114.1
Glycotyping of Serum Transferrin Isoforms by Capillary Zone Electrophoresis in Healthy Humans
F. J. Legros1,2, V. Nuyens1, and J.-P. Henry2
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Sera from 50 healthy humans were submitted to capillary zone electrophoresis (CZE) using a capillary double-coating method with polyanions and polycations and cleaning lipoproteins with sodium dodecylsulfate inside the capillary. The separating tris-borate buffer pH was 8.5. The peaks recorded on the electropherograms were identified using an anti-human transferrin serum. Five immunoreactive peaks were visualized. The predominant tetrasialotransferrin isoform, known as exhibiting two N-glycan chains and 4 antennae averaged 80% of total transferrin (retention time 6 min). The two earlier forms likely represented trisialotransferrin (mean percentage 5; retention time 5.8 min), and disialotransferrin (1%; retention time 5.7 min). A pentasialiform (12%; retention time 6.1 min) and a last form (2.5%; retention time 6.2 min) were encountered. Migration times offered coefficients of variation inferior to 1%. The five isoforms disappeared after 3 h treatment with neuraminidase 0.2 U/ml and N-glycosidase 300 U/ml. Five immunoreactive peaks were observed in healthy adults, as already observed. The five isoforms disappeared after 3 h treatment with neuraminidase 0.2 U/ml, while less sialylated forms developed. Treatment with N-glycosidase suppressed these forms, and one single peak was observed after 24 h, presumably corresponding to the aglycosylated transferrin aminoacid sequence. Seven immunoreactive isoforms were visualized in cord blood sera, 4 of them being less sialylated than tetrasialotransferrin. All except the earliest one disappeared during hydrolysis with neuraminidase. The last one disappeared with N-glycosidase while a new peak developed.

114.2
Development of Human Serum Transferrin Isoforms from Newborns to Adults
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Sera from 50 healthy adults and 50 newborns' cord blood were submitted to capillary zone electrophoresis. Separation of the isoforms occurred at pH 8.5 and pH 8.25. The electropherograms were identified using anti-human transferrin antiserum. Sialylation and glycosylation were evidenced by successive 24 h hydrolysis with neuraminidase 0.2 U/ml and N-glycosidase 300 U/ml. Five immunoreactive peaks were observed in healthy adults, as already observed. The five isoforms disappeared after 3 h treatment with neuraminidase 0.2 U/ml, while less sialylated forms developed. Treatment with N-glycosidase suppressed these forms, and one single peak was observed after 24 h, presumably corresponding to the aglycosylated transferrin aminoacid sequence. Seven immunoreactive isoforms were visualized in cord blood sera, 4 of them being less sialylated than tetrasialotransferrin. All except the earliest one disappeared during hydrolysis with neuraminidase. The last one disappeared with N-glycosidase while a new peak developed.

114.3
Characterization of the Low Molecular Weight Serum Proteome
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Serum is attracting increasing interest in proteomics, which is striving to broadly characterize its protein constituents with the goal to discover an increasing number of reliable disease biomarkers. While serum has a high protein content, these proteins are present across an extraordinary dynamic range of concentration that spans more than 10 orders of magnitude. This large dynamic range exceeds the analytical capabilities of traditional proteomic methods making the detection of lower abundance serum proteins extremely challenging. Affinity methods have been developed to remove abundant proteins from serum prior to mass spectrometry (MS) analysis, however, this procedure also results in the loss of many important low molecular weight (LMW) proteins. We have developed a simple method for the removal of high molecular weight species from serum without the concomitant loss of LMW components. This method employs centrifugal ultrafiltration using solvent conditions that disrupt protein-protein interactions so that LMW components that may be bound to larger species are released and are free to pass through the molecular weight cutoff membrane. The LMW serum proteome was digested with trypsin and subsequently fractionated by strong cation exchange chromatography. Each of these fractions was analyzed by microcapillary reversed-phase liquid chromatography coupled online with electrospray ionization tandem MS (LC-MS/MS). Complete analysis of the resulting MS/MS spectra resulted in the confident identification of more than 300 proteins. No peptides originating from human serum albumin were identified in any of the fractions analyzed. The large number of proteins identified demonstrates the efficacy of this depletion method combined with multi-dimensional fractionation and ESI-MS/MS analysis for the characterization of the LMW fraction of serum.
114.4
High-throughput Identification of Plasma Proteins Using Multidimensional Liquid Chromatography Coupled With Ion Trap Mass Spectrometry

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The large dynamic range of protein concentrations found in human plasma poses a serious analytical challenge in studies aimed at mapping the plasma proteome. In this report we discuss the implementation of multidimensional LC separations (off- and on-line) coupled with electrospray ion trap mass spectrometry as an analytical strategy for high-throughput, high-confidence identification of plasma proteins. The methodology described involves two discrete stages: (1) off-line preparative-scale, semi-automated protein fractionation and albumin/IgG depletion followed by (2) proteolysis and on-line automated MultiDimensional Protein Identification Technology (MuDPIT). Whereas the details of the off-line step are discussed elsewhere in an accompanying presentation (see A. Jorsback et al., HUPO 2003), this work focuses on front-end enhancements made to existing MuDPIT technology in order to extend its dynamic range, as well as on the use of internal standards to normalize variations in inter- and intra-assay response.

114.5
Immunoaffinity Depletion of High-abundant Proteins from Human Serum for Proteomic Sample Preparation—Method Evaluation

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Human serum is a valuable sample in proteomic analysis for the discovery of new biomarkers for disease or novel drug targets. This fluid represents the most complex sample of the human proteome. Difficulties in the analysis of serum arise from the fact that proteins range in concentration over 12 orders of magnitude. The high-abundant proteins such as albumin, IgG, transferrin, haptoglobin, IgA and anti-trypsin represent up to 90% of the total protein mass in serum. These major protein constituents interfere with identification and characterization of the important low-abundant proteins by limiting the dynamic range of mass spectral and electrophoretic analysis. We have developed an immunoaffinity column for simultaneously selecting and removing six high-abundant proteins from human serum. Affinity purified polyclonal antibodies, covalently coupled to porous particles, are used as a selection media. Optimized use conditions permit the column to be used multiple times (200 injections) with total column run cycle of 20 minutes. We have assessed column reproducibility by analysis of the flow-through fractions from more than 200 consecutive column runs. The column provides consistent and robust depletion of high-abundant proteins as determined by 1D gel patterns, MALDI-TOF and LC/MS analysis of tryptic digests. ELISA results indicate that the multiple affinity removal column depletes 98–99% of targeted high-abundant proteins in a single pass through the column.

We have compared the performance of the immunoaffinity column to dye-affinity chromatography (Cibacron Blue). Bound fractions were resolved electrophoretically, protein bands were excised, digested by trypsin, and then analyzed by LC/MS/MS. We found that the immunoaffinity system exhibits superior specificity of depletion in comparison to Cibacron Blue.
114.6
Proteomic Analysis of Human Plasma/Serum
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Human plasma/serum is the most readily accessible clinical sample source for the investigation of disease progress and drug effect. Our proteomic core facility has been participating in the Human Plasma Proteome (HPP) initiative organized by Human Proteome Organization (HUPO) to comprehensively analyze human plasma/serum protein constituents, to consolidate national and regional proteome organizations into a worldwide organization, and to identify the potential protein biomarkers for early diagnosis of the risk, onset and progression of many diseases. To identify more low abundant proteins, albumin (Alb) and immunoglobulin (Ig) were first depleted from plasma/serum samples prior to LC- or gel-LC-based proteomic analysis. For LC-based approach, the Alb/Ig-depleted protein sample was reduced, alkylated and digested, and the resulting peptides separated by 1D-LC (reverse phase) or 2D-LC (ion exchange-reverse phase) and identified by ESI-Q-TOF or MALDI-TOF/TOF. For gel-LC-based approach, the Alb/Ig-depleted protein sample was separated by SDS-PAGE, in-gel digested and separated by 1D-LC (reverse-phase), and also identified by ESI-Q-TOF or MALDI-TOF/TOF. Three plasma and two serum samples were initially analyzed in our facility to evaluate the relative advantages and limitations of technology platforms in current pilot study status. In next execution phase study (Jul-Sep, 2003), more HUPO plasma and serum samples will be analyzed by our standardized proteomic platforms. The progress, problems and challenges will be summarized in our presentation to share and discuss with HPP research groups.

114.7
Iron Chelators Prevent Inhibition of Tartrate-Resistant Acid Phosphatase From Human Blood by Hydrogen Peroxide
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The tartrate-resistant acid phosphatases (TRAPs) are a class of metalloenzymes that catalyze the hydrolysis of various phosphate esters under acid reaction conditions. These enzymes can be used as biomarker for several diseases, including bone resorption. The purpose of this work was to determine the effect of hydrogen peroxide (H2O2) and in the TRAP from human blood serum and a correlation with iron, since this enzyme contains two iron atoms at its active site [Fe(III-FeII)]. The peripheral blood from healthy volunteers was collected and diluted in physiological solution (1:6). The enzyme activity was determined using p-nitrophenylphosphate as substrate (5mM) and hepes buffer (100mM), pH 7.4. After 30 minutes the reaction was stopped with NaOH (1.0M), and p-nitrophenol was measured at 405nm. The TRAP was inhibited 50% by H2O2 (60mM), and this effect was prevented in the presence of GS (20mM), phenanthrolin (10 mM) and uric acid (5 mM). Despite the TRAP sensitivity to H2O2, the enzyme was not inhibited by a typical protein tyrosine phosphatase inhibitor, pervanadate, which acts as oxidant. Since TRAP has no SH-residue in the active site, our results suggest a possible participation of this enzyme in the Fenton reaction in the presence of H2O2. Uric acid acts as hydroxyl scavenger, therefore, the enzyme activity protection observed in the presence of this compound confirmed that the TRAP catalyses the generation of reactive oxygen species.

114.8
Imunoaffinity Techniques for Human Plasma Proteomics
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Two-dimensional gel electrophoresis (2DE) of protein samples of human tissues and blood plasma and the subsequent immunoblotting with antibodies have been used for epitopic analyses on antigens of tumours such as hepatocellular carcinomas with HCV-infection or of infected-microorganisms such as H. pylori. Recent advances in mass spectrometry enabled us to determine the structure of epitope by combination of 2DE. Furthermore, newly developed technique of affinity electrophoresis (AEP) made it possible to separate a tiny amount of proteins by specific interactions with their ligands and to determine the affinity constants of those interactions without purification of the proteins in crude samples. The technique of AEP can be used not only for epitopic analyses on autoantigens of immune diseases but also for functional proteomics of human plasma proteome.
114.10

Identification of Protein-Protein Interactions within Human Serum

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Serum contains a broad spectrum of molecular information whose presence may indicate metabolic states, cellular responses to variety of stresses, hormonal stimulation and diseases. For these reasons, blood represents possibly the most insightful proteomic sample for disease diagnosis. The detection of biomarkers in serum, however, is largely hindered by the most abundant proteins in serum, which represent up to 99% of the total protein mass in serum. Traditionally, biomarker investigations of serum typically rely on depletion of the most abundant proteins, however, since many of these proteins function as carrier proteins, it is reasonable to assume that many small associated proteins or protein fragments are depleted as well. In this study the individual most abundant proteins were isolated using specific antibodies or affinity resins under mild buffer conditions to preserve possible protein-protein interactions. The proteins or protein fragments associated with albumin, IgG, IgM, IgA, transferrin, and apolipoprotein were then identified. Remarkably, many of the species identified as being bound to these high abundance proteins have not been previously identified in any of the published global serum proteomic investigations, likely due to their low abundance. Many of the proteins identified are derived from diverse cellular compartments and previously known to be involved in signal transduction, apoptosis, and hormone biochemistry, demonstrating that a potential archive of disease related proteins or protein fragments are associated with the most abundant proteins in serum. Furthermore, we propose that these highly abundant serum carrier proteins may in fact act as biomarker scaffolds onto which important disease related proteins or their fragments partition.

114.11

High-throughput Plasma Depletion with Chicken Antibodies for Proteomic Analysis

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Plasma from different species remains to be the most accessible and valuable source for biomarker discovery in clinical and animal samples. However, due to the high abundance protein expression of some proteins such as albumin and immunoglobulins, low abundant proteins are often undetectable in proteomic analysis of plasma. Thus far, we have established a high-throughput and automated plasma depletion scheme using chicken antibodies (GenWay Biotech) against various abundant proteins. This immunofinity purification procedure is able to deplete albumin from different species of plasma or serum in minutes. The high binding capacity and specificity of the chicken antibody allows efficient capture of its ligand from micro-liter volume of plasma sample. The resulting 2D gel analyses of the depleted and captured samples show significant enhancement of the low abundant proteins and specific capture of the abundant ligand. By utilizing this sample preparation scheme, it is now possible to analyze the plasma proteome in a rapid and large scale for biomarker discovery, drug target discovery or toxicology studies.

114.12

Depletion of High Abundant Proteins for Human Plasma Proteome Studies


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It is often difficult to elucidate low abundant biomarkers in the presence of a large amount of high abundant proteins in blood. By removing them, thereby enabling the detection of the remaining proteins present in much lower concentrations. In this study, we evaluated a simple in-house affinity spin tube filter approach on its removal efficiency and several commercially available HSA depletion methods including POROS Affinity Depletion Cartridges and Montage™ Albumin Deplete kit. Our approach is not a classical column-based affinity chromatography. Instead, a one-step Spin-X tube filter is used to assemble protein G beads. Protein G has the ability to bind specifically to IgG and when anti-human serum albumin antibody is used, this method can deplete high abundant HSA and serum IgG simultaneously. A two-site ELISA for human albumin was utilized to accurately quantitate specific albumin removal efficiency. The BCA protein assay was used for total protein concentration determination to calculate the protein loss by the process. The affinity spin tube filter depletes the albumin over 80% in average and less than 5% of the protein is lost. Compared to other methods, our approach provides optimized reagents and disposables in a convenient format for manual processing and potential automation. In conclusion, our method is more efficient, easier to use, and in a high-through platform. It will facilitate human plasma proteome analysis.

114.13

Comprehensive Profiling of Human Plasma and Serum Proteomes Using Microsol-IEF Prefractionation and Major Protein Depletion


The Wistar Institute, Philadelphia, PA, USA

Human plasma and serum proteomes offer promising resources for discovery of potential disease biomarkers. To date, two-dimensional (2-D) gel electrophoresis has been the most common method for quantitative analysis of protein profiles. However, sample complexity usually makes reproducible analyses difficult and comprehensive profiling of most proteins present is impractical. A very wide dynamic range of protein concentrations further complicates analysis of serum and plasma proteomes. In cooperation with the HUPO Plasma Proteome Project, we are evaluating methods to detect as many proteins as possible in human serum and plasma. The strategy we are using is to combine albumin and IgG depletion with MicroSol-IEF separation, a prefractionation method developed in our laboratory and commercialized as the ZOOM IEF fractionator by Invitrogen Corp. Several methods to deplete major proteins were evaluated, and we optimized the methods to result in a total unbound fraction containing minimal albumin and IgG and a bound fraction with minimal non-specific protein contamination. Both fractions were subsequently fractionated using the Zoom IEF fractionator into 5 well-separated pools based upon the proteins pls. The effects of major protein depletion and IEF prefractionation were evaluated using subsequent analyses on narrow range 2D gels to determine the total number of proteins that can be detected. The optimized method will be used to compare differences between plasma and serum as well as to detect disease markers.
114.14 Proteome Analysis of Plasma and Serum Proteins Using a 3-D Strategy Combining Microsol-IEF, 1-D Gels, and LC-MS/MS
The Wistar Institute, Philadelphia, PA, USA

Protein profiling of plasma is complicated by the wide range in protein concentrations, which limits the capacity to consistently detect and quantify low abundance proteins using available technologies. In cooperation with the HUPO Plasma Proteome Project we are developing and evaluating a 3-D protein profile analysis approach to dig deeper into plasma/serum proteomes. One strategy being developed in our laboratory initially fractionates human plasma or serum into five well resolved pools using Microsol-IEF separation, a prefractionation method developed in our laboratory and commercialized as the ZOOM IEF fractionator by Invitrogen Corp. These fractions are then separated on short 1-D gels and the entire lane is cut into uniform slices, resulting in a “batch 2-D gel.” These batch 2-D gels provide low to moderate resolution information about proteins’ pIs and molecular weights. Each slice is then digested with trypsin and analyzed by LC-MS/MS to identify the proteins in each sector of this 2-D separation. Quantitative comparisons can be obtained by incorporating stable isotope tags, although an affinity purification to simplify the peptide mixture is not needed. In some experiments, the advantages and disadvantages of major protein depletion on detection of low abundance proteins and reproducibility are being assessed.

114.15 Analysis of Peptides and Phospho-Peptides from Protein and Phospho-Proteins Mixtures by Offline C18 LC-MALDI-Qq-TOF
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Phospho-proteins are of central importance in mammalian signal transduction. However, identifying phospho proteins and mapping their phosphorylation sites has presented a significant technical and bio-informatic challenge. The abundance of non-phosphorylated peptides often exceeds the phosphorylated peptide from a digested protein sample thus suppressing the signal of the phospho-peptides. To avoid this problem, phospho-peptides have been selectively purified by immobilized metal affinity chromatography (IMAC). However, analyzing the resulting purified phospho peptides presents a bio-informatic problem as the resulting mass spectra must be searched against all possible phosphorylation sites of all known proteins producing a significant computation challenge. Standing to the presence of huge quantity of several proteins such as albumin, which do not provide any diagnosis but complicate the analysis and to the lack of any appropriate analysis methodology. In this work, we are more particularly interested in the study of a family of proteins widely found in plasma, the apolipoproteins A. Indeed, these proteins are involved in many cardiovascular diseases and they could be interesting biomarkers easily found and analysable in a proteomics approach since they are found in high quantity in plasma (1mg/ml ?). After careful selection based on medical and clinical chemistry data, we have recruited patients who do not present any pathology and patients with different cardiovascular diseases. Aliquots from each individual were kept unpooled for follow-up studies. The study of the normal individual plasma reveals that these proteins present a very specific phosphorylation profiles, that we have characterized (phosphorylation level and localization) combining dephosphorylation by an alkaline phosphatase and the use of IMAC column integrated on a Q-TOF instrument. The second step is the study of the different case of the pathologies and we will present preliminary results on the changes appearing in these post-translational modification profiles in function of the existing pathologies. These results will be confirmed by a differential proteomics study (DIGE and ICAT) of the most interesting pathologies.

114.16 Apolipoproteins A: A Good Candidate Family of Cardiovascular Disease Biomarkers in Plasma
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Plasma is a privileged biofluid for analysis of human sample by proteomics. However, the identification of proteomic biomarkers in plasma is a real challenge due to the thousands of proteins present in such a medium, due to the presence of huge quantity of several proteins such as albumin, which do not present any pathology and patients with different cardiovascular diseases. In this work, we are more particularly interested in the study of a family of proteins widely found in plasma, the apolipoproteins A. Indeed, these proteins are involved in many cardiovascular diseases and they could be interesting biomarkers easily found and analysable in a proteomics approach since they are found in high quantity in plasma (1mg/ml ?). After careful selection based on medical and clinical chemistry data, we have recruited patients who do not present any pathology and patients with different cardiovascular diseases. Aliquots from each individual were kept unpooled for follow-up studies. The study of the normal individual plasma reveals that these proteins present a very specific phosphorylation profiles, that we have characterized (phosphorylation level and localization) combining dephosphorylation by an alkaline phosphatase and the use of IMAC column integrated on a Q-TOF instrument. The second step is the study of the different case of the pathologies and we will present preliminary results on the changes appearing in these post-translational modification profiles in function of the existing pathologies. These results will be confirmed by a differential proteomics study (DIGE and ICAT) of the most interesting pathologies.

114.17 Human Serum Proteins Pre-separated by Electrophoresis or Chromatography Followed by Tandem Mass Spectrometry
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The presence of more than 600 types of protein products were identified in human serum. Proteins from crude serum or proteins resolved by ionic electrophoresis or prepared by chromatography were analyzed. Proteins were digested with trypsin or chymotrypsin. Naturally-occurring peptides were also collected by reversed-phase chromatography. The resulting peptides were identified by tandem mass spectrometry. The peptides were either desorbed by a laser from a metal chip into a quadrupole-time-of-flight mass spectrometer or ionized as an electro-spray from reversed-phase chromatography. The resulting peptides were identified by tandem mass spectrometry. The peptides were either desorbed by a laser from a metal chip into a quadrupole-time-of-flight mass spectrometer or ionized as an electro-spray from reversed-phase chromatography via a metal needle under voltage into an ion-trap mass spectrometer. All of the commonly known proteins associated with serum were detected and the two mass spectrometers agreed on the identity of abundant serum proteins. Pre-separation of serum proteins prior to digestion markedly enhanced the capacity to detect un-common proteins from blood. Preparing serum with multiple chromatography columns yielded some 420 significant proteins. De-albuminization followed by reduction, trypsin and 2D peptide separation yielded some 260 proteins. De-albuminization followed by trypsin/chymotrypsin and 2D peptide separation yielded 480/220 proteins. Electrophoretic separation followed by 1D peptide separation yielded 140 proteins. Electrophoretic and multiple chromatography based
115.1

**Dutpase in the Drosophila Proteome**


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The enzyme dUTPase functions as a preventive DNA repair protein ensuring low cellular dUTP/dTTP ratio to avoid misincorporation of uracil into newly synthesized DNA during replication or repair of genetic information. High uracil content of DNA leads to chromosome-fragmentation and programmed cell death due to futile hyperactivation of the base excision repair system. Antagonism of dUTPase, as the major controller of low cellular dUTP/dTTP ratio, may provide efficient antiviral and anticancer therapeutic possibilities. In this poster a proteomical search for macromolecular interacting partners of this essential DNA repair enzyme is presented using Drosophila melanogaster as an appropriate eukaryotic model. Surface plasmon resonance together with far-Western experiments confirmed existence of Drosophila proteins physically interacting with dUTPase. Some of the macromolecular partners were shown to be heat stable. Affinity and immuno-affinity chromatography as well as co-immunoprecipitation proved to be efficient for isolation of the dUTPase partners, which were identified by mass spectrometry after in-gel digestion. Homologues of human proteins RpR0, hRNP A1, and actin, previously described as apoptosis-associated proteins together with dUTPase, were also pulled down by our methodology. Some of our protein hits are involved in DNA repair. Previous studies regarding Drosophila dUTPase in our laboratory suggested multilevel regulation of the enzyme. Accordingly, we propose that some of the interacting proteins may function as regulator of dUTPase localization and/or activity.

115.2

**Isolation and Mass Spectrometry of Multi-Proteins Complexes Regulating the Ras Signalling in Saccharomyces cerevisiae**

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With the completion of genome sequencing for more than 50 prokaryotic organisms as well as several important eukaryotic ones, including a draft of the human genome, the challenging task now is to decipher relationships between individual genes and to understand the molecular organization of cellular networks. Deciphering protein complexes is a real challenge and the strategies, we have apply and develop to investigate the topology of multi-protein complexes, result of the combination of different techniques and consist in three main steps: (i) complex purification by TAP-TAG, (ii) identification of the complex constituent proteins and their post-translational modifications. Despite the simplicity of the concept, each step requires the development or the optimization of specific and original methodology. We will illustrate this strategy on multi-proteins complexes regulating the Ras signalling in Saccharomyces cerevisiae. Since we have recently shown that Tfs1, a PEBP, activates in vivo Ras signalling and cAMP/PKA cascade, we will focus here on the study of Tfs1 complex. We have used TAP-TAG (tandem affinity purification) to purify the endogenous proteins complex. To characterize the composition of protein complexes, we have performed a standard but automated proteomic analysis with robots of the 1D and 2D gel obtained with these purified fractions. Analysis of the data reveals a family of serine/threonine protein kinase. This is particularly interesting since human PEBP have been shown to interact with this particular family. Moreover, the biological function of Tfs1 is investigated by the differential proteomic study of yeast cell deleted or not of Tfs1 gene.

116.1

**Correlation Analyses of Changes of Hepatocytes Chromatin Phospholipid Component After Vagotomy and Solarectomy**

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The goal of our study was the revealing of molecular biological mechanisms of phospholipids qualitative and quantitative changes in hepatocytes chromatin after bilateral sub-diaphragmatic vagotomy, solarectomy (removal of plexus solaris) and their combination. Data obtained show considerable changes in content of total and individual phospholipids, particularly of phosphatidylethanolamines and phosphatidylcholines, in solarectomized rat liver as well as in combination of solarectomy and vagotomy. These changes of some neutral phospholipids to some of acidic also take place. It can be a cause of changes of structural organization and functional activity of membranes and sub cellular formations. Above-mentioned disorders are more expressed after solarectomy and its combination with vagotomy. These investigations once more indicate the significant role of Vegetative Nervous System and its peripheral parts in regulation of subcellular structures and cells functioning. There also were the changes of hepatocytes mitochondrial fractions’ phospholipids. The results of related investigations can be as a new qualitative step in confirming of L. A. Orbeli’s biological conception reality about the adaptational-trophical role of Autonomic Nervous System on subcellular structures and cells in whole.

116.2

**Serum Cysteine, Zinc and Copper in Cirrhosis**

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It has been known that biosynthesis of cysteine from methionine via the hepatic transsulfuration pathway is impaired in cirrhotic patients, who might require cysteine in the diet. Therefore, this study was based on the investigation of cysteine levels as well as zinc and copper alteration in cirrhotic patients. We studied 32 cirrhotic patients (12 females, and 20 men), aged 45 ± 11 and 32 control subjects (20 men, and 12 women), aged 39 ± 9 in the department of gastroenterology at the F.U. School of Medicine. Blood samples were collected from subjects between 8.00am and 10.00am following a 12-hour fast. Serum levels of copper and zinc were measured by atomic absorption spectrophotometry. Cysteine levels were determined in the serum by high-performance liquid chromatography (HPLC). The levels of copper, were associated with antioxidant enzyme increases, whereas zinc decreased significantly in cirrhotic patients. Serum copper, was found to increase slightly in cirrhotics but not in a statistically significant way. A positive correlation was seen between Cu/Zn ratio and Cu in controls (r = 0.690; p < 0.01), but the correlation of between Cu/Zn ratio and Cu was not significant in the cirrhotic group. Negative correlations were seen between the serum concentration of zinc and Cu/Zn ratio in controls and cirrhotic patients (r = 0.618; p < 0.01 and r = -0.670; p < 0.01, respectively) it was concluded that cirrhotic patients displayed multiple abnormalities such as cysteine metabolism as well as zinc, copper, and zinc may have some beneficial effect in the treatment of liver cirrhosis.
The Level of Homocysteine in the Cirrhotic Patients

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Homocysteine (Hcy) is a sulfur bearing amino acid that is converted to cysteine or is remethylated to methionine by methylenetetrahydrofolate reductase (MTHFR). MTHFR plays a central role in the metabolism of folate. It has been hypothesized that the increased plasma Hcy can induce liver diseases and may play remarkable role in hepatic disorders. Therefore, the purpose of the present study was to investigate the relationship among plasma vitamin B12, folate, and Hcy level alterations in patients with cirrhotic patients and healthy subjects.

We studied 32 cirrhotic patients (12 females, and 20 men), aged 45 and 32 control subjects (20 men, and 12 women), aged 39 in the department of gastroenterology at the F.U. School of Medicine. Blood samples were collected from subjects between 8.00am and 10.00am following a 12-hour fast. The level of homocysteine was measured by HPLC. Folate and vitamin B12 concentrations were measured by immunoassay method.

There was an important invers correlation between homocysteine and vitamin B12 in controls (r = -0.442; p < 0.011), but not in cirrhotic patients (r = -0.147; not significant). Also, the mean plasma folat was decreased in cirrotic patients when compared to controls (p < 0.001).

Although hyperhomocysteinemia was known as an atherogenic and thrombogenic risk factors for cardiovascular disease it might also be a risk factor for cirrhotic patients. Plasma homocysteine level, vitamin B12 and folic acid measurement may improve the evaluation of cirrhotic patients and might be used as markers related to hepatic dysfunction.

Chronic Alcohol Consumption Inhibits Oxygenation Changes in the Livers of Live Rats—Noninvasive In Vivo Dynamic CT, Functional MRI and 31P MRS Studies

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Hypoxia is central to alcohol-induced liver damage. Metabolism of ethanol by ADH and CYP2E1 increases O2 demand. Long-term alcohol may decrease O2 supply via disruption of microvascular structure or responsiveness. A high-fat Lieber-DeCarli diet (5% v/v ethanol or dextrin maltose) was given to male Wistar rats for 6–8 weeks. For CT, MRI or MRS studies, rats were anesthetized (2% isoflurane), mechanically ventilated and challenged with 10% O2 (hypoxia), 98% O2 (hyperoxia), 5% CO2 (hypercapnia) and 93% O2/5% CO2 (carbogen). Functional MRI and 31P MRS were done on a 3 Tesla IMRIS MRI. Two weeks later, dynamic contrast enhanced CT was done on the same rats with a clinical slip-ring Lightspeed scanner (GE) after 1 ml/kg i.v. iohexaol (Omnipaque). Functional MRI is based on deoxyhemoglobin’s paramagnetic broadening of 1H tissue water resonances, decreasing MRI signal intensities. MRI intensities of livers decreased with hypoxia and increased with hyperoxia & hypercapnia. MRI changes correlated with pulse oximetry (r² = 0.95, n = 30). In all physiological challenges, control rat livers responded more and faster than chronic alcoholic rats, indicating ethanol-induced micro-vascular dysfunction. For example, carbogen caused significantly increased T2* weighted fMRI changes (72 ± 30%), CT measured blood flow (29 ± 11%) and blood volume (40 ± 15%) changes in controls, versus nonsignificant changes of 9.0 ± 3.0%, 6.8 ± 11% and 6.5 ± 15% respectively in alcoholic rats (n = 6 pairs, p < 0.05). In alcoholic rats, ATP/ Pi was significantly increased with hypoxia and decreased with hypoxia compared to controls (31 P MRS), again indicating insufficient O2 in alcoholic livers. (Funding: NIH & NSERC)
117.2 Phosphorylated Proteins Profiling of Human Fetal Liver
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Although the genome and transcriptome for the foetal liver during 22 weeks of gestation (HFL22w) supplied abundant information to realize the functions of foetal liver on development and hematopoiesis, a comprehensive protein expression profiling is necessary to the discovery of key molecular, understanding of the unique functional characteristics and probe the regulation mechanisms from genome to transcriptome and proteome of HFL22w. The protein expression profile of HFL22w was developed using two strategies. With 2DE strategy, 5487 protein spots were visualized and more than 1000 proteins were identified by MALDI-TOF/MS. Relative abundances of the identified proteins were calculated through the image analysis of the profile. In the second strategy, total proteins from HFL22w were separated by 7.5%, 10% and 15% SDS-PAGE, followed by full-gel slicing, enzyme digestion and LC-ESI-MS/MS identification, another more than 400 proteins were identified, in which many proteins were in the range of high molecular weight and high basic pi on the gel, which fall out of the capability of 2-DE separation. Also with this methods, proteins in subcellular organelles of HFL22w that were separated by centrifuge in sucrose buffer were characterized, which resulted in the characterization and localization of 117 proteins in nuclear, 143 proteins in endoplasmic reticulum, 130 proteins in golgi body and 201 proteins in membrane. Functional categories of the identified proteins showed that most of these proteins involved in the human body metabolism, gene and protein synthesis, which is consistent with the high proliferation and differentiation characters of the foetal liver.

117.3 Proteomic Analysis of Different Area of Different Aged Human Brains
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Proteins in the brain of different ages are very important for us to understand the brain function and the pathology of neurodegenerative diseases. In this study we used two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) to investigate the different protein expression pattern in different aged human brains. We found that four 14-3-3 proteins in old brains were down-regulated compared with young brains including 14-3-3 protein zeta, beta/alpha, gamma and epsilon. While 14-3-3 protein expression level in neurodegenerative brains was even lower than that of the normal aging brains. The expression of 14-3-3 protein beta/alpha and protein gamma was relatively lower. We also found human brain creatine kinase B (CKB) was sharply down-regulated in disease brains compared with that of normal brains. Other eight down-regulated and ten up-regulated proteins were found in old brains compared with young brains. Our results may supply the evidence for the possible molecular mechanism of protein expression changes in the aging of human brain and neurodegenerative diseases. Supported by the national program for key basic research projects of China.
117.4 Profiling of the Proteomes in Human Tissues by In-gel Isoelectric Focusing and Mass Spectrometry
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Two-dimensional gel electrophoresis (2DE) has been the most widely used method for the separation of complex protein mixtures. However, the 2DE-based approach suffers from a number of limitations, and therefore a large effort has been focused on the development of novel proteomics methods. In the study presented here, an alternative strategy for the analysis of complex proteomes was developed and tested. The strategy combines isoelectric focusing (IEF) in immobilized pH gradient strips, mass spectrometry, and bioinformatics. Protein mixtures are separated by in-gel IEF, and the strip is divided into a set of gel sections. Proteins in each section are digested with trypsin, and the tryptic peptides are subjected to liquid chromatography-nanoelectrospray-ion trap tandem mass spectrometry (LC-MS/MS). The LC-MS/MS data are used to identify the proteins through searches of protein sequence databases. The in-gel IEF-LC-MS/MS method was applied to the analysis of whole proteomes in the human pituitary and prostate, and to the investigation of the human pituitary phosphoproteome. A total of 127 proteins from the human pituitary were identified; the mapped proteins included cytoplasmic proteins and proteins from various subcellular locations (e.g., nucleus, mitochondria). The largest functional groups sampled were proteins involved in basic metabolism, cell structure, protein processing/folding, and cell defence. Using in-gel IEF-LC-MS/MS in combination with IMAC-based phosphopeptide enrichment, 19 phosphoproteins from the human pituitary were identified. These phosphoproteins included the human growth hormone, galanin, secretogranins, and other important regulatory proteins.

