin, epoxomicin) and synthetic (peptide aldehydes, vinyl sulfones). The peptide vinyl sulfones and natural inhibitors of proteasome bind to 20S core particle in an irreversible manner such that proteolytic activity cannot be restored upon their removal. That's why this problem was solved by replacing the aldehyde group with boronic acid - peptide boronates dissociate more slowly from the proteasome. Dipeptide boronic acid - bortezomib is of particular interest from a clinical perspective. Bortezomib is first molecule in this class to reach clinical trials in cancer patients.

Bortezomib is highly toxic against a broad range of cancer cell lines in vitro, induce apoptosis in spheroid cell cultures as in monolayer cultures (so it can be effective against solid tumors and hematological malignancies). What's the most important proteasome inhibition with bortezomib also sensitized cancer cells to ionizing radiation. Bortezomib inhibits blood vessel formation in embryonic CAM (densely vascularized tissue commonly used to assess antiangiogenic drugs) and what's the most important can be used in therapy of those cancers where the NFκB pathway is constitutively active and is associated with resistance to anticancer therapy (adenocarcinoma, melanoma, lymphoma).Bortezomib is the only proteasome inhibitor to have progressed to clinical trials in cancer therapy (data from the May of 2004).

Proteasome inhibitors can be used in cancer therapy. The proteasomal inhibitor – bortezomib in preclinical studies was found as an excellent compound with antitumor activity. There is a chance that bortezomib can be used in more type of cancer therapies.

Construction and application of plasmid vectors for the expression of recombinant chemokine CCL28 in mammalian cells, yeasts and bacteria

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The chemokine CCL28 called also Mucosae-associated Ephitelial Chemokine (MEC) is a novel chemokine belonging to the second group of chemokines (CC or β - chemokines).It is expressed by epithelial cells. MEC regulates T-lymhocytes and blood eosinophile migration signaling through CCR10 and CCR3 receptor, respectively. It was known from preliminary observations (C. Kieda *et al.,* unpublished) that CCL28 recognised lung endothelial cells better than endothelial cells from other origin. Therefore, it could be used as a tool in gene therapy for treatment of Cystic Fibrosis (CF) for targeting the gene of interest to the lung tissue with the aim of helping with the transcitosis of the DNA complex used for gene therapy. The aim of this study was production and purification of the recombinant human chemokine CCL28. Several expression vectors were constructed in order to produce the chemokine of interest either in bacteria or in yeast.

Studies on the proteome of cytokine-stimulated HepG2 cells

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Liver cell response to inflammatory cytokines is manifested by a characteristic change in the profile of synthesized plasma proteins (acute phase reactants). Paradoxically, much less is known about cytokine-induced changes in the metabolic rates of the hepatocyte cellular proteins.

Interleukin-1 (IL-1) and interleukin-6 (IL-6) are principal proinflammatory cytokines inducing the acute phase response of various tissues, including liver.

We report the results of studies concerning the effects of IL-1 and IL-6 on the cellular proteome of human hepatoma HepG2 evaluated by 2D electrophoresis and mass spectrometry.

HepG2 cells were cultured in DMEM and in order to simulate the *in vivo* situation relatively low doses of cytokines were used in agreement with sequential appearance of these cytokines in the inflamed tissue.

Cells were collected, disrupted by sonication in the lysis buffer containing 8 M urea and the soluble proteins were separated by 2D polyacrylamide gel electrophoresis.

The gels were stained with Coomassie Brilliant Blue R-250 or SYPRO Ruby and in additional experiment *de novo* synthesized proteins were labeled with [^{35}S]-methionine. All results showed a similar pattern of protein changes. The protein spots showing different intensity in comparison to control unstimulated cells were subjected to mass spectrometry analysis by LC-MS/MS.

We identified 7 proteins that amount increased by IL-1/IL-6 treatment (calreticulin, Mn-dependent superoxide dismutase, electron transfer flavoprotein, heterogenous nuclear ribonucleoproteins A2/B1, 60 kDa heat shock protein, protein disulphide isomerase and cophilin), and 2 proteins with their level decreased by cytokine stimulation (fatty acid binding protein and radixin). Functionally, all these proteins either participate in cellular metabolic processes or are engaged in gene expression, protein synthesis and folding.

Inhibition of premature ageing of sugar-treated human fibroblasts via heat shock

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Chronic exposure of cell to sugars seems to be the main cause of cellular ageing. Reducing sugars such as glucose, fructose and their derivatives e.g. glyoxal can not only generate free radicals, but also react non-enzymatically with proteins, nucleic acids and lipids giving protein-sugar, lipid-sugar and nucleic acid-sugar adducts. These adducts can rearrange to the so called Amadori products, whose subsequent rearrangement can lead to advanced glycation end products (AGEs). AGEs accumulate within the cell causing its senescence and impairment of its functioning. For that reason diabetic patients exposed to high glucose doses in the blood display hallmarks of premature ageing.

