Transfusion Medicine and Hemotherapy

Abstracts

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Wednesday, September 22, 2004: Plenary Session

PL1: Stem Cell Therapy

PL101

Blood stem cell, 40 years old, seeking new challenges

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Advances in stem cell technology raise high hopes that we are at the beginning of a new era of regenerative medicine. Modern day stem cell research began with the discovery of assays to detect hematopoietic stem cells (HSC) in 1963. In the meantime, blood progenitor cell transplantation has been proven to be a curative strategy for patients with malignant and hereditary diseases. Stem cells can be isolated from embryonic, fetal or adult tissues. Parallel to the progress made in embryonic stem cell research, adult stem cells, especially those derived from the bone marrow, have also been shown to exhibit developmental potentials heretofore not considered possible. Knowledge gained from blood stem cell transplantation has shown that adult stem cells are ableto migrate to sites where they are needed - a phenomenon that is designated as "homing." Once settled in a niche after homing, surrounding cells in the microenvironment play a major role in defining their long-term fate. We have demonstrated that symmetry of divisions and self-renewal capacity of blood stem cells are governed by direct contact with the cellular microenvironment. In the bone marrow, direct contact with mesenchymal cells increased the absolute number of blood stem cells undergoing asymmetric divisions and with self-renewing capacity. To characterize the interactions between stem cells and cellular determinants we have analyzed the molecular composition of these interactions using a panel of antibodies specific for various components of tight junctions, gap junctions, adhaerens junctions and desmosomes by immunofluorescence microscopy, protein biochemical methods, RT-PCR, as well as with electron microscopy. We made two fundamental observations that are completely novel and of key importance for stem cell biology. In addition to the typical *puncta adhaerentia* junctions we have noted the occurrence of another type of adhering junctions. These consist of slender, villiform-to-vermiform cell protrusions of up to several micrometers, tightly inserted into invaginations of neighboring cells of *recessus penitus* type. This novel type of adhering junction is comparable to other junctions of the *adhaerens* category and bear similarity to those described in primary mesenchymal stem cells of day 7 to 8.5 mouse embryos. This discovery opens up another dimension for characterization of stem cells and indicates that adult stem cells communicate with each other through junctions and junction complexes. Characterization of and identifying the role of such cell-cell contacts in specific differentiation stages will be of fundamental significance for understanding the molecular mechanisms of plasticity potential of stem cells.

PL102

The Oocyte – Embryonic Stem Cell Cycle

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Mouse embryonic stem cells in culture can develop into oogonia that enter meiosis and recruit adjacent cells to form follicle-like structures and later develop into blastocysts (Hübner et al. 2003). Oogenesis in culture should contribute to various areas including somatic cell nuclear transfer. Reprogramming, in the context of the transfer of a differentiated nucleus into an oocyte, can be defined as the transformation of a somatic cell nucleus into a functional embryonic nucleus capable of forming a viable organism. Correct expression of embryonic genes is a prerequisite for development and is indicative of nuclear reprogramming. *Oct4* is a gene essential for peri-implantation development and embryonic pluripotency. It is expressed during cleavage stages and essential for the differentiation of the blastocyst. *Oct4* expression becomes restricted to the inner cell mass and epiblast. After gastrulation *Oct4* is active only in germ cells and silent in somatic cells. *Oct4* was used as markers for which gene reprogramming could be directly related to developmental potential of somatic cell clones (Boiani et al. 2002). Cumulus cell clones initiate *Oct-4* expression at the correct stage but exhibit an incorrect spatial expression in the majority of blastocysts. The quality of an *Oct4* GFP transgenic signal in blastocysts correlates with the ability to generate outgrowths maintaining GFP expression, and frequency of ES cell derivation. Mouse clone blastocysts have less than half the normal cell number, and often a faulty expression of *Oct4*. Clone-clone aggregates of genetically identical, but epigenetically different embryos do not form more blastocysts, but the majority expresses *Oct4* normally and has higher rates of fetal and postnatal development (Boiani et al. 2003). Although cloning efficiency is augmented 8-fold, ES cell derivation is not significantly improved. Our results indicate that the derivation of ES cell lines from clone blastocysts and reproductive cloning are distinct in their requirements.

Boiani M, et al. Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev*16: 1209, 2002.

Boiani M, et al. Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? *EMBO J*. 22: 5304, 2003.

Hübner, K et al. Mouse Embryonic Stem Cells in Culture give rise to Oocytes. *Science:* 300, 1251, 2003.