118.1 Alkyl-lysosphospholipid Induces Apoptosis After Lipid Raft-mediated Endocytosis, Whereas Lysosphatidylcholine, Preventing This Apoptosis, Internalizes by Trans-Membrane Flipping
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The synthetic alkyl-lysosphospholipid, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ALP; Et-18-OCH₃) can induce apoptosis in tumor cells. Unlike conventional chemotherapeutic drugs, ALP acts at the cell membrane level and interferes a.o. with de novo phosphatidylcholine (PC) biosynthesis, which is essential for (tumour) cell survival [Van der Luit et al. (2002) J. Biol. Chem. 277, 39541–39547]. ALP accumulates in lipid rafts and then undergoes a process of internalization is inhibited, which is inhibited by low temperature, monensin, disruption of lipid rafts or expression of a dominant-negative mutant (K44A) of dynamin. Thus, ALP is internalized by raft- and dynamin-mediated endocytosis. Dynamin-K44A alleviated the ALP-induced inhibition of PC synthesis and rescued the cells from apoptosis induction. Additional cell rescue was attained by exogenous lysoPC, which after internalization serves as an alternative substrate for PC synthesis (through acylation). Contrary to ALP, and despite the high structural similarity to ALP, lysoPC uptake did not occur via lipid rafts and did not depend on functional dynamin, suggesting no involvement of endocytosis. Albumin back-extraction experiments suggest that (radiolabeled) lysoPC undergoes transbilayer movement (flipping). We conclude that ALP is internalized by endocytosis via lipid rafts and endocytosis to cause apoptosis, while exogenous cell-rescuing lysoPC traverses the plasma membrane outside rafts, most probably by flipping, and is then rapidly acylated to PC. (Van der Luit et al., Biochem. J. 2003, in press)
118.2
Isolation and Characterization of Different Subsets of Lipid Rafts from the RBL-2H3 (Rat Basophilic Leukemia) Cell Line

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Lipid rafts are microdomains within the plasma membrane that are enriched in certain lipids (sphingolipids, glycosphingolipids, and cholesterol), as well as various lipid-modified proteins. Rafts have been implicated as platforms for signalling through diverse cellular receptors, and also act as portals for entry of toxins, viruses, and pathogenic bacteria. Raft microdomains appear to exist in the liquid-ordered phase, which contributes to their partitioning from the surrounding liquid-disordered glycerophospholipid environment. Thy-1 is a GPI-anchored protein that is highly expressed in RBL-2H3 (rat basophilic leukemia) cells, in conjunction with the Src-family protein tyrosine kinase, Lyn, thus making the RBL-2H3 cell line an excellent model for studying lipid rafts. We have employed extraction by two different non-ionic detergents (0.5% Brij-96 and 1.0% Triton X-100) and subsequent sucrose density centrifugation to isolate lipid rafts and compare their properties. The molecular composition and density of the isolated lipid rafts depended on the type of detergent used. However, in both cases the detergent-resistant membrane (DRM) structures were isolated as sealed unilamellar vesicles (most likely as a result of coalescence of individual raft domains during the procedure), which were of similar size. Interestingly, the isolated DRM vesicles differed in their orientation, and this phenomenon was related to the nature of the detergent used. Lipid rafts isolated using Brij-96 were substantially more enriched in the lipid raft constituents Thy-1, Lyn and cholesterol, were of lower density, and formed vesicles of right-side-out orientation, while Triton X-100 lipid rafts were of higher density and formed mainly inside-out vesicles. The combined data indicates that Brij-96 and Triton X-100 are probably isolating different subsets of detergent-insoluble membrane microdomains from RBL-2H3 cells.

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118.3
Cathepsin B Localizes to Plasma Membrane Caveolae in Differentiating Myoblasts: A Role for Cathepsin B

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An increasing number of lysosomal and non-lysosomal proteases have been implicated in the differentiation of skeletal muscle cells (myogenesis). During myogenesis, cathepsin B protein and activity decreased when cells became post-mitotic, then increased as myoblasts fused to form myotubes. Using immunocytochemistry and confocal analysis, we demonstrated that cathepsin B protein relocates from perinuclear regions of proliferating myoblasts to plasma membrane caveolae of fusing myoblasts. Using Triton X-100-discontinuous sucrose density gradient fractionation and western blot analysis we demonstrated that the 31 kDa single chain form but not the 25/26 kDa doublet form of active cathepsin B was localized predominantly to the dense lysosomal fractions of proliferating myoblasts. Conversely, the 25/26 kDa form but not the 31 kDa form of active cathepsin B was localized to both the light plasma membrane fractions and the dense lysosomal fractions of fusing myoblasts. These fractions also contained the 21-kDa form of caveolin-3, a marker protein for skeletal muscle caveolae, and the 52 kDa and 47 kDa isoforms of annexin VII, a protein found to be associated with caveolin-3 in the plasma membrane of differentiating skeletal muscle cells. Finally, using "real-time" cathepsin B activity assays we demonstrated increases in both pericellular (membrane-associated plus secreted) and secreted cathepsin B activities as proliferating myoblasts fused to form multinucleated myotubes. Collectively, these data support a role for active cathepsin B at the plasma membrane during myoblast-myoblast fusion. Supported by NSERC (MJD) and by CA 56586 (BFS).
118.4
Association of LDL Receptor Related Protein (LRP1) with the Detergent Insoluble Membranes in 3T3-L1 Fibroblasts and Adipocytes Treated with Insulin

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The multifunctional receptor LRP1 is not only an endocytic receptor involved in the clearance of a variety of structurally unrelated ligands, but may also play a role in signalling. We showed previously that insulin treatment increased cell surface presentation of LRP1 in 3T3-L1 adipocytes. Since lipid rafts are membrane microdomains that harbor various signaling molecules, we hypothesized that insulin treatment might result in LRP1 association with lipid rafts in adipocytes. To test this hypothesis, we determined distribution of LRP1 between the plasma membrane and intracellular membrane in 3T3-L1 fibroblasts (undifferentiated) and 3T3-L1 adipocytes (differentiated) under basal and insulin-treated conditions. In fibroblasts, 40% of total LRP1 under basal condition was associated with the plasma membrane, and the remainder with high-density microsomes. In adipocytes, a new pool of LRP1 (~20% of total) was found in low-density microsomes (LDM). In both fibroblasts and adipocytes, insulin treatment (0.01 "M, 10 min) caused rapid LRP1 association with lipid rafts. While the raft-association of LRP1 (~15% of total) in fibroblasts resulted mainly from lateral movement of LRP1 from bulk plasma membrane, in adipocytes the raft-LRP1 was also derived from translocation of intracellular LDM-associated LRP1 onto the plasma membrane. In both fibroblasts and adipocytes, the insulin-stimulated LRP1 raft-association was blocked by pre-treatment with the phosphatidylinositide (PI) 3-kinase inhibitor wortmannin. The effect of insulin treatment on raft-association was rather specific to LRP1; other receptors such as transferrin receptor, platelet-derived growth factor (PDGF) receptor or GLUT4 showed no or marginal response to insulin in adipocytes. Although LRP1 raft-association was induced only by the treatment of insulin in adipocytes (but not in fibroblasts), substituting insulin by PDGF-BB, a ligand known to activate the PI 3-kinase pathway, exerted similar effect on LRP1 raft-association in fibroblasts. These results provide first evidence that raft-association of LRP1 upon insulin treatment is achieved via the PI 3-kinase pathway, which may play a signalling role during adipogenesis.

118.6
The Proteome of Phagosome Lipid Microdomains: Further Insights into Phagosome Functions

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Macrophages are cells of the immune system specialized in the destruction of invading pathogens, and the elaboration of an efficient immune response. Phagocytosis, the process by which pathogens are internalized by host cells, leads to the formation of phagosomes formed at the cell surface by the direct recruitment of the endoplasmic reticulum. Phagosomes engage in a complex maturation process leading to the formation of phagolysosomes, allowing the killing and degradation of microorganisms. Unfortunately, several microorganisms have evolved strategies to alter phagolysosome biogenesis, a process still poorly understood. Proteomics analyses revealed that phagosomes are made of hundreds of proteins, highlighting the complexity of the molecular mechanisms involved in phagosome functions. We have shown recently that the phagosome membrane is not homogeneous but rather made of lipid microdomains. The functions of these cholesterol-enriched microdomains on phagosomes is unknown. To understand these functions, we initiated the systematic characterization of these domains using a proteomics approach. Phagosome microdomains were isolated based on their insolubility in Triton X-100 and their flotation on OptiprepTM step gradients. MS/MS analyses of these microdomains led to the identification of 264 proteins indicating that functions such as acidification, cholesterol metabolism, signal transduction, and membrane fusion are likely to take place on the phagosome membrane microdomains. Interestingly, our results also indicate that the intracellular pathogen Leishmania donovani survives in macrophages by disturbing the proper organization of phagosome lipid rafts, emphasizing the importance of these structures in our ability to fight infection.

118.7
Assembly of CXCR1 During Chemotaxis in Response to CXCL8

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Cell adhesion molecules and soluble factors play a critical role in tumor metastasis. While much is known about the role of cell adhesion molecules, little is known as to the cause of directional migration through the endothelium during diapedesis. We previously determined that metastatic melanoma cells undergo chemotactic migration towards CXCL8, which is mediated by the receptor CXCR1. Immunofluorescence studies show that CXCR1 is concentrated in the leading edges of the migrating cell. To further analyze the dynamic role of CXCR1 in chemotaxis, we have identified the presence of CXCR1 in a Triton-insoluble floating fraction, implication its association with lipid rafts. Additionally, antibodies can be used to cap CXCR1 on the surface of WM239 melanoma cells and this process was inhibited by methyl-beta-cyclodextrin (MCD) or latrunculin B. After centrifugation in sucrose density gradients, CXCR1 was found in the low density fractions, co-localizing with G-proteins and the ganglioside GM-1, a marker for mammalian rafts. Pretreatment of cells with CXCL8 caused a shift of CXCR1 to higher density fractions, with a corresponding co-localization with clathrin and beta-arrestin. The data suggest that ligand binding triggers the endocytosis of CXCR1. Treatment of cells with MCD, prevented CXCR1 from being associated with the low density fractions. MCD treatment also inhibited chemotaxis towards CXCL8 and transendothelial migration of melanoma cells. Taken together, our results indicate the presence of CXCR1 in rafts, which may play a role in the assembly of CXCR1 and in the diapedesis process. Supported by the CIHR, Canada.
119.1
Effect of UV-C Irradiation on the Activity Modulation of Phospholipase D in Vero76 Cells
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Phospholipase D (PLD) activity is known to be modulated through cellular signalings linked to oxidative stress and membrane perturbation. In this study, we examined effect of UV-C (100–280 nm) on the PLD activity in monkey kidney epithelial (Vero76) cells. At the dose of 10000 J/m² of UV-C irradiation the PLD activity was induced approximately 10 folds over the basal activity. This UV-C induced PLD activity was dependent on the presence of extracellular calcium and inhibited by amastin, an intracellular thiol antioxidant, and catalase. By pretreatments of genistein, a protein tyrosine kinase inhibitor, and Ro32-0432, protein kinase C inhibitor, the UV-C induced PLD activity was significantly inhibited. The PKC signaling linked to the UV-C induced PLD activity was further confirmed by down-regulation of PKC by PMA preincubation. Among Vero76 cells expressed with PLD isoforms, mouse PLD2 (mPLD2) gene overexpressed Vero76 cells responded more sensitively to the UV-C irradiation than PLD1b gene expressed cells. The mPLD2 immunoprecipitated was found to be phosphorylation at serine residue(s). However, this phosphorylated serine residue(s) was immediately dephosphorylated upon UV-C irradiation, followed by dephosphorylation of serine residue(s) at 2–15 min after irradiation. In the same period of 2–15 min, tyrosine residue(s) in mPLD2 was simultaneously phosphorylated. The status of phosphorylation of tyrosine residue(s) coincided with the time course of induction by UV-C irradiation. Our results suggest that UV-C irradiation induced PLD activity is mediated through extracellular calcium as well as intracellular oxidative signals which appears to be dependent on the phosphorylation of serine and tyrosine residue in PLD2.

119.2
Haploinsufficiency of the Pten Lipid Phosphatase Induces Insulin Hypersensitivity
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An impairment of insulin production or function has been implicated in the etiology of diabetes mellitus. Signalling through phosphatidylinositol 3-kinase (PI3K) is a key pathway whereby insulin regulates the uptake and storage of glucose. Pten, an inositol 3-phosphatase, negatively regulates the PI3K pathway by catalyzing the reverse enzymatic reaction of PI3K; however, its role in insulin sensitivity in vivo is unclear.

Here we report that Pten heterozygous (Pten-/-) mice, which have decreased Pten protein levels and activity, display insulin hypersensitivity. Pten-/- mice exhibit increased glucose tolerance and insulin sensitivity. Following glucose challenge, blood glucose in the Pten-/- mice returned to fasting levels in approximately half the time as wild type mice whereas insulin production and pancreas histology were similar in both sets of mice. Moreover, during an insulin tolerance test, Pten +/- mice displayed delayed recovery to glycemia. Uptake of [3H]-deoxyglucose was monitored in cultured primary myocytes derived from Pten-/- and wild type mice. In the presence and absence of insulin, glucose uptake by Pten +/- myocytes was significantly enhanced as compared to wild type cells. An early downstream effector of PI3K signalling is Akt, which is phosphorylated and activated in a PI3K-dependent manner. In the presence of insulin, Akt remained highly phosphorylated in Pten +/- myocytes for up to 4 h, whereas phosphorylation peaked and began to decrease after 2 h in wild type cells. Our results show that Pten plays a role in regulating insulin sensitivity and may be a potential drug target for treatment of Type II diabetes.
Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two-second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol mediates the activation of protein kinase C (PKC), and inositol 1,4,5-trisphosphate releases calcium from intracellular stores. The delta-type PLCs are thought to be the primary forms expressed in mammals. Among these, PLC delta1 is expressed abundantly in most tissues and has been studied most intensively. However, its biological and physiological functions are still poorly understood. Here we report that PLC delta1-deficient mice undergo progressive hair loss in the first postnatal hair cycle. Epidermal hyperplasia was observed, and many hairs in the skin of PLC delta1-deficient mice failed to penetrate the epidermis and become zig-zagged owing to occlusion of the hair canal. Two major downstream signals of PLC, calcium elevation and PKC activation, were impaired in keratinocytes and skin of PLC delta1-deficient mice. In addition, many cysts that had remarkable similarities to interfollicular epidermis as well as hyperplasia of sebaceous glands were observed. From these results, we conclude that PLC delta1 is required for hair follicle formation.

**Phospholipase C Delta1 Is Required for Hair Follicle Formation**

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Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover and is involved in a variety of physiological functions. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two-second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol mediates the activation of protein kinase C (PKC), and inositol 1,4,5-trisphosphate releases calcium from intracellular stores. The delta-type PLCs are thought to be the primary forms expressed in mammals. Among these, PLC delta1 is expressed abundantly in most tissues and has been studied most intensively. However, its biological and physiological functions are still poorly understood. Here we report that PLC delta1-deficient mice undergo progressive hair loss in the first postnatal hair cycle. Epidermal hyperplasia was observed, and many hairs in the skin of PLC delta1-deficient mice failed to penetrate the epidermis and become zig-zagged owing to occlusion of the hair canal. Two major downstream signals of PLC, calcium elevation and PKC activation, were impaired in keratinocytes and skin of PLC delta1-deficient mice. In addition, many cysts that had remarkable similarities to interfollicular epidermis as well as hyperplasia of sebaceous glands were observed. From these results, we conclude that PLC delta1 is required for hair follicle formation.
Necessary for Lysophosphatic Acid-induced NIH3T3 Cell Motility

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Lysophosphatic acid (LPA) is a lipid mediator that may play an important role in growth and survival of carcinomas. LPA binding to G protein-coupled receptors encoded by the Edg gene family promotes growth and motility in numerous cell lines. Rho family members have been demonstrated as key modulator during LPA-induced cell motility, but the mechanisms remain to be elucidated. In the present study, we found that activation of p21-activated kinase 1 (PAK1) was required for LPA-induced cell motility. These findings strongly indicate that PAK1 is located downstream of Gi, P3K\(^\gamma\), Rac1, Cdc42, and plays a critical role in LPA-induced NIH3T3 cell motility. Supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG3-20801–0001).

HIF-1alpha Protein Is a Potential Target for S-Nitrosation

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Hypoxia inducible factor 1 (HIF-1) is a heterodimeric protein, which consists of HIF-1\(\alpha\) and HIF-1\(\beta\) subunits. HIF-1\(\beta\) subunit is constitutively expressed, whereas HIF-1\(\alpha\) expression is inducible. HIF-1\(\alpha\) is a transcription factor that controls the expression of more than 40 target genes, which are essential for angiogenesis, hypoxic signal transduction, tumour progression et al. Considering that reactive nitrogen species are recognized for post translation protein modifications, among others S-nitrosation, we asked whether HIF-1\(\alpha\) is a target for S-nitrosation. In vitro NO\(\neg\) donating NO species such as GSNO and SNAP provoked massive S-nitrosation of purified HIF-1\(\alpha\). However, thiol modification of purified protein was marginal when treated with spermine-NO\(\neg\)GSH, a NO radical donating compound. However, spermine-NO\(\neg\)GSH in the presence of O2\(-\), generated by xanthine/xanthine oxidase, regained S-nitrosation, most likely via formation of N2O3-like species. In vitro, S-nitrosation of HIF-1\(\alpha\) was attenuated by the addition of GSH or ascorbate. In renal carcinoma cells (RCC) 4 and human embryonal kidney (HEK) 293 cells GSNO or SNAP reproduced S-nitrosation of HIF-1\(\alpha\), however with a significantly reduced potency that amounted to modification of 3 to 4 SH-groups only. Importantly, endogenous formation of NO in RCC4 cells via inducible NO-synthase (NOS) elicited S-nitrosation of HIF-1\(\alpha\) that was sensitive to inhibition of iNOS activity with NMMA. NO stabilized HIF-1\(\alpha\) via inducible NO-synthase (NOS) elicited S-nitrosation of HIF-1\(\alpha\) that was sensitive to inhibition of inosine with NMMA. NO stabilized HIF-1\(\alpha\) susceptible to the addition of N-acetyl-cysteine that destabilized the protein in close correlation to the disappearance of S-nitrosated HIF-1\(\alpha\). In conclusion, HIF-1\(\alpha\) is a target for S-nitrosation by exogenously and endogenously produced NO as well as to N2O3-like species.

Signaling Pathways Controlling Low Density Lipoprotein Expression by Intracellular Calcium in Hepatic Cells

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We have previously shown that different extracellular stimuli require signaling through Raf-1/MEK/p42/44MAPK signaling pathway to induce LDL receptor expression in hepatic cell types. The cellular signaling pathway and the molecular mechanism that underlie LDL receptor induction by intracellular calcium have now been examined. We show that increase in calcium dramatically induce LDL receptor expression in a sterol-resistant manner, without significantly affecting squalene synthase expression. Pharmacological reduction of intracellular and extracellular free calcium reversed the induction process. Although increase in intracellular calcium led to sustained activation of p42/44MAPK, induction of LDL receptor expression is independent of this pathway, since it was only slightly inhibited by MEK-1/2 inhibitors at doses which completely blocked p42/44MAPK activation. However, inhibition of protein kinase C by specific inhibitors completely abrogated LDL receptor induction without at the same time inhibiting p42/44MAPK activation. Studies to investigate the role of sterol response element-binding proteins and histones modifications in order to define the mechanism of induction will also be presented. The above results highlights the presence of a novel mechanism that has the ability to differentially regulate genes involved in cholesterol uptake and biosynthesis. Such a mechanism may be functional when a separate control of cholesterol biosynthesis and uptake via LDL receptor is required.
Increased fatty acid flux has been suggested to be strongly associated with insulin resistant states. We have investigated the effect of free fatty acids, oleate and palmitate, on insulin sensitivity and insulin signaling pathways in a muscle cell line, C2C12. First insulin sensitivity was assessed by measuring glucose uptake. While insulin treatment of control cells induced a 50% increase over basal glucose uptake, cells treated with either oleate or palmitate showed a statistically significant reduction in insulin stimulated glucose uptake ($P < 0.05$). To investigate the mechanisms involved, we examined the effects of oleate and palmitate on insulin sensitivity. There was no significant difference in insulin receptor substrate (IRS-1) protein levels of the control compared to cells treated with oleate or palmitate. IRS-1 tyrosine phosphorylation showed an increasing trend in the cells treated with oleate in the basal state in comparison with controls and no further increase was observed upon stimulation with insulin. However, cells treated with palmitate showed no change in basal but a clear reduction in the insulin stimulated tyrosine phosphorylation of IRS-1. We also tested the levels of protein tyrosine phosphatase (PTP-1B) and found no differences in cells treated with either oleate or palmitate. The phosphosatase screen based on a proteomics approach clearly showed a significant increase in the phosphorylation of PKC $\alpha$, $\beta$, $\delta$ and $\epsilon$ isoforms in cells treated with oleate. In addition, the general PKC inhibitor, Bis-I, was found to reverse the oleate-induced reduction in insulin-stimulated glucose uptake but not that of palmitate. The data suggest that the monounsaturated fatty acid, oleate, may directly induce muscle insulin resistance via a PKC dependent mechanism.

### 119.13
Lipid Phosphate Phosphatase-2 Regulates Proliferation and Cell Cycle Progression in Rat 2 Fibroblasts

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Bioactive lipids such as lysophosphatidic acid (LPA), sphingosine-1-phosphate (SIP), ceramide-1-phosphate (C1P), and phosphatidic acid (PA) are important regulators of physiological processes such as angiogenesis and tumor invasiveness by regulating proliferation, survival, and chemotaxis of cells. The lipid phosphate phosphatasases (LPPs) de-phosphorylate these lipid mediators, thus regulating the balance between lipid phosphates and their products in the cell or at the plasma membrane, and thereby regulating the signalling cascades initiated by these bioactive lipids. In vivo studies have indicated that LPPs are important in development and tumorigenesis, but the exact mechanisms by which the LPPs elicit many of their effects are not well understood. LPP2 (Pap2c) has been overexpressed in Rat2 fibroblasts. Stable cell lines overexpressing the enzyme have a significantly decreased proliferation rate compared to control cells. Using flow cytometry, it has been determined that overexpressing cells enter S-phase prematurely after attachment to culture dishes and subsequently arrest in G2/M phases. Additionally, cells overexpressing LPP2 fail to arrest in G1 in response to UV irradiation, demonstrating a failure in the G1/S checkpoint. Western blots show that levels of all three D-type cyclins and cyclin E are significantly elevated in cycling and arrested LPP2-overexpressing cells. This may overwhelm the inhibition by downstream targets of p53 and be sufficient to cause cells to proceed into S phase even after DNA damage. An inactive mutant version of LPP2 has no effect on cell cycle progression or growth, therefore the phenotype can be attributed to the lipid phosphate activity of the enzyme. Additionally, the phenotype observed is characteristic of cells transfected with LPP2 and not LPP1 or LPP3 isoforms.

### 119.14
Cdk5 Is Involved in Neuregulin-Dependent Activation of PI-3 Kinase and Akt Activity Mediating Neuronal Survival

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The PI-3 kinase/Akt signaling pathway plays an important role in mediating survival signals in wide variety of neurons and cells. Recent studies show that Akt also regulates metabolic pathways to regulate cell survival. In this study, we reported that cyclin-dependent kinase-5 (Cdk5) regulates Akt activity and cell survival through the neuregulin--mediated PI3-Kinase signaling pathway. We found that brain extracts of Cdk5-/− mice display a lower PI-3 kinase activity and phosphorylation of Akt compared to that in wild type mice. Moreover, we demonstrated that Cdk5 phosphorylated Ser-1176 in the neuregulin receptor ErbB2, and Thr-871 and Ser-1204 in the ErbB3 receptor. We identified the Ser-1120 sequence RSRFRP in ErbB3 as a novel phosphorylation consensus sequence of cdk5. Finally, we found that Cdk5 activity is involved in neuregulin-induced Akt activity and neuregulin-mediated neuronal survival. These findings suggest that cdk5 may exert a key role in promoting neuronal survival by regulating Akt activity through the neuregulin/PI3-K signaling pathway.

### 119.15
PC-1 Phosphodiesterase Activity in a Type II Diabetic Animal Model

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Membrane glycoprotein PC-1 inhibits insulin signaling and when overexpressed, plays a role in human insulin resistance. The present study investigated the involvement of nitric oxide (NO) in the etiology of type II diabetes by measuring the levels of glycoprotein PC-1 in muscle and adipose tissues of a type II diabetic rat model. Adult Wistar rats (120–300 g) were injected with streptozotocin (STZ; 75 mg/kg) and nicotinamide (150 mg/kg) to induce type II diabetes, with controls, injected with saline. After four weeks, an oral glucose tolerance test was performed, and diabetic and control rats sacrificed. Mean plasma glucose levels in type II diabetic rats was 13.52 ± 2.92 mmol/L after 2 h. PC-1 phosphodiesterase activity in the adipose tissues of the rats treated with STZ and nicotinamide (0.34 ± 0.12 nmol of PNTP hydrolyzed/mg protein/min) was markedly increased to a level of 4-fold greater than that in the controls; ($P < 0.05$). However, the PC-1 phosphodiesterase activity of the muscle cytosol fractions of the diabetic rats was not different from controls (3.84 ± 0.35 vs 3.75 ± 0.54 nmol of PNTP hydrolyzed/mg protein/min; $P > 0.05$). This study indicates that NO released from streptozocin has a greater effect on the PC-1 phosphodiesterase activity in adipose tissues than in muscle of type II diabetics. This suggests that weight loss and the associated decrease in PC-1 phosphodiesterase activity should improve blood sugar control in diabetics. There is also a possible role of NO in the etiology of type II diabetes mellitus.
120.1

Contribution of Negatively and Positively Charged Residues in S2, S3 and S4 to the Final Membrane Topology of the Voltage Sensor in the Potassium Channel, KAT1

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KAT1 is a member of the Shaker family voltage-dependent potassium channels, which has six transmembrane segments (S1–S6), including a highly positively charged voltage-sensing segment, S4 (Uozumi et al., PNAS 95, 9773–9778, 1998). The process involved in the integration of the protein into the membrane remains to be elucidated. We have used in vitro translation and translocation experiments to evaluate the interactions between residues in the voltage sensor of KAT1, and their effect on the final topology in the endoplasmic reticulum (ER) membrane. The results obtained are as follows: A pair of transmembrane segments, S3 and S4 is integrated synergistically (Sato et al., PNAS 99, 60–65, 2002). D95 in S2 assists in the membrane insertion of S3-S4. D105 helps to prevent S4 from being released into the ER lumen. Transient interactions between D105 in S2 and R171 in S4 and between negative residues in S2 or S3 and R174 in S4 should occur during membrane integration. These data show the role of charged residues in S2, S3 and S4, and identify posttranslational electrostatic interactions between charged residues that are required to achieve the correct voltage sensor topology in the ER membrane (Sato et al., JBC 278, 13227–13234, 2003).

120.2

NMR Investigations of the Structure of Myelin Basic Protein

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Myelin basic protein (MBP) is a family of developmentally-regulated, and highly post-translationally modified, isoforms and charge isoforms involved in formation and maintenance of the myelin sheath. The 18.5 kDa isoform of MBP is the most common in adult humans and has been the most studied. In its deiminated form (arginyl residues are converted to citrulline), MBP is also a candidate autoantigen in the autoimmune disease multiple sclerosis (MS). However, MBP’s 3D structure is still unknown, almost 40 years after its first isolation. Traditional structural approaches such as X-ray crystallography have been unsuccessful because all MBP isoforms are ‘intrinsically unstructured’, or ‘natively-unfolded’, to facilitate their interactions with ligands. We have expressed a recombinant form of the murine 18.5 kDa isoform of MBP in E. coli BL21 (DE3)pLySS (Novagen) cells. Upon growth in M9 minimal media supplemented with 15NH4Cl, a yield of 10 mg of purified MBP was obtained per liter of culture. NMR studies were undertaken on a Bruker Avance 600 MHz spectrometer, at 298K. The protein undergoes rapid rotational correlation resulting in narrow lines. We present heteronuclear multidimensional NMR spectra (HSQC) and 3D ENDOR studies in 250 mM NaCl, or in 30% TFE-d2 in water. In either case, the protein preparation could be concentrated to a maximum of 2 mM. Two-dimensional HSQC data were far superior in 30% TFE than in phosphate buffer alone. Moreover, TFE has several further advantages for NMR studies of MBP. Firstly, it mimics the protein’s in vivo environment of the cytoplasmic leaflet of the myelin membrane. Secondly, it appears to stabilize the protein in solution, and HSQC spectra have demonstrated no degradation or precipitation of the sample after 3 months. Preliminary peak assignments using 3D NMR methods on 13C15N-labelled MBP will be presented. This work was supported by NSERC, CIHR, and the MS Society of Canada.

120.3

Structural and Functional Characterization of P-Glycoprotein Catalytic Transition State Complexes

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The P-glycoprotein multidrug transporter (Pgp) couples ATP hydrolysis at two cytoplasmic nucleotide binding domains (NBDs) to the transport of many different hydrophobic compounds. Vanadate, beryllium fluoride, and fluoride-trap nucleotide in one of the NBDs by forming a stable complex (Pgp-M2-ADP-X), which is proposed to resemble the catalytic transition state. In myosin, BeFx traps nucleotide in an immediately pre-hydrolysis conformation, while AIF4 and Vi trap slightly different post-hydrolysis conformations. Here, we report a study of the structure and function of Pgp transition state complexes formed via pre-incubation with ATP (catalytically forward) or ADP (reverse) and BeFx, AIF4, or Vi. Pgp ATPase activity was stably inhibited when pre-incubated with ATP or ADP and BeFx, AIF4, or Vi. Acylamide quenching of Pgp intrinsic Trp fluorescence, and binding of TNP-ADP/TNP-ADP and Hoechst 33342 (H33342) indicates that all three trapping reagents lead to the formation of Pgp transition state complexes with distinct conformations. Trapping in the forward direction (ATP) leads to a different conformation than that observed for trapping in the reverse direction (ADP). All transition state complexes bound TNP-ATP/TNP-ADP (Kd = 0.6 micromolar) and H33342 (Kd = 1 micromolar) with similar affinities compared to native state Pgp, however the fluorescence enhancement of bound TNP-nucleotide and H33342 varies with the trapping reagent as well as the trapping direction. This work demonstrates that the conformation of the Pgp transition state complex likely varies due to trapping at different points in the catalytic cycle, and that the forward and reverse-trapped conformations are not structurally identical.

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120.4

Heteronuclear NMR Spectroscopy of the Integral Membrane Protein Glycerol Facilitator

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One result of the worldwide effort to sequence genomes is that we now know that membrane proteins constitute approximately 25% of all cellular proteins. Unfortunately, relatively little high-resolution NMR structure and dynamics data have been reported for helix bundle membrane proteins. This is due to low levels of cellular expression coupled with poor solubility characteristics and slow molecular tumbling of detergent- and lipid-solubilized protein. To address the first problem, we have been developing methods for over-expression and purification of the E. coli integral membrane protein glycerol facilitator. We are able to produce up to 5 mg of 95% pure protein per litre of rich medium. The protein can be concentrated to approximately 0.5 mM in sodium dodecyl sulfate solution suitable for 1H NMR spectroscopy. One-dimensional 1H NMR spectra exhibit features typical of protein methyl, methylene, and alpha hydrogens, in addition to an amide and aromatic region, suggesting that at least parts of the protein undergo rapid rotational correlation resulting in narrow lines. Expression of the glycerol facilitator in 15N-enriched minimal medium lowers the yields to 0.6 mg of uniformly labelled protein per liter of growth medium. We present heteronuclear multidimensional NMR spectra (HSQC and HSO-C-TROSY) collected on uniformly 15N enriched glycerol facilitator protein in different detergents. pH titrations of the one- and two-dimensional spectra collected with pre-saturation of solvent reveal a subset of slowly exchanging amides indicating the presence of secondary structure. The spectra and hydrogen-exchange data are interpreted in terms of previously published models of the conformation of the protein based on circular dichroism and fluorescence measurements.
Spin Label Study of the Interaction Between Triton X-100 and Red Blood Cells

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The nonionic surfactant Triton X-100 is a very important surfactant, because the most common use in biomembrane studies. In the present study we investigated the interaction of the nonionic surfactant Triton X-100 with erythrocyte membranes by electron paramagnetic resonance (EPR). Analysis of EPR spectra of spin label (5-doxyl stearic acid D 2 mol% v/v) incorporated into lipid bilayer of the erythrocyte membrane (hematocrit 40%) and in presence of Triton X-100 [0.1 to 50 mM], indicates an increase in the mobility and decrease in the order of membrane. The hemolytic effect was measured by hemoglobin and phosphate leakage in the supernatant and the results were compared to the EPR data. The hemolysis phenomena was characterized as a membrane to micelle transition, evaluated by the spin label incorporation into different (membrane, mixed membrane, mixed micelle, micelle) aggregates. EPR technique also allowed the determination of the cmc of Triton X100 (cmc = 2.5x10^-4 M) in good agreement with the literature.