In the present study we addressed the question, whether heat shock has protective effects on sugars-treated cells in culture, which was shown previously to be a very potent anti-aging agent (Rattan, 1992).

First part of our experiments was to assess pro-ageing effects of sugars using cell model, the second was to investigate, if heat shock (HS) has protective effects on cells, which would allow to inhibit of premature aging induced by sugars. Following assays were performed: one step growth curve experiment, "counting" experiment, Giemsa staining, β-galactose staining and apoptosis assay. The results obtained confirmed that sugars are strong inducer of premature cell senescence. Importantly, heat shock pretreatment seems to prevent cells from undergoing sugar-induced ageing caused by sugars.

Further investigation is needed to disclose the molecular pathways responsible for protection.

Molecular typing of *Staphylococcus aureus* strains; comparison of well-established with new genotyping methods

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Staphylococcus aureus, especially its methicillin-resistant variants (MRSA) is a frequent cause of nosocomial infections. Rapid and efficient epidemiologic typing systems may be useful to investigate dissemination of the lineages of *S. aureus*. The usefulness of well-established to those of newly developed rapid typing methods as epidemiological tools were compared in our project.

A set of 59 S. aureus isolates was analysed by pulsed-field gel electrophoresis (PFGR), multilocus sequence typing (MLST), repetitive-element PCR techniwue (rep-PCR) based on the presence of DNA sequence that are homologous to MP3 repeat in *Mycoplasma pneumoniae*, multiple-locus variable-number tandem repeat analysis (MLVA), and multiplex PCR-based method with primer mix of the *spa* gene, the *coa* gene, and the hypervariable region adjacent to *mec*A gene.

A total of 59 *S. aureus* isolates clustered by PFGE in 50 different genotypes. MLVA, which had the highest compability with PFGE of all testing methods in this study, clustered into 38 different genotypes, multiplex PCR-based methods clustered into 23, and rep-PCR clustered into 16 different genotypes. Rep-PCR differentiared S. aureus isolates in a way similar to MLST that clustered analysed isolated in 19 grups.

However PFGE still seems to be the gold standard, owing its high discriminatory power amongst molecular typing methods, genotyping methods based on PCR may be useful in respect of speed and easy of performance. MLVA, multiplex PCR-based methodology and rep-PCR are rapid, reproducible, and easy to perform. However MLVA and multiplex PCR-based method generate more unambigous results than those of rep-PCR.

Subpopulation of Rh 123 dim human keratinocytes is highly enriched in skin stem cells

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The aim of our studies was to develop an efficient strategy to isolate and effectively expand human epidermal stem cells. For the purpose of separating the fraction of stem cells enriched in cells able to develop holoclones containing early keratinocytes, we stained the isolated epidermal cells with antibodies against β1-integrin (CD29) and in the presence of rhodamin123 (Rh123). Rh 123 which is metabolical dye that stains active mitochondria and it is removed from cells by multidrug resistance efflux pump. Cells which are metabolically quiescent (stem cells) have low level of mitochondrial activation and low uptake of rhodamin 123. This small pointed cell could be an epidermal stem cell based on the level of rhodamin123 uptake. We observed that Rh123 dull and β1-integrin bright cells isolated by FACS are highly enriched in cells growing in vitro holoclones (the most primitive population of keratinocytes). We performed also image cytometry analysis of cells growing holo- and paraclones. We found, that parameters characterizing cell morphology for rhodamin 123 dim and beta1integrin bright cells are typical for early undifferentiated cells. We evaluated motile activity and colony forming efficiency of both cell types. We found that cells forming holoclones migrate significantly faster than differentiating cells forming paraclones.

New role of IL-6 family cytokines in skin development

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Objective*:* Human skin is an organ highly enriched in stem cells, which are responsible for its self-renewal and homeostasis. There is a need to find optimal combination of cytokines and growth factors which will stimulate proliferation and inhibit differentiation of early keratinocytes *in vitro*.

Biology of skin stem cells is still poorly understood. However, IL-6 family cytokines was shown to have some influence on their behavior.