PL103

Einsatz von Stammzellen für die Rekonstruktion im Nervensystem

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Unabhängig von der intensiven öffentlichen Debatte zählt das Gebiet der Stammzellen zu den faszinierendsten Themen der modernen Lebenswissenschaften. Dieses hat insbesondere damit zu tun, dass es zahlreiche Sparten der Biomedizin berührt. Häufig wird dabei die Möglichkeit, Stammzellen zur Spenderzell-Gewinnung für eine Transplantationsbehandlung einzusetzen, an erster Stelle genannt. Attraktiv erscheint dieses Vorgehen in nicht-regenerativen Geweben wie dem Nervensystem und bei solchen Erkrankungen, denen nur eine bestimmte Zellform zum Opfer fällt. Beispiele sind die Parkinson Krankheit, die Multiple Sklerose, bestimmte Formen von Epilepsie, der Diabetes mellitus, Herzmuskelleiden sowie Knochenmarkserkrankungen. Es werden jedoch zahlreiche weitere Gebiete von der Stammzellbiologie großen Nutzen ziehen, unter anderem die Entwicklungsbiologie und Entwicklungsmedizin, die Krebsforschung sowie die pharmazeutische Industrie durch neue Verfahren der Wirkstoffprüfung. Im Rahmen des Vortrags sollen ausgewählte Perspektiven aufgezeigt werden.

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SP1: Experimental Cell Therapy

SP101

The Cell Therapy Program at the University of Minnesota: A Collaborative Approach Facilitating the Delivery of Therapies from Bench to Bedside

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Background: The Cell Therapy Program at the University of Minnesota is very active with several ongoing studies involving hematopoietic stem cells (with an emphasis on umbilical cord blood), natural killer cells, tumor vaccines, multipotent adult progenitor cells, and T-regulatory cells and genetically-modified T-cells, both for management of graft-versus-host disease. **Methods:** The Cell Therapy Program utilizes a three-tiered approach in the development of cell therapy products to make efficient use of specialized talents, to provide economy of resources, and to insure high quality standards for the delivery of therapies. This approach requires a collaborative effort from the basic researchers, clinical investigators, and all laboratories involved with a given project. **Discussion:** Tier 1 involves interaction between the basic research laboratory and the Translational Research Laboratory at the Cancer Center. Procedural modifications are identified, and evaluation initially takes place on the small-scale production (research) model. Laboratory read-out assays are further developed. With a feasible method for large-scale production in place, the project moves to tier 2, the Translational Research Laboratory at the Minnesota Molecular & Cellular Therapeutics (MMCT) Facility. Methods are further scaled-up, and standard operating procedures (SOPs), batch production records (BPRs), and QA (quality assurance)/QC (quality control) plans are developed. With reproducible large-scale production using clinical-grade reagents and clinically acceptable methods, the project moves to tier 3, the Clinical Cell Therapy Laboratory within the MMCT, where final SOPs/BPRs are written and validation runs occur prior to clinical production. **Conclusion:** This collaborative three-tiered approach to cell therapy translational research and clinical scale-up is efficient and has been successfully used with several clinical protocols at the University of Minnesota.

SP102

Cord Blood derived Mesenchymal Stem Cells: Defining Criteria for Efficient Isolation

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Purpose: Evidence has emerged that mesenchymal stem cells (MSC) represent a promising population for supporting new clinical concepts in cellular therapy. However, as the number and differentiation capacity of MSC decline with age, their therapeutic potential might be diminished as well. Since umbilical cord blood (UCB) has recently emerged as an alternative source of hematopoietic stem cells, the same might apply for MSC. Therefore we investigated, whether cells with MSC characteristics and multilineage differentiation potential can be cultivated from UCB of healthy newborns, and whether yields might be maximized by optimal culture conditions or by defining UCB quality criteria. **Methods:** UCB units from full term deliveries were collected from the unborn placenta. Mononucleated cells (MNC) were obtained by Ficoll density gradient centrifugation, resuspended in MSC growth promoting medium (MSCGM) and seeded into 6-well plates. Non-adherent cells were removed after 12-18h, and the adherent cells were cultivated until fibroblastoid cell clones appeared (14-24 days of culture). These were subjected to osteogenic, chondrogenic and adipogenic differentiation assays and flow cytometric analysis within the first 1-2 passages and at later passage 7-8. **Results:** Using optimized isolation and culture conditions, in up to 63% of 59 low volume UCB units, cells were isolated showing a characteristic fibroblastoid morphology and immune phenotype (MSC-like cells). These were similar to control MSC from adult bone marrow (BM). The frequency of MSC-like cells ranged from 0 to 2.3 clones per $1x10^8$ MNC. The clones proliferated extensively with at least 20 population doublings within eight passages. In addition, osteogenic and chondrogenic differentiation demonstrated a multilineage capacity comparable to BM MSC. However, in contrast to MSC, MSC-like cells showed a reduced sensitivity to undergo adipogenic differentiation. Crucial points to isolate MSC-like cells from UCB were both the time from collection to isolation of less than 15 hours, a net volume of more than 33 ml and a MNC count of more than $1x10^8$ MNC. **Conclusions:** UCB can not only be used as alternative source for hematopoietic but also as demonstrated herein for mesenchymal stem cells. Since cells with a high proliferative and differentiating potential can be isolated at high efficacy from full term UCB donations, we regard UCB as an additional valuable stem cell source for experimental and potentially clinical purposes.