Molecular Cloning of a Novel K+/Ca2+ Exchanger

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Na+/Ca2+ exchangers are a crucial component of the Ca2+ efflux system across the plasma membrane in a wide range of tissues and are encoded by a superfamily of genes that all share duplicated hydrophobic sequences known as a-repeats. Here, by using bioinformatics and molecular cloning tools, we have identified a novel gene and isolated cDNA clones from various mouse tissues, which encode the sixth member of the K+-dependent Na+/Ca2+ exchanger family, NCKX6. The NCKX6 protein shares about 47% sequence identity in the putative a-repeat regions with previously identified Na+/Ca2+ exchangers. NCKX6 transcripts of 4 kb are widely and abundantly expressed in all tissues examined. At least two alternatively spliced isoforms of this novel gene were identified that resulted from deletion of one or two exons and caused a frame shift in the coding region. The spliced isoforms differ in the C-terminal hydrophobic domain and therefore possibly display distinctive functional properties and/or physiological regulation. Functional analysis by digital imaging of fura-2 loaded transfected HEK cells demonstrated that the short isoform exhibited K+-dependent Na+/Ca2+ exchange activity while the long isoform did not. A FLAG epitope was introduced into the loop between TM0 and TM1 of NCKX6. Immunofluorescence studies indicated that the long isoform was retained within ER membrane in transfected cells. The discovery of NCKX6 therefore reveals a novel member of the Na+/Ca2+ exchanger superfamily whose ubiquitous expression in all tissues suggests an important role for K+-dependent Na+/Ca2+ exchange in maintaining cellular Ca2+ homeostasis in diverse tissues and cell types. Supported by the Canadian Institutes of Health Research and the Alberta Heritage Foundation for Medical Research.

Structure of the Yeast Saccharomyces cerevisiae Protoporphyrinogen Oxidase: Homology Modeling and Biochemical Studies of the Active Site Topology

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Protoporphyrinogen oxidase is the penultimate enzyme of the heme biosynthetic pathway. It catalyzes the oxidative O2-dependent aromatization of the protoporphyrinogen IX to the protoporphyrin IX, the precursor of both hemes and chlorophylls. Protoporphyrinogen oxidase is an atypical membrane bound flavoprotein with FAD as a cofactor. Diphenyl ether-type herbicides are very potent inhibitors of protoporphyrinogen oxidase activity. We are presently investigating the structure of Saccharomyces cerevisiae protoporphyrinogen oxidase by homology modeling. As templates, we used three proteins of a same structural family that shares with protoporphyrinogen oxidase several structural and functional features (N-terminal ADP binding fold, oxidases O2-dependent with FAD as cofactor etc): L-Amino Acid Oxidase from Calloselasma rhodostoma, Polyamine Oxidase from Zea mays and Monoamine oxidase B from human. Identity scores between these proteins and yeast protoporphyrinogen oxidase are very low (less than 15%). Therefore, we investigated sequences by manual alignment using mainly the hydrophobic cluster analysis method. We are performing experiments to validate the model. Hence, we try to identify the substrate binding site by using surface plasmon resonance and by coupling affinity photolabeling to mass spectrometry. We have already demonstrated that the protoporphyrinogen oxidase active site is embedded into a catalytic tunnel as described in the structures of the templates we chose. The preliminary data showed that the tunnel extends for 15 Å at least. Moreover some crystallization experiments with recombinant purified yeast protoporphyrinogen oxidase are presently performed.

The Molecular Environment Surrounding Yeast Mitochondrial F1F0-ATP Synthase Subunit 8

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The F1F0-ATP synthase is a remarkable molecular motor divided into two distinct units, F1 and F0, which couple the energy produced by respiratory metabolism to the synthesis of ATP. The well-characterised F1 moiety contains the catalytic headpiece (on which reside the sites for the synthesis of ATP) and a central stalk that functions as the rotor of the engine. The F0 moiety contains the proton translocation pathway and the mechanical ‘stator’. In yeast mitochondrial ATP synthase (mtATPase), subunit 8 (Y8) is a small intrinsic membrane protein essential for the assembly and stability of F0. We examined the structural arrangement and local environment around Y8 in intact mtATPase. The stoichiometry of Y8 in mtATPase was determined by co-expression in yeast cells of two different Y8 variants, each bearing either an HA or FLAG epitope at their N-termini. Immunoprecipitation of intact mtATPase complexes via these tags demonstrated that the single copy of Y8 is present in mtATPase. Cysteine scanning mutagenesis was used to further explore Y8 in intact mtATPase. Expression of mutated forms of Y8, bearing single cysteine replacements for 45 of its native amino acids, allowed restoration of respiratory function in yeast cells lacking endogenous Y8. Thiol-specific chemical labelling was used to probe the structure of the single transmembrane stem of Y8 across the inner mitochondrial membrane (N-terminus facing out). Site-directed cross-linking revealed interactions between Y8 and mtATPase proteins 6, b, d and f, which contribute to the stator stalk. In conclusion, the single copy of Y8 is an essential component of the mtATPase stator, necessary for physically and functionally coupling proton pumping to the synthesis of ATP. Supported by Australian Research Council.
120.9
Solution Behaviour of TonB from Escherichia coli: Self Association and Interactions with an Outer Membrane Receptor

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TonB from Escherichia coli, an energy-transducing cytoplasmic membrane protein (Mr: 26 kDa) extends into the periplasm and interacts with outer membrane (OM) receptors. H6.88-TonB, a recombinant N-terminal hexahistidine-tagged variant with no signal anchor sequence, was subjected to analytical ultracentrifugation (AUC). Sedimentation velocity experiments indicated that H6.88-TonB adopts different conformations in solution. At a protein concentration of 0.30 mg/ml, a slower sedimenting species was observed at 1.22 S; a more abundant, faster sedimenting species was observed at 2.88 S. At 0.90 mg/ml, the slower species sedimented at 1.34 S and became the abundant form, indicating a concentration-dependent conformational change. The addition of detergent lauryldimethylamine-N-oxide (LDAO) prevented H6.88-TonB from adopting multiple conformational states in solution, suggesting that detergent interacts with the protein. The C-terminal domain of TonB, residues 155–239 expressed with an N-terminal hexahistidine tag, was also examined. At 0.63 mg/ml, H6.88-TonB(155–239) sedimented as a monomer, whereas at 1.25 mg/ml it sedimented as a dimer; concentration-dependent dimerization of full-length TonB was not observed by AUC. H6.88-TonB(155–239) was complexed with FhuA-ligand-ferricodin, followed by crosslinking with dimethyl suberimidate. Electro- phoretic analysis of the reaction products indicated a major band, consistent with a 1:1 stoichiometry of [H6.88-TonB(155–239)–FhuA]. A minor band corresponding to a [H6.88-TonB(155–239)–FhuA] stoichiometry of 2:1 was also observed. In the absence of ligand, complexation occurred but was inefficient. Ligand binding to FhuA thus enhances its ability to complex with H6.88-TonB(155–239), and in solution the monomeric form of TonB is preferred for interactions with the OM receptor.

120.10
Interaction Analysis of the Bacterial Outer Membrane Protein FhuA and Its Energy Transducing Partner TonB

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The FhuA (ferric hydroxamate uptake) receptor in the outer membrane of Escherichia coli facilitates the high affinity transport of siderophores including ferricodin (Fc). Energy for siderophore transport into the periplasm is provided by the cytoplasmic membrane protein complex TonB/ExbB/ExbD. Exploiting surface plasmon resonance (BIACORE), interactions between FhuA and TonB were analyzed. Two TonB proteins were expressed, each with an N-terminal hexahistidine tag, their signal anchor regions having been genetically deleted. H6.88-TonB (a.a. 33–239 of mature TonB sequence) and a C-terminal (CT) variant of H6.88-TonB (a.a. 153–239 of mature TonB sequence) were each immobilized on the surface of CM4 sensor chips using amine coupling. FhuA/Fc over a range of concentrations was injected and interactions with the TonB proteins were measured in real time. Equilibrium analysis of FhuA and H6.88-TonB (CT) demonstrated a single interaction site with a two-fold decrease in affinity in the presence of Fc (Kd = 1490 nM) compared to the absence of Fc (Kd = 842 nM). In contrast, the H6.88-TonB protein displayed two distinct interaction sites, one having Kd values consistent with the C-terminal site with Fc (Kd = 1530 nM) and without Fc (Kd = 564 nM). Another higher affinity site was positioned outside the C-terminal segment through which Fc enhanced the interaction (Kd = 5 nM) compared to interactions without Fc (Kd = 37 nM). Global analysis using SPRevolution software revealed that experimental data for the H6.88-TonB protein matches both conformational change and two-population models. The combination of global analysis with equilibrium analysis demonstrates intricate interactions between FhuA and TonB, incorporating multiple binding sites and a rearrangement of the complex.

120.11
Molecular Interactions of Bacterial Outer Membrane Transporters and the Energy-Transducer TonB

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Siderophores and vitamin B12 require high affinity receptors for their uptake across the outer membrane (OM) of Escherichia coli. Energy necessary for transport of these molecules is provided by the proton motive force, delivered by physical contact with TonB-ExbB-ExbD, a complex anchored in the cytoplasmic membrane. Crystallographic structures of FhuA, FepA and FecA, three OM receptors, demonstrate their common structural features: a beta-barrel and a globular cork domain. The cork inserts into the barrel, facing both the external binding pocket and the periplasm. TonB boxes of OM receptors are recognized as sites of interaction between OM receptors and TonB but little is known about the mechanism by which the TonB-ExbB-ExbD complex allows energy transport for siderophores and vitamin B12. To identify other regions of OM receptors interacting with TonB, we selected TonB-binding sequences from a random library of peptides displayed on phages. Eight peptides mapped to identical or similar amino acid sequences of OM receptors of known structure. For FhuA, FepA and FecA, a loop of the cork domain that extends toward the external binding pocket was identified as a potential interaction site for TonB. Superposition of these loops in FepA and FecA revealed their similar C-alpha trace. In addition, three different peptides were mapped to periplasmic turns, one each of FhuA, FepA and FecA. These findings demonstrate that more than one region of OM receptors is involved in interactions with TonB.

120.12
The Na+/H+ Exchanger Cytoplasmic Tail: Structure, Function and Interactions with Tescalcin

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We characterized the regulatory cytoplasmic tail of the Na+/H+ exchanger using a Histidine tagged protein of the C-terminal 182 amino acids (His182). Both Tescalcin and calmodulin were demonstrated to bind to the His182 protein. Calcium caused an increase in fluorescence of Cascade blue labeled His 182 protein suggesting of exposure of the label to a more hydrophilic environment. Decreasing external pH caused a transient increase in cascad blue fluorescence followed by a decrease in fluorescence of cascade blue labeled Na+/H+ exchanger. Tescalcin caused a decrease in fluorescence by labeled His182 protein and calcium was able to reverse this effect. Expression of tescalcin in vivo inhibited activity of the Na+/H+ exchanger. We examined the CD spectra of His182 in the presence or absence of tescalcin. The C-terminal amino acids demonstrated a very small amount of alpha-helical structure and much more beta-sheet and beta-turn. This was not greatly affected by the presence of tescalcin but calcium caused an increase in the amount of beta structure and a decrease in the unstructured proportion of protein. Sedimentation equilibrium analysis demonstrated that the C-terminal 182 amino acids exist predominantly as a monomer. The results suggest that the C-terminal of the Na+/H+ exchanger exists primarily as a monomeric protein that binds regulatory proteins and can change conformation depending on pH and calcium. Conformation changes in this region of the protein may be responsible for altering the pH sensitivity of the intact Na+/H+ exchanger. Supported by CIHR.
120.13    Mutation of Structurally Important Residues Abolishes the Activity of Na+/H+ Exchanger Isoform

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The mammalian Na+/H+ exchanger isoform 1 (NHE1) is a ubiquitously expressed integral membrane protein that functions to remove one intracellular proton in exchange for one extracellular sodium ion. To date, several residues in transmembrane helix four (TM IV) have been implicated in ion binding and transport. The importance of TM IV for NHE1 function is extremely interesting because this proposed transmembrane helix contains highly conserved proline residues. These proline residues may increase the flexibility within TM IV, cause a ~267 kink in the helix, or allow for the presence of free backbone carbonyls that can directly interact with transported cations. We examined the functional importance of the proline residues in TM IV of NHE1 by using site-directed mutagenesis to mutate Pro167 and Pro168 to alanine, glycine, and cysteine and to mutate Pro170 to alanine. The activity of each mutated NHE1 protein was assessed by measuring intracellular pH changes in stably transfected cells that lacked an endogenous Na+/H+ - exchanger. The Pro168 and Pro170 mutant proteins were expressed at levels similar to wild-type NHE1 and were targeted to the plasma membrane. However, the mutants Pro167Gly and Pro167Ala were expressed at lower levels than wild-type NHE1, and a significant portion of Pro167Ala was retained intracellularly. The Pro167Gly, Pro168Ala, Pro167Gly, and Pro168Cyts mutant abolished Na+/H+ - exchange activity, while the Pro168Gly and Pro167Ala mutations caused significantly reduced activity. In contrast, the Pro170Ala mutant retained normal Na+/H+ - exchange activity. The results indicate that both Pro167 and Pro168 in TM IV of NHE1 are required for normal Na+/H+ exchange activity, and that mutation of Pro167 affects expression and membrane targeting of the exchanger. Supported by CIHR, AHFMR, and HSFC.

120.14    Kinetic Analysis of Protein Palmitoylation: Is Exponential Decay an Appropriate Description?
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Protein palmitoylation is the only known reversible lipid modification of proteins. The reversibility of the reaction is indicative of a potential regulatory role for this modification. Traditionally, the half-life of protein-bound palmitate has been estimated by metabolically labelling cells with 3H palmitate then following the decay of protein-bound radioactivity over time after label withdrawal. Fitting experimental data from 3H palmitate pulse/chase experiments to a model of exponential decay often presents well-documented mathematical and logical anomalies. An example of a mathematical anomaly is an increase in the initial portion of the curve instead of the decrease expected for exponential decay. An example of a logical anomaly is half-life estimates of the palmitoylation exceeding the half-life of the carrier protein backbone itself. In this work, we assessed whether the conditions permitting the use of exponential decay were respected under the experimental conditions used to measure the half-life of protein-bound palmitate. For this purpose, we measured 3H palmitate uptake, time-dependent changes in 3H palmitate incorporation into proteins, and total cellular palmitoyl CoA. We found that the dynamic evolution of these parameters is incompatible with an exponential decay description, accounting for both the mathematical and logical anomalies encountered when such a fit is forced on data sets. Based on the experimental data collected, we propose an alternative mathematical representation of protein-palmitoylation dynamics.

120.15    Structural Characterization of Equilibrative Nucleoside Transporters
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Nucleoside transporters (NTs) are transmembrane proteins responsible for the movement of nucleosides across cell membranes. Since NTs also transport clinically important nucleoside analog drugs, detailed structural analyses will provide increased understanding of the mechanism of substrate translocation and will facilitate drug design. However, NTs are hydrophobic and typically expressed at low levels, making structural analysis particularly challenging. We identified (Acimovic & Coe 2002) a bacterial protein (Tsx) that shows similarities in both sequence and function to the predominant NT in humans, hENT. We propose Tsx as a model for overexpression and structural analysis of NTs. The tsx gene was cloned into pET-30alpha (+) and the overexpressed recombinant protein, Tsx-His, was confirmed by Western analysis and fingerprinting by MALDI mass spectrometry. Recombinant Tsx-His was purified by metal chelate chromatography, solubilized and concentrated for crystallization trials. To extend our findings, we are pursuing parallel studies of hENT1 over-expressed in bacteria. A number of modifications (e.g. in the hENT1 sequence) were necessary for optimal over-expression and purification, as previously observed for other human transporters expressed in bacteria (Quick & Wright 2002). A better understanding of the structural organization of NTs, and particularly hENT1, will help define substrate binding and transport mechanisms and significantly aid in the development of nucleoside analog drugs.

120.16    Expression, Purification and Characterization of Bacillus subtilis BsSCO, a Membrane Protein Involved in a Cytochrome C Oxidase Assembly
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BsSCO from Bacillus subtilis, similarly to its yeast homologue Sco1, is proposed to play a functional role in the assembly of cytochrome c oxidase. We have previously shown that BsSCO is involved specifically in the assembly of CuO, but is not required for the assembly of CuQ. The putative copper-binding domain of BsSCO was expressed as a soluble GST-fusion protein in Escherichia coli strain DH5α, using the expression plasmid pGEX-4T3. After cell lysis the recombinant protein was purified from the supernatant by affinity chromatography using Glutathione Sepharose 4B. Soluble recombinant BsSCO (BsSCO+) was released by 16hour-colon thrombin cleavage at room temperature. The molecular mass of BsSCO+, as determined by mass spectrometry, is 19 721 ± 0.18. The CD spectrum of the purified BsSCO demonstrates that the protein adopts a defined folded conformation. Cu (II) induces no change in the CD spectrum of BsSCO+ . It is found that the cysteines in the BsSCO are fully oxidized, and after reduction with Tris(2-carboxyethyl) phosphine hydrochloride, two free thiols per protein are detected. Fluorescence emission spectra of the reduced BsSCO, without and with addition of Cu(II) are identical, however in the presence of Cu(II) the quantum yield of the fluorescence intensity decreases, suggesting that BsSCO, binds Cu(II). Our future studies of BsSCO+ will focus on the protein crystal structure and further understanding of copper binding by BsSCO and on the role of BsSCO in the mechanism of copper transport to the CuQ center of cytochrome c oxidase in B. subtilis.
120.17
Insights Into the Model of the Integral Membrane Protein EmrE Through Lipid Face Prediction and Disulfide Crosslinking

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The small multidrug resistance protein EmrE is responsible for transport of a diverse group of lipophilic cations in Escherichia coli. EmrE is a four transmembrane helix (TMH) anti-parallel bundle containing 110 amino acids of which there are three cysteine residues: two in TMH II (C39 and C41) and one in TMH IV (C95). This protein has been shown to be a homo-oligomer and has been modeled as various multimeric forms. The sequence analysis program Vector Variability Analysis of Membrane Protein Structure (VWAMPS) was used to predict the probable lipid facing residues of each EmrE TMH from variance in amino acid sequence from a homology alignment. VWAMPS is based on the observation that lipid facing TMH residues are less conserved and larger than protein facing TMH residues. Tricine SDS-PAGE analysis of reduced and non-reduced populations of purified EmrE revealed an intra-molecular disulfide between TMH II and IV. Size Exclusion HPLC in 5% methanol or 5% trifluorethanol with or without varied amounts of reducing agent confirmed the presence of an intra-molecular disulfide in EmrE. Since the cysteine residues must be in close contact to form the disulfide, additional restriction can be placed on the possible proximity of TMH II and IV. The steric constraints imposed by the disulfide in addition to the predicted lipid faces of EmrE allow a model to be postulated.

120.18
Structural Analysis of Neurospora crassa Mitochondrial Porin in Detergent Systems
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Mitochondrial porins, otherwise known as voltage-dependent anion selective channels (VDAC), are mitochondrial outer membrane proteins that display high beta-strand content, similar to bacterial porins. These channels form beta-strands in detergent micelles but to date, characterization of their folding is incomplete. In this study, the folding of His-tagged recombinant Neurospora crassa mitochondrial porin has been examined in non-ionic and ionic detergents by several spectroscopic methods. The recombinant porin was analyzed in single ionic, non-ionic, or zwitterionic detergents, as well as in mixed non-ionic/ ionic detergent systems. In specific detergent porin systems reproducible spectra were observed for far UV (205–250 nm), fluorescence, and UV absorption analysis. The systems that induced beta-strand character in porin as analysed by far UV CD spectroscopy also generated UV absorption and fluorescence spectra similar to those observed for bacterial porins. It is noteworthy that the detergent systems producing beta-strand characteristics in N. crassa recombinant mitochondrial porin were different from those yielding high beta-strand content in bacterial porins. Near UV (250–330 nm) CD spectropolarimetry also revealed significantly different spectra for recombinant mitochondrial porin in this region as compared its bacterial counterparts. In conclusion, despite their similarities to bacterial porins, mitochondrial porins require very different folding conditions.

120.19
Elucidating the Functional Mechanics of ABC Transporters
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Membrane proteins known as ATP Binding Cassette (ABC) transporters use energy derived from the hydrolysis of ATP to translocate a wide variety of molecules across cell membranes. Members of this ubiquitous class of proteins have been shown to confer resistance to antibiotics and anti-cancer agents by pumping drug molecules out of the cell. It is also known that malfunctioning induced by mutations in ABC transporters is the cause of a number of genetic diseases like cystic fibrosis, adrenoleukodystrophy and macular degeneration. In order to gain a clearer understanding of the functional mechanics of ABC proteins, we performed a computer simulation study of the real time dynamics of BtuCD, the vitamin B12 transporter from E. coli, in a lipid bilayer. Our molecular dynamics simulation identified key patterns of correlated motion relevant to the transmission of conformational change from the nucleotide-binding pocket to the transmembrane domain. Based on these results, we predict the sequence of events likely to be important for the functional coupling of nucleotide recruitment, hydrolysis and release to the translocation of molecules through the transmembrane segment. Insights gained may in future be useful in the formulation of drug discovery efforts aimed at yielding therapeutic results against diseases in which ABC proteins have been implicated.

120.20
Fluorescence Characterization of the Interaction of LDS-751 and Rhodamine 123 with P-Glycoprotein: Evidence for Simultaneous Binding of Both Drugs
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The P-glycoprotein multidrug transporter (Pgp) carries out ATP-driven cellular efflux of many hydrophobic drugs, natural products, and peptides. The protein appears to interact with its substrates after they have partitioned into the lipid bilayer. The number, nature, and location of the drug binding sites of Pgp have been the subject of much speculation. Pgp is proposed to contain two drug transport sites, referred to as the H site and the R site for their respective preferences for Hoechst 33342 (H) and rhodamine 123 (R). The fluorescent dye LDS-751, which appears to interact preferentially with the R site, undergoes a large increase in fluorescence emission upon interaction with Pgp, making it possible to monitor the binding of this compound in the presence of the competitor, rhodamine 123. In the presence of rhodamine 123, the apparent binding affinity of LDS-751 (as measured by the dissociation constant, KD) decreased in a non-linear manner, contrary to the linearity expected according to a direct competition model. These results suggest that rhodamine 123 is present on the protein simultaneously with LDS-751, but is not located in exactly the same binding site. The data obtained from two different experimental approaches fitted well to a model in which both compounds bind simultaneously and randomly to separate binding sites within the protein, with a reciprocal negative interaction of approximately 5-fold with respect to their dissociation constants. These results provide evidence that the Pgp molecule can accommodate two different drugs in separate, possibly overlapping, binding sites within its structure.
120.21
Structural Analysis of the HflKC Complex of Escherichia coli
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HflK and HflC, members of the PID superfamily, are integral plasma membrane proteins of prokaryotes. These proteins associate to form a complex, HflKC, of unknown subunit stoichiometry. Each protein contains a single N-terminally located membrane-spanning segment and a large C-terminal domain exposed to the periplasmic space. In Escherichia coli, the HflKC complex binds to, and negatively regulates the activity of the membrane-bound, ATP-dependent metalloprotease, FtsH. The FtsH/HflKC heterooligomeric complex is required for the proteolytic degradation of several uncomplexed integral membrane proteins (including SecY and the a subunit of ATP synthase) and soluble regulatory proteins such as phage lambda CI (a key regulator of λ lysogenization). In E. coli, HflK and HflC are 45.5 kDa and 37.6 kDa, respectively. During the purification of the HflKC complex from Escherichia coli, we have isolated fractions containing two additional proteins, which appear to be in complex with HflKC. One of these is likely FtsH (70.7 kDa), and the other is the 39 kDa porin OmpF, which was identified based on tryptic peptides using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF). We have also identified sequences within the C-terminal domains of both HflK and HflC that contain 11-residue (undecad) repeats with small residues in the a and h positions, characteristic of two-stranded right-handed coiled coils, suggesting that the protein exists as a complex made of extended dimeric protomers. The additional recurrence of small, hydrophobic residues at the j and f positions of HflK and HflC alpha-helices, respectively, allows us to predict that the undecad-containing helices of each protein are packed together to form a right-handed barrel.

120.22
Functional Expression and Cellular Localization of the Na+/H+ Exchanger, Sod2, of the Fission Yeast Schizosaccharomyces pombe
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The Na+/H+ exchanger is a ubiquitous protein that transports Na+ and H+ in opposite directions across cell membranes. In the fission yeast, Schizosaccharomyces pombe, the Na+/H+ exchanger, sod2, plays a major role in the removal of excess intracellular sodium. Disruption of the sod2 gene results in an inability to extrude cytoplasmic Na+ and Li+ and a salt sensitive phenotype. We examined the subcellular distribution and dynamics of sod2 expression in S. pombe. A sod2-GFP fusion protein was constructed for use as a reporter of sod2 expression, abundance and localization. Expression of sod2-GFP was under the control of an attenuated version of the inducible nmt promoter, to avoid mistargeting the protein by overexpression. Sod2 was localized to the plasma membrane, as well as the nuclear envelope. In addition it was found in an as yet unidentified intracellular organelle. The sod2 transcript was localized to discrete regions of the plasma membrane during subsequent cell growth and division. Over time, localization was predominantly limited to the lateral regions of the plasma membrane of growing cells. This provided an estimate of sod2 protein turnover/degradation rates as well as a structural marker of old vs. newly synthesized plasma membrane. Sod2 localization was not affected by salt or pH stress. Further studies are required to determine the function of the intracellular sod2 and its role in ion transport. Supported by NSERC & CIHR.

120.23
Structural Studies of the Neuropeptide Methionine-Enkephalin in Monoolein Cubic Phases Using Nuclear Magnetic Resonance Spectroscopy
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Monoolein cubic phases have been of interest to structural biologists in recent years following their application in the successful crystallization of the membrane protein bacteriorhodopsin. The use of the cubic phase as a matrix from which protein crystals could be grown indicated that a) the native structure of a membrane protein could be maintained and b) diffusion of a membrane protein could occur, within this lipid matrix. Previous studies of monoolein cubic phases in the presence and absence of proteins had indicated that the monoolein cubic phase a) has a local bilayer structure, b) that both the lipid and aqueous components of the cubic phase undergo rapid diffusion, c) can support high lipid to peptide/protein ratios and d) has a large degree of plasticity and stability. This information led us to believe that monoolein cubic phases may be a suitable matrix for structural studies of membrane binding peptides and proteins using heteronuclear solution NMR techniques. The results of our previous NMR experiments on transmembrane peptides bound to monoolein cubic phases indicated that peptide residues found at the surface and in the interfacial regions of the cubic phase could be observed using solution NMR techniques. We continued our NMR studies of peptides in monoolein cubic phases using the neuropeptide methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) whose structure has been previously studied in various lipid environments. The NMR data that we have collected on 15N- and 13C-labelled methionine-enkephalin in monoolein cubic phases indicates that the peptide becomes structured upon binding to the monoolein cubic phase. Our data provide the first example of peptide structure determination using solution NMR in cubic phase lipids.

120.24
Heteromeric Versus Homomeric Expression of Inwardly Rectifying K+ Channels Suggests Different Microenvironments Near the Pore
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Potassium flux through inwardly rectifying (Kir) channels is thought to provide a driving force for formation of the hepatic exocrine secretion bile. Kir4.2a, Kir 4.2 and Kir 5.1 have been cloned from rat hepatocytes. Studies using the Xenopus oocyte expression system reported that Kir4.2 activity is sensitive to cytosolic pH (pHi), and that coexpression of Kir4.2 with Kir5.1 results in reduced accessibility of the homo- and heteromeric Kir channels to changes in pHi. HEK293T cells were transfected with the open reading frame of Kir4.2, or Kir4.2a alone or in combination with Kir5.1 as a fusion construct. The patch electrode was used to dialyse cells with solutions buffered between pH 8.1 and 6.6. Voltage ramps between +150 and -150mV (400ms duration, 0.016Hz) were applied for 10min. Kir4.2 activity was significantly affected by pHi 1min following attainment of the whole-cell configuration. Over the succeeding 10min, current from cells dialyzed with solutions buffered above pHi 6.6 were stable, whereas those at pHi 6.6 cells decayed to 21% of the initial activity (t 0.5 4.0±0.2min). Conversely, currents from cells expressing the monomeric channels were stable over the recording time at all pHi values. These results demonstrate that the pH titration of the heteromer, but not the homomer, occurs in a delayed manner. We suggest that coexpression of Kir5.1 with Kir4.2 results in reduced accessibility of bulk solvent to a site involved in potassium conduction.
120.25
Lipid-Protein Interactions of a Mechanosensitive Ion Channel (MscL)
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MscL, a large mechanosensitive ion channel of known high resolution structure, forms a gated pore in the membrane that opens in response to mechanical deformation or stretch from within the bilayer, an effect that may ultimately rely on interactions between the hydrophobic surface of the channel and the surrounding shell of lipid molecules. We have characterised the lipid-protein interactions of the MscL channel from *M. tuberculosis*, by reconstituting Trp mutated MscL into mixtures of brominated and non-brominated lipid by dilution, and using fluorescence quenching to determine lipid binding constants relative to dioleoylphosphatidylcholine (d(C18:1)PC). The strongest binding was observed with d(C16:1)PC for MscL, with the binding affinity decreasing for shorter and longer acyl chain lengths. The total change in affinity for MscL was 1.6-fold in the range C16-C24, much less than expected if hydrophobic matching only involves distortion of the lipid bilayer. The size of the head group (phosphatidylcholine (PC) or phosphatidylethanolamine) had no affect on the binding affinity. In contrast, anionic lipids such as phosphatidylserine and phosphatidylinositol bind with greater affinity than PC (~3.5-fold increase), an effect that was largely salt independent, suggesting H-bonds or other interactions at the lipid-protein interface may be more important in transducing the mechanical signal to one that gates the channel.

121.1
Modification of Cryptic Splice Site in β-Glucosidase cDNA Increases Expression Yield: Implication for Treatment of Gaucher Disease
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Gaucher’s disease is caused by deficiency in the lysosomal enzyme β-glucosidase (β-glucocerebrosidase, GCC). Currently treatment of most patients is done using enzyme replacement therapy, with enzyme produced in transfected COS cells. This has proved to be extremely costly, in the order of $150000/patient/year. Evidence from RT-PCR, supported by low protein and enzyme activity yields in transfected CHO cells have shown that a significant portion of the mRNA produced from the GCC cDNA is misspliced due to the presence of a cryptic splice site. PCR mutagenesis was used to abolish the cryptic splice site and to also correct a potential aberrant start site. Following sequencing the old construct and the “fixed” construct were transferred into the retroviral vector pFB-Neo. Transfection into the PhoenixAmpho packaging cell line followed by virus isolation and subsequent infection into the FlyRD18 cell line produced retrovirus ready for infection into human cells. The human rhabdomyosarcoma cell line, TE671, was sequentially infected up to five times with each construct. Cells harvested after each sequential infection showed an increasing level of GCC expression with the “fixed” construct producing 2-4-fold higher levels as compared to the old wild-type construct (15–20-fold above background) Infection was confirmed using PCR from genomic DNA, and protein production confirmed using Western Blot analysis. Evidence that this “repaired” construct produces higher levels of protein and enzyme activity suggests that its use in transfecting CHO cells could lower cost overall of enzyme replacement therapy, and be more effective when used in retroviral or naked DNA based gene therapy protocols.

121.2
Evaluation of Human Serum Albumins Produced in Iran Based on Total Protein
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Albumin is the major protein of human and makes up approximately 60% of the total plasma protein. Albumin solution is prepared as a concentrated solution containing 15.0 to 25.0% W/V of total protein or as an isotonic solution containing 4.0 to 5.0% W/V of total protein. In this study produced albumins were evaluated based on their protein content. Total of 49 samples of albumin 5% & 20% were studied. Twenty-three of 5% concentration albumin solution showed mean value 5.05 ± 0.12 (4.93 – 5.17) g/100. Only one of the samples (4.35%) was out of the British Pharmacopoeia (BP) range. Twenty-six of 20% concentration albumin samples had mean value 19.87 ± 0.83 (19.04 – 20.70) g/100. Two of 25 samples (7.69%) were out of the BP range. Results of this study showed majority of albumin produced by Iranian producer are in the BP range.
121.3
Catalytic Activities of Platelet Enzymes (Glutamate Dehydrogenase, Rotenone-Insensitive Cytochrome C Reductase and Isocitrate Dehydrogenase) in Relation to Alzheimer’s Disease

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In this study, the importance of the catalytic activities of rotenone-insensitive NADH-cytochrome C reductase (RINCR), glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (ISCD), in relation to Alzheimer disease, was investigated. This work was undertaken due to the fact that a deficiency of cytochrome C oxidase (forth complex) in the platelets and nervous tissues of patients suffering from this disease had already been demonstrated. Also a number of mitochondrial enzymes including GDH, in Parkinson patients, which is pathogenetically similar to Alzheimer’s, has already been investigated.

A study involving 16 normal controls 16 patients suffering from Alzheimer’s was carried out as detailed below with their respective ages: Normal 56–81, Patients 54–83.

ISCD and GDH activities in normal and Alzheimer patients were almost the same while RINCR showed substantially lower activities in patients suffering from this disease (0.95 ± 0.38 OD/mgprot in normal and 0.24 ± 0.12 OD/mgprot in patients). It is suggested that this lowering of activity may be due to malfunctioning of mitochondria of the platelets due to some pathological changes in these patients.