Aim*:* The aim of this study was to shed more light on the role of IL6 family cytokines on proliferation and locomotion of early keratinocytes. This could help in a future to develop more efficient expansion strategies of skin stem cells *in vitro* and broader their clinical application *in vivo.*

Methods*:* Activation of STAT family members, as well as MAPK and Akt kinases, was evaluated by Western blotting. Proliferation potential of early keratinocytes was estimated by cell counting. Motile activity was recorded and analysed by computer-aided methods.

Results and Conclusion*:* OSM and IL-6 at the molecular level stimulated phosphorylation of MAPK p42/44, AKT and STAT proteins in early keratinocytes. Nevertheless, locomotion of cells treated by IL6 family cytokines does not change significantly.

Among members of IL6 cytokine family, oncostatin M exerted the most significant impact on proliferation of early keratinocytes. We also found that keratinocytes produce OSM (real-time PCR) and we speculate on autocrine mechanism of its action. OSM is a potential new component of culture medium for improving keratinocytes expansion *in vitro.*

Application of green fluorescent protein in the study of actin cytoskeleton organisation and dynamics in plant cells

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The aim of our ongoing project was to create a stable, transgenic lines of *Arabidopsis thaliana* plants, harboring genes and effectively expressing plastin-GFP (green fluorescent protein) fusion protein. Actin bundling proteins, linked to GFP are valuable tools in investigating architecture and function of actin cytoskeleton in a number of intracellular processes such as cell division and expansion, organelle movement and positioning.

In this study a genetically modified *Agrobacterium tumefaciens* strain carrying a human plastin (one of the actin bundling proteins) fused with GFP encoding genes was used to transform *Arabidopsis thaliana* and to introduce genes of interest into the plant genome. Transformation was performed via infiltration of flowering plants with bacteria and transformants were recovered from the progeny. Previously, the same construct was successfully introduced into *Nicotiana tabacum* using cocultivation of tobacco leaf discs with *Agrobacterium* prior to generation of callus tissue and regeneration of plants (A. Anielska-Mazur *et al.*2004). A stable transgenic line of tobacco, with approximately uniform expression level of plastin-GFP in leaf mesophyll was obtained.

Here we present images of plastin-GFP tagged actin fibres, from *Arabidopsis thaliana*, visualised with confocal laser scanning microscopy. However we observed expression and colocalisation of our marker protein with actin cytoskeleton in siliques and sepals, the level of labelling in leaf mesophyll was insufficient to investigate organization and activity of cytoskeleton. This corresponds with recently published results (T. Ketelaar *et al.*2004). The authors achieved expression of mouse talin-GFP chimeric protein (mouse talin is another actin bundling protein) only in some percentage of *Arabidopsis* root hair cells.

It is an open question why actin labeling with plastin-GFP is so diverse. We suggest that either gene silencing, improper protein folding or presence of different types of actin in particular *Arabidopsis thaliana* tissues may explain these results but further research is required to answer this question.

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Light independent protochlorophyllide oxidoreductase

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Chlorophyll is produced in biosynthetic pathway in which protochlorophyllide oxidoreductase (POR) plays crucial role as the key enzyme which activity in angiosperms is controlled by light (Lebedev & Timko, 1998). POR controls the chlorophyll biosynthesis preventing plants from accumulation of large pool of free chlorophyll and its precursors that may have phototoxic effects on plant cells in case of sudden exposure to light after growing in the darkness (Skinner & Timko, 1999).

In contrast to nuclear-encoded, single peptide POR, dark POR (DPOR) is plastid-encoded enzyme composed of three subunits. These subunits are coded by genes *ChlL, ChlN* and *ChlB* which are highly conservative and present in plastid genome of algae, bryophytes, pteridophytes and gymnosperms as well as in the genome of photosynthetic Prokaryotes such as cyanobacteria and anoxygenic photosynthetic bacteria. (Armstrong, 1998). DPOR has the ability to convert protochlorophyllide to chlorophyllide without involvement of light.

So far no detailed information about DPOR structure and its mechanism of the reaction has been provided. Available hypothetical models of molecule architecture and proposed reaction mechanism are only based on sequence similarity to nitrogenase. (Fujita & Bauer, 2000).

We are showing the results of the expression of three genes *chlL, chlN* and *chlB* from *Synechocystis spp*. in heterologous system. We have designed pairs of specific primers, amplified genes, cloned into pGEX-4-T1 expression vectors with glutathione S-transferase (GST) fusion peptide and introduced to *E.coli* BL21 strain. Fusion protein expression was induced with IPTG. Peptides were overexpressed and purified using affinity chromatography on glutathione agarose. The expressed ChlL peptide was located in inclusion bodies so the denaturation in 8M urea and refolding steps prior to chromatography was necessary. Other two peptides also appear in inclusion bodies, but quite large pool of them remains soluble, so that the denaturation is not required. Thrombin digestion to remove GST was performed.

Characterisation of physical and biological properties and *in vitro* reconstitution of whole heterohexameric enzyme will be proceed. Some attempts of reconstitution of the active enzyme has already been taken, but so far no positive results were obtained presumably due to steric obstacle caused by GST peptide attached to N-end of enzyme peptides.

Some structural studies will be also considered.

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Changes in accumulation of different forms of protochlorophyllide in etiolated seedlings of photoreceptor mutant of *Arabidopsis thaliana*

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In etiolated plant tissues protochlorophyllide (Pchlide) - a chlorophyll precursor - is accumulated in two different forms: photoactive and nonphotoactive ones. The former, phototransformable Pchlide is bound to NADPH:protochlorophyllide:oxidoreductase (POR) and has a low-temperature fluorescence emission maximum at 655 nm. The latter, nonphotoactive Pchlide with a fluorescence peak at 632 nm. After illumination, the photoactive Pchlide is reduced to chlorophyllide (Chlide, 692nm at 77K). This photoconversion is catalysed by POR and is a key regulatory step in the chlorophyll biosynthetic pathway.

The accumulation of the initial pool of the Pchlide is a prerequisite of successful transition of etiolated seedlings to photoautotrophy (deetiolation). On the other hand, bulk amounts of free Pchlide accumulated in cells may cause lethal damage upon light exposure due to photooxidation. Therefore, a balance between the initial Pchlide pool and the capacity of its enzymatic photoreduction driven by POR's is particularly important during deetiolation.

The light signals that induce the deetiolation transition are perceived by a photoreceptor system including red/far red light-absorbing pigments – phytochromes (*phy*) and blue light-absorbing cryptochromes (*cry*).

The effect of mutations in loci encoding phytochromes (*phyA, phyB)* and cryptochromes *(cry1, cry2)* on the ratio of nonphotoactive to photoactive form of Pchlide as well as on the total amount of Pchlide was studied in etiolated seedlings of *Arabidopsis*.

The ratio of Pchlide-632 to Pchlide-655 in *cry1, phyA* and in double mutants *phyA/phyB* and *phyA/cry1* showed slight differences (±3-9% of control) in comparison to wild type (WT*)* seedlings. However, in double mutants the total amount of Pchlide was significantly lower (17-20%). On the contrary, the Pchlide-632 to Pchlide-655 ratio increased in *phyB* (30%) and *cry2* (50%) mutants. The total amount of Pchlide in *phyB* decrased (30%), whereas in *cry2* increased (20%).

In conclusion, our results indicate that the "inactive" form of *phyB* and *cry2* receptors negatively regulate chlorophyll biosynthesis processes in normal genetic background (WT). The Pr form of *phyB* and *cry2* receptors may be responsible for the inhibition of chlorophyll biosynthesis in darkness.

The temperature effect on the violaxanthin de-epoxidation reaction *in vivo* and in model system

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The xanthophyll cycle, one of the most important processes for stress adaptation in plants, consists of two reactions. First of them is the violaxanthin de-epoxidation, occurring in light or after lumen acidification which leads to antheraxanthin and zeaxanthin formation. The reverse reaction of zeaxanthin epoxidation proceeds from zeaxanthin *via* antheraxanthin to violaxanthin. Violaxanthin de-epoxidation is catalysed by the enzyme violaxanthin de-epoxidase localised in the lumen of thylakoids.

We examined the heat treatment influence (25^0C-45^0C) on violaxanthin de-epoxidation in leaves of 7-day old wheat plants. An increase of reaction rate after incubation up to 40^0C occures, and in higher temperature a decrease of reaction rate was observed. Additionally, we found that the reaction rate depends also on the duration of high temperature treatment. Examining the effect of heat on the activity of isolated violaxanthin de-epoxidase, we found non-significant changes in rate of catalysed reaction, in previously given temperature scale. Measurable decrease of activity occurs only after 20 min. incubation in temperature higher than 60^0C, what well corresponds to the calorimetric profile of the enzyme extract.

The obtained results allowed us to conclude, that in the temperature range of 25^0C-45^0C the observed effect of heat treatment on violaxanthin de-epoxidation are connected rather with changes in membrane than in the violaxanthin de-epoxidation protein properties.