121.4
Liposomes Containing Epidermal Growth Factor (EGF): Encapsulation Characteristics and the Immune Response in Mice

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One of the strategies for cancer treatment is the blocking of epidermal growth factor (EGF) and its receptor interaction trough EGF elimination by specific antibodies and consequently the reduction of tumor cells. Liposomes could be an alternative adjuvant to present EGF to the immune system in order to improve the immune response against this protein. In this study we investigated the encapsulation efficiency of human recombinant EGF such as or conjugated to P64k protein from Neisseria meningitidis, into dehydation and rehydration vesicles (DRV) and the stability of these vesicles in the experimental conditions. We evaluated else the immune response against EGF encapsulated into dipalmitylophosphatidylcholine:cholesterol (DPPC:Cho) liposomes in comparison with other adjuvants. Liposomes of DPPC:Cho exhibited the highest EGF encapsulation and the lowest EGF release during one month storage. The inclusion of P64k with EGF, conjugated or not, decreased the encapsulation efficiency of the latter protein. Characterization of anti-EGF IgG subclasses revealed not only high IgG1 titres but also the presence of relevant IgG2a and IgG2b levels. Mice immunized with EGF immobilized into liposomes showed similar levels of IgG2a and IgG2b to those observed in the groups immunized with the conjugate EGF-P64k in Aluminium hydroxide (Alumin) or in PBS, and higher than that treated with Alumin-EGF. The immunization with Liposome-EGF induced a delayed type hypersensitivity response (DTH) specific for this antigen and improved the survival of mice challenged with Ehrlich ascites tumor cells in comparison with the group immunized with Alumin-EGF.

121.5
DNA-based Vaccination Using Multiple Schistosoma mansoni Genes

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DNA vaccines have been called the Third Wave in Vaccine Development because of their ease of construction and potential for stimulating strong humoral and cell mediated immune responses against many different pathogens including parasitic infections. Schistosomiasis is a liver disease affects more than 200 Million patients worldwide and is due to infection with the schistosome parasites. To study the efficiency of DNA vaccination using several S. mansoni genes constructs for induction of protective immunity against challenge infection. Swiss albino mice were immunized by intramuscularly injection with a mixture of pcDNA1 plasmid constructs encoding Smfim1, Sm21.7 and Smaldolo, parallel with non recombinant constructs as a control (blank pcDNA1/Amp). ELISA analysis indicated that immunization induced specific antibodies for these proteins. The specific antibodies were able to mediate a significant killing of schistosome moularia using peritoneal macrophages as effectors cells. Immunization with the mixture conferred a significant level of protection (47%) against challenge in Swiss Albino mice. Histopathological examination of the vaccinated liver revealed a decreased in the number, size and change in the cellularity of the granuloma. In addition reductions in worm viability, worm fecundity and egg hatching ability have been observed following challenge with S. mansoni cercariae. The number of eggs in the liver and intestine was reduced by 42% and 47% respectively. The results suggested that the mixture might be a candidate antigen for the generation of antipathology vaccine against schistosome parasite infection. (Supported by TBRI Grant # 74M)

121.6
Effect of Alcohols and Organic Solvent on Neocarzinostatin—A Molecular View of the Drug Delivery System

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Neocarzinostatin is a potent enediyene antitumor antibiotic complex in which a non-protein chromophore (NCS-chr) with a novel structure that is responsible for DNA cleavage and is stabilized by binding to the protein, apo Neocarzinostatin (apo NCS). Our previous studies using Trifluoroethanol revealed that the release of the NCS-chr from its holoprotein does not require major conformational changes. So the major factor affecting the release of the chromophore from holoprotein is an interesting question to be addressed. For this, we have examined the effect of various alcohols and organic solvent on the neocarzinostatin using circular dichroism and fluorescence. We also compared the kinetic release of chromophore using different salts, alcohols and organic solvent. We observed that the alcohols and organic solvent enhance the release rate, where as in the case of salts (sulphate and perchlorate) the release rate was very slow. Studies with the series of different alcohols also confirmed that the release of the chromophore from the holoprotein does not require the conformational change in the apo NCS. In general, these studies are expected to shed light towards the long-standing puzzle of how proteins regulate the availability of its ligand such as the NCS-chr for its action.
121.7
Shigella Enterotoxin Alters Enterocyte Differentiation and Is Associated with Declined Intestinal Antioxidant Status in Mice

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Shigella enterotoxins are widely known to mediate ileal fluid accumulation and colonic epithelial injury in shigellosis and the mechanism of gut susceptibility to Shigella enterogeneity seems to involve a myriad of events, which are not completely understood. In this study, sixteen Shigella strains (4 strains each of S. dysenteriae, S. flexneri, S. boydii, S. sonnei), isolated from diarrhoeic stools of patients attending hospitals in Lagos, Nigeria were investigated for their enterogenicity in mice. The association between this virulence factor and intestinal vitamins A, E, and catalase activity was also studied. Suckling mouse assay revealed significant enterogenic differences among the Shigella serogroups (P = 1.7 x 10^-9) in which only one S. boydii strain was enterogenic and none of the tested S. sonnei strains elicited ileal fluid accumulation. Compared with sterile water inoculated mice the enterotoxin fractions of the Shigella strains tested caused significant decreases in intestinal catalase activity (6.88 ± 1.9 units/mg protein vs. 15.30 units/mg protein) and levels of vitamins A (2.67 ± 3.68 vs. 3.94 mg/g) and E (2.38 ± 2.96 vs. 3.20 mg/g) (P < 0.05). Generally, the magnitude of fluid accumulation was inversely and significantly related to intestinal antioxidant level (P = 8.52 x 10^-15 Δ 12.21 x 10^-5). Cell culture experiments showed that S. flexneri and S. dysenteriae enterotoxin fractions inhibited growth and elicited cytopathic effect on Caco-2 cell-line in vitro, suggesting the ability of this virulence factor to impair enterocyte differentiation and cause cell injury. The results of this study strongly support antioxidant supplementation as an adjunct to the management of severe shigellosis.

121.8
Analysis of Tumour Suppressor p53 Protein Binding Properties by New ELISA Technique

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The function of tumour suppressor protein p53 is the maintenance of homeostasis by induction of growth arrest and DNA repair, terminal differentiation or apoptosis. The major regulation functions are controlled by sequence-specific transcriptional target genes. Protein p53 binds to the DNA consensus sequence 5'-PuPuPuCA(T/T)AGPyPyPy-3' (p53CON) and also to supercoiled DNA (scDNA). To study activation of p53 to sequence specific binding we used supershifting of p53/DNA complexes by monoclonal antibodies. C-terminal specific antibodies Bp53-6.1, Bp53-10.1, Bp53-30.1 activated the sequence-specific binding of p53 to p53CON in scDNA in a selective manner, while other MAbs did not influence the p53CON binding. However, incubation of p53 protein with MAbs DO-1, ICA 9 prior to addition of Bp53-6.1, Bp53-10.1, Bp53-30.1 can inhibit activation effects of these MAbs. We have developed a rapid and reliable method for analysing sequence specific binding of p53 protein to DNA using a modified enzyme-linked immunosorbent assay (ELISA). In this p53/DNA - ELISA we use streptavidin-coated microplates to capture biotinylated oligonucleotides containing p53 consensus sequences. Using this method we can detect binding of endogenous p53 to p53CON and activation of p53 protein for sequence-specific DNA binding. Variations of the basic protocol have also been developed to perform competition experiments and to study p53 binding to natural binding sequences. This modified DNA - ELISA is applicable for screening p53 binding properties from various sources in a short time. Acknowledgements: This work was supported with grants No. 301/00/D001 from GACR and B5004203 from GA AV.

121.9
Comparative Analysis Between Two Tests for Determining Fetal Lung Maturity

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Objective: Preterm birth is accountable for significant mortality and morbidity. The use of fetal lung maturity tests is aimed to assist the clinician in determining the optimal time of delivery in cases of preterm labor, pregnancy-induced hypertension, diabetic pregnancy, intraterine growth restriction, placenta previa and previous abruptio placenta. Due to the fact that the predictive value of mature test results is close to 100%, it is needed for Lecitin/sfingomielin test to be done for all the pregnancies that are before 37 weeks of gestation. This test should be done in combination with other rapid, inexpensive tests, such as the test for determination of Lamellar Bodies (LBs). LBs are concentrically layered packages of phospholipids, which represent the storage form of pulmonary surfactant that are extruded into the alveoli from type II pneumocytes.

Materials and methods: In this study we examined 209 women with normal pregnancy from 28th to the 41st week of gestation, as well as the L/S and LBs in amnion fluid, gained through amniocentesis. The similarity of the size of LBs to platelet size, allow us to determine the LBs concentration by reading off the platelet channel using a standard hematological counter Cobas Micros. L/S is determined with thin-layered chromatography by using the phosphorous through the Method Gluck 1971.

Results: The obtained results show us that the value of L/S is rising with proximity of 1.2 from 28 to 10 in the 41st gestation week, LBs values are in the range from 1000/μl in 28th to 207000/μl in 41 g.w. The coefficient of correlation of L/S and LBs is r = 0.80.

Conclusion: The obtained results of L/S and LBs show a high degree of correlation, which indicates that the measurement of LBs can be taken as a parameter for fast determination of fetal lung maturity. The rising values of L/S and LBs indicate that it is achieved fetal lung maturity and it is impossible the respiratory distress syndrome to be develop.

121.10
Induction of Neutralizing Antibodies Against Herpes Simplex Virus Type 1 in BALB/c Mice Inoculated with Recombinant pCDNA3-gD or pCDNA3-gB or Both of Them

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E.coli DH5a cells were first transformed by either pCDNA 3-gB carrying glycoprotein B gene of Herpes Simplex Virus Type-1(HSV-1) or pCDNA 3-gD carrying glycoprotein gene of HSV-1. Large amount of each plasmid was then prepared and purified. Three groups of BALB/c mice each containing 10 mice were inoculated intramuscularly with pCDNA3-gD, pCDNA3-gB, or a combination of both plasmids respectively. Each mouse received 90 microgram of the inoculum. The fourth group of mice were injected intraperitoneally with 3000 TCID50 of HSV-1, KOS strain and used as positive control. The negative control group comprising 6 mice each received 100 microliter PBS.A group of nine mice also received empty pCDNA3 and used as negative control. Second and third injections were done at 21 day intervals and bleeding was done before each injection and after the last one. Viral Neutralization Test (VNT) using serum samples from all test and control animal groups was done. It was shown that each individual mouse which had received any of the constructs or combination of them produced antibody against HSV-1 which reached the highest titer after the last inoculation. The third group of mice showed higher anti HSV-1 neutralizing antibody compared with other test groups. Negative control groups showed no such antibody. The highest antibody titer belonged to the mice which received the whole virus particles.
Autoantibodies Against DNA Topoisomerase I from Human Systemic Sclerosis Patients Cross-React with a Fibroblast Surface Antigen

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Human systemic sclerosis (SSc) patients are characterized by autoantibodies against nuclear antigens that allow their classification into distinct clinical groups. One of these antigens is DNA topoisomerase I (topo). The occurrence of fibroblast dysregulation leading to lung fibrosis in SSc patients is highly correlated with the presence of antitopo. However, no pathophysiological connection between these two events has been discovered. We have found that IgGs from SSc patients with antitopo displayed a cell-type specific surface fibroblast binding activity (FBA) by flow cytometry. No binding to endothelial cells was detected. FBA were absent from the sera of SSc patients which did not possess antitopo, as well as normal and other disease controls. A strong correlation was observed between antitopo titers by ELISA and FBA mean fluorescence intensity. Affinity-purified antitopo from SSc sera were found to display FBA of intensities similar to those of unfraccionated IgGs. Furthermore, a commercial monoclonal IgM antibody to topo was found to bind to fibroblast surfaces, and be competitively inhibited by IgGs from antitopo patients. However, no antitopo binding occurred on fibroblasts attached and spread on culture dishes. Taken together, these results support the hypothesis that either topo itself or an antigen sharing one or more epitopes with topo is expressed at the fibroblast surface, and may be involved in cell adhesion. Experiments are under way to identify the cross-reactive fibroblast surface antigen and to determine the physiological effects of antitopo binding to fibroblasts. This is the first demonstration of a direct link between an SSc antinuclear autoantibody and a cell surface fibroblast target, thus opening the way to novel pathophysiological studies of SSc.

Protection Against Hypoxic Insult in Cardiac Cell by HSP70 Protein Transduction

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Inducible heat shock protein 70 (HSP70) is an intracellular stress protein that confers cytoprotection to a variety of cellular stressors. Recent reports have established that the overexpression of HSP70 mammalian cell lines confers protection against several noxious stresses like hyperthermia, hypertonic stress, and various cytotoxic agents, including cytoskeletal disruptors. Several lines of evidence have suggested that augmentation of the heat shock response by increasing the expression of HSP70 represents a potential therapeutic strategy for the treatment of critically ill patients. In this study, we used a novel approach to study the potential cytoprotective effect of HSP70 as a therapeutic agent in rat myogenic cell line H9c2 and primary neonatal cardiomyocytes. In the current study, we cloned a human HSP70 cDNA gene and fused it with a gene fragment encoding the 11-amino acid transduction domain of HIV-1 TAT protein. We then analyzed the efficiency of transduction of the resulting TAT-HSP70 fusion protein into rat myogenic cell line H9c2. Finally we determined the ability of TAT-mediated transduction of the HSP70 protein to confer cytoprotection against a serum starvation and hypoxic insult. The experimental data we reported here suggest that the TAT-mediated delivery of HSP70 may represent a novel strategy of augmenting intracellular HSP70 levels. [This work was supported by the BK21 Project for Medical Sciences, Yonsei University]
121.14
Isolation and Characterization of Mycobacterium bovis 19 kDa Native Protein Distinct from MBP 70/80
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Dissection and study of mycobacterial components has led to better understanding of tuberculosis development and the host immune response against mycobacteria. Isolation of specific antigens could be useful in the development of more sensitive in vitro tests and new vaccines production. We purified two different 19 kDa antigens from M. bovis culture filtrate protein extract by isoelectric focusing. In this work we analyzed differences between these two proteins with same molecular weight, but with different isoelectric point. Amino terminal sequence showed that one of them is the well characterized secretion protein MBP70/80 with 100% homology, and the other protein show 100% homology with hypothetical mature protein Rv1174c of M tuberculosis. We demonstrated that this protein in native form is formed by a homo-dimer, but one of the monomers has sugar molecules attached to it. Our results showed this protein is not MBP70/80, and its specific of mycobacteria genus. We speculate about the possible role of this protein in mycobacteria biology.

121.15
Peptide Decoys as a Potential Solution to Block Anti-FVIII Antibodies in Hemophilia Patients?
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Objectives: A significant proportion of hemophilia A patients develop inhibitor antibodies (Abs) following factor VIII (FVIII) infusion. Such Abs inhibit the procoagulant activity of infused FVIII and strongly complicate patients’ medical care. To try to circumvent this difficulty, we devised an approach based on blocking of the pernicious activity of these Abs by low molecular weight peptide decoys mimicking epitopes of anti-FVIII Abs. Methods: To validate our concept, the anti-FVIII human mAb Bo2C11 was selected. This choice was motivated by the fact that Bo2C11 recognizes a conformation-dependent epitope on the C2 domain of FVIII, as many inhibitors do, and is extremely potent in inhibiting FVIII activity. To select peptide decoys, the phage peptide display methodology was chosen. Several phage peptide libraries, displaying random peptides at the surface of the bacteriophage phill or pHVIII protein were screened for binding specifically to Bo2C11. Results: After three rounds of selection, peptides mimicking the Bo2C11 epitope were identified. Among the 88 positive phage clones, 27 different sequences were obtained. All the peptides were constrained dodecapeptides, that have the core sequence W-R. These peptides mimic the epitope recognized by Bo2C11 and were thus able to inhibit specifically and in a dose-dependent manner the binding of Bo2C11 to FVIII. One peptide neutralized very efficiently the activity of Bo2C11 in vitro and restored as well in vivo coagulation in a murine model of hemophilia A. Conclusion: This study clearly demonstrates the potential of peptides able to mimic complex conformational epitopes and to neutralize the inhibitory activity of Abs arising in hemophilic patients.

121.16
Tissue Inhibitor of Metalloproteinases 4 (TIMP-4) Expression and Function in Cervical Carcinoma
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Extracellular matrix turnover has an important role during physiological and pathological processes. Matrix metalloproteinases (MMP) are responsible for the degradation of extracellular matrix components. Tissue inhibitors of metalloproteinases (TIMP's) are multifunctional proteins that regulate MMP activity. TIMP-4 expression has been studied in several tumors and its function has been shown to be dependent on cellular type. However, TIMP-4 participation in human cervical carcinoma, which is an important health problem in Mexico, is unknown. We have investigated TIMP-4 expression in human cervical samples and studied its function in human cervical carcinoma cell lines (HCCCL). TIMP-4 mRNA and protein were found in several HCCCL such as HeLa, Siha and Caski. Human cervical carcinoma biopsies of different disease stages were analyzed and TIMP-4 mRNA was found in 91.66% of them. A positive correlation between mRNA expression frequency and disease progression was found. However, no clear correlation was found between the expression level and the disease stage. TIMP-4 mRNA was not found in healthy patients control samples. To study TIMP-4 function, we have generated a TIMP-4 tetrazycline-regulated overexpression system, a TIMP-4 retroviral overexpression system and a TIMP-4 siRNA silencing system. With the tetrazycline system, we have found that TIMP-4 overexpression decrease cell viability and induce cell migration in HeLa cells which suggests that this protein might participate in the regulation of cell growth and invasion.

121.17
Free and IgG-bounded Fibronectin in Scleroderma Systematica
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Fibronectin (FN), multidomain glycoprotein of blood plasma and extracellular matrix, takes a part in pathogenesis of scleroderma systematica (SS). The role of fibronectin in immunopathology of SS is predetermined not only by structural changes of FN but its ability to bind and eliminate immune complexes. The aim of this work was study of degradation and interaction of FN with IgG in composition of immune complexes in SS. The following methods are used: the affinity purification of FN-IgG complexes on Protein G sepharose and gelatin-agarose, the determination of FN and IgG by ELISA and immunoturbidimetry, correspondently. The degradation of FN was studied by immunoblotting. Results. The significant increase of plasma IgG level from patients with SS was observed. The FN/IgG ratio in composition of isolated immune complexes was authentically twice higher than in norm. The tendency to inverse correlation between plasma FN concentration and FN/IgG ratio in immune complexes was revealed. There was wider spectrum of plasma FN fragments and particularly of lowmolecular fragments in SS than in norm. The higher rate of occurrence of IgG-bounded FN fragments with molecular weight 220, 190, 140, 130, 120 and 95–105 kDa was observed in comparison with free FN in SS. The FN fragments being bounded with IgG with molecular weight 200, 190, 170, 140, 95–105 and 85 kDa were revealed more often in SS than in norm. Besides, the FN fragments being bounded with IgG with molecular weight 230 and 150 kDa were observed only in SS, but not in norm. In conclusion one should say that the state of FN degradation is an additional marker of SS.
121.18
Liposomes as an Attractive Adjuvant to Modulate the Immune Response Against Human Recombinant EGF (hrEGF)
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Liposomes constitute an alternative to present tumour-associated antigens to the immune system. Epidermal growth factor (EGF) and its receptors are attractive targets for active immunotherapy against tumour cells over expressing EGF-receptor (R-EGF). We have get insight into the ability of liposomes to modulate the immune response against hrEGF conjugated or not to P64k protein from Neisseria meningitidis in experimental animals. hrEGF such as or conjugated to P64k (EGF-P64k) was encapsulated into dehydrated-rehydrated vesicles (DRV). It was possible to observe that the sensibilization with hrEGF encapsulated into DRV was enough to prepare immune system for the induction of equivalent antibody titers to those obtained with EGF-P64k encapsulated into liposomes or adsorbed in Alumin. The evaluation of IgG subclasses showed a (IgG2a/IgG2b)/IgG1 ratio significantly higher in the groups sensitized with DRV/EGF or DRV/EGF-P64k preparations than that immunized with EGF-P64k in Alumin. The lymphocyte proliferation assay demonstrated an expansion of hrEGF specific T-cells only in those groups, which received the antigen, encapsulated into liposomes. The study of effect of liposomal phospholipid composition showed that saturated phospholipids enhanced more efficiently the immune response against hrEGF than unsaturated phospholipids, in terms of: total IgG, IgG2a and IgG2b titers and antibody neutralization ability to block the hrEGF and R-EGF interaction. Liposomes were able to enhance the stimulation of EGF specific immune cells and qualitatively improve the humoral immune response against hrEGF, even when this antigen was encapsulated without P64k as immunostimulated protein.

121.19
The TGF-beta1 Promoter Is Induced by HPV-16 E6 and E7 Oncoproteins in Cervical Cancer
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TGF-beta1 is a multifunctional cytokine involved in several immunoregulatory processes and plays a critical role in the escape of some cancer from host immunity. HPV is the main etiologic agent in cervical cancer, and E6 and E7 oncoproteins have properties of transforming cell and trans-regulation of cellular genes. We have identified a correlation between TGF-beta1 gene expression and the advance of the malignancy, which suggest that HPV induces TGF-beta1 gene expression. However, the mechanisms by which HPV induce TGF-beta1 gene expression are unclear. In this study we analyzed whether HPV-16 E6 and E7 oncoproteins are involved in the molecular mechanisms of TGF-beta1 gene expression. For that end, we amplified the human 5’-TGF-beta1 promoter by POR from peripheral blood lymphocyte DNA, which was cloned in pBlueScript and sequenced. This DNA fragment was subcloned in pBLCAT3 and several constructs generated by deletion of TGF-beta1 promoter. C33A cells were transfected with these constructs and evaluated the effect of HPV-16 E6 and E7 oncoproteins by cotransfection with pSV2E6 and pSV2E7. We observed that E6 and E7 oncoproteins induced twofold promoter activity inside TGF-beta1 core promoter, while had not significant effect in other TGF-beta1 regulatory regions. At the present, we are studying the TGF-beta1 core promoter by EMSA and Footprinting analysis to identify DNA-protein complexes responsible for this transactivation. Preliminary results suggest several interactions between viral transcriptional factors inside of TGF-beta1 core promoter. These results may explain the molecular mechanisms of TGF-beta1 gene regulation in cervical cancer as well as tumor escape mechanisms from host anti-tumoral immune response.

121.20
IgG Glycosylation in Composition of IgG-Fibronectin Complexes in Normal Pregnancy and Pre-eclampsia
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Pre-eclampsia is a frequent, unpredictable syndrome, which might have an immune etiology. There are increasing level of both fibronectin (FN) and immune complexes (IC) in this pathology. It is known that FN is one of the opsonic factors of IC which can bind IgG through Fc-fragment. We supposed that changes of IgG glycosylation have an influence on forming and elimination of IC from blood circulation. The aim of our work was investigation of IgG glycosylation in composition of IgG-FN complexes. IC were allocated from maternal plasma and umbilical cord serum of normal pregnant (n=15) and in pre-eclampsia (n=30) by affinity chromatography on ProteinA-Sepharose. IgG-binding FN was separated by following chromatography on gelatin-Sepharose. The study of IgG glycosylation was carried out by lectin-blot analysis using LcL, Con A, WGA and SNA, PCA. Our results testify that there are changes in structure of a carbohydrate component of IgG in pre-eclampsia. It was shown, that heavy chains of IgG in IgG-FN complexes contained complex type glycans in mother and fetus in pheisiological and pre-eclamptic pregnancy. In normal pregnancy the branches of these N-glycans were always galactosylated, but sialic acids were absent on their termini. On the contrary, N-glycans of IgG heavy chains of mother and fetus were always degalactosylated in pre-eclampsia. Possibly, the interaction of such IgG with FN breaks an opsonic activity of FN and clearance of IC from blood circulation.

121.21
Interactions of Connective Tissue Growth Factor and Vascular Endothelial Growth Factor in Human Airway Smooth Muscle
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Extracellular matrix (ECM) proteins are altered in airway wall remodelling associated with persistent asthma. Connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) play a role in the ECM remodelling and wound healing in some fibrotic and inflammatory diseases. TGFβ is increased in asthma and increases the release of ECM proteins from airway smooth muscle (ASM). We have previously shown that TGFβ induces enhanced production of both CTGF mRNA and protein in asmatistic ASM cells (Burgess AJRCCM 167, 71–77, 2003). ASM cells release VEGF. CTGF binds VEGF thereby inhibiting the angiogenic activity of VEGF. Little is known about the role of the interaction of CTGF and VEGF in asthma. In this study we have shown that in the presence of PGE2, a negative regulator of CTGF, the significant increase in CTGF mRNA induced by TGFβ in both the asmatistic and non asmatistic cells was lost. The release of VEGF was significantly increased in the presence of TGFβ and PGE2, compared to TGFβ alone (P<0.007) in both cell types. Stimulation with a CTGF recombinant protein significantly increased (p<0.01) the ECM proteins fibronectin and collagen I mRNA in the asmatistic (0.84±0.15, 3.17±1.41) compared to non-asthmatic (0.82±0.39, 0.71±0.12) ASM cells respectively (results expressed as fold increase) but not VEGF release. The effect of VEGF on the ECM proteins is being examined. Further studies are necessary to understand the interaction of CTGF, VEGF, ECM proteins and their role in the pathogenesis of asthma.
121.22
Surface Immobilized BMP-2 on Implants—A Novel Application
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In the past Bone morphogenetic proteins (BMPs) have been the subject of widespread research and clinical trials as stimulants of bone growth. Recently recombinant human BMP-2 (rhBMP-2) has been chemically immobilized on implant surfaces leading to enhanced bone growth and accelerated integration in vivo [1, 2]. However the noncovalent immobilization of proteins on metal surfaces is still poorly understood, since the oxide layers on metals like titanium, stainless steel or cobalt chromium alloys are poor adsortents of proteins. In this paper the preparation of protein adsorbing surfaces on titanium and cobalt chromium molybdenum alloy for the adsorption of rhBMP-2 and ubiquitin will be described. rhBMP-2 and ubiquitin are bound extremely tight to surfaces containing propyl or hexyl groups [3] of a certain surface concentration and are slowly released over a range of at least 24–100 days making such surfaces applicable as long-term drug delivery devices for enhancing bone growth or implant integration.


122.1
Survey of Chitinase Genes Polymorphism in Iranian Rice Varieties
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Every year about 25–51.6% of the rice yeilds is lost by sheat blight disease caused by Rizoctonia solani. The general defense proteins such as chitinase and glucanase can ruin the cell wall of this fungus. The potential approach PBR (PCR-based-RFLP) was used for recognition of alleric polymorphism among cht-1, cht-2 and cht-3 genes in Iranian rice varieties. All the three genes encode class I chitinase that are inductive with fungal elicitors. Chitinases to be involved in defense against sheat blight that causes by rizoctonia solani and it is the most important fungal disease in rice. We design two set of primers that one specificity amplifies catalytic domain of cht-3 (RICCH-3) and another amplified catalytic domains of cht-1, cht-2 and cht-3 in 30 varieties. cht-2 and cht-1 showed polymorphism in some varieties. We digest these amplified fragment with four restriction enzymes Rsa I, Taq I, Sac I and Ava I. Digest with Rsa I and Taq I showed polymorphism in cht-1 and cht-2 in some varieties. Because of the same approximate size of cht-1 and cht-3, it’s not possible to separate these two genes on agarose gel. Thereby amplification of cht-3 in separate reaction was necessary for interpretation of the results. For interpretation of the digestion patterns, referring to database and founding probable restriction sites was necessary.

122.2
The Related Species Revert Us to the Problem of Abundant Eukaryotic Genomes
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The problem of abundant of eukaryotic genomes remains unsolved in modern molecular biology. A perspective direction of examinations for comprehension of this problem is the comparative analysis of related species. Perch (Perca fluviatilis) and Balkhash perch (Perca schrenki) are very close species. The origin of Balkhash perch is conditioned by isolation of basin of lake Balkhash. The size and the structure of genomes had been analysed by rate of reassociation of short fragments (300–400 base pairs) of DNA. The size of genome of perch is 1.9 pg and of Balkhash perch is 1.1 pg. Thus, the genome of the common perch is 1.7 times more. About 20% repetitive DNA and 20% of single DNA have no analogies in a genome of other species. It is not possible to ascertain had Balkhash perch lost of a part of genome or had perch increased its genome in time of separate evolution? Comparative analysis of changes in genomes of two species of perch as analysis of the literary data for other species allow us to suppose nonrandom character of evolutionary changes of genome sizes. Probably, there are evolutionary mechanisms of archiving (packaging) genetic information.

122.3
A Novel Carboxypeptidase-like Enzyme and Its Inhibitor from the Annelid Marine Sabellastarte magnifica
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Metallocarboxypeptidases (CP) are an important class of enzymes with different digestive and regulatory functions. Consequently, their inhibitors are molecules with potential effectiveness in Biotechnology and Biomedicine. A novel inhibitor of carboxypeptidases has been isolated from tentacles crown from the annelid marine Sabellastarte magnifica. The inhibitor (SmCl) was purified to homogeneity combining heat treatment, affinity chromatography (CPA immobilized on glioxyl agarose) and RP-HPLC (C8). Its molecular mass is 19670 Da (MALDI-TOF) and isoelectric point is 4.6. The amino acid sequence has not revealed homology with other CPA inhibitors described, but it showed homology with Kunitz like domains. The inhibitor is activated, not only against pancreatic CPA and other CP like protease, but also against serine proteinase such as, trypsin, elastase and chymotrypsin. On the other hand, a CPA like protease (SmCPA) was purified from the animal body extract combining ionic exchange, gel filtration and affinity chromatography (PCI immobilized on glioxyl agarose). It is a protease of 34 kDa and high homology (N terminal sequence) with other CP. SmCPA is inhibited by different specific CP inhibitors.
122.4
Isolation, Sequencing and Cloning of a Newfound Hyperthermostable Alpha-Amylase Gene From a Native Strain of Bacillus licheniformis and Its Expression in Escherichia coli
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A hyperthermostable α-amylase activity was detected from a Bacillus licheniformis native strain, isolated from waste-water of flour-mills of Kishan vicinity in Iran. For high yield production the gene was isolated and cloned in E. coli following the application of PCR protocols by using designed primers according to the gene databank analyses. In this context specific bases were inserted in the primer in Shine and Dalgarno region. The PCR product was cloned in pBluescript II KS cloning vector and pET24d expression vector. Transformation of E. coli BL21 (DE3) cells with the latter construct resulted in the production of recombinant enzyme in presence of IPTG and also different concentrations of lactose as inducers. After confirming the enzyme activity with specific activity staining method on SDS-PAGE gel, the yield of the product in two separated intra and inter-cellular phases was determined and compared by using enzymatic assay methods. Moreover, automated DNA sequencing method determined the nucleotide sequence of the gene. The data show that the gene is 1539 bp long and its deduced protein consists of 483 amino acids, similar to what has been reported for this gene. Comparison of the inferred nucleotide sequence with those from other strains of B. licheniformis, reported in databanks, revealed about 6.5% differences resulting in 20 different amino acids which two of them, Glu133 (instead of His) and Thr209 (instead of Ala), can easily reflect more thermostability of the enzyme in comparison with the reference one.

122.5
Molecular Evolution of Bioelectron Transfer: Hydropophicity Conservation in the Docking Surfaces of Redox Protein Partners
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An interesting evolutionary aspect of electron transfer in biological systems is the conservation of the molecular structures which are directly involved in the electron flight from the donor protein to the acceptor redox partner. For example, in the cytochrome c family of metalloproteins from bacteria (e.g., Thermus thermophilus) to the rice plant (Oryza sativa) to the horse (Equus caballus), the proteins backbone is characterized by the presence of similar structural elements. In this work, the search for structural and/or functional homologies is extended to redox pairs performing similar electron transfer steps but presenting large structural differences. This approach was instrumental in disclosing new structure-function aspects of bioelectron transfer pointing to the conservation of protein surface clusters in molecular evolution. The example discussed here is the photosynthetic electron transfer between cytochrome f (cyt f) and the reaction center in photosystem I (PSI). The electrons are shuttled between cyt f and PSI by the mobile carriers plastocyanin (pc) and cytochrome c6 (cyt c6). Note first that cyt c6 and pc have molecular structures largely unrelated, thereby indicating that the observed convergence of function implies the presence in pc and cyt c6 of structural elements capable of performing similar interactions with cyt f. It is shown that the hydrophobic clusters in pc and cyt c6 are arranged as a docking cleft with the Cu-atom in pc and the Fe-atom in cyt c6 surrounded by an almost circular hydrophobic region. The evolutionary value of these clusters is indicated by calculations showing that the total hydrophobicity of the docking surfaces in cyt f, cyt c6 and pc is conserved.

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Different Methylation Status of Human-
specific LTRs That Integrated in
Regulatory Gene Regions
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Humans share about 99% of their genomic DNA with chimpanzee and bonobo, thus the differences between these species are unlikely to be in gene content, but could be caused by inherited changes in regulatory systems. Endogenous retroviruses (ERVs) are one of the best candidates, which could be part of such systems. ERVs comprise ~5% of the human genome, and are mainly represented by single Long Terminal Repeats (LTRs) which contain many regulatory sequences (promoter, enhancer, polyadenylation signal etc.), thus, they can influence on gene expression. All Human-specific (Hs) LTRs belong to the HERV-K family, the most active ERV family in human genome. Some of these HERVs, which had integrated in regulatory regions of the human genome, could influence on the expression of adjacent genes and, consequently, contribute to human evolution. Recently, we obtained and analysed a whole-genome library enriched in Hs LTRs HERV-K. Some of them had integrated in regulatory regions of known genes. We have investigated methylation pattern for such Hs LTRs in seven tissues. We found that most of them have different methylation status in different tissues, which could be linked with expression level of the neighbouring genes. In MedLine journals we found that expression level for most of such genes correlated with methylation status of the corresponding LTRs. These LTRs could be expression markers for neighbouring genes. The functional role of analysing LTRs is discussed.