Fluorescence quenching studies of subunit 7 of the Cop9 Signalosome. Implications for the structure of the protein

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Cop9 signalosome (CSN) is a multisubunit protein complex first identified as a repressor of photomorphogenesis in plants. It is involved in whole array of cellular processes, from proteolysis to diverse signalling pathways. The complex is conserved among all Eukaryotes and bears significant similarity with 26S proteasome and eIF3 complex. The complex consists of eight different subunits denoted as CSN 1-8. A distinctive feature of all the subunits is a novel structural motiff called PCI domain[1]. The precise structure of the signalosome has yet to be elucidated, no high resolution images of the PCI domain has been available so far.

CSN7, a 25kDa signalosome component, has been chosen as a model for investigation on the structure of the PCI domain, and the overall signalosome composition. Since CSN7 contains three tryptophan residues, quenching of the intrinsic fluorescence of the protein was a method of choice for preliminary structural studies.

Using iodide as a quencher and iterative, non-linear, least-squares fitting procedure (FQRS)[2] the fluorescence emission spectrum of the studied protein was resolved into two components. One of the components corresponds to a totally unquenchable, probably buried, class of tryptophans while the other represents a quenched fluorophore, presumably a solvent exposed tryptophanyl residue. Based on the known location of tryptophans in the amino acid sequence, tentative conclusions were drawn as to the position of these residues in the tertiary structure of the protein.

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Synthesis and activity of oxytocin and vasopressin analogs modified with glycoamino acid

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The synthesis of glycopeptides require stereoselective formation of the glycosyl bond between a carbohydrate and a peptide (amino acid) part, and also an appropriate protecting group methodology that allows a selective deblocking of only one functional group in these polyfunctional molecules. Despite the large number of naturally occurring glycoproteins, the types of covalent bonds between the protein and the saccharide moiety show limited variation. One of the linkage types between sugar and amino acid residue (peptide) is *O*-linked glycoside [1].

Because of the need for glycopeptides in our laboratory we have decided to examine different possibilities of transformations of Fmoc-protected serine derivatives into appropriate *O*-glycosylated precursors suitable for solid phase peptide synthesis (SPPS). The crucial step in the synthesis of these derivatives is glycosylation [2]. For the synthesis of α- and β-*O*-glycosides following substrates were used as glycosyl donors: per-*O*-acetyl-mannopiranosyl bromide and per-*O*-acetyl-glucopiranosyl bromide. Fmoc-serine allyl ester was glycosylated with glucosyl and mannosyl donor in the presence of silver trifluoromethanosulphonate (AgOTf) resulting in: allyl *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serinate and allyl *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serinate. The selective cleavage of the allyl ester was achieved by tetrakis(triphenylphosphine)palladium (0) – catalyzed allyl transfer [3].

These Fmoc-serine derivatives was used in synthesis of glycopeptides. Four analogs of oxytocin modified in position 4 were obtained.

Moreover, we are interested in the syntheses of compounds with nonglycosyl bond between a carbohydrate and a peptide part. The derivative of glucuronic acid with the Fmoc-lysine allyl ester attached to its 6^{th} carbon atom was obtained. These Fmoc-lysine derivative was used in synthesis of analogs vasopressin modified in position 8.

The biological activity of all analogs of oxytocin and vasopressin will be determined.

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Comparison of the aggregation properities of the beta amyloid fragment 11-28 and its mutants in the positions 21-22

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Cerebral amyloid angiopathy (CAA) due to β-amyloid (Aβ) formation is one of the key pathological features of the Alzheimer disease (AD). In hereditary AD CAA is linked to mutations at positions 21-23 within the Aβ sequence.

To gain more insight into conformational and aggregational properties of the Aβ peptide and its mutants, we have studied their behaviour by means of CD, FT-IR and ThT assay.

As working with full length Aβ peptides is difficult due to their high propensity for aggregation in our studies we have used the 11-28 fragment of each peptides, which is reported to have amyloidogenic and neurotoxic properties .

To study solvent effects on aggregation process of Aβ peptides, we used FT-IR spectroscopy. The peptides were studied in hexafluoroisopropanol and D_2O mixtures. To verify the obtained results, CD spectra were registered in the same conditions and analysed.

The CD and IR experiments revealed that the peptides studied have a tendency to adopt helical structure in hydrophobic environment. Increase of D_2O content in HFIP/D_2Omixtures forces significant changes towards β-sheet structure.

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A new class of peptide antibiotics structurally based upon the aminoterminal segment of cystatin C

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The increasing rate of appearance of bacterial pathogens resistance to conventional antibiotics has resulted in efforts to develop antimicrobial compounds with new mechanism of action. Antimicrobial peptides that are lethal to bacteria and fungi though non-toxic to mammalian cells are ubiquitously produced in nature and most of these peptides kill microorganisms by forming pores in their cell membranes. Some peptidomimetics and cyclic peptides structurally based upon the N-terminal binding fragment of human cystatin C was found to suppress the growth of streptococcal and staphylococcal species of bacteria [1,2]. The widest antibacterial spectrum and the highest activity against several clinically important Gram-positive pathogens, including multi resistant staphylococci, was displayed by the compound called Cystapep1, of structure Z-Arg-Leu-NH-CH(iPr)-CH_2-NH-Cin (where Cin = cinamoyl). Cystapep1 displays comparatively low MIC and MBC values for both *Streptococcus pyogenes* and *Staphylococcus aureus*. Futhermore, a strong mouse protective capacity of the compound against lethal streptococcal infections was noted. Our preliminary results indicate that mechanism of action of Cystapep1 may differ from those of most antibiotics currently in clinical use.

In the present work synthesis and antibacterial properties of new compounds based on the structure of Cystapep1 will be shown.

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Application of biosensor to study ligand-receptor interactions

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Investigation of ligand-receptor interactions is essential to fully understand the formation and dissociation of individual bonds between biological macromolecules. Atomic force microscopy (AFM) can be used to measure attractive or repulsive forces between the cantilever tip and the sample surface, elucidating local chemical and mechanical properties like adhesion and elasticity. We have applied this technique in our studies preparing avidin or streptavidin functionalized tips called biosensors. Such biosensors have interacted with the sample coated by biotin forming highly specific bonds. This approach allows us to measure bond-rupture forces on the level of single pairs of molecules.

Our aim was to compare intermolecular interactions in two model complexes well-known for creating one of the strongest non-covalent bonds among biological molecules. For each system the unbinding force of a single bond was determined from the center of Gaussian distribution fitted to the force histogram. Under our experimental conditions we have observed that the rupture force of the avidin-biotin complex was stronger than of the streptavidin-biotin complex by the same value of loading rate. Some differences in molecular structure of streptavidin and avidin are responsible for their various affinity for biotin binding.

Moreover, we present loading rate dependence of the rupture force of a single bond in these two systems in a range of loading rate between 420 and 6000 pN/s. Using Bell model we have been able to calculate a dissociation rate and further to estimate an activation energy barrier of the interaction between biotin and streptavidin or avidin.

The results confirm the theoretically predicted differences in biotin binding sites in avidin and streptavidin.

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Synthesis of "Hao" derivatives – potential inhibitors of human cystain C dimerization

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Human cystatin C (hCC) is a natural protein inhibitor of cysteine proteases, ubiquitous in human tissues and body fluids which acts in extra- and intracellular environment. A human cystatin variant Leu68Gln is directly involved in the formation of amyloid, which leads to brain hemorrages [1]. The aggregation is supposed to start from forming the stable dimer of hCC. This dimerization occurs through 3D domain swapping mechanism and starts with conformational changes within β-sheet structure of the inhibitor [2]. Our purpose was to find low-molecular mass ligands of hCC able to stabilize the inhibitor.

We have synthesized a series of compounds designed on the basis of "Hao" (5-HO_2CCONH-2-MeO-C_6H_3-$CONHNH_2$) which is β-strand mimics proposed by Nowick [3]. "Hao" could be envisioned as the unnatural amino acid mimicking the hydrogen-bonding functionality of one edge of a tripeptide β-strand. We expect our ligands to bind to β2- or β5-strand of hCC and stabilize the β-sheet structure of the hCC monomer through hydrogen bonds. They are also expected to block interactions between β-structures in the hCC oligomers.

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Search of potential inhibitors of cathepsin B including *E*-3-(benzylsulphonyl)acroyl moiety

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There are being ceaselessly demanded inhibitors of cathepsin B, which would be used to investigations extracellular protein degradation in various pathological processes, as well as to become the potential medicines. The vinyl sulfones derivatives have been known among other things as specific irreversible cysteine protease inhibitors with high inhibition potency [1].

We found in our earlier research several potent and selective lisosomal inhibitors, including *E*-3-(benzylsulphonyl)acroyl moiety [2]. We affirmed that they show the reversible inhibition. From the viewpoint of pharmacological value they are valuable and promising substances leading in design medicines, especially to fight against chronic diseases.

In this paper there is show synthesis and initial conformation research series potential inhibitors of cathepsin B in general structures: Ph-CH_2-SO_2-CH=CH-CO-Phe-Asn(R)-OH (R: -H or –CH_2-CH_2-NH-Bz) and Ph-CH_2-SO_2-CH=CH-CO-Ile-X(OH)-N(CH_3)$_2$ (X: -Asp, -DAsp or -DGlu). Peptide segment structurally based upon chosen units of CA074 structure and the part responsible for bind inhibitor to enzyme constitute *E*-3-(benzylsulphonyl)acrylic acid residue.