Structural Analysis of Monomeric
Isocitrate Dehydrogenase from
Corynebacterium glutamicum
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Isocitrate dehydrogenase (IDH) is one of the Krebs cycle enzymes, which catalyzes the oxidative decarboxylation of 2R, 3S-isocitrate with NAD(P)⁺ to yield α-ketoglutarate, CO₂, and NAD(P)H in a reversible manner. It requires a divalent ion such as Mg²⁺ or Mn²⁺ for its catalytic reaction. While NAD⁺-dependent IDHs are oligomeric present only in eukaryotes, NADP⁺-dependent idh is divided into two classes: homodimeric and monomeric NADP⁺-dependent IDHs. Homodimeric NADP⁺-dependent IDH is ubiquitously present in both eukaryotes and prokaryotes whereas the monomeric form is limited to few bacteria such as Vibrio species, Mycobacterium bovis, and Corynebacterium glutamicum. Although identity of amino acid sequence between homodimeric and monomeric NADP⁺-dependent IDHs is less than 10%, the structural analysis shows that the catalytic sites are well conserved between them. The diffraction data of C. glutamicum monomeric NADP⁺-dependent IDH were collected to a resolution of 1.8 Å. The crystal belongs to monoclinic C2 space group with unit cell dimensions of a = 129.0 Å, b = 52.7 Å, c = 124.0 Å, and β = 108.9°. The substrate-free monomeric NADP⁺-dependent IDH structure was solved by molecular replacement (MR) using AmoRe in CCP4 and was refined to an R-factor of 0.223 using X-PLOR. It consists of two distinctive domains, which form a similar catalytic cleft to the one present at the interface of two subunits in homodimeric NADP⁺-dependent IDH. Such structural analysis reveals that a single polypeptide can function similarly to the homodimeric enzyme via its domain duplication with the conserved catalytic site. Detail regulatory mechanisms and the cofactor-binding site would be investigated for further understanding of monomeric NADP⁺-dependent IDHs. Supported by the NSERC operating grant to LTJD and Saskatchewan Synchrotron Institute for a graduate fellowship to Fi.

DTT-induced In Vitro Autocleavage of
Human Hedgehog Protein’s Hint Domain
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The conserved HINT (Hedgehog and INTein) domain of intein and Hedgehog protein carries out post-translational modification reactions named protein splicing and autoprocessing, respectively. Despite the fact that inteins have been found only in unicellular organisms, and the HINT domain of a Hedgehog protein solely in multicellular organisms, many similarities between these two protein domains are recognized, such as their cleavage mechanisms, conserved motifs and three dimensional crystal structures. The objective of this study is to gain more insights into the structure, function, and evolutionary relationship of an intein and a human HINT domain. In this study, the wild type human Sonic HINT domain was expressed in an expression vector that was commonly used in intein studies, and many mutations (conserved motifs deletion, intein-human HINT chimera, and site directed mutation) were introduced in the same vector. Proteins of interest were purified, DTT-induced in vitro autocleavage reactions were tested under different reaction conditions, and resulting samples were analyzed on SDS-PAGE gel. The results show that human HINT domain has certain extein specificity, and deletion of any conserved motifs abolished the autocleavage activity. Neither the intein-human HINT chimera nor the split human HINT domain showed any detectable cleavage activity. Zinc ion at 2 mM concentration exhibited inhibitory effect on the autocleavage reaction of the human HINT domain, as also observed in inteins. This study shows that the human HINT domain possesses many characteristics of an intein, which confirms their close evolutionary relationship.

Melting of DNA Complexes with Cis-
Diaminedichlorplatinum (II) at Acidic pH
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Deprotonation of DNA at basic pH in the presence of cis-diaminodichloro-
platinum (II) (cis-DDP) has been explored in some detail [1]. In particular, it has been found that the proton binding constants (pKₐ’s) of AT and GC base pairs decrease upon DNA complexation with cis-DDP. For example, at 25 °C, the value of pKₐ for a GC base pair in double helical DNA decreases from 10.7 (in the drug-free state) up to 9.5 (in the drug-bound state). In contrast to alkaline pH region, the influence of cis-DDP binding on DNA protonation at low pH has not been investigated. To address this deficiency, we have used temperature-dependent UV light absorption spectrophotometry to study the thermodynamics of helix-to-coil transition of calf thymus DNA in the presence and absence of cis-DDP at acidic pH. Our results reveal that, at low pH, the melting behaviour of free calf thymus DNA is distinct from that of the cis-DDP-DNA complex. We interpret the observed differential melting profile of free DNA and the drug-DNA complex, in particular, the differential pH-dependence of denaturation temperature, TM, in terms of structural alteration of DNA caused by its covalent linking with cis-DDP.
122.11

Binding of New Meso-Tetra-(3N-Pyridil) Porphyrins to DNA

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We have used UV light absorption and circular dichroism spectroscopy to characterize the binding of new meso-tetra-(3N-pyridyl) porphyrins to calf thymus DNA. In particular, we have investigated the role of peripheral substituents of meso-tetra-(3N-pyridyl) porphyrins and their metallocomplexes. Our results reveal the following significant features: In metal-free porphyrins, peripheral substituents do not influence the drug-DNA binding; In nonmetalloporphyrins (MetAl), branching may occur as the result of a limited number of binding sites on DNA. For Zn-containing metalloporphyrins (ZnMet) and nonmetalloporphyrins (MetAl), branching may occur as the result of the presence of a relatively weak motif, the WD repeat. This sequence repeat, approximately 40 amino acids long, features a Gly-His pair and a Trp-Asp pair with some intervening residues of conserved hydrophobic character. WD domains exist with different numbers of repeats, ranging from 4 to 8. It is predicted that proteins with other number of WD repeats also fold into the propeller architecture, though no discrete single peaks. CD and fluorescence spectra suggest that these binding affinity of Zn-containing metalloporphyrins follows the following hierarchy: ZnTOEPyP (3) > ZnButPyP (3) > ZnAlPyP (3).

122.12

Protein Evolution In Vitro: Design, Cloning and Expression of Modular B-Propeller Domains Using an Idealized WD Repeat

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Many proteins involved in key biological processes are modular in nature. A group of these, the %“-propeller proteins, fold by packing 4-stranded %“-sheets in a circular array. The largest group of %“-propeller proteins, are characterized by the presence of a relatively weak motif, the WD repeat. This sequence repeat, approximately 40 amino acids long, features a Gly-His pair and a Trp-Asp pair with some intervening residues of conserved hydrophobic character. WD domains exist with different numbers of repeats, ranging from 4 to 8. It is predicted that proteins with other number of WD repeats also fold into the propeller architecture, though no structural evidence is yet available. We have designed an idealized WD repeat, maximizing the %“-turn forming interactions on the basis of the %“%“ structure (a 7-bladed %“-propeller). DNA for the prototype WD module was prepared by PCR using 4 oligonucleotides. After cloning this fragment into a vector with suitable restriction sites, concatemers with up to 10 repeats were prepared. All proteins with 4 to 10 repeats were successfully over expressed in a soluble form. These proteins could also be purified by metal-affinity chromatography; when run on a size-exclusion column, no aggregation was observed and the proteins eluted in discrete single peaks. CD and fluorescence spectra suggest that these proteins do not have a unique tertiary structure but presumably, as expected, a molten globular-like structure. Our results support the idea that these proteins may have evolved from an ancestral gene, via a process of multiple gene duplication and fusion.

122.13

Split Inteins with Protein Trans-Splicing Activities

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Inteins are protein equivalents of introns and exist in various precursor proteins in a wide range of organisms. Inteins are capable of protein splicing in which the intein sequence is excised and the flanking peptide sequences are joined with a new peptide bond. Although other intein sequences are contiguous, we found split DnaE intein sequences in several cyanobacterium species that were capable of protein trans-splicing when tested in E. coli cells, in which a mature protein was produced by splicing together two separate proteins or polypeptides. These split DnaE inteins most likely originated from a contiguous intein sequence, suggesting that inteins may potentially evolve into various split forms. This was tested experimentally by breaking a contiguous intein sequence at various selected split sites and testing for protein trans-splicing activity of the resulting split inteins. At least two novel split sites were found to support efficient protein trans-splicing function. In addition, a 3-piece split intein was found to be capable of protein trans-splicing for the first time. These findings produced new understandings of intein structure-function, protein trans-splicing, and intein evolution.

122.14

NMR Studies of TcUBP-1, a mRNA Destabilizing Factor from Trypanosoma cruzi

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Trypanosomes, protozoan parasites causing worldwide infections in human and animals, mostly regulate protein expression through post-transcriptional mechanisms and not at the transcription initiation level. We have previously identified a Trypanosoma cruzi RNA-binding protein named TcUBP-1. This protein is involved in mRNA destabilization in vivo through binding to AU-rich and GU-rich elements in the 3′-untranslated region of SMUG mucin mRNAs (D’Orso I. and A. C. Frasch (2001) J Biol Chem 276, 34801–34808). This protein consists of a single RRM-type motif and Glycine and Glutamine-rich regions at the C-terminal region, and resembles the protein Sex-lateral from Drosophila. We wanted to determine the RNA-binding mechanism mediated by TcUBP-1 and compare it with the ones described with other U-rich RNA-binding proteins, including Sex-lateral. For these propose, we performed chemical shift mapping upon titration with a short GU-rich sequence, derived from SMUG 3′-UTR region. NMR resonance assignments of TcUBP-1 were determined using standard triple resonance techniques on a 13C, 15N-labelled region of SMUG mucin mRNAs. This protein consists of a single RRM-type motif and Glycine and Glutamine-rich regions at the C-terminal region, which resembles the protein Sex-lateral from Drosophila. We wanted to determine the RNA-binding mechanism mediated by TcUBP-1 and compare it with the ones described with other U-rich RNA-binding proteins, including Sex-lateral. For these propose, we performed chemical shift mapping upon titration with a short GU-rich sequence, derived from SMUG 3′-UTR region. NMR resonance assignments of TcUBP-1 were determined using standard triple resonance techniques on a 13C, 15N-labelled sample. A comparison of the amide 1H and 15N chemical shifts between free and bound states has highlighted residues, which respond to RNA-binding. Strikingly, the b-sheet, specially b2, b3 and b4, form an RNA interaction surface. Residues within these regions that might interact with RNA were identified. Of these residues, some of them showed differences compared with other RNA-binding proteins.
122.15
Structural and Functional Studies of Nitrite Reductase Type I Copper Site Mutants Bound with Exogenous Ligands

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Alcaligenes faecalis nitrite reductase (AFNir) catalyses the single electron reduction of nitrite to nitric oxide. AFNir has a type I copper site at the surface of the protein that receives electrons from an external donor and transfers them to a type II copper, the site of nitrite reduction. The unique structure of type I copper sites in blue copper proteins allows for rapid electron transfers between these sites. A goal of this project is to replace wild-type AFNir type I copper ligands with exogenous ligands, and obtain detailed x-ray diffraction maps to better understand the role of ligand coordination in electron transfer through type I copper sites. H145A AFNir can accommodate exogenous ligands such as imidazole and chloride. Other exogenous ligands such as histidine are currently being tested, however the previously used crystalization conditions contain small anions that can compete as exogenous ligands. The crystalization conditions for AFNir have been modified to minimize the presence of competing ligands. Bulkier ethylbuthylrate can replace acetate as a pH 4.5 buffer. Also, sulfate can replace chloride to maintain ionic strength in the mother liquor. An additional goal of this project is to measure enzymatic activity in E. coli upon exogenous ligand binding. The ssoXRS regulon of E. coli, which provides for AfNir have been modified to minimize the presence of competing anions. Bulkier ethylbuthylrate can replace acetate as a pH 4.5 buffer. Also, sulfate can replace chloride to maintain ionic strength in the mother liquor. An additional goal of this project is to measure enzymatic activity in E. coli upon exogenous ligand binding. The ssoXRS regulon of E. coli, which provides

122.16
Conformational Changes of a Peptide from HIV-1 p24 Protein Induced by Ionic Micelles

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It has been recently shown that peptides from HIV proteins can either inhibit the fusion process, prevent the interaction of the virus with receptors, or induce changes on the cell surface. This has shown promise in the characterization of the mechanisms by which HIV-1 interacts with cells and facilitate the discovery of new ways for the prevention and treatment of HIV-1 disease. In this sense, we studied the interaction and micelle-induced conformational changes of the peptide AAMQLKETI-NEEAAEWDVHRPVHAGPIA, from HIV-1 p24 protein (196–224) in the presence of SDS (anionic), CTABr (caticionic) and HPS (zwitterionic) micelles at pH 4, 7 and 10, monitored by Circular Dichroism, fluorescence and Electron Spin Resonance (ESR). The SDS micelles induced an alpha-helix structure on the peptide in all pH values and a blue shift in the fluorescence emission, indicating an internalization of its tryptophan residue. Similar results were observed with CTABr micelles at neutral and basic pH. HPS micelles did not induce structural modifications on the peptide. ESR studies showed that p24 peptide decreases the mobility of nitroxide spin labels at positions C5 and C12, while causing only small changes at the polar head group region. These results suggested that one part of the peptide, the last ten residues (VHPHAGPIA), can be located at the hydrophobic core of the micelles, whereas the first eighteen residues form an alpha helix bent over the polar head group.

122.17
Use of Phage P2 a Protein for the Display of Random Peptide Libraries

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The protein produced by A-gene of Phage P2 (P2 A) is an endonuclease that initiates a rolling circle replication by introducing a single strand discontinuity (nick) in the DNA and the attaching covalently in cis to the same molecule of DNA from which it was expressed. We are determining whether this cis-binding activity can be used as a new in vitro display system for protein evolution. In order to determine if this technology will actually work, we generated a library in which 90-nucleotides of random sequence was inserted between P2A and HA epitope coding sequences as a linker by 3 steps of PCR. After sequencing, the library DNA was input to an E. coli S30 lysate for in vitro transcription and translation (ITT) reaction. The ITT reaction containing protein-DNA complexes was diluted and subjected to affinity selection against HA antibody to see if we can identity a sequence that enhances the HA binding to the antibody. After 2 rounds of selection direct sequencing of the PCR products revealed that the sequences recovered were still pretty random. Control experiments revealed that both wild-type P2A (without random sequence and HA tag) can be recovered from HA antibody coated tubes indicating some non-specific binding of our library. But a quantitative assay for a sample containing a mixture of library and wild-type P2A showed that our library may have the potential to compete with wild-type P2A under some conditions. Overall our results suggest that P2 A could be developed into a very useful library display tool for research or drug discovery.

122.18
Purification and Characterization of a Novel NADP+ -linked Alcohol Dehydrogenase from Euglena gracilisZ

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Here, we report the purification of a novel NADP+ -linked cytosolic alcohol dehydrogenase from 1-hexanol-grown Euglena gracilisZ, which is active towards the mid and long-chain primary alcohols but not the primary short-chain and secondary alcohols. The native enzyme was found to be a homo-tetramer of 53-kDa subunits. The pK values of the enzyme were determined as 45.8 kJ mol-1, 116 J mol-1 K-1, and 83.7 kJ mol -1, respectively. Furthermore, the turn over (Kcat) was 20 min-1 at 55°C, pH 8.8. The thermodynamic of the reaction catalysed by the ADH i.e. the enthalpy change (ΔH°), entropy (ΔS°) and the change in Gibbs free energy (ΔG°) for 1-hexanol break down were determined as 45.8 kJ mol-1, 116 J mol-1 K-1, and 83.7 kJ mol -1, respectively. Accordingly, the novel enzyme, which is located in the cytosol and active towards the mid and long-chain fatty alcohols, is suggested to be involved in the assimilation of fatty alcohols formed from wax esters under aerobic conditions, which are synthesized in anoxia (wax ester fermentation).
123.1 Production of Herpes Simplex Type-I Thymidine Kinase Recombinant Protein in E. coli
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Herpes simplex virus type-I (HSV-I) was first propagated in HeLa cells. When TCID50 of the virus reached 107 inoculated cells were frozen and thawed 3 times. The resultant was subjected to DNase and proteinase K treatment and the virus DNA was then extracted using phenol-chloroform method. In order to amplify the thymidine kinase gene a pair of primers were designed in which BamHI and EcoRI enzyme sites were also inserted. The amplified gene was first purified before being digested with the above mentioned enzymes. PTrcHis2C plasmid was also digested with the same enzymes and after purification of both digested gene and the plasmid, ligation was done using T4 DNA ligase. Competent E.coli strain DH5α cells were transformed by the constructed plasmid and grown on agar containing LB medium having ampicillin. Clones having plasmids with the gene of interest were prepared in large scale and the plasmid was extracted and purified. Thymidine kinase was produced in the same cells after being induced by IPTG. SDS-DPAGE showed that the protein of interest was produced in transformed cells. In order to identify the protein an indirect fluorescent antibody technique as well as western blotting were done. Results showed that the protein reacted with the immune serum against HSV-I perfectly.

123.2 Application of Baculovirus Expression System to Produce Recombinant Glycoprotein G of Herpes Simplex Virus Type—I In Order to Develop an ELISA Test
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Recombinant baculovirus carrying protein GgG-1)of Herpes Simplex Virus Virus Type-I (HSV-I) was first inoculated to Sf9 cells with MOI of 10. Inoculated cells as well as control ones (which had received only growth medium) were collected 72 hours post inoculation. Cells were washed with 20mM Tris buffer (pH~8) after being frozen and thawed three times. Cell extracts were treated with 1% Triton X-100 and centrifuged at 10000 rpm for 10 minutes at 4 degree centigrade. SDS-PAGE was done for each of the supernatants to see the band of the recombinant gG-1. The protein of interest was identified in Western blotting method using specific anti gG-1 monoclonal antibody. Large amount of the recombinant gG-1 was then prepared and purified by anion exchange chromatography. In order to run an ELISA test 35 ng of the protein was coated on a Maxisorp ELISA plate. The cut off value of the test was determined as 0.218. The first antibody was a human serum collected from a seropositive person and the second antibody was mouse-antihuman antibody conjugated with HRP. A total 90 human sera were tested with this method of which 80% showed anti-gG-1 antibody with the titer ranging 1/100 to 1/1500. Fifty seven of tested sera were also subjected to Virus Neutralization Test (VNT). The sensitivity and specificity of the ELISA test were 100% and 94.7% respectively.
123.4
From Stem Cells to the Skeleton—Differentiation of ES Cells Into Osteoblasts and Chondrocytes
N. Zur Nieden\textsuperscript{1}, G. Kempka\textsuperscript{2}, D. E. Rancourt\textsuperscript{1}, and H. J. Ahr\textsuperscript{2}
\textsuperscript{1}University of Calgary, Calgary, AB, Canada; and \textsuperscript{2}Bayer AG, Wuppertal, Germany

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of mouse blastocysts and have been shown to differentiate spontaneously into cell types representing all three germ layers. These cells can be forced to undergo various differentiation pathways in response to growth factors and culture additives. Mineralized osteoblasts could be cultured from ES cells under the influence of ascorbic acid, $\delta$-glycerophosphate and 1a,25-dihydroxyvitamin D3. The essential components that led to cartilage formation were found to be bone morphogenetic protein 2, transforming growth factor $\beta$, ascorbic acid and insulin. Osteogenic mineralization and cartilageous proteoglycan synthesis were detected by histochemical staining methods. Four major components of bone matrix, osteocalcin, -pontin, -nectin and bone sialoprotein and the two major components of cartilage matrix, collagen II and aggrecan, could be localized to the differentiated cells by immunofluorescence. Real-time quantitative PCR was applied to analyze the expression of a wide variety of bone and cartilage matrix genes, respectively, e.g. collagens I and II, which are expressed in a time-dependent pattern in vitro corresponding to the in vivo development of the skeleton. The in vitro differentiation of mouse embryonic stem cells into these two skeletal elements may provide a suitable model to learn more about the challenging topic of molecular regulations of osteoblastic development in vivo.

123.6
Characterization of Transgenic Drosophila Synthesizing a Small Heat Shock/Alpha-Crystallin Protein from Artemia franciscana
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Small heat shock/alpha-crystallin proteins function as molecular chaperones, preventing irreversible protein denaturation during stress. The brine shrimp, Artemia franciscana, produces large amounts of the small heat shock/alpha-crystallin protein p26 during oviparous development. Oviparously derived, encysted Artemia embryos enter diapause, characterized by arrested development, greatly reduced metabolism and tolerance of long-term anoxia, desiccation and temperature fluctuation. p26 has been implicated in the remarkable stress tolerance of Artemia embryos. Transgenic Drosophila producing p26 were successfully generated by P-element mediated germ line transformation and the protein was selectively expressed in adults and embryos using the GAL4/UAS system. Constitutive expression of p26 did not adversely affect growth and development of transgenic Drosophila. Preliminary analysis indicated transgenic Drosophila adults expressing p26 were more heat tolerant than normal flies and age-specific survival of older flies may be increased. However, p26 appeared not to enhance anoxia resistance of transgenic embryos, a function ascribed to the protein in Artemia embryos where it is produced in much greater amounts. Transgenic Drosophila are being used to further examine p26 function upon exposure to thermal and oxidative stress, and to determine if intracellular localization of p26 is influenced by stress, as occurs in Artemia. The work was supported by NSERC Discovery Grants to THM and VL, an NSHRF New Opportunity Grant to THM and an NSHRF Graduate Fellowship to YS.

123.7
Regulation of FOXC1 Expression by PAX3, GLI2 and $\beta$-Catenin During Myogenesis
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Myogenesis is a multi-step process in which muscle precursor cells become committed to the myogenic lineage prior to differentiation, and is thought to involve several transcription factors such as Pax3, Gli2 and $\beta$-catenin. In order to identify other transcription factors that might be important, this study examines the role of Foxc1 during skeletal muscle development. Foxc1 belongs to the forkhead/winged-helix transcription factor family, whose members have been shown to regulate developmental processes such as cell-fate specification. Mice lacking Foxc1 and its homologue Foxc2 show abnormalities in eye and kidney development, cardiovascular remodeling and somitogenesis. In order to further characterize the biochemical changes regulated by Foxc1, P19 embryonal carcinoma (EC) stem cells were chosen as a model system. As revealed by Northern blot analysis, Foxc1 is expressed transiently during muscle specification. In a P19 cell line expressing a dominant-negative Pax3, P19(Pax3-Engr), myogenesis is disrupted and expression of Foxc1 transcripts is decreased when compared to control cells. Furthermore, transcript levels appear to be reduced in P19(Gli2-Engr) cells, which also exhibit impaired myogenesis. These results suggest a role for Pax3 and Gli2 in the regulation of Foxc1 expression. Finally, in P19($\beta$-catenin-Engr) cell lines which show a loss of myogenin, Pax3 and Gli2 expression, Foxc1 transcripts are undetectable, suggesting that $\beta$-catenin might be an upstream activator of Foxc1 transcription. Studies are currently underway to determine whether or not Foxc1 is essential and/or sufficient for myogenesis, by overexpression of wild-type and dominant-negative forms of Foxc1 in P19 cells. Taken together, this information will allow us to place Foxc1 within the complex hierarchy of transcription factors regulating muscle development.
**123.8**

**Bmp Regulates Skeletal Myogenesis at Two Steps**

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The ability to manipulate the timing and direction of adult and embryonic stem cell differentiation has enormous potential for use in cell regeneration therapy. We are investigating the ability of Bone Morphogenic Protein-4 (BMP-4) to suppress skeletal myogenesis in P19 cells. The P19 cell system, an embryonal, murine line of carcinoma cells, provides an attractive alternative to the embryo for elucidating the molecular mechanisms involved in the early stages of skeletal muscle development. Our data indicates that BMP-4 inhibits skeletal myogenesis at two steps. P19 cells over-expressing BMP-4 fail to form terminally differentiated skeletal myocytes and the early markers of skeletal myogenesis were never expressed, suggesting that the over-expression of BMP-4 interfered with the specification of cells to the myogenic lineage. In addition when these BMP-4 over-expressing cells were co-cultured with wild-type P19 cells that were at the myoblast stage, there was significant reduction in the conversion of this proliferating population to myocytes. Blocking BMP signaling using noggin, a BMP antagonist, was able to rescue skeletal myogenesis in these cells. Hence BMP inhibits myogenesis in P19 cells at both an early and a late stage of differentiation. The ability of BMP-4 to reversibly halt myogenesis at late stages can be exploited to provide an abundant supply of undifferentiated skeletal precursor cells. Further studies are underway to give insights into the mechanism by which BMP-4 is capable of regulating myogenesis in our system. Canadian Institute of Health Research (CIHR) has supported this work.

**123.9**

**Characterization of a Novel Function of Bone Morphogenetic Proteins Type II Receptor (BMPRII) in Neuronal Development**

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The transforming growth factor-beta (TGF-b) superfamily is a family of growth and differentiation factors that are grouped into three classes: TGF-bs, activins, and Bone Morphogenetic Proteins (BMPs). These factors transduce signals through type I and type II transmembrane serine/threonine kinase receptors that activate intracellular proteins termed Smads which subsequently regulate transcription of specific genes. BMP ligands play significant roles during multiple stages of neuronal development. BMPRII, a Type II receptors, binds BMP ligands and is predominantly expressed throughout the brain. Recently, a Drosophila TGFb family Type II receptor, Wishful thinking (Wit) most closely related to BMPRII, has been shown to be crucial for various aspects of neuronal development. This suggests that BMP ligands regulate neuronal development at least in part via signaling through BMPRII. Both BMPRII and Wit possess a C-terminal extension absent in other type II receptors. To investigate whether this unique domain in BMPRII may have an important function, we first examined the role of BMPs in primary mouse cortical cells. We observed that incubation of cortical cultures with BMP7 induces neurite outgrowth and that adenovirus-mediated overexpression of wild type BMPRII enhances this effect. Interestingly, expression of a truncated BMPRII lacking the tail significantly decreased the number of neurites formed in response to BMP7. As deletion of the BMPRII tail did not alter Smad-dependent transcription responses, we postulate that the BMPRII carboxyl terminal tail may exert its positive effects on neurite induction through the recruitment of novel proteins. We are currently screening for BMPRII interacting molecules and are investigating their contribution to neurite outgrowth. It is hoped that this work will provide insights into Smad-independent signaling pathways emanating from the BMPRII receptor.
123.11
Injection of DNA Into Both Pronuclei Decreases Mosaicism in Early Mouse Embryos
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The ability to introduce foreign genes into mammalian germline has proved to be one of the powerful tools in modern biology. The major problem in this way is the low rate of germline transmission of transferred genes due to mosaic distribution in early embryos after pronuclear injection. In this experiment, both male and female pronuclei were injected with growth hormone gene construct using a fine micro needle. PCR analysis of single blastomeres derived from cleaved embryos revealed that 8% of 2-cell, 21% of 4-cell and 52% of 8-cell embryos have mosaicism, whereas, 38%, 67% and 100% of 2-, 4- and 8-cell embryos in which only one pronucleus was injected were mosaic. This data suggest that microinjection of both pronuclei can reduce mosaicism in the mouse embryo.

123.12
Development of a Clone of BK Cells and Its Usage for Propagation of Parainfluenza 3 Virus and Herpes Simplex Virus Type 1
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Primary cells were taken from kidneys of a healthy Holestone calf and cultured in DMEM medium supplied with 10% heat inactivated calf serum. After several passages some cells changed morphologically. These cells were cultured separately and a continuous cell line was prepared. The Karyotype of the cell line was studied and it was shown that its chromosome number was as 2n=46. Attempts were made to make a clone of the above cell line derived from only one cell. Therefore vero cells were chosen as feeder. These cells were cultured in the same medium before being treated with 10 ug/ml Mitomycin C to stop their mitosis. Decreasing dilutions of the BK cell line suspension were then cultured in different wells each containing a monolayer of treated vero cells. Wells in which a clone of the BK cell was formed were selected and each clone was trypsinized and collected very carefully in order not to allow any contamination with the feeder. The cloned cells were passed several times and the karyotype of each passage was determined. It was shown that the chromosome number of cloned cells remained much less changeable compared with that of the original cell line. Parainfluenza 3 virus (PI3V) and Herpes Simplex Virus type 1(HSV-1) were propagated in the original cell lines as well as cloned cells derived from them. The titer of the PI3V in the original cell line was significantly higher than that of the clone one, while titers of HSV-1 remained the same in both cells.

123.13
Proteomic Analysis of Early Craniofacial Development in Chicken Embryos
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Craniofacial regions (craniums and faces including facial bones) are formed through complicated steps during the embryonic development. These processes require many unidentified molecules and genes to complete the facial and cranial developments in programmed time sequences. Due to limitation of technology, past studies had difficulty to have the whole profile of reacting molecules involved in the craniofacial development. Using a new innovative technology for protein analysis, we are able to analyze the whole protein profile that might reflect differently at each developmental stage. Here we tried to identify the proteins expressed differently at the critical stages of patterning and outgrowth of craniofacial regions. We made the proteomic map of each developmental stage of chicken embryos and compared the regional and interval changes of expressed proteins. From these results, we are able to set up the protein profiles in the craniofacial development. These accumulated data help us understand the molecular mechanism in craniofacial development in the near future.
An indicator of reactive nitrogen species, protein nitrotyrosine, increased with lowered cytokinins had half LFP concentration compared to controls. Increased level of cytokinins leads to double increase in LFP whereas plants composed of fractions differing in their chromatographic mobility. In-spectral methods. With HPLC we proved that individual fluorophores are with increased, and 16 in leaves with lowered cytokinin content using leaves with modified cytokinin content. We distinguished 15 fluorophores in which cytokinin levels are decreased due to increased degradation. We genes (kind gift of Prof. Amasino). We compared the results with tobacco
ployed tobacco plants whith prolonged life span of their leaves due to attack of free nitrogen species producing protein nitrotyrosine. We em-
kinesis to free radicals is extremely important. We studied characteristic role in regulation of senescence, the question of the relationship of cyto-
growth regulators cytokinins control senescence. Because of their central
role in regulation of senescence, the question of the relationship of cyto-
kinins to free radicals is extremely important. We studied characteristic
end products of lipid peroxidation, lipofuscin-like pigments (LFP) ad the
attack of free nitrogen species producing protein nitrotyrosine. We em-
ployed tobacco plants with prolonged life span of their leaves due to
coupling of cytokinin synthesis to the promoter of senescence related
genes (kind gift of Prof. Amasino). We compared the results with tobacco
in which cytokinin levels are decreased due to increased degradation. We
found changes in composition and content of LFP during aging of tobacco
leaves with modified cytokinin content. We distinguished 15 fluorophores
with increased, and 16 in leaves with lowered cytokinin content using
spectral methods. With HPLC we proved that individual fluorophores are
composed of fractions differing in their chromatographic mobility. In-
creased level of cytokinins leads to double increase in LFP whereas plants with
lowered cytokinins had half LFP concentration compared to controls.
An indicator of reactive nitrogen species, protein nitrotyrosine, increased
in senescence in plants with elevated cytokinins by 2.5 fold. In plants with
lowered cytokinins the concentration of nitrotyrosine in senescence did not
change. These results indicate that senescence in plants with in-
creased cytokinin is accompanied with higher production of free radicals
derived from both oxygen and nitrogen.
Nucleotide Sequence of Creatine Kinase From the Cephalochordate Branchiostoma belcheri and the Properties of the Gene Product by Escherichia coli

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To further our knowledge of creatine kinase (CK; EC 2.7.3.2) in the fields of molecular evolution and comparative enzymology, we analyzed the CK gene of the Branchiostoma belcheri (B. belcheri). The complete sequence of 1,140 bases in the CK gene of B. belcheri was revealed, with 71.5% being homologous with the CK gene of other organisms. The amino acid sequence corresponding to the nucleotide sequence of the CK protein was determined from this sequence, and the secondary structure of this protein was estimated in comparison with that of other organisms. Although the region involved in the enzymatic function of CK was completely conserved, some regions around the active site varied in different organisms. The molecular phylogenetic tree, the preparation of which was based on the amino acid sequence, revealed that the CK of B. belcheri may correspond to the phylogenetic predecessor of the vertebrate, i.e., the stage prior to the branching of CK into CK-M and CK-B. In addition, we attempted to synthesize the CK protein of B. belcheri by using an expression system obtained from Escherichia coli (E. coli) and to determine its partial characterization. The CK protein was purified from an E. coli suspension, and the purified enzyme was demonstrated to be homogeneous by 2D-electrophoresis. The CK was enzymologically characterized as having a molecular weight of 8.1 kDa, an isoelectric point of 5.8, and optimum pHs of 8.5 and 7.0 in forward and reverse reactions, respectively. The apparent Km of CK for creatine (Cr) and creatine phosphate (Cr-P) were $4.2 \times 10^{-4} \text{mol/l}$ and $9.3 \times 10^{-4} \text{mol/l}$, respectively.
123.20

Purification and Characterization of Major Gelatin-Cleavage Activities in the Apically Located Extracellular Matrix of the Sea Urchin Embryo

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The hyaline layer is an apically located extracellular matrix that surrounds the sea urchin embryo from the time of fertilization until metamorphosis occurs. Gelatin-cleavage activities were absent from freshly prepared hyaline layers but a dynamic pattern of activities developed when layers were incubated at 15°C or 37°C in Millipore-filtered seawater. A 55 kDa species was identified as the major activity at 37°C whereas 41 and 32 kDa species were the major species at 15°C. After 92 hours of incubation at 15°C, the 55 kDa and other higher molecular weight species appeared. The 55, 41 & 32 kDa activities could be dissociated from the hyaline layer. All three species were purified using gel filtration chromatography. In both qualitative and quantitative assays, the 55 kDa gelatinase activity was inhibited by 1,10 phenanthroline (a Zn²⁺ - specific chelator) and ethyl enebis (oxoethylenenitrito) acetic acid. Calcium reconstituted the activity of the ethylenes (oxoethylenenitrito) acetic acid-inhibited 55 kDa with an apparent dissociation constant of 1.2 mM. Since the hyaline layer is in direct contact with seawater that contains 10 mM CaCl₂, 50 mM MgCl₂ and 500 mM NaCl, the effects of MgCl₂ and NaCl were also studied. MgCl₂ was found to activate the 55 kDa species, while NaCl caused inhibition. Developmental substrate gel analysis was performed using eggs and various stage embryos. The 55 kDa species comigrated with a gelatin-cleavage activity present in 69 hours old embryo. We conclude that the 55 kDa belongs to the matrix metalloproteinase class of extracellular matrix remodeling enzymes. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to J.J.R.