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Mitochondrial dysfunction in heart failure as monitored by following semiquinone EPR signal

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Transgenic mice with cardiomyocyte-specific overexpression of activated Gαq protein (Tgαq*44 mice), represent an unique model of heart failure[1]. Importantly, time-course of development of end-stage pathology in Tgαq*44 mice is protracted over several months as death of animals due to overt heart failure occurs at 14-15 months of age. Here we characterized changes in EPR signals from hearts of 4, 8, 10 and 14 months old Tgαq*44 mice as compared to age-matched FVB mice.

Our results show that the pathology of the heart in Tgαq*44 mice is associated with c.a. 40% decrease of the intrinsic EPR signal at g= 2.005, attributed to the semiquinone free radicals of Q10, and NADH-dehydrogenase of the mitochondrial respiratory chain. This effect was observed in 10- and 14 months old Tgαq*44 mice but not in age-matched FVB hearts. Also in hearts from younger Tgαq*44 mice no alteration of heart EPR signal was detected. No difference in EPR-detectable signals between Tgαq*44 mice and FVB mice in other organs was found.

Alterations in EPR signal in older Tgαq*44 hearts may be due to the impairment of oxygen supply, and/or to the direct destruction of mitochondria in cardiomyocytes, that is know to occur along with the progression of heart failure pathology. Paradoxically, increased oxidant stress associated with the progression of heart failure[2, 3] results in a decrease of the immanent EPR signal of the tissue. This signal, which directly indicates the steady-state level of "metabolic" semiquinone free radicals[4, 5], may serve as a convenient indicator of the efficiency of mitochondrial respiration. We conclude that EPR can be useful tool in monitoring the metabolic impairment of cardiomyocyte function in heart failure.

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EPR spin trapping of nitric oxide in S-91 melanoma tumors *in vivo*

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Nitric oxide is a free radical with many biological functions, such as modulating vasorelaxation, neurotransmission, inhibition of platelet aggregation, and immune defense. In tumors, NO can be both an activator and inhibitor of cancer growth and development. NO can inhibit tumor growth in NO-sensitive cell lines and facilitate disease progression in NO-resistant cell lines [1]. The differential sensitivity of tumor cells to NO depends on the genetic make-up of the cells, the local concentration of NO, and the presence of NO scavengers [1, 2]. In order to apply NO-mediated mechanisms in antitumor therapies, a nonivasive method of NO detection in tumor tissue *in vivo* is needed. We propose to employ EPR spin trapping for that purpose.

S-91 Cloudman melanoma cells ($2.5x10^6$) were injected sc into the tail of DBA/2 mice. When tumors reached volume $0.5cm^3$, mice were anaesthesized using ketamine (150 mg/kg) and xylazine (10 mg/kg). Two egzogenous NO spin traps, diethyldithiocarbamate (DETC)[3] and N-(dithiocarboxy)sarcosine (DTCS)[4] were compared. DETC at 500 mg/kg was injected ip with a 50 mg/kg solution of $FeSO_4$ and 250 mg/kg citrate given subcutaneously. DTCS at 500 mg/kg was administered together with $FeSO_4$ at 50 mg/kg and 250 mg/kg citrate and injected ip. An hour after injection of a spin trap, the animal was restrained and tail with the tumor placed in the EPR resonator. Throughout the measurement the temperature of the animal was monitored and maintained at 35-37C using a heating lamp. The parameters of the EPR measurement were: field 910 G, field sweep 100G, frequency 2.5 GHz, 30 mW of power, modulation amplitude 2G, sensitivity 10mV, 3 scans, scan time 32 s.

S-band EPR spin trapping using egzogenous DETC and DTCS spin traps allows non-invasive detection of nitric oxide generated in tumor tissue in living animal.

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Application of proton magnetic relaxation and hydration kinetics in studies of bound and unbound water pools in *Usnea antarctica*

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Water maintains the structure of the biological membranes and biochemical processes taking place in living cells. Knowledge about the number and distribution of water binding sites, sequence and kinetics of their saturation, as well as the formation of tightly and loosely bound water fractions at different steps of hydration processes is crucial for understanding of the molecular mechanism of the metabolic activity recovery during rehydration.

Antarctic lichens are living organisms extremely resistant to water and temperature stress conditions. Therefore it is very interesting to monitor dehydration limits of these organisms. In our experiments we focused on the hydration processes, the nature of binding sites, and water fractions bound at subsequent stages of early hydration process for *Usnea antarctica*. We have applied two independent methods: nuclear magnetic resonance (NMR) and hydrations kinetics in our studies. The first one allows us to detect directly various water pools via the proton relaxation spin- spin times. The proton relaxation results differentiated tightly and loosely bound water fraction. The second method gives quantitative information on various strenght bound water fractions. To monitor early hydration processes, the hydration courses from the gaseous phase were performed and fitted using double exponential function. From the sorption isotherm , using Dent model we are able to distinguish very tightly bound water fraction.

Both methods: nuclear magnetic resonance and hydration kinetics gave us well correlated informations on different water pools.

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Hydroxyapatite extracted from bovine bones - material for tooth implants? Biocompatibility and resorption properties checked with CAL-72 cells

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Hydroxyapatite is the basic mineral component of the bones. The majority of materials used in dentistry for preparation of tooth implants contain hydroxyapatite as base ingredient. The biocompatibility and resorption properties of hydoxyapatite prepared at Academy of Mining and Metallurgy was checked using CAL-72 (human osteosarcoma) cells. The ability of cell growth on the surface of hydroxyapatite discs was confirmed during prolonged (11 days) incubation of cells under standard conditions (37^0C, 5% CO_2, DMEM with supplements). First experiments indicated that one of the key factors affecting the cell growth was the pH. Throught removal of strong bases, used in the process of hydroxyapatite purification was necessery. Hydroxyapatite discs were repeatedly rinsed with distilled water until pH was stable. The cloning experiment resulted in forming colonies on the surface of hydroxyapatite with efficiency equal to the controls ie. growth on the bottom of plastic wells without hydroxyapatite discs. The final stage of hydroxyapatite preparation prior to biological experiments consists of exposing the material to elevated temperature. The properties of the resulting material vary, high temperature (above 450^0C) causing shrinkage of microcanals and microcavities present in ”raw” hydroxyapatite discs. The cell colonies growing on the discs prepared under different temperature conditions varied. The observations was performed with both optical microscope suited for non-transparent samples and Environment Scanning Electron Microscope (ESEM). Cells growing on hydroxyapatite which was never exposed to temperatures above 400^0C were sparsely distributed over the surface of sample discs and appeared to penetrate the internal volumes of discs, whereas cells seeded on the surface of ”high temperature” (600^0C, 800^0C, 1000^0C) processed material were forming dense colonies without signs of penetration to the interior of discs. This different cell growth correlate with the resorption measured by weight analysis of the discs.

Selected biophysical properties of murine C57BL/6 spleen and skin in telogen – the "resting" phase of the hair growth cycle

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Recent investigations on the murine hair growth cycling revealed that the final part of telogen ending up with hair shedding forms a separate stage - exogen, an active process of ultimate hair follicle remodeling. The maximal intensity of exogen is coupled to anagen IV-V of the subsequent hair generation but some hair shafts remain in skin till telogen of the next cycle. One hair follicle may, therefore, contain two hair shafts being in different stages of different hair cycles. Consequently, elimination of exogen by depilation may result in the change of hair and skin properties of the induced hair cycle on every subsequent stages, including telogen, as compared to the spontaneous cycle.

Using the model of C57BL/6 murine hair cycling, it was checked whether the difference between spontaneous and depilation–induced hair cycle is reflected in the thickness of skin, and in the intensity of its EPR signal originating from hair shaft melanin. The putative differences in physical features of spleen were another subject of the study.

EPR signals of telogen skin were found slightly more intensive in spontaneous, than in depilation–induced cycles. The skin in the telogen of depilation–induced cycle was also thinner than of the spontaneous one. This difference might be brought about by the on-going residual exogen, which the depilated skin was devoid of. Spleens differed in their mass, which was the highest in the initial stage of spontaneous telogen of the second hair cycle, and the lowest in the case of telogen of the depilation-induced cycle. The dark pigment present in some spleens was shown to be eumelanin, and the intensity of its EPR signal was reverse-proportional to the time between the onset of telogen and the moment of skin harvesting. It is supposed that the spleen melanin may originate from the follicular melanocytes, which undergo apoptosis in catagen. Also biophysical properties of hair and spleen melanin differed – the spleen melanin revealed more heterogeneous population of paramagnetic centers, as judged by the shape of its power saturation curve.

The study supports the notion that physiology of telogen skin in C57BL/6 mice depends on the way of hair cycle induction. As some physiological and biophysical parameters change with the progress of telogen, most probably due to the on-going, parallel exogen of the preceding hair cycle, telogen cannot be treated as a homogenous and static (“resting”) state of skin.