123.21

Kinetics of Hoxd1 Protein Binding to the Promoter Region of Myelin Oligodendrocyte Glycoprotein

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The identification of Hox protein interactions in multiple germ layers to coordinate cell division and cellular functions during development has generated a keen interest in determining their precise functions since their discovery. In order to study these Hox protein interactions, it will be necessary to first identify and characterize their downstream target gene(s). The objectives of this study were: (i) to determine putative targets of Hoxd1 using the Hoxd1 consensus binding sequence and GenBank, (ii) to characterize the binding specificity of Hoxd1 to its putative target. GenBank data search has revealed that the promoter region of the myelin oligodendrocyte glycoprotein gene (MOG) contains the consensus-bind ing site for Hoxd1. The binding specificity of the Hoxd1-MOG complex was characterized. The dissociation coefficient constant (Kd) was estimated to be 1.5 x 10⁻⁷ M, the dissociation rate constant (kd) was found to be 1.3 x 10⁻³ s⁻¹ and the DNA-Hoxd1 homeodomain complex has a half-life (t₁/₂) of 12.5 min determined by equilibrium and kinetics studies. Mutational analysis of the binding specificity of Hoxd1-MOG complexes revealed that M1 (a mutation from TAAT to TACT within the consensus binding site) and M2 (a mutation from TAATG to TAATTG within the consensus binding site) probes? binding affinity to MOG promoter were severely affected. Thus the TAATTG core of the binding sequence appears to be significant for Hoxd1 specificity. However, the TAAT sites flanking the putative binding site also influenced the binding activity of Hoxd1 protein as indicated by mutant probe, M3 (mutation from TAAT to TACT in three neighbouring TAAT sites outside Hoxd1 consensus site). Our data suggest that MOG may be a target of Hoxd1.

123.22

Neurospora crassa Catalase-Peroxidase in Fungal Differentiation and Stress Tolerance

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Neurospora crassa morphogenetic transitions involve hypoxiderator states. Antioxidant enzymes are developmentally regulated and participate in the regulation of cell differentiation. Here we study the regulation and function of N. crassa Catatalase-2 (CAT-2). The enzyme has catalase and peroxidase activity with similar kinetic constants to that of bacterial catalase-peroxidases (CPs). It is a homodimer of 83.4 kDa subunits with a prosthetic group different from protoporphyrin IX. Based on the sequence of an internal peptide, the cat-2 cDNA sequence was determined. The cat-2 gene revealed an ORF with no introns encoding a 753 amino acid protein that corresponds to a typical CP. Phylogenetic analysis indicates that CAT-2, together with other fungal CPs, diverged from a b- proteobacteria clade, suggesting a bacterial origin for fungal CPs. CAT-2 mRNA and activity were associated with late stationary-phase mycelia, when arthroconidia are formed and hyphae undergo autolysis. CAT-2 was induced during stress conditions such as carbon deprivation, H₂O₂-generated oxidative stress and heat shock. CAT-2 activity was found to be regulated during the macroconidiation process as well. Regulation is exerted at the transcriptional and possibly at the posttranscriptional level: cat-2 mRNA accumulated rapidly after induction of conidiation by air exposure of a mycelial mat, however, CAT-2 activity was detected latter, until aerial hyphae are formed. Both, mRNA and CAT-2 increase further in aerial hyphae and conidia. CAT-2 mutant strains obtained by the RIP procedure showed an increase in the formation of arthroconidia during late stationary-phasemycelia and are sensitive to elevated temperatures. In conclusion, N. crassa CAT-2 was found to be part of the conidiation processes of the fungus and is essential for stress tolerance.

123.23

Tid-1, a Human DnaJ Protein, Associates with the Trk Family of Receptor Tyrosine Kinases

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Tid-1, tumorous imaginal disc 1, is a member of the DnaJ family of chaperone proteins, was first identified as a tumor suppressor which could inactivate the E7 oncoprotein of human papilloma virus 16. Tid-1 affects cell survival/apoptosis in mammalian cells and interacts with the signaling proteins Ras GAP and JAK. We have identified Tid-1 as a novel binding protein of the Trk family of receptor tyrosine kinases by yeast two-hybrid screening. Tid-1 binds to and is tyrosine phosphorylated by TrkA, TrkB and TrkC receptors in a phosphotyrosine-dependent manner in mammalian cells. The binding sites for Tid-1 were mapped to Tyr683 and Tyr684 of the activation loop of rat TrkA. Tid-1 also binds to the adaptor proteins Grb2, SH-2B and APS. Tid-1, in association with HSP70 and HSP90/CD93, is expressed widely in mouse cortical tissues and in various cell lines including COS, rat adrenal pheochromocytoma PC12, human neuroblastoma SY5Y and human medulloblastoma Med283 cells. Tid-1 localizes to both cytosol and mitochondria-rich regions when overexpressed in mammalian cells. Tid-1 overexpression in nrr5-TrkA cells enhances the cells more responsive to NGF stimulation. Taken together, Tid-1 represents a novel signaling protein for the Trk receptors suggesting a role for this protein in neurotrophin dependent signal transduction.
Proteomic Analysis of Human Embryonic Stem Cells

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The application of embryonic stem (ES) cells in biomedical field provides us new therapeutic paradigm to cure unsoloving human diseases. Above all, to establish and develop ES cell therapeutics, the mass production of undifferentiated ES cells and the characterization of ES cells is strongly needed. However in spite of the possibility of ES cell therapeutics, its basic characteristics still remains unclear probably due to shortage of our knowledge about proteins functioning in the ES cells. There have been few studies about proteome of mouse ES cells, however, the proteome of human ES cells has never been reported. In this study, we performed proteomic analysis of undifferentiated human ES cells based on 2D gel electrophoresis with Coomassie and silver staining and mass-spectrometry. We detected 675 individual spots, some of which we identified by mass spectrometry and database searching. We present here a first set of 85 proteins identified which are the normally expressed of human embryonic stem cells. They include house keeping genes, all type of t-complex gene, cytoskeleton-related proteins, a protein previously shown to be involved in cell proliferation, enzymes, oxidative-stress related proteins, and a few of transcription-regulating factors. We may suggest that these proteins may have certain roles in the maintenance of the undifferentiated state of human ES cells. This the first report about the proteome of human ES cells.

Identification of KOX1 Specific Target Genes by Microarray-analysis


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In total 813 C2H2 zinc finger genes (IPR000822) and 291 KRAB containing C2H2 zinc finger genes (IPR001900) have been identified and annotated (www.ensembl.org/Homo_sapiens/diameterview). The KRAB domain initially described as heptad repeat of leucines in KOX1 (New Biol. 363–74, 1990) had been found to be one of the strongest repressor domains in mammalian cells (PNAS 91, 4509–4513, 1994). The repression of the KOX1-KRAB domain was postulated to be mediated by SMPI (MB 15, 1907–1914, 1995). This factor cloned as transcriptional intermediary factor 1 beta (TIF1b, synonyms: KAP-1, KRIPI-1, TRIM28, SMPI) is thought to be essential for mediating repression initialized by KRAB-containing C2H2 zinc finger proteins. Genes that are regulated through TIF1b in vivo are possibly putative target genes of KRAB zinc finger proteins. 176 Affymetrix Hu133A Chips monitored the expression of 42 KRAB zinc finger genes on 27 human tissues. KRAB zinc finger gene expressions representing a subset of these genes were validated by TaqMan analysis. Gene expression profiles were correlated with phylogenetic relationship of zinc finger genes and with their chromosomal location within zinc finger gene clusters. Genes regulated by the expression of KOX1, a KRAB containing zinc finger gene, were studied in a cell culture system. Substituting the zinc finger region of KOX1 partly with zinc fingers of ZNF136 indicated that different endogenous gene sets could be up regulated in their expression. Thus, gene expression of endogenous genes can be directed in their expression by in vivo expression of novel combinations (rearrangements) of individual zinc finger domains already existing in nature. In summary, the functional analysis of KRAB zinc finger genes reveal coordinate gene expression patterns evolved during mammalian evolution.

Molecular Biology and Gene Transformation in Brassica napus by Agrobacterium

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Brassica napus cultivation has being developing in Iran since last few years. Rapseed breeding for oil quality is very important and necessary. The usage of classical methods for rapseed breeding is time consuming and sometimes unsuccessful. By genetic engineering can produce resistant cultivars e.g. to herbicides, insects or diseases. The aim of this project was optimizing transformation and regeneration systems in Brassica napus using GUS reporter gene. Some genotype (e.g. Maluka, Hyola 308, Global, PF, NSA, westar and falcon) was chosen for this study. In the first step of this research we recognized that the best genotype was, Global and the best explant was 3-days old cotyledon and the best medium was SIM containing 4.5 mgl-1 BAP of different doses. Three strains of Agrobacterium (LBA 4404, CS8 (pGV3101) containing, pVW 432 and pB1121 plasmid were tested. To increase the efficiency of transformation, we used phenolic compound (such as acetosyringone) and no differences were observed between media with acetylpyrione and without (control) for transformation and was studied on the activity of CS8 (pGV3101) promoter also. GUS staining solution tested the transformed plants. As a result blue colour were observed in root, Leaf, and flower. The blue colour in transformed tissues was the result of successful transformation by GUS gene, and we obtained 37% complete plants.

X-ray Crystallographic and Scanning Electron Microscopic Analysis of Starches from Five Yam (Dioscorea spp.) Varieties Grown in Jamaica

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Yams (Dioscorea spp.) are important sources of carbohydrates, where 70–80% of the dry mass is starch. The utilization of starch by the form of glucose is dependent on the starch source and associated physiochemical properties (Imad et al., 1999). There are over 15 cultivated varieties of Jamaican yams; however there is a lack of information with respect to the properties of the starches from these yams. This study was designed to investigate the physiochemical properties of five local yam cultivars by application of X-ray crystallographic and scanning electron microscopic techniques. Starches from five yam cultivars belonging to five different species grown in Jamaica were extracted and analyzed. X-ray diffractograms of Round leaf yellow yam (D. cayenensis), Renta yam (D. alata), and Negro yam (D. rotundata) showed open hydrated hexagonal crystallites (type-B). However, Bitter yam (D. polygonoides) had denser crystallites, with staggered monoclinic packing (type-A), and Chinese yam (D. esculenta) the intermediate type crystallites (type-C). It is interesting to note that Bitter yam (D. polygonoides) a wild variety that is usually used in traditional medicine has a crystalline structure (Type-A) typical to that of corn (Zea mays). The sizes of the granules varied from a low of 1-3 µm for Chinese yam and a high of 16–42 µm for Round leaf yellow yam. Variations in the granule shapes were also observed. Granules from Chinese and Bitter yams were rounded, while Round leaf yellow yam, Negro yam and Renta yam displayed ellipsoid, polyhedral and triangular shapes respectively. The differences in the physiochemical properties of the different yam starches may affect the digestibility.

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HUPO 2nd Annual & IUBMB XIX World Congress, October 8–11, Montreal
Myostatin Signals Through a TGFβ-like Signalling Pathway to Block Adipogenesis and Osteogenesis

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Myostatin, a Transforming Growth Factor-β (TGFβ) family member, (previously known as growth and differentiation factor 8, GDF8) is a potent negative regulator of skeletal muscle growth. Myostatin null mice display a two fold increase in muscle mass due to widespread hyperplasia and hypertrophy. This profound muscle growth is accompanied by decreased body fat accumulation. In order to decipher the mechanisms mediating the biological role of myostatin, we have identified the myostatin receptors and its intracellular signalling pathway. We demonstrate that myostatin binds the type II ser/thr kinase receptor, ActRIIB, but not the closely related ActRII, indicating that these two receptors display distinct ligand binding preferences. Myostatin-bound ActRIIB forms a heteromeric complex with the type I receptor ALK4 or ALK5, to induce Smad2 phosphorylation and activate a TGFβ-like signalling pathway. This represents the first demonstration that ALK5 can mediate signals for a ligand other than TGFβ. Furthermore, we reveal a strikingly specific antagonism of BMP7, but not BMP2-mediated processes by myostatin. While both BMP7 and BMP2 activated transcription from the BMP-responsive I-BRE-Lux reporter and induced adipogenic and osteogenic differentiation of mesenchymal cells, myostatin inhibited BMP7 but not BMP2-mediated responses. We elucidated the molecular mechanism of this antagonism by demonstrating that myostatin blocks BMP7-induced heteromeric receptor complex formation by competing for the common type II receptor, ActRIIB. These findings suggest that myostatin commands exquisite control of complex events underlying mesenchymal cell differentiation in development and homeostasis by differentially regulating BMPs and by functioning only in the context of appropriate receptor combinations.
124.1
The Effect of Continued Diabetes on the Formalin Induced Pain and Baclofen Analgesia in Rat
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AIM: one of the side effects of diabetes epidemic today in the world is painful neuropathy, the reasons and treatments of which are unknown. Due to the importance of the problem of pain treatment as one of the harmful phenomena in life, this research studies focused on the effect of continued diabetes on the formalin induced pain and baclofen analgesia in rat. Moreover effect of Baclofen as a nonopiate, analgesic drug on the decreased pain in the quiescent phase as the model of diabetic pain was investigated.

METHODS: The method is experimental by evaluating the pain level through conducting the formalin test in 3 groups of rats. The first group was divided to control (injection normal salin) and diabetic (injection aloxan 100 mg/kg) which were tested, following the observation of the routine signals of diabetes, after one to four weeks from the beginning of diabetes, the second one was divided to a new control and diabetic group and before performing formalin test, the baclofen (10 mg/kg) was injected to them and third one was divided to two diabetic groups that received baclofen and normal saline and then the pain of the quiescent phase was compared between them.

RESULTS: The results indicated that diabetes increases formalin induced pain (p <0.05) and remained with continued diabetes. It also indicated that diabetes established increased pain in the quiescent phase (p<0.05). Diabetes had no influence on the baclofen analgesic effect, on the first phase of formalin test and increased it on the second phase and also baclofen could quiet the increased pain in quiescent phase (p<0.05) very well.

CONCLUSION: Due to the results of this study it seems that diabetes with the changes in the central and preferal pathways of the pain and also pain control, increases the pain. More studies are required for determining its mechanism. These changes are accompanied with weakening the internal antipain systems such as Gabaergic, that it can treat with baclofen. Diabetes has no interaction with the baclofen’s analgesic effect, so baclofen may be recommend as an effective drug to comfort painful diabetic neuropathy.

KEY WORDS: diabetic painful neuropathy-baclofen-formalin test.

124.2
Ethanol and Transferrin Glycosylation
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We have recently demonstrated that ethanol abuse leads to alterations of transferrin glycosylation by increasing the level of poorly sialylated, monoglycosylated isoforms. Sera from 67 alcohol abusers entering and out-going a 10 day withdrawal treatment were analyzed by capillary zone electrophoresis using polyanions and polycations double-coated capillaries, and adding sodium dodecylsulfate to eliminate interfering lipoproteins. Separation of the isoforms occurred at pH 8.5. Evolution of the transferrin isoforms was compared with common markers of alcoholism, gamma-glutamyltransferase, transaminases and erythrocyte mean volume. Although all markers levels or activities decreased during abstinence, no statistical correlations (Pearson) were found between enzymatic activities and decrease of monoglycosylated transferrin forms. When entering or out-going the withdrawal program, a positive correlation was found between the monoglycosylated isoforms levels related to alcoholism. An inverse correlation related those forms to the trisialylated one in alcohol abusers. This negative correlation was not found in abstainers, moderate drinkers nor at the outgoing of the withdrawal program. The level of the trisialylated isoform was statistically similar (Wilcoxon) in abstainers, moderate or excessive alcohol consumers, before and after withdrawal. The first conclusion was that glycosylation of transferrin modification occurred independently of the leakage of hepatic enzymes into the blood own to hepatic plasma membrane damage or to modification of erythrocyte membrane fluidity induced by chronic ethanol consumption. Additionally, it may be hypothesized that ethanol did not modify the alteration of the mannopy core to the elaborating transferrin in the rough endoplasmic reticulum. The difficulty to add a third oligosacharidyl antenna to transferrin N-glycans suggests alterations in the expression/activity of N-acetylglucosaminyltransferase III and/or IV.

124.3
The Influence of Sarcolizin on Electrical Resistance of Bilayer Membranes and Free Radical Oxidation
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Background: The development of cancers immediately connects to the destroying of free radical oxidation of lipids of biological systems, which is caused by alterations in membrane structures of cells and reflected by alterations of intensity of chemiluminescence. Aim: The aim of these investigations is to discover possible alterations caused by the action of sarcolizin on lipid peroxidation (LPO) of biological target.

Methods: It were used the method of bilayer lipids and the method of investigation of intensity of chemiluminescence. As biological target were used solutions of total phospholipids of brain of cow for investigations of membrane structures and homogenates of brain of cow for chemiluminescence analysis. Results: During investigations of the influence of sarcolizin on membrane structures it was found out that sarcolizin behaved itself as modicator of bilayer membranes. The effect of sarcolizin was considered in following solutions: 0.1M KCl, 0.1M NaCl and 0.1M CaCl2. It was shown that value of electrical resistance of biological target for monovalent ions was 4.546*1010 ± 0.27*1010 Om (for K+3) and 3.014*1010 ± 0.376*1010 Om (for Na+), and for bivalent ion Ca2+ 5.564*107 ± 3.55*1010 Om. Adding of sarcolizin caused the decreasing of value of electrical resistance till 1,149*108 ± 0.11*108 Om (for K+3). 5.564*107 ± 0.083*107 Om (for Na+) and 1,394*108 ± 0.087*108 Om (for Ca2+). Also it was investigated the influence of sarcolizin on the level of chemiluminescence. This analysis showed the suppressing activity of sarcolizin on free radical oxidation (35% inhibition). These investigations pointed out that in the base of inhibition of LPO by sarcolizin there lie alterations of electrical resistance of biomembranes. These results may be used for more detailed learning of mechanisms of action of sarcolizin during chemotherapy.
124.4

The Alteration of Electrical Conduction of Bilayer Membranes by Action of Some Anti-inflammatory Drug Plants

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In the base of the development of inflammatory processes lies the alteration of antioxidant conditions of biological systems, which leads to the intensification of lipid peroxidation (LPO). With this aim, it was investigated the antioxidant activity of water extracts of some anti-inflammatory drug plants from Armenia. There were used two methods: 1. The method of investigation of the level of LPO by determination of concentration of malondialdehyde (MDA) as one of the last products of LPO. 2. The method of investigation of value of conduction of bilayer membranes. As biological target there were used homogenates (for the first method) and total phospholipids (for the second method) from cow brain.

Results showed that used extracts behaved as stabilizators of membrane structures, which is reflected by the alteration of value of the conduction of bilayer membranes. For example, the extract of Cortex Quercus (ECQ) decreased the conduction of control membranes by 34% in 0,1 M KCl and 30% in 0,1 M CaCl2 and the extract of Hypericum Perforatum (EHP) increased this value on 2 orders and 1 order respectively. These results are thought to connect to antioxidant properties of used drug plants. That is why there were investigated alterations of concentration of MDA of biological target during action by plant extracts. It was found out that ECQ suppressed the intensivity of LPO on 82% and EHP on 88%. So it was shown that antioxidant activities of investigated extracts of drug plants might be provided by work of mono- and bi-valent ions. These results can be useful in evaluation of activities of drug plants in antioxidant therapy.

124.5

Blindness: The Special Schools Serious Need for Genetic Counseling in Iran

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Blindness is a major problem in most developing countries. It occurs at ten times the rate seen in the developed countries and in over 80% of cases is either preventable or curable. The study was carried on 560 blindness students (79.66% male and 29.34% female) in three special schools and their families in Tehran. The results are as follows: (2) About 62% consanguinity among students’ parents in which 75% are belonged to first cousins and 25% to second and farther degrees. (2) More than 6% of parents (3.5% fathers and 2.4% mothers), 26% and 25% to second and farther degrees. (2) More than 6% of parents (3.5% fathers and 2.4% mothers), 26% and 21% of patients’ brothers and sisters are affected, respectively. (3) There were 11.97% cataract, 10.04% retinal degeneration, 9.45% optic atrophy, 9.45% glaucoma and 0.96% albinism. In this, study the blindness symptoms e.g. cataract, 10.04% retinal degeneration, 9.45% optic atrophy, 9.45% glaucoma and 0.96% albinism. The study was carried on 560 blindness students (79.66% male and 29.34% female) in three special schools and their families in Tehran. The results are as follows: (2) About 62% consanguinity among students’ parents in which 75% are belonged to first cousins and 25% to second and farther degrees. (2) More than 6% of parents (3.5% fathers and 2.4% mothers), 26% and 25% to second and farther degrees. (2) More than 6% of parents (3.5% fathers and 2.4% mothers), 26% and 21% of patients’ brothers and sisters are affected, respectively. (3) There were 11.97% cataract, 10.04% retinal degeneration, 9.45% optic atrophy, 9.45% glaucoma and 0.96% albinism. In this, study the blindness symptoms e.g. cataract, 10.04% retinal degeneration, 9.45% optic atrophy, 9.45% glaucoma and 0.96% albinism. In this, study the blindness symptoms e.g. cataract, 10.04% retinal degeneration, 9.45% optic atrophy, 9.45% glaucoma and 0.96% albinism.

124.6

Mapping Genes for Resistance to Gastrointestinal Nematode Infection in Mice

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Gastrointestinal nematodes are mainly controlled by chemotherapy but resistance is emerging. Understanding the natural genetic resistance to infection in mice can enhance decoding of resistance genes in mammals. We used an F2 cross between the resistant SWR and the susceptible CBA infected with Heligmosomoides polygyrus. Resistance traits measured were fecal egg counts, worm burden and granuloma on intestinal mucosa, IgG1 and IgE titers and mucosal mast cell protease 1. Interval mapping analysis after selective genotyping detected 15 QTL some of which have pleiotropic effects on resistance traits.

Reference to http://www.informatics.jax.org, identifies several candidate genes within the QTL that may regulate immunity to H. polygyrus. These include, on MMU1, those regulating Stat4, CD28 and IL1 receptors, RAG1, RAG2 and integrin γ4 on chromosome 2. The QTL on MMU17 encompasses the MHC, supporting earlier work implicating MHC-linked genes, those for mast cell proteases 6 and 7, trefoil factors 1–3 and TNF superfamily member 6 on MMU9. The QTL on MMU8 encompasses genes for cadherin isoforms which together with integrin γ4 regulate T-cell movement in the mucosa. The QTL on MMU13 contain T-cell receptor γ-chain, genes for mast cell production and IL-9. Fine mapping of these QTL is ongoing using the advanced intercross lines to facilitate determination of sequence and expression level polymorphisms of positional candidate genes.

124.7

Cytotoxic and Genotoxic Effects of Action of Porphyrins and Their Metal Complexes on Human Blood Lymphocytes

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The investigation of porphyrin action mechanisms is very important for elaboration of new more effective antitumor drugs, e.g. in photodynamic therapy of tumors. Also, it is known that porphyrins induce different DNA alterations. To evaluate DNA damage by porphyrins and their metal complexes in human leukocytes and DNA repair single-cell electrophoresis (the comet assay) was applied. The whole blood was treated by porphyrins (10–5M, 10–6M and 10–7M) during 0.5–3.5 hours. The effects were investigated either immediately after treatment with porphyrin or after incubation in RPMI 1640 medium. First results indicate that porphyrins (10–5M) induce some increase of DNA damage in non-irradiated cells immediately after treatment when DNA damage was repaired after 30 min or 1 hour of incubation. No significant DNA damage was detected in case of drug-only treatments in all other variants (10–6M, 10–7M). After cell treatment by porphyrins (10–5M) and light irradiation with further incubation (1 h), increase of DNA migration in the comet assay has been revealed.

Comparative estimation of cytotoxic and genotoxic effects of newly designed anticancer substances (photosensitizers and cis-platin) will be investigated to select substances with optimal anticancer properties.
124.8
CpG Methylation in the Promoters of ER α and ER β Frequently Occur at or in Proximity to the Binding Sites of Transcriptional Factors in the Breast Cancer
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Aberrant hypermethylation is known to be responsible for a significant proportion of many types of tumors. The data presented here described that most CpG methylation of ERα and ERβ were found at or near the transcriptional factor binding sites in the breast cancer tissues, and this type of methylation more strongly exerted effects on gene silencing, investigated by the bisulfite genomic sequencing and RT-PCR. Methylation of ERα and ERβ gene was found in 71.1% and 50.0% of 38 breast cancers, respectively. The -375 CpG site in the ERα gene was the most frequent methylation site, while the ERβ gene methylation often at E217. Of note, the frequent methylation sites were located near the CCAAT box (-363 and -375) in the ERα gene, and at or adjacent to binding sites of GATA (-302, -217) and Sp1 (+160, +224, +227) in the ERβ. The tumors with CpG methylation at or near the binding sites of ERα and ERβ gene did not express mRNA, whereas those outside the binding sites showed moderate expression. Our results suggest that DNA methylation in ERα and ERβ appears to be most often present in the CpG site at or near the transcriptional factor binding sites and this type of methylation exerts more significant effect on transcriptional silencing.

124.9
Anti Sense GM-CSF Construct Using Shuttle Vector
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Antisense technology is the method of choice for gene therapy and analysis the autocrine function of protein. Several methods are available for construction of antisense molecules including synthesis of oligonucleotides and vector containing antisense oriented DNA. Some of these vector providing the benefit of constitutive production of antisense molecule in the cell. We have produced a shuttle vector containing a part of Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA oriented in the antisense direction with respect to the promoter. GM-CSF is a multipotent hematopoietic growth factor which is also produced by nonhematopoietic malignant cells. We used pK-CMV as the shuttle vector. We placed the GM-CSF cDNA in antisense direction with respect to the CMV promoter. The CMV promoter is a strong eukaryotic promoter which drives the production of antisense molecules in eukaryotic cells. The antisense direction was confirmed by DNA sequencing using the T3 and T7 promoter of the pBK-CMV vector. The pBK-CMV vector containing the antisense of GM-CSF molecule can be used for transfection of a variety of normal and malignant cell types and study the role of GM-CSF in the function of these cells.

124.10
Suppression of Metastatic Cancer Cell Invasion by Myosin Light Chain Kinase Inhibitor
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The role of myosin phosphorylation by myosin light chain kinase (MLCK) in regulating the invasiveness of metastatic cancer cells was investigated using three Dunning rat prostate adenocarcinoma cell lines with different metastatic abilities, AT-01, AT-02 and MLL, and a MLCK inhibitor, ML-7. Treatment with ML-7 resulted in marked reduction of invasiveness of all three cell lines with similar inhibition profiles. Inhibition of invasiveness was principally due to impaired cellular motility, whereas the ability to survive and proliferate, to adhere to matrix, and to secrete gelatinases were unchanged. Suppression of invasion by ML-7 shows increasing IC50 values in AT-01, AT-02 and MLL cells, respectively, correlating with the increasing metastatic abilities of these cells, and suggesting that the metastatic abilities of the Dunning cells are, at least in part, attributed by the degree of MLCK-catalyzed myosin phosphorylation.

124.11
Effect of Acute Hypoxia on Lipid Alterations of Rats’ Brain, Liver and Myocardium During Autolysis In Vitro
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Lipids content of the tissues of myocardium, liver, and grey and white brain matter was studied when incubated in vitro at 37°C for 10 min, 1 h, 4 h and 24 h. Rats were underwent an acute hypobaric hypoxia in an altitude chamber (1 h 30 min, height 10500 m). In myocardium, under forgoing oxygen deficiency a hydrolytic degradation of phospholipids (PL) and triacylglycerols (TAG) was shown to occur during both early and late terms of autolysis and to be most evident up to 24 h. The cleavage of PL and TAG was accompanied by the increase in the content of free fatty acids and diacylglycerols, likely, as a result of a hyperactivity of phospholipase C and triacylglycerol lipase. In the liver under hypoxia, it was observed transacylase reaction of lipid fractions together with hydrolytic ones in the system of PL-TAG during an early period of autolysis as well as in the system of free and esterified cholesterol (Ch-ECh) during initial and late incubation. In grey and white matter of test rats, hydrolytic lipid degradation intensified in early (10 min) and late (24 h) terms of autolysis in vitro. In grey and white matter preceding hypoxia activated also transacylase reaction of lipid component within late incubation (24 h), being in grey matter in the systems of PLECh, PhosphatidylethanolaminePhosphatidylserine and PhosphatidylcholineSphingomyelin, and in white matter in the systems of PLTAG (24 h), PhosphatidylethanolaminePhosphatidylcholine (10 min, 1 h, 4 h). Thus, hypoxia have late effects on lipids component revealed during autolysis.
124.12 Survival and Natural Killer Cell Activity in Tumor-bearing Mice Treated with Dehydrocrotonin, a Diterpene Lactone Isolated from Croton cajucara

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Croton cajucara is a plant found in the Amazonian region, where local populations as a medicinal plant use it. A clerodane nor-diterpene lactone, trans-dehydrocrotonin (DHC) is an important bioactive compound of Croton cajucara (Saccaca), including hypoglycaemic, antilucre and anti-inflammatory activities. Diterpenes have shown a significant activity against carcinoma cell lines. In order to obtain more information about diterpenes lactones in cancer chemotherapy, in the present study we show the evaluation of in vivo and in vitro antitumoral activity of dehydrocrotonin, previously isolated from the bark of Saccaca, against Ehrlich carcinoma. The effects of DHC on the survival of Ehrlich carcinoma ascitic tumour, on the Natural Killer (NK) cell cytolytic assay and on the proliferation of cultured Ehrlich cells were determined. When the mice were treated with 20 mg/kg of DHC a significant antitumoral activity was obtained (%/T/C = 80%) verified by survival increase. Treatment of the animals with DHC significantly increased NK cell function, restoring normal values of activity at all effector: target cell ratios. The cytotoxicity of DHC against Ehrlich carcinoma was evaluated through the MTT reduction and protein phospha- tase assays in 72h-cell culture. However DHC was not cytotoxic against Ehrlich carcinoma until 1 mM (in vitro). The experimental data in vivo provided a basis for the potential therapeutic application of dehydro- crotonin in cancer therapy. These results analysed together indicate that the effect produced by DHC on NK cells may contribute to the antitumor effect of this compound.

124.13 Central Role of the Proteasome in Senescence and Survival of Human Fibroblasts: Induction of a Senescence-like Phenotype Upon Its Inhibition and Resistance to Stress Upon Its Activation

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Normal human fibroblasts undergo a limited number of divisions in culture and progressively they reach a state of irreversible growth arrest, a process termed as replicative senescence. The proteasome is the major cellular proteolytic machinery, the function of which is impaired during replicative senescence. However the exact causes of its malfunction in these conditions is unknown. Using WI38 fibroblasts as a model for cellular senescence we have observed reduced levels of proteasomal peptidase activities coupled with increased levels of both oxidized and ubiquitinated proteins in senescent cells. We have found the catalytic subunits of the 20S complex and subunits of the 19S regulatory complex to be down-regulated in senescent cells. This is accompanied by a decrease in the level of both 20S and 26S complexes. Inhibition of proteasomes in young cells caused by treatment with specific inhibitors induced a senescence-like phenotype, thus demonstrating the fundamental importance of the proteasome for retaining cellular maintenance and homeo- stasis. Stable overexpression of β1 and β5 subunits in WI38 established cell lines was shown to induce elevated expression levels of β1, subunit in β5 transfec- tants and vice-versa. Transfectants possess increased proteasome activities and most importantly, increased capacity to cope better with various stresses. In summary these data demonstrate the central role of the proteasome during cellular senescence and survival as well as provide insights towards a better understanding of proteasome regulation.

124.14 Inhibiting Virus Replication at the DNA Level Using Group II Introns for Anti-HIV Gene Therapy

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As a new approach to HIV-1 gene therapy, we try to target HIV-1 provirus DNA with a modified group II intron. The advantage of this strategy over others, which target the HIV-1 RNA/proteins, is that it may confer a complete and permanent inhibition of HIV-1 replication in an infected cell. Group II introns targeting three sites, one at LTR (nucleotide 54, at anti-sense strand: 54α), and two at the pol region (nucleotides 4021 and 4069, at sense strand: 4021S and 4069S) in the HIV-1 provirus DNA have been modified by cloning a designed cassette into the domain V of the introns. The cassette, which contains a neo gene, a multiple cloning site, and a terminator, is cloned in antisense orientation into the pPACD-54α (a vector expressing the intron that targets the 54α site). The cassette without the terminator was cloned in sense orientation into pPACD-4021S and pPACD- 4069S (vectors expressing the introns that target the 4021S and 4069S sites). To test the mobility of the introns, E. coli cells are co-transformed with both modified introns and recipient vectors containing fragments of HIV-1 DNA. It was shown that the introns could splice and insert them- selves into the target sites. Next, E. coli cells are co-transformed by modified introns and pNL4–3 (a vector containing the whole HIV-1 ge- nome) to test the insertion of modified intron to the real sequence of HIV-1 genome. The experiments that confirm the intron insertion are under way. Then, the intron-inserted pNL4–3 will be used for producing HIV-1 viruses. If the HIV-1 DNA is inactivated, studies will be performed to determine the effectiveness and frequency of insertional inactivation in mammalian cells.

124.15 Age-dependent Changes of Glucose Metabolism in Rat Liver During Immobilization Stress

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Senescence is accompanied by increase of tissues sensitivity to stress injury. In order to learn the reasons for the age-dependent sensitivity of the liver to the damaging action of stress, the status of glucose metabolism in the liver of adult and old rats during immobilized stress has been studied. Fifty Wistar male rats 10 D12-month- old (adult) and 22 D 26-month D old (old) were used. Each group was divided into two subgroups: intact ones and those, affected by an immobilization stress by fixing them in dorsal position for 30 minutes. The effectiveness of stress was controlled patho- morphologically and by measuring level of glucocorticoid hormones and epinephrine in the blood. Obtained data indicate that immobilization of both rat groups was accompanied by the stimulation of glycolysis velocity in liver. In adult rats this change was accompanied by a decrease in oxidative deacboxylation of pyruvate. Old rats have not shown analogous change in pyruvate metabolism during the stress. In other hand, immobi- lization of old rat was accompanied by an increase in glucose utilization in pentose phosphate pathway. Based on present data, we propose that old rats have more value of liver metabolic adaptation to stress injury than adult ones. The importance of changing in glucose catabolic pathways in maintaining of liver resistance to stress injury in senescence has been discussed.
Preparation of Human Preproinsulin cDNA from Human WBC Genome
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Regarding therapeutic issues related to insulin and diabetics essential needs to this product, producing insulin is regarded as one of the most basic requirements. Besides, we are interested to gain cDNA from a more suitable and available way that it may be to gain some RNA and cDNA from another available cells. Therefore, in this research the human preproinsulin cDNA was produced in a new way. In this study, the human genome was totally extracted and after designing specific primers for the human preproinsulin gene, Polymerized Chain Reaction was done. In the next stage, the resultant gene was cloned in a bacterial vector namely pBluescript II KS (\textregistered/H11001) and therefore pFP3bluins was produced. Then, the gene was extracted from the plasmid and was taken into a potent eukaryotic expression vector, pcDNA3, and pFP3ins was yielded. These eukaryotic plasmids were gotten to transfect cultured eukaryotic cells. In addition to being eukaryotic, these plasmids have CMV (cytomegalovirus) promoter that gives them a high transcriptional potency. After then, RT-PCR was done and the human preproinsulin cDNA was produced. The cDNA was cloned into prokaryotic plasmid, pBluescript II KS (\textregistered); and in this way some plasmids carrying the cDNA were obtained such as pcBFP3ins. The direction and sequence of the cDNA in this plasmid were identified which were correct ones.

Comparison of Western Blotting Method with Virus Neutralization Test for Measuring Herpers Simlex Virus Type 1 (HSV-1) Antibodies
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Herpes simplex virus type 1(HSV-1) was adapted to a continuous cell line of BK. One thousand TCID50 of the virus combined with Freund’s complete adjuvant was injected to each of 2 healthy and susceptible rabbits. The same amount of the virus was injected to the rabbits at 21day intervals with freud’s incomplete and complete adjuvants, respectively. Using the HSV-1 antiserum prepared as mentioned above virus neutralization test (VNT) and Western blotting (WB) method were applied. In order to compare the titer of the antibody against the virus in both tests serial two fold dilutions of the antiserum were tested against the virus. Western blotting was performed under denaturing conditions using whole virus particles as the antigen and 100 TCID50 of the same virus was used in the VNT test. The experiment was repeated with 61 human serum samples whose neutralizing antibodies ranged from 1/32 to 1/128. Western blotting done on these sera showed that serum dilutions of 1/1000, 1/2000, and 1/4000 with positive results in WB corresponded with 1/32, 1/64, and 1/128 in VNT.

Alpha-1-Antitrypsin Phenotypes Presenting as Chronic Liver Disease in Children in Northern India
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Objective: To determine the alpha-1-antitrypsin (AAT) phenotypes among children with chronic liver disease (CLD). Design: Clinic based cross sectional survey. Setting: A tertiary care referral and teaching institution in North India. Methodology: Children with suspected chronic liver disease were recruited from April 1991 to November 2002 for workup. Phenotypic characterization by isoelectric focusing (IEF) was done for AAT allele characterization. Results: A total of 1635 children (<3months: n-319; 3mo-15 yr: n-1316) were investigated for CLD. 483 (29.5%) cases with incomplete workup or absent liver disease were excluded from the analysis. 261/1152 (22.6%) had metabolic liver disease (MLD). Alpha-1-antitrypsin was suspected in 6.9% (80/1152). Suspicion was based on absent alpha-1 globulin band on agarose electrophoresis and/ or reduced serum AAT levels and/ or PAS positive diastase resistant granules on liver biopsy. IEF identified AAT deficient phenotypes in 36 out of 80 (45%) subjects. Protease inhibitor (PI) Z allele was the most common mutation. The frequency of AAT alleles was PIZZ: 15 (41.6%); PIMZ: 9 (25%); PISZ: 7 (19.4%); other alleles: 5 (13.9%). Conclusions: AAT deficient alleles are prevalent in North Indian population and associated with childhood CLD. The finding has implication in adult idiopathic liver disease and chronic obstructive lung disease. Further mutational and phenotypic analysis in larger population group will determine AAT allele frequency in Indian population.
124.19
Oxidative Stress-induced Alterations of Gene Expression in Human Skin Fibroblasts Harboring A8344G Mitochondrial DNA Mutation

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The effect on gene expression of the well-known pathogenic A8344G mutation in a MERRF (myoclonic epilepsy with ragged-red fibers) patient and her family members was investigated by Agilent’s human 1 cDNA microarray and RT-PCR technologies. The activation of transcriptional responses, such as the induction of inflammation-related proteins, matrix metalloproteases, and markers of oxidative stress were observed in the patient’s fibroblasts. By contrast, the gene expression of enzymes involved in energy metabolism, cytoskeleton-related proteins, extracellular matrix proteins (e.g., MMPs), and translation-related proteins were all down-regulated. We also found that the mRNA and activity levels of Mn-SOD but not catalase and glutathione peroxidase were significantly increased in skin fibroblasts of the MERRF patient. The intracellular level of hydrogen peroxide was increased in skin fibroblasts of the MERRF patient as compared to those of her family members and age-matched controls. As a result, ROS may not be efficiently removed and in turn elicit an elevation of oxidative stress in skin fibroblasts of the MERRF patient. These findings suggest that the alterations in gene expression were the results of cell response to the increased oxidative stress. On the other hand, we observed dramatic change in mitochondrial network structure and distribution in the fibroblasts of the patient. Taken together, the changes in gene expression imply that inflammation, disruption of mitochondrial reticulum together with degradation of muscle proteins and oxidative damage to affected tissues are all involved in the pathophysiology of MERRF syndrome.

124.20
Inhibition of Androgen Action in Prostate Cancer Cell Lines by the Dual 5α-Reductase Inhibitor, Dutasteride

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5α-Reductase (5αR) catalysis of the reduction of testosterone (T) to dihydrotestosterone (DHT) enhances androgen action in tissues such as the prostate. We have shown that the novel 4-azasteroid 5αR inhibitor dutasteride inhibits T action in LNCaP prostate cancer cells, consistent with 5αR inhibition. However, dutasteride also inhibits DHT-induced accumulation of PSA (prostate specific antigen) with an IC50 of 0.5–1 μM after incubation of LNCaP cells with 0.1 nM DHT for 18 hr or 6 days. This cannot be due to 5αR inhibition and may reflect dutasteride binding of the androgen receptor (AR) (IC50 ~ 1.5 μM). This concentration of drug has no effect on cell viability, although higher concentrations (≥ 10 μM) do so, accompanied by a decrease in AR protein levels and enhanced apoptosis, as shown by Annexin V staining and release of histone-associated-DNA fragments. Both LNCaP cells, which contain mutant AR (T877A) and LAPC-4 cells, which contain wild-type AR, show decreased viability and proliferation after 48–72 hr incubation with 10 μM dutasteride in the absence of DHT. PC-3 cells, which lack AR, are only affected by the drug at 50 μM. Thus, prostate cells containing mutant or wild-type AR are more sensitive to the cytotoxic effects of dutasteride than cells lacking AR. In LNCaP cells, the dose of dutasteride required for its antiandrogenic effect on DHT-induced PSA expression is about 10-fold less than that required for inhibition of viability or proliferation, suggesting that different mechanisms are involved.

124.21
Molecular Characterization of Hemagglutinin Gene of Influenza A/H1N1 Virus in the 2001–2002 Season

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Influenza viruses are negative stranded RNA viruses, which exhibit two surface antigens hemagglutinin (HA) and neuraminidase (NA). The ability of influenza virus to alter its antigenic properties by genetic mutations accounts for its ability to evade pre-existing antiviral immunity, thereby leading to re-infection. Among the three types of influenza viruses (A, B, and C), influenza A is the one most often associated with epidemics. It is also well established that the glycoprotein HA is the critical viral antigen for immune defense against influenza viruses, particularly the N-terminal globular HA1 region. Determining the amino acid sequence of the HA1 gene by DNA sequencing offers an efficient means for studying viral antigenic drift and antigenic shift. Nasal specimens collected from subjects with respiratory infections enrolled in a multi-nation, multi-continent, influenza vaccine clinical trial during the Y2001–2002 influenza season were cultured once in MDCK cells to obtain influenza isolates. Viral RNA was isolated from approximately 100 influenza A/H1N1 isolates using a robotic nucleic acid workstation. The typing and subtyping of these viral isolates were determined by RT-PCR, and the HA1 gene sequences were determined by DNA sequencing. Using A/New Caledonia/20/99, the recommended vaccine strain for Y2001–2002, as the reference, HA1 amino acid variations of these clinical isolates were investigated. Phylogenetic analysis of the nucleic acid sequences of these isolates reveals that they fall into six distinct groups each with signature amino acid residues, suggesting their common origin. The structural and antigenic implications of our findings will be also be presented and discussed.
124.22
p53 Polymorphisms and Haplotypes in Jordanian Ethnic Groups, Breast Cancer and Lung Adenocarcinoma Patients

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Three biallelic polymorphisms in the p53 gene (a 16-bp duplication in intron 3, BstUI RFLP in exon 4 and MspI RFLP in intron 6) and haplotype combinations were studied in Jordanian general population, two ethnic groups; Bedwing and Charkas and in two groups of cancer patients; breast cancer and lung adenocarcinoma groups. Differences in allele frequencies for all three polymorphisms were observed among the various Jordanian population and cancer patient groups. The absence of the 16-bp duplication was common among the studied groups, being highest in Charkas (0.886). The MspI RFLP allele frequency in Bedwing group (0.300) was significantly higher in comparison with other groups. In the two studied cancer groups, the extended haplotype that includes the absence of the 16-bp duplication combined with the BstUI Pro and absence of MspI restriction site were the most frequent. The Jordanian general population and both ethnic groups differed significantly from each other with respect to their haplotype distributions, thus p53 alleles and haplotypes could be used as anthropological markers.

124.23
Development of a High-throughput Molecular Assay for Assessing Cell-mediated Immunity

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Development of quantifiable biomarkers using rapid and reliable molecular methods is needed for screening large numbers of clinical trial specimens. Here, we developed a high-throughput cytokine mRNA quantitation method combining automated RNA isolation and real-time PCR assays. We also compared our method for measuring intracellular mRNA with ELISPOT assays for secreted protein as a means of identifying surrogate markers of immunity. Interferon-gamma (IFN-γ), a putative marker cytokine for cell-mediated immune response, presumably plays a key role in resolving viral infections. Our initial objective of this study was to test and validate the methodology and to evaluate the potential of IFN-γ and its induced gene product(s) as surrogate markers for assessing the effectiveness of influenza vaccines. Peripheral blood mononuclear cells (PBMC) were isolated from a group of elderly clinical trial subjects immunized either with trivalent inactivated influenza vaccine (TIV) or an experimental trivalent live cold-adapted influenza vaccine (CAIV-T). PBMC were stimulated in vitro with each of the three monovalent vaccine components. Phytohemagglutinin (PHA) treatment was included as a positive control for monitoring mitogenic response and the inducibility of IFN-γ gene. Agilent RNA chips assessed RNA qualities. ELISPOT assays for IFN-γ secretions were performed in parallel by an independent laboratory. Our results show that most PBMC tested responded to PHA stimulation, albeit to varying degrees. A subset of PBMC tested also responded to antigen stimulation by upregulating the IFN-γ gene. In addition, the prevalence of antigen responsiveness in elderly subjects, as well as the correlation between the number of IFN-γ Elispots and the quantity of IFN-γ mRNA normalized against control PGK mRNA will also be presented and discussed.

124.24
Construction of a hFab Phage Display Library from Patients with Osteosarcoma

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Osteosarcoma is the most common primary malignant bone tumour, frequently occurring in adolescent at sites of rapid bone growth. Despite current management protocols, up to half of patients with osteosarcoma succumb to the disease. Until the present moment there is no well characterized molecular marker for diagnosis and prognosis of this illness. In order to select antibodies that recognize osteosarcoma antigens we constructed a recombinant hFab (human antigen binding fragment) library and selected it against osteosarcoma cell line surfaces, using the recently described methodology BRASIL Total RNA extracted from peripheral blood lymphocytes of eleven patients with osteosarcoma were pooled together. RT-PCR was used to amplify the heavy chain and light chain and the amplification products were inserted successively into the vector pComb3X to construct the hFab library. The library size was 1.7 x 108 different forms. Aiming the depletion of the ligands to the normal cell receptors we had pre-panned the library with the human fetal osteoblast cell hFab and then selected the specific Fab in the surface of three osteosarcoma lineages: U2-OS, MG 63 and Saos-2 in different combinations. After three or four rounds of selection we obtained an enrichment of 104 times and considered to obtain the specifics Fab. That is the first description of a phage display library from osteosarcoma patients and also the first time that an antibody fragment library is selected using BRASIL methodology and the method proved to be efficient in obtaining specific osteosarcoma antibodies.

124.25
The Apolipoprotein E e4 Allele Is No Risk Factor for Turkish Breast Cancer Patients

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Apolipoprotein E (ApoE) was shown to have a central regulatory role in cholesterol metabolism and recently it was also reported that as it is a potential inhibitor of cell proliferation it has a considerable role in antioxidant activity and thus tumor growth and proliferation. ApoE has three common isoforms E2, E3, E4 coded by three variant alleles e2, e3, and e4 and the presence of different isoforms is reported to affect tumor growth and proliferation in different level. Our study was designed to determine whether Apo E polymorphism is associated to breast cancer and tumor cell proliferation. ApoE polymorphism has been studied among 32 breast cancer patients and 20 control patients and genotyping was carried out by the Apo E genotyping Kit commercially available from Roche Diagnostics on the Light Cycler. We ended with 6.3% E23, 90.6% E33 and 1.9% E34 in breast patient group and 100% E33 in control group. This result shows that the allelic frequency for e2 is 3.1% for e3 95.3% and for e4 1.6% in breast patient group and 100% E33 in control group. That is the first description of a phage display library from osteosarcoma patients and also the first time that an antibody fragment library is selected using BRASIL methodology and the method proved to be efficient in obtaining specific osteosarcoma antibodies.
124.26
Ras and p53 Gene Mutations in Iranian Bladder Cancer Patients
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Bladder cancer is one of the leading causative deaths in most developed countries. Studies on Ras family activation in bladder cancer showed a significantly change in the expression of these genes. In this work DNAs from 30 bladder cancer fresh specimens were examined for presence of H-Ras and N-Ras activation using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Detection of mutations of p53 gene exons 4, 5–6 on the same specimens was also considered in this study, using a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). Less than 10% of patients were detected to have RAS mutations. Nearly 50% of patients had possible mutations in exon 4, and a few showed mutations in exon 5–6. These results can be used for further molecular oncology studies for identifying those causative factors responsible for the development of bladder cancer around the world.

124.27
Mutant UvrB Recognition and Incision Characteristic of Polypeptide-DNA Cross-link
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Purpose: Nucleotide excision repair (NER) plays a crucial role in maintaining DNA integrity and fidelity. There are three main proteins: UvrA, UvrB, and UvrC involved in recognition and incision in E. Coli NER process. The wild type UvrB protein has litter recognition ability, there are no any reports concerning mutant UvrB protein recognition and incision efficiency of polypeptide-DNA cross-link substrate in vitro. Experiments: UvrB cDNA was sub-cloned into pTYB2 vector of IMPACT system. The T7 promoter worked as an initiator in the protein expression. Using site-direct mutagenesis method three mutants UvrB were produced. Mutant UvrB were then over-expressed in BL21 competent cell. The chitin beads affinity chromatography were used to purify the mutant UvrB proteins by one step purification method. UvrA and UvrC were also sub-cloned into IMPACT system and purified using one-step chitin beads purification procedure. The three mutant UvrB proteins, Y92, Y95, and Y101 recognition and incision ability of damaged polypeptide-DNA cross-link substrates were investigated by reconstitute the reaction in vitro. And the compared wild type with these mutants UvrB binding affinity and incision ability were also screened in vitro condition. Polypeptide-DNA cross-link substrates were prepared according to the previous report procedure. The binding and incision of DNA-protein cross-link data were obtained by using Fuji FLA-5000 imaging system to integrate percentage of wild type and mutant UvrB binding and incision with the substrates. Results: The mutant UvrB can significantly change binding and incision efficiency to DNA-protein cross-link substrates. The gel mobility shelf assay results show the binding

124.28
Proteome Analysis of Regulatory T Cells—Promising Targets for Innovative Immunotherapeutical Approaches
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Several mechanisms control the differentiation between self and non-self, including the thymic deletion of auto-reactive T cells and the induction of anergy in the periphery. In addition to these passive mechanisms, evidence has accumulated for the active suppression of auto-reactivity by a population of regulatory T cells that co-express CD4 and CD25. Regulatory T cells play a central role in the maintenance of the immunological balance and are powerful inhibitors of T cell activation both in vivo and in vitro. The enhancement of suppressor-cell function might be a target for immunotherapeutical approaches for the treatment of immune-mediated diseases like multiple sclerosis and Crohn’s disease. By contrast, the elimination of regulatory T cells or the inhibition of their functions might prove to be beneficial for the induction of tumour immunity or the treatment of chronic infectious diseases. To elucidate the still unclear effectors functions of regulatory T cells we performed differential proteome analyses with diverse human and murine T cell populations. The whole protein extracts of conventional and regulatory T cells were separated by high resolution two dimensional gel electrophoresis according to Klose. The proteomes of stimulated and resting CD4+ and CD4+CD25+ T cells were compared. The identification of candidate proteins which are of functional relevance for both human and murine regulatory T cells contributes to the understanding of the causes and mechanisms involved in autoimmune diseases, allergy and cancer and will lead to the development of new drugs to manipulate the activity of regulatory T cells.

124.29
Detection of bcr/abl Fusion in CML Patients by Interphase Fluorescence In Situ Hybridization
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Rapid and accurate detection of chromosomal abnormality in Leukemia plays an important role in its prognosis. Chronic Myelogenous Leukemia (CML) Patients, observed with abnormal chromosome in WBC nucleus called as Philadelphia chromosome (ph), are proclaimed to have disorder as a result of proto-oncogen fusion of bcr gene on chromosome 22 with abl gene on chromosome 9. The present study was carried out to optimize the technique of Dual-Colour Fluorescence In Situ Hybridization (FISH) for detection of bcr/abl transfusion in CML patients. The study was primarily started by uni-colour FISH using centromeric probes specific for chromosomes X and Y, followed by Dual colour FISH using the same probes and then carried by application of commercially available probe m-bcr/abl translocation on metaphase spreads prepared from normal individuals. Following to the localization of the indirectly labelled probes on the expected areas, the technique was used to study the fusion of bcr and abl proto-ontocogens in 16 CML patients. In 12 specimens a yellow signal specific for bcr/abl fusion gene and, a green and a red signal specific for proto-oncogenes in 16 CML patients. In 12 specimens a yellow signal specific for bcr/abl fusion gene and, a green and a red signal specific for bcr and abl genes respectively, were observed on each cell. The detection efficiency of the probe was 91 percent. Four cells failed to show any signal. These cells were from the patients who underwent radiotherapy and treatment with IFN-alpha. Our study indicates that Dual colour interphase Fluorescence In Situ Hybridization is a rapid and sensitive method for detection of Philadelphia chromosome (Ph) in CML patients.
124.30
Cytokine Responses of Bone and Cartilage Cells
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Rheumatoid arthritis and osteoarthritis are associated with changes in apoptosis and cell proliferation and the production of cytokines in inflamed joints. Cytokines have been shown to alter the sugar chains of glycoproteins and proteoglycans. On the cell surface, these glycan play a critical role in cell surface functions. In this study, bone and cartilage cells, i.e. human and bovine chondrocytes, osteosarcoma cells and prostate cancer PC-3 cells from bone metastases were grown in culture and treated with the inflammatory cytokines, TNFalpha that induced apoptosis, and TGF-beta that induced cell proliferation in all cell types. Lectin binding assays showed characteristic glycosylation patterns in each cell type. The changes in lectin binding patterns could be partially explained by changes in glycosyltransferase activities, and were cell type and species specific. This is the first study establishing the biosynthetic pathways of O- and N-linked sugar chains of glycoproteins in bone and cartilage cells, and the effect of cytokines on glycosylation synthesis. All cell types were capable of synthesizing complex N-glycans and simple O-glycans. PC-3 and osteosarcoma cells, as well as human but not bovine chondrocytes were capable of synthesizing complex O-glycans. This study shows that the inflammatory environment in the arthritic joint can significantly alter cell surface glycosylation, and that these changes are related to altered apoptosis and cell proliferation. Supported by the Arthritis Society and Materials and Manufacturing Ontario.

124.32
Phosphorylation of the Potyvirus Capsid Protein by Protein Kinase CK2 Is Essential for Viral Infection
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We have previously reported that the capsid protein (CP) of potato virus A (PVA; genus Potyvirus) is phosphorylated both in virus-infected plants and in vitro (Ivanov et al. 2001, J. Biol. Chem. 276, 13530–13540). In this report, a protein kinase that specifically phosphorylates PVA CP was identified as casein kinase 2 (CK2). DRB, a specific cell-permeable inhibitor of CK2, was shown to inhibit phosphorylation of PVA CP in infected tobacco plants. The α-catalytic subunit of CK2 (CK2α) was purified from tobacco and characterized using in-gel kinase assays and LC-MS/MS sequence tag analysis. Tobacco CK2α was cloned, expressed and purified from E. coli, and specific antibodies were raised against the recombinant enzyme. Immunofluorescent microscopy revealed that PVA CP co-localized with CK2α in infected protoplasts. The major site of CK2 phosphorylation was mapped to a threonine residue at position 242 of PVA CP within a triple CK2 consensus sequence. A full-length infectious cDNA clone of PVA tagged with the green fluorescent protein (GFP) reporter gene was constructed to study the phenotypic effects of point mutations affecting the CK2 consensus sequence in PVA CP. Analysis of these mutants in tobacco plants showed that they were either restricted to individual cells or greatly delayed in systemic movement. Using in vitro assays, we showed that CK2 phosphorylation inhibited binding of PVA CP to RNA suggesting a molecular mechanism of CK2 action. These results indicate that phosphorylation of PVA CP by CK2 may have an important regulatory role in viral infection.

124.31
Biochemical Characterisation of Pseudomonas Species from Semi Arid Region Uzbekistan
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Biochemical characterization of microorganisms is important in order to understand the microbial ecology in soil and other ecosystems. The purpose of this study was to characterize biochemical and physiological activity of Pseudomonas species isolated from different agricultural crops grown in semi arid climate. Pseudomonas species were isolated from the soil under cotton, wheat, alfalfa, melon, tomato and maize grown at the field location in Surchandarya region Uzbekistan. Isolated strains identified using fundamental selection methods, the standard battery of biochemical and physiological characterization tests. It was found that the most commonly genera were Pseudomonas, P. mendocina, and P. stutzeri encompassing 60% of all Pseudomonas strains. The strains were divided into groups on the basis of their carbohydrate fermentation patterns. Group A included P. alcaligenes PsA15, P. fluorescens PsF54, P. mendocina PsM38, P. stutzeri PsS25 that produced acid from saccharose and did not produce acid from mannitol. Group B included P. mendocina PsM13, P. rathonis PsR20, P. stutzeri PsS23 strains did not produce acid from saccharose and produce acid from mannitol. Strains P. alcaligenes PsA15, P. rathonis PsR20 hydrolyzed Tween 20, Tween 60, and lecithin. P. alcaligenes PsA15 produced 3.2 μg IAA 100 ml–1 filtrate, isolate P. alcaligenes PsD6 produced 3.0 μg IAA 100 ml–1 filtrate but IAA was not detected in filtrates of isolates P. mendocina PsM13, P. rathonis PsR20. P. denitrificans PsD6 produced vitamins- thiamine, pantothenic acid, biotin and P. stutzeri PsS23 produced only nicotinic acid. Most of isolated strains produced amino acids - asparagine, histidine, glutamine and enzymes lipase, nitratreduc-tase, amylase, and arginine dihydrolase. All of the strains oligotrophil, and they have ability to survive N-deficient soils. Most of Pseudomonas species were salt tolerant and heat resistance. These abilities contributed the isolated bacteria to survive in the environmentally stressed conditions.

124.33
Anoxia Causes Accumulation, Transcriptional Activation and Cross-talk Between p53 and HIF-1
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HIF-α and p53 are transcription factors known to accumulate under conditions of low oxygen tension. Under normoxic conditions, both proteins are ubiquitinated via specific E3 ligases (pVHL and Mdm-2, respectively) and subsequently are degraded via the 26S-proteasome pathway. Recent data suggest that there might be direct interactions between both proteins, with the consequence that binding of p53 to HIF-1α causes pVHL-independent degradation of HIF-1α. Here we show in colon carcinoma cells (RKO) accumulation of HIF-1α and p53 in anoxia (0% O2). HIF-1α protein reached maximal levels at 8h and slightly decreased thereafter. The transcriptional activity of HIF-1 increased time-dependently to a maximum level at 24h, p53 accumulation started at 16h and its level increased until 24h, at the same time being transcriptionally active. Transient overexpression of p53 resulted in an accelerated disappearance of HIF-1α protein at 16 and 24h, but did not influence the initial accumulation up to 8h. Enforced expression of p53 resulted in transcriptionally active p53 and significantly blocked the transcriptional activity of HIF-1 under both normoxic and anoxic conditions at all time points.

Thus, our results indicate that low level p53 expression completely attenuates HIF-1α transactivation. This might be due to competition for co-factors such as p300, because p53 itself is fully active at low expression rates. On the other hand, p53 at higher levels causes HIF-1α degradation which possibly results from a direct interaction of p53 and HIF-1α and subsequent Mdm2-dependent HIF-1α degradation.
phy1 we purified 3 proteins with molecular masses of 60, 65 and 67 kDa by affinity elution chromatography. These proteins were analysed and characterized by proteomic analytical methods.

Results: Using the above approaches, three proteins from the LTR binding complex were purified. One of the constituent proteins was identified as a 70 kDa heat shock protein (HSP70).


125.3 QSAR Models of the 3-Aminoflavonoid Group: Linear Regression vs. Neurofuzzy Logic

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Quantum chemical approach has been usually used in QSAR modelling with multiple linear regression as a frame work. In QSAR study for a series of cytotoxic 3-aminoflavonoids, quantum chemically calculated data were obtained by SCF calculations, using PM3 semi-empirical method in HyperChem 6.0, as the input, the neurofuzzy logic technique integrated in INForm 3.0 intelligent software was applied in comparison with the multiple linear regression method provided by BMDP new system 2.0. The application of artificial intelligence in QSAR research has been reviewed. Neurofuzzy logic technique - combining the learning power of neural networks with the flexibility of fuzzy logic- has been introduced to be powerful for discovering the subtle and non-linear relationships in the experimental data. The aims of this work were to apply both multiple linear regression approach and the neurofuzzy logic technique in QSAR study for the 3-aminoflavonoids and to compare the prediction power between the linear and non-linear models. The linear QSARs were poor in prediction. The non-linear QSARs was versatile and the non-linear models were more predictive than the linear ones. The neurofuzzy logic technique seems to be a powerful tool in QSAR modelling. QS1 position in 3-aminoflavonoids related anticancer activity. Five newly 3-aminoflavonoid molecules were designed with higher activity and lower toxicity than lead compound.

125.4 Fab End of a Fascinating Story of the Development of Novel Anti-malarials

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Malarial parasite, Plasmodium falciparum which causes the most severe form of malaria was shown earlier to lack the ability to synthesize fatty acids de novo. Contrary to these beliefs our studies showed that P. falciparum makes fatty acids de novo. Fatty acid synthesis (FAS) occurs maximally during the trophozoite stage of parasite development.

Triclosan, an inhibitor of type II FAS, potently compromised the growth of both the drug resistant and sensitive parasite strains suggesting that it has type II FAS which was proven further when enoyl-ACP reductase (FabI) from the parasite (PfFabI) was purified. PfFabI reduced enoyl-CoA and enoyl-ACP and was inhibited by triclosan. Bacterially expressed enzyme has been characterized further to elucidate the mechanism of its inhibition by triclosan. Mechanistically triclosan acts as a slow-tight binding inhibitor for PfFabI. Also, triclosan was able to completely cure mice of malaria. Since the FAS machinery of parasite is very distinct from that of its host. It opens avenues for designing of novel anti-malarialals without harmful effects on the host. As triclosan is used widely in human consumer products it should be possible to introduce it for treating malaria. Recently we have also expressed FabZ and FabG enzymes of the parasite. The unique structural features of the parasite enzymes allow us to develop novel inhibitors of this pathway as anti-malarial agents.

125.5
Histone H1 as a Target for Anthracycline Antibiotics
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Anthracycline antibiotics such as daunomycin and Adriamycin are potent antitumour agents widely used in cancer chemotherapy. The binding of these drugs to DNA has been studied in detail, however, in the cell nucleus, DNA is associated with histones in a complex that is known as nucleosomes. Thus chromatin and not DNA is the major target for these drugs. We have studied the effects of anthracycline antibiotics on histone H1 using equilibrium dialysis and UV/Vis spectroscopy at different ionic strength and pHs. The results show that the interaction of H1 with daunomycin reduces the absorbance at 210 and 480 nm and produces hypochromicity in the difference spectra. Binding isotherms showed that the binding process is positive cooperative with two binding sites. The binding affinity is reduced as the ionic strength was increased and the optimum pH was 7. Also the interaction of different parts of histone H1 obtained from trypsin digestion with daunomycin revealed that the intact protein is necessary for drug binding. It is suggested that in chromatin not only DNA but also histone H1 should be considered as a main target for anthracyclines action.

125.6
Gene Expression Profile of SOCS2 Knockout: Defining the Set of Genes Involved in Somatic Growth Using Microarray Assay
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Suppressors of cytokine signalling (SOCS) proteins are a family of proteins involved in the negative regulation of cytokine action through inhibition of the Jak/Stat pathway. Mice that are unable to express SOCS2 (SOCS2-/-) grow significantly larger that their littermates with some characteristics as the increased lengths of long bones, the enlargement of some organs, increase in the local IGF-1 production and collagen accumulation in the dermis. These characteristics are also observed in people with over secretion of Growth Hormone (GH), indicating that SOCS2 has an essential role in the negative regulation of the GH/IGF-1 axis. Using microarray assay we have found 61 differentially regulated genes in the absence of SOCS2 in liver (45% up regulation; 55% down regulation). Interestingly, many of the well-known GH-dependent genes, such IGF-1, were not up regulated by the absence of SOCS2. Quantitative Real-Time PCR was used to verify some of the results found in the microarray. Genes as Lpl, IGFBP-3 and SCAD were confirmed to be up regulated, while PEPCK, CYP8B1 and KID-1 were confirmed as down regulated genes in SOCS2-/-.

Very interesting, we found that 31% of the total of regulated genes are regulated by sex and even more, 74% of them indicate a partial feminization in SOCS2-/-.

Taken together these results and other studies made in our lab we could suggest the existence of GH down regulated genes that can influence the sensitivity to GH. Further characterization of these genes is needed in order to define their role in the regulation of the somatic growth.

125.7
Trace Elements Distribution in a Shrimp Species from Persian Gulf and Possible Role of Metallothionein in Their Redistribution During Refrigerated Storage
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The study was conducted between May and December 2002. The main objectives of this study were assessment of distribution of three trace elements (Cd, Cu and Zn) in tissues (exoskeleton, abdominal muscle and hepatopancreas) of a shrimp species (Penaeus merguiensis) as well as evaluation of the possible role of metallothionein in redistribution of the elements during refrigerated storage. The specimens were sampled from northeastern (near the Hormuz Strait) part of the Persian Gulf. The concentrations of metals in the tissues were measured using Inductively Coupled Plasma-Atomic Emission Spectrophotometer (ICP-AES) at the University of Bradford. Metallothionein levels were determined by Differential Pulse Polarography (DPP) method. pH of the muscle samples was also measured using a puncture electrode in different stages. Only in the case of Zn significant differences between sexes could be detected. Concentrations of the metals in the muscle samples were considerably below the most guidelines for human consumption. The results were in general agreement with those obtained by some other researchers in other world areas. The highest Cu and Zn concentrations were found in hepatopancreas samples, but the highest level of Cd was observed in exoskeleton. There were significant differences between the fresh and refrigerated samples from accumulation of Zn in tissues point of view, which can be attributed to the metal binding properties of metallothioneins as well as their degradation during the storage. The mean pH values measured in muscles of the specimens in different conditions (fresh, one day- and three days-refrigerated samples) indicated an ascending order (7.12, 7.14 and 7.37, respectively) and since thiol groups are more stable toward oxidation at neutral than at alkaline pH, it can be considered as a reason for possible transportation of the metals from hepatopancreas to other parts of the body. This project was funded by Iran Fisheries Research Institute (IFRI).
125.8
DNA Methylation and Nuclear Envelope During Cereals Germination
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Plants have a complex battery of genes encoding putative DNA methyltransferases, only three of which have been shown to have methyltransferase activity. Given the detrimental effects of genome-wide demethylation, one might predict that plants would have evolved mechanisms to avoid global demethylation in response to a mutation in a key component of the methylation machinery. Perhaps chromomethylases play a role not only in the methylation of the ribosomal and centromeric repeats of DNA, but in the methylation of heterochromatin. The changes in some physicochemical characteristics of cereals seeds DNA and chromatin during germination and activation under influence of gibberellic acid have been obtained. We investigated such DNA characteristic as differential melting curves (DMC), the level of DNA methylation and the number of repeating sequence. We have suggested that the high methylated region of repeated DNA commonly lies in heterochromatin region adjacent to the NE. From other side during germination of the cereal seeds we have obtained the changes in electrophoretic properties of the intact nucleus surface. From above mentioned results we suggested correlation between regulator role of DNA methylation in construction of chromatin in region bordering to the inner nuclear membrane. The filamentous nucleosomal proteins are attached to the nuclear pore complexes nuclear face and extend into the nuclear interior, and have been implicated in anchoring telomers to the nuclear envelopes. Further analyses should resolve this suggestion and reveal relationship between chromatin organization, DNA methylation and regulation of permeability of the nuclear envelope.

125.9
The State of the Antioxidant System in the Liver of Rats Under Experimental Hypertension Conditions
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The oxidative stress which lead to depletion of NO content may caused the hypertension. The increasing of reactive oxygen species formation can inhibit the NO-syntase reaction. In such conditions the state of the antioxidant system is significant for cell defense. The antioxidative status of rats fed some amino acids under 3-month hypertension conditions is the matter of the great interest. Experiments were performed on female Wistar rats fed some amino acids under 3-month hypertension conditions. The antioxidative status of rats fed some amino acids under 3-month hypertension conditions is the matter of the great interest. Experiments were performed on female Wistar rats fed some amino acids under 3-month hypertension conditions. The antioxidative status of rats fed some amino acids under 3-month hypertension conditions is the.

125.10
Effects of Anti-tumor Antibiotics Daunorubicin on Histone Chromosomal Proteins
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Anthracycin antibiotics, Daunorubicin is a potent anti tumour drugs wildly used in cancer chemotherapy. DNA has been reported as a main target of these drugs but the role of chromosomal proteins especially histones remains unknown. In this study, the effect of Daunorubicin on histones was investigated using uv/vis spectroscopy and equilibrium dialysis. The complex of histones was prepared by dimethylsulphamide (DMS) core linking, and purified by gel electrophoresis and electrophoresion procedure. The binding was carried out at 250c and in the dark. In equilibrium dialysis, histone complex was dialyzed 72 hours at 250c against various concentrations of drug and the binding parameters were determined according to the Scatchard method. The results show that a gradual increase in Daunorubicin concentration reduced the absorbance of the protein at 210nm indicated by both absorbance and difference spectra. The complex showed a positive cooperative binding behaviour, confirmed by slope of Hill plot. The binding constant was 3.71e+5 1/mol . The negative amount of free energy indicated the binding process to be exothermic. Also results obtained from the interaction of core complex with Daunorubicin in the presence of DNA showed that increasing drug concentrations increased absorbatid of 210, 260 and 480 nm. The results suggest that in the cell, apart from DNA, histones may play a critical role in drug binding. It is suggested that interaction of Daunorubicin with the chromatin produces histone-histone or histone-DNA complexes, which precedes the compaction of chromatin.
125.11
Concerted Mass Spectrometry Strategies for Glycomics and Glycoproteomics
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A major technical limitation in implementing glycoproteomics analysis by advanced mass spectrometry is the heterogeneity and uncertainty of glycan structures that may be attached to any one glycosylation site. Experimental approach in which glycans were cleaved off after glycopeptide or glycoprotein capture may facilitate identification and sequencing of the deglycosylated peptide but information on the site-specific glycan is lost. Alternative strategies rely on detection of glycan specific fragments by precursor ion scan functions followed by MS/MS of the selected glycopeptides. In the absence of peptide or protein sequence as in shotgun proteomics approach, determination of the mass contribution by the glycan moiety can however be difficult and simultaneous sequencing and identification of both glycan and peptide is often not possible. MALDI MS and CID MS/MS provide significant advantages over LC-ESI-MS/MS in rapid profiling of the glycome. Based on a wide range of novel and standard glycans analyzed, the characteristic MS/MS fragmentation pattern established for the permethyl derivatives of each class of glycans is presented herein. Emphasis is given to successful discrimination among different core structures, branching pattern, type 1 versus type 2 chain, and various fucosylated terminal epitopes as in H and Lewis antigens. In addition to providing a rich glycobiological context, such ‘first-screen’ glycomics mapping enables one to focus on targeted analysis of novel or specific terminal epitope thus identified, based on more specific precursor ion detection strategy coupled with prior lectin or antibody capture enrichment step. We demonstrate here our concerted strategies from glycomics analysis to implementation of LC-ESI-MS/MS based glycoproteomics analysis on Q-TOF instruments controlled by latest operational software release.

125.12
Proteomic Analysis of L6 Rat Skeletal Muscle Cells Following 6-Aminonicotinamide Treatments
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Neurotoxin 6-aminonicotinamide (6-AN) has been known to cause a number of biochemical alterations such as a reduction of the concentration of ATP, glycolytic flux, catecholamines, RNA, nucleotide pool and poly(ADP-ribose). We observed proteome alterations of L6 rat skeletal muscle cells treated with 100 μM 6-AN alone or combination of either 1 mM ATP or 1 mM NAD. Proteome alterations were investigated using two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The results showed that the treatment of 100 μM 6-AN alone caused the alternation of 29 proteins or enzymes compared to the control group. Furthermore, the inclusion of 1 mM ATP in 6-AN treated group also caused 31 altered proteins or enzymes and the inclusion of 1 mM NAD in 6-AN treated group caused 33 altered proteins or enzymes. Of these proteins, the expressions of heat shock protein, fructose-bisphosphate aldolase, glyceraldehydes-3-phosphate dehydrogenase and PARP were markedly affected by 6-AN treatment. The proteins altered in the present study can be used in the future for studies pertaining to apoptosis, or such specific disorders as myopathy and injury for therapeutic aims.

125.13
Identification of CFTR Interacting Proteins and Genes Responding to CFTR Expression in Yeast
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Cystic Fibrosis (CF) is the most prevalent lethal autosomal recessive disorder in populations of European descent. It is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). The most common mutation is a deletion of phenylalanine at position 508 (delta F508) in the first nucleotide binding domain of CFTR. Essentially all of this mutated form of the protein as well as about 80% of wild type CFTR are retained in the ER and targeted for ER-associated degradation (ERAD) by the 26S proteasome. The folding and degradation of proteins, including integral membrane proteins like CFTR, require molecular chaperones. Previously, the Brodsky laboratory demonstrated that the cytosolic Hsp70, Ssa1p, facilitates CFTR degradation in yeast, whereas the ER luminal chaperones calnexin and BiP do not. In order to identify the complete spectrum of factors associated with CFTR at each step during its folding and degradation we have taken a proteomic and genomic approach. In the proteomic approach, differences in protein expression between yeast strains expressing CFTR and control strains are detected by SDS PAGE and identified by peptide mass fingerprinting utilizing MALDI-TOF analysis. In the genomic approach, differential expression is detected at the level of mRNA by microarray analysis. Candidate genes are currently being genetically and biochemically confirmed.
125.14
Electron Microscopy Cell Fraction Preparation Robot
R. G. Waterbury, K. Punwani, J. J. M. Bergeron, and R. E. Kearney

A high-throughput device was developed to expedite and standardize cell fraction sample preparation for electron microscopic examination. It provides a means for mass, parallel validation of sub-cellular sample purity and confirmation of protein localization in isolated organelles. Due to the inherent fragile nature of cell fraction specimens, the device was designed to handle all aspects of chemical and mechanical manipulation necessary to prepare organelles for electron microscopic examination. Its modular design permits sequential, automated filtration, chemical processing, delivery and embedding of 96 cell fraction samples in parallel. The automated system minimizes mechanical stress to the samples, controls delivery and removal of processing reagents and regulates temperature by integrating five sub-systems: (1) a core mechanism composed of four modular plates, (2) a 5-axis motion control system (X, Y, Z, α, β), (3) an electromagnetic plate transfer arm, (4) a cooling platform, and (5) an automated fluids handling sub-system. As part of the supporting technology developing for proteomics, the automated device will allow, for the first time, massive, parallel electron microscopy screening and subsequent statistical analysis of sub-cellular and protein targets necessary for high-throughput proteomics.

125.15
Re-thinking of Adleman’s Computing Model
T. Chen, J. H. Li, and T. M. Chen

Biological macromolecules, such as DNA, have extraordinary potential to process biological information in a manner that contemporary silicon-based supercomputer can hardly compete with regarding computing speed, data storage and energy efficiency. Adleman proposed a DNA computation model using ligation of two DNA fragments as a single operation at the concentration of micromole to address the directed Hamiltonian Path problem. (Adleman, Science 266: 1021–1024, 1994) When 20mers oligonucleotides which were designed based on the four distinct nucleotides were chosen for encoding the graph, the theory that algorithm of 4.sup.n is applicable to any region of DNA sequences were assumed spontaneously. However, the genetic information was actually stored in the DNA chemical composition of Adenine, Thymine, Guanine and Cytosine. The genetic code was arranged in sequential orderly forms in DNA that could be measured quantitatively. The codon-based algorithm of 61.sup.(n-1) predicts all the possible existing oligonucleotides with n codon-length long sequences. The codon-based algorithm of 61.sup.(n-1) predicts all the possible existing oligonucleotides with n codon-length long sequences for 5'-UTR or 3'-UTR respectively. The number of all possible 21mers oligonucleotides: 61.sup.(7–1) = 51,520,374,361 whereas 4.sup.(21) = 4,398,046,511,104. 64.sup.(7–1) = 68,719,476,736 whereas 4.sup.(21) = 4,398,046,511,104. Although a given 21mers oligonucleotide designed by nucleotide may or may not have the redundant nucleotide(s), there are 85.3 times more redundant 21mers sequences of Adleman’s model than all possible 21mers sequences of coding region with 5'-start codon orientation and 64 times more redundancy than all possible 21mers sequences of 5'-UTR with 3-start codon or 3'-UTR with 5'-stop codon orientation respectively in total. Therefore, we propose to rethink and redesign the oligonucleotides in Adleman’s experiment to explore the possibility of resolving the directed Hamiltonian Path problem.

125.16
Proteomic and Genomic Analyses of Androgen Response in Mouse Prostate and Liver Tissue

We used a proteomic approach to identify proteins responsive to dihydrotestosterone (DHT) in the mouse prostate and liver. The tissues were collected from control castrated, and castrated animals treated for 24h with a single physiological dose of DHT. Total protein extracts and specific sub-cellular fractions were prepared from these tissues. Proteins were analyzed by comparative two-dimensional gel electrophoresis (2-DE) and identified by peptide mass fingerprinting and/or tandem mass spectrometry. We identified, for the first time, 42 proteins (32 in prostate; 10 in liver) whose expression levels are modulated by DHT. Based upon their reported functions, 79% of these proteins are involved in chaperone/stress (22%), signal transduction (22%), metabolism (19%) and cytoskeletal organization (16%). The remaining less abundant proteins detected after specific sub-cellular fractionation are implicated in translation (9%), protein synthesis (3%), transport (3%) or have unknown functions (6%). The DHT-induced changes in protein levels correlate with a similar change in the corresponding transcript in 63% of cases as determined by Q RT-PCR and in 57% of cases as measured by microarray analysis. Additionally, we have confirmed by immunoblotting that level of one of the identified prostate proteins namely the Rho GDP dissociation inhibitor (RhoGDI), a protein involved in a signaling cascade implicated in cytoskeletal dynamics, is indeed decreased by DHT. The information obtained by such proteomic-based methodologies may well uncover markers of potential use for the detection, and potentially, the treatment of cancer.
**125.17**

**Identification of O-GlcNAc Modified Proteins, Peptides and Their Signaling Roles in Mammalian Systems**

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N-Acetyl Glucosamine linked O-glycosidically (O-GlcNAc) to serine and threonine residues of proteins is a post translational modification that is being investigated recently, however, the specific functionality associated with this modification still remains elusive. Recent reports are clearly indicative of a complex yet dynamic interplay between phosphorylation and O-GlcNAc modifications implicated on cytoskeletal proteins, signaling proteins and transcription factors. In the present study, monoclonal antibody against O-GlcNAc residues linked to serine and threonines of peptides (CTD110.6) was used to immunoprecipitate proteins from Whole cell extracts, nuclear and cytosolic fractions from mammalian cells. Proteins selectively extracted in this fashion were resolved by 1 and 2D gel electrophoresis. Six prominent bands seen on 1D resolved into 70 selectively extracted in this fashion were resolved by 1 and 2D gel electrophoresis. Six prominent bands seen on 1D resolved into 70 + bands on 2D gels. MALDI-MS was used to identify random protein spots from 2D gels following trypptic digestion. Different categories of proteins were identified that included Intestinal Fatty Acid Binding Protein, Olfactory Receptor 6B1, Receptor Tyrosine Kinase, and Septin-1 differentiation 6 (Deoxyguanosine triphosphate triphosphohydrolase). Apart from these, proteins such as transcription factor Sp1, alpha-tubulin and Receptor tyrosine kinase implicated in glucose metabolism, phosphorylation associated and insulin signaling pathways were also identified. Treatment with PNGase F (N-Glycosidase inhibitor) had no effect on the protein profiles, validating the O-linked presence of glycosyl groups on Ser/Thr residues. On the contrary, strong increased and decreased levels of O-GlcNAc expression were detected on treatment with Streptozotocin/PUGNAc (O-GlcNAc hydrolase inhibitor) and Alloxan (OGT inhibitor) respectively suggesting that a working model has been established for further inhibitor related studies. The specific sites of modification on proteins/peptides and their sequence determination by precursor ion scanning based on reporter ions specific for the O-GlcNAc moiety are currently being pursued.

**125.18**

**Heat Shock Protein Expression in Waste Incineration Workers Exposed to Dioxin**

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Heat shock proteins (HSPs) are families of highly conserved proteins that are induced in cells and tissues upon exposure to extreme conditions causing acute or chronic stress. They have distinct functions and have been implicated in various diseases, including cancer. In order to investigate the relationship between HSPs expression and exposure of environmental pollutants, we have experimented proteomic analysis to evaluate expression changes of HSPs of plasma protein of waste incinerating workers exposed to various pyrolysis products including polycyclic aromatic hydrocarbons (PAHs) and dioxin. About 30 workers from waste incinerating company, Ansan, South Korea and 30 matched, unexposed healthy subjects who received their annual check at Medical Center of Korea University, Seoul, South Korea were selected. All subjects completed a questionnaire, which included questions of smoking, drinking, age, dedication, etc. Plasma proteins of workers and unexposed subjects were analyzed by 1-DE and 2-DE with pH 3-10 NL/4-7 L IPG Dry strip with anti-HSP27, 60, 70, 90 monoclonal antibodies. HSP 70 and 90 showed no significant changes between both subjects. But expressions of HSP27 and 60 were increased to 2.5 fold and 1.2 fold, respectively in workers. In conclusion, certain families of HSPs are implicated in a cellular process that is relative to pollutants including PAHs and dioxin. Further studies will be necessary to find out the mechanism and relationship between HSP and its cellular function to environmental pollutants.

**125.19**

**Analysis of Serine-/Threonine-Phosphorylation Sites Via Peptide Derivatization**

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Serine and threonine phosphorylation is a key mechanism in enzyme regulation. It is relevant in many important cellular signal transduction pathways, in the cellular division cycle or the regulation of metabolism. Our project goal was the development of a fast and reliable method for the identification of serine-/threonine-phosphorylated peptides from digested proteins and protein mixtures. In addition, the reaction products should be well observable in mass spectra. The phosphorylated peptides were derivatized with &#61538;elimination and Michael-addition of different thiol compounds either directly in a tryptic digest or after reversed-phase HPLC. The related ions were selected for MS/MS-measurements to identify the peptide, the derivatized amino acid and, therefore, the previous site of phosphorylation. Using thiokalanines of different chain length as alkylation agents, a 14 Da mass difference was introduced into the peptide map, which was sufficient to select the individual peptide species with the timed ion selector and do the MS/MS-analysis. Bovine phosducin was analysed with the new technology. After HPLC fractionation of the digest, each fraction was subjected to derivatization and the respective MS spectrum was screened for a mass difference of 14 Da. 6 phosphorylation sites were identified of which only 1 was previously known. 5 out of 6 selected peptides were actually phosphorylated, one of them was doubly phosphorylated. The application of this method provided a stable and reliable chemical platform for the analysis of the O-phosphates phosphoserine and phosphothreonine. The method can be used for the detection and localisation of protein phosphorylation.
125.20
The Alternations in Human Bronchial Epithelial BEAS-2B Cell Proteome Upon the Treatment of Air Pollutants
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Chronic exposure of second-hand smoke containing various carcinogens, polycyclic aromatic hydrocarbons (PAHs) in air pollution, and cooking oil fumes, were considered as important environmental risk factors for lung diseases. Three toxic chemical compounds, benzo[a]pyrene (BaP), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), and trans-trans-2,4-decatadienal (ttDDE), frequently detected in high quantity in aforementioned environmental risk factors, were shown to be carcinogenic. The objective of this work is to use proteomic experimental approaches to study how these three toxic chemical compounds may alter the proteome in BEAS-2B normal human bronchial epithelium cells. The difference in the levels of proteins extracted from human bronchial epithelial BEAS-2B cells with and without exposure to BaP, NNK, and ttDDE, were investigated using 2D-gel electrophoresis (2D GE) display. Roughly 1000 protein spots were observed after silver staining applied to the 2D GE of the protein extract from BEAS-2B cells. The identities of the proteins with altered levels were determined by reverse phase high performance liquid chromatography-ion trap mass spectrometry (RPHPLC-ITMS) analysis of gel tryptic digests and protein sequence database search. With BaP or NNK treatment, the levels of fewer than 6 proteins were considered altered. Among these proteins, the level of the protein disulfide isomerase ER-60 was elevated at least 400% with BaP treatment. In general, the number of proteins that varied in abundance was not large. This is contrast to what was observed when the BEAS-2B cells were treated with ttDDE. With ttDDE treatment, the levels of 31 proteins were considered up-regulated, and 11 were down-regulated. That is, 42 proteins, accounting for 4.3% of 966 proteins revealed by 2D GE.

125.21
Post-translational Modifications of Choline Acetyltransferase
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Choline acetyltransferase (Chat) catalyzes the reaction between choline and acetyl-CoA to produce the neurotransmitter acetylcholine. It has been hypothesized that the catalytic activity of Chat is decreased or nonfunctional in the brains of people with Alzheimer’s disease and other cognitive disorders. Two-dimensional SDS-PAGE analysis of Chat revealed that there are numerous acidic isoforms ranging from a pI of 6.3 to 8.0. These isoforms are approximately the same apparent molecular mass, and are not produced by alternative splicing. Although Chat has been found to be phosphorylated, these isoforms are known not to be modified in this way. Other post-translational modifications are currently being investigated by mass spectrometry (MS). Pure Chat was alkylated, digested with trypsin and a peptide map was produced using RP-HPLC. As the peptide mixture was complex, the digest was fractionated by RP-HPLC and each fraction was analyzed by matrix assisted laser desorption ionization (MALDI) MS. Protein coverage was 95%. Comparisons between the theoretical digest and experimentally measured masses allowed us to identify peptides with potential post-translational modifications. Electrospray ionization MS/MS (ESI-MS/MS) is being used to obtain sequence data on the peptides and the exact placement and identification of the modification.
125.23
Toxico Proteomic Analysis of IC-21 Cells Treated with Propanil
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Propanil is a post-emergent amide class herbicide used extensively on rice crops worldwide. In vivo and in vitro propanil exposure causes a decrease in cytokine production in IC-21 cells, a macrophage cell line, which can be linked to multiple targets in the signaling pathways associated with cytokine production. For this toxico-proteomic study, IC-21 cells were exposed to 100 microM propanil or ethanol vehicle and simultaneously stimulated with lipopolysaccharide. After a 30 min exposure, cytosolic fractions were isolated by standard methods. Additional extracts were obtained from unstimulated, untreated, i.e., not propanil- or ethanol-treated, cells. 1- and 2D-gel electrophoresis profiles were established by running 3 independent gels on extracts from each treatment group. The propanil-treated cell extract gel had an average of 1,042 spots and the vehicle-treated cell extract gels had an average of 990 spots. Differential statistical analysis of the spots detected on both gel sets using Phoretix Pro identified 12 spots that were differentially expressed between the propanil- and ethanol-treated groups. In all cases the protein concentration was decreased in the propanil-treated cells versus the vehicle-treated cells. These spots were picked and trypsin-digested for mass spectrometry (MS) identification. This analysis is currently in progress and until the identity of these proteins is confirmed, it is impossible to interpret these findings. Additional comparisons between cytosolic fractions from LPS-stimulated and unstimulated cells, that were neither propanil- nor ethanol-treated, are also in progress to determine the number of differentially expressed proteins induced solely by the LPS-stimulation. Supported by the USPHS, NIEHS.

125.24
Characterisation of Apolipoproteines a Phosphorylation Sites Using IMAC Affinity Column in a Gyros CD Microlaboratory
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Analysis of post-translational modifications in protein is an important step in understanding the changed functional properties of many proteins upon such a modification. Thus one of the challenges now facing proteomic research is the characterization, analysis and localization of these post-translational modifications. Phosphorylation, a post-translational modification which is of greater interest in many biological processes and notably in signalling cascade, is very difficult to analyze due to the very high instability of the phosphates during MS/MS analysis, their low ionisation yield, the difficult detection of phosphopeptides and their low retention time in nano-LC experiment. So we have developed a strategy based first on the detection of the phosphorylation sites using an alkali phosphatase and secondly on the localization of the various phosphorylation sites with the help of IMAC affinity column in a gyros CD microlaboratory. We illustrated this strategy with the study of the different phosphorylation profiles of various apolipoproteins A isoforms, isolated from human plasma. More precisely, we have first characterized the phosphorylation level combining dephosphorylation and analysis by 2D gels of the different fractions. Once the phosphorylation detected, we have performed a structural proteomic analysis eluting the digestats of native proteins on IMAC affinity column integrated on a GYROS CD microlaboratory to specifically concentrate the phosphopeptides and so allowing precise investigation of the phosphorylation level and above all precise localization of the modified sites.

125.25
Use of CAPLC Q-TOF Combination for Site Specific Analysis of N-linked Glycosylation
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Defining the extent and type of post-translational modification of a protein is often key in understanding its structure and function. Protein bound carbohydrate can play an important role in the structure, pharmacokinetics and functional activity of a therapeutic protein. Conversely, malfunction in the cell can also be reported changes in protein glycosylation. The location of glycan is often as important as structural information, a factor not often accounted for in most standard glycosylation analyses. Here a method for detection, characterization and location of glycosylation in a single HPLC-MS/MS experiment is demonstrated. A proteolytic digest has been analysed by reverse phase HPLC-ESI using a Q-Tof mass spectrometer. The instrument runs two MS surveys at different gas cell collision energies. The low energy survey (7eV) shows only the intact or parent ions. The high-energy survey (35eV) shows the fragments of these ions. Upon detection of the carbohydrate oxonium ions at m/z 204 (HexNAC), 366 (HexHexNAC) and 274/292 (NeuAc) the instrument is switched into MS/MS mode and ions from the low energy spectra are selected by the quadrupole for fragmentation. The high mass of glycopeptides gave rise to glycopeptide ions, typically of 4 or more positive charges, a feature that is exploited for their preferential selection for MS/MS. The mass measurement of the parent ion is enhanced by the use of nano-lockspray. The glycosidic bonds tend to be more labile than the peptide bonds hence MS/MS spectra produced predominately glycopeptide Y-type fragment ions. The interpretation of complex MS/MS spectra containing multiply charged ions are assisted by the use of the MaxEnt3 algorithm and Carbotools software.

125.26
Directed Evolution of Cystatins-modifying Protease Inhibition
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We have used phage display to direct the evolution of cystatins for the benefit of plant biotechnology. Cysteine protease inhibitors are implicated in protecting plants from the fungal, viral, and pest infestations. We have evolved an inhibitor of cysteine proteases in order to find efficacious and specific inhibitors of proteases from major insect pests, and plant pathogens. Based upon the co-crystallized structure of the cystatin from chicken eggwhite and the model cysteine protease papain, the amino acids of a cystatin implicated in the binding were varied. We were able to direct the evolution of this protein to obtain novel variants with high affinity and lower Ki against papain. We choose to perform competitive elution with the small cysteine protease inhibitor E64, and have found several variant proteins with novel properties. This method of competitive elution has allowed us to direct the evolution of this molecule, and select novel amino acid residues at the active site cysteine binding domain.
125.27
A General Approach to the Analysis of Post-translational Modifications Combining Ion Trap Technology, Accurate Mass Detection, and Data Mining Software
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Protein analysis by peptide mass maps has become an important technique for proteomic studies. A protein from a gel spot or a separated subcellular fraction is proteolytically digested and analyzed by LC-MS/MS. Proteins are identified by comparison of measured peptide fragment ions with those predicted by in silico digestion of a protein database. This technique can account for some modifications to the protein primary sequence, i.e. post-translational modifications (PTMs), but the protein must be present in the database and the modification must be known a priori. Locations and structures of unexpected PTMs can be determined by further fragmentation of the modified peptide (e.g. MS to 3 in an ion trap). They can also be identified by analysis of existing data using enhanced software tools for spectral interpretation, de novo sequencing, and spectral pattern analysis. In this report, we will demonstrate use of an ion trap mass spectrometer (LTQ FT), coupled with protein identification (TurboSEQUEST), de novo sequencing (DeNovoXTM), and pattern analysis (SALSA) software to study phosphorylation, glycosylation, and methylation in a complex biological sample mixture. The confidence of all of these techniques is enhanced with access to data sets with very high mass accuracy, e.g. <2ppm.

125.28
Alteration of the Insulin Binding and Glucose Uptake Capability at the Hyperglycemia States
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Background and aim; Biologic action of insulin on the tissue begins after it’s bound with specific receptor on the cell surface and one of the main effect are glucose transport (GT) into the cells. GT may be altered at the many disorders and states, but relationship between GT and cells insulin bind capability (IBC) is not clear. The aim of our study is investigation of the erythrocytes GT and IBC in patients with hyperglycemia. Materials and methods; GT and IBC was investigated at the in vitro study by erythrocytes incubation in Krebs-Ringer-Phosphat buffer, pH7.4, for 15 minutes at 37°C at the presence of H3-D-glucose and H3-insulin. There are 10 patients with DM, 9 persons with Impaired Glucose Tolerances (IGT), 8 patients with acromegaly (A), 8 with obesity (Ob), 9 with Polycystic Ovarian Syndrome (PCOS), and 9 healthy (control group) subjects with normal glucose tolerances were observed. Results. Erythrocyte H3 GT increased on 49.3% (P<0.05) in DM, on 21% in IGT, and on 20% in Ob, as compared with control subjects. Erythrocyte H3-IBC was higher on 18.77% in DM, 9.8% in IGT, and 8.8% in Ob. GT was not significantly increased in patients with A and PCOS, but IBC had no difference when compared to control subjects. An increasing of GT into erythrocytes probably indicates the presence of insulin resistance in the hyperglycemia states. Conclusions. GT and IBC alterations mechanisms at the DM and other hyperglycemia states are difference and may be considered as a result of the partially or general insulin resistance.

125.29
Semi-pilot Scale Fermentation of Citric Acid by Aspergillus niger
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Twenty-five different strains of Aspergillus niger were isolated from the soil samples collected from various localities of GC University, Lahore, Pakistan and screened for citric acid production. Out of these, BT-10, the best citric acid producing isolate (yield, 58.96 g/l) was subjected to UV-irradiation for different time intervals. Thirty mutants were obtained and screened for citric acid production. Out of these, UV-20 was the best citric acid producing mutant (yield 64.33 g/l). Shake flask technique was employed for the screening of Aspergillus niger isolates and mutant strains. The nutritional study was carried out in a semi-pilot scale stirred bioreactor of 15-L capacity using the strain, UV-20. Ferrocyanide treated (200 ppm) cane molasses medium containing sugar 150 g/l, was used as the basal fermentation medium. NH4NO3 (0.20%) and K2HPO4 (0.1%) were optimized as nutrient parameters for maximum yield of the acid. Highest yield of citric acid obtained during the course of study was 82.34 g/l with the sugar consumption of 97.00 g/l. The mycelia were intermediate pellets having dry cell mass 16.55 g/l.
Key phrases: Aspergillus niger, ammonium nitrate, cane molasses, citric acid, dipotassium hydrogen phosphate.
125.31
The Influence of Polyethylene Glycol 400 on Biochemical Structure and Function of Haptoglobin
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Haptoglobin (Hp) is a haemoglobin-binding glycoprotein. This polymeric protein consists of two alpha and beta chains. On the chain there is a center of linkage of haemoglobin. In the organism the main Hp function is haemoglobin catabolism and storage of iron gem. Besides Hp fulfils a lot of other functions connected with the immune status of organism. Hp is used as the element of biochemical test system reacting to a health state.

The structural and functional features of native Hp, Hp in composition fresh plasma of blood, after its storage at liquid nitrogen temperature (-196 degrees C) and interaction of Hp with 15% polyethylene glycol 400 (PEG-400) were investigated by determining of peroxidase activity of the complex Hp with haemoglobin and fluorescence spectrophotometry methods.

The parameters of fluorescence native Hp were determined.

The 6 nanometer shift of fluorescence maximum of Hp to the long-wave diapason occurs as a result of freezing Hp up to temperature ~196C degrees at both lengths of excitation waves (296 nm and 280 nm) and half width increases, that testifies to the rise in availability the tyrosine and typtophane rests.

The presence of PEG-400 results in the small shift of fluorescence (2 nm) of the native Hp, both at the excitation wave 296nm and 289nm, however, freezing Hp at presence PEG-400 does not result in the further shifts of fluorescence Hp. Thus the presence PRG-4000 stabilizes molecular structure Hp.

The decrease in Hp functional capability for binding haemoglobin (up to 80%) was observed after cryopreservation of plasma without cryoprotectant. There is no reduction of functional activity Hp both in the fresh plasma, and after it freezing thawing with PEG-400.

Thus, PEG-400 stabilizes structure Hp, without significant lowering this glycoproteid functions ability. Apparently, during the interaction Hp between PEG-400 the center of linkage Hp, is not involved in structural changes.

125.32
Studies of Functions and Biochemical Mechanisms of Telomerase in Avian Marek’s Disease
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We use Avian Marek’s disease (MD) tumor as a model to study telomerase functions both in vitro (cell line) and in vivo (Marek’s disease tissues) in order to understand the functions and the biochemical mechanisms of this unique reverse transcriptase. We hope to identify a novel mechanism that telomerase may play in cancer cells. Set up of tumor model in Marek’s disease of chickens. 160 chicks were divided randomly into two groups, experiment group (80) was inoculated with 0.5ml MDV solution, while control group was inoculated with 0.5ml 0.9% NaCl solution. The tissues of kidney, liver, heart, spleen, lung, ovary, spermary, and Bursa of Fabricus were collected in each other day from inoculated, and then put in liquids nitrogen for using. Detection of telomerase activity in Marek’s disease tissues. Telomeric repeat amplification protocol TRAP reaction) was used. Elongation/amplification. After each sample was lysised, PCR amplification was done: First, telomerase adds telomeric repeats (TTAGGG) to the 3’-end of the biotin-labeled synthetic P1-TS-primer. Second, the elongation products are amplified by PCR using the P1-TS and P2, generation PCR product with the telomerase-specific 6 nucleotide increments. Detection by ELISA An aliquot of the PCR product is denatured and hybridized to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. The resulting product is immobilized via the biotin labeled primer to a streptavidin-coated microtiter plate. The immobilized PCR product is then detected with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase. Finally, the probe is visualized by virtue of peroxidase metabolizing TMB to form a colored reaction product. Results: Telomerase activity was present early in kidney (8th day after inoculated), liver (10th day), spleen (8th day).