SP103

Mesenchymal Stem Cells (MSC) display coordinated rolling and adhesion behaviour on endothelial cells

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Purpose: MSC have been applied clinically both via local delivery and intravenously. Intravenously administered MSC have also been shown to home and engraft in distinct organs in animal models. To better characterize the mechanisms which MSC use for controlled extravasation, we analyzed the rolling and adhesion behaviour of MSC under continuous flow. **Methods:** MSC were isolated from human bone marrow according to standard protocols. Cell surface marker were analysed by flow cytometry. For adhesion assays a parallel flow chamber system was used. Cells were flushed at defined shear forces in HBSS with $MgCl_2$, $CaCl_2$, and 0.1% of BSA. Human umbilical vein endothelial cells (HUVEC) were cultivated on fibronectin-coated glass sides and were stimulated with recombinant human TNF-alpha. **Results:** Flow cytometric analysis showed expression of homing molecules CD29, CD44, and (variably) CD62P, but not CD62L or CD31. MSC displayed coordinated rolling on endothelium with a velocity ca. 7-50 µm/s, comparable to hematopoietic cells. The typical rolling on preformed "streets" as has been described for human neutrophils was also observed with MSC, and rolling resulted in either firm adherence or reentry into the circulation flow. MSC adhered to HUVEC at shear forces of 0.1 dyn/cm^2 with a similar frequency as peripheral blood mononuclear cells (PBMNC). Once bound, MSC resisted shear forces of up to 2 dyn/cm^2. After prestimulation of HUVEC with TNF-alpha, binding of both MSC and PBMNC was increased (6.2 +/- 2.5-fold and 3.2 +/- 1.1-fold, respectively (means +/- SD)). In contrast to HUVEC-coated surfaces, coating with fibronectin, laminin or collagen IV, or analysis on uncoated tissue culture plastic did not induce binding of MSC under flow. Although MSC rolled at shear forces of up to 2 dyn/cm^2 as did PBMNC, MSC showed strongly decreased binding to endothelia above shear stresses of > 0.1 dyn/cm^2. **Conclusion:** MSC roll on endothelium under flow in our model. In addition, MSC adhesion is increased on inflammatory endothelium, but MSC display lower binding strength than hematopoietic cells. Our results show that MSC are capable of performing initial steps of endothelial interaction which are necessary for a coordinated, tissue-specific homing process.

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SP104

Methylguanine methyltransferase-mediated in vivo selection and chemoprotection of allogeneic stem cells in a large-animal model

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Clinical application of gene therapy for genetic and malignant diseases has been limited by inefficient stem cell gene transfer. This limitation could potentially be circumvented by efficient in vivo selection of gene modified stem cells. Here we studied in a clinically relevant canine model a strategy based on genetic chemoprotection mediated by a mutant of the DNA-repair enzyme methylguanine methyltransferase. We hypothesized that genetic chemoprotection might also be used to enhance allogeneic stem cell transplantation, and thus we evaluated methylguanine methyltransferase-mediated chemoprotection in an allogeneic transplant setting. We demonstrate that gene-modified allogeneic canine CD34+ cells can engraft even after low-dose total body irradiation conditioning. We also show that cytotoxic drug treatment produced a significant and sustained multilineage increase in gene-modified repopulating cells. Marking in granulocytes rose to levels of up to 98%, the highest in vivo marking reported to date to our knowledge in any large-animal or human study. Increases in transgene-expressing cells after in vivo selection provided protection from chemotherapy-induced myelosuppression, and proviral integration site analysis demonstrated the protection of multiple repopulating clones. Drug treatment also resulted in an increase in donor chimerism. These data demonstrate that durable, therapeutically relevant in vivo selection and chemoprotection of gene modified cells can be achieved in a large-animal model and suggest that chemoprotection can also be used to enhance allogeneic stem cell transplantation.

SP105

ADAR 1 in human erythropoiesis

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Purpose: Recently, the requirement of adenosine deaminases acting on RNA 1 (ADAR1) for mouse erythropoiesis was described. Embryos with a defect ADAR1 allel died before d14 with a failure in enucleation of erythroid progenitors. We want to investigate the influence of ADAR1 on human red cells in an in-vitro model of erythropoiesis. Therefore, two culture techniques were tested for optimal proliferation and differentiation of CD34+ cells to reticulocytes. Furthermore the expression of ADAR1 mRNA in CD34+ cells and erythroid progenitors was determined. **Methods:** Purified CD34+ cells from leukapheresis were cultured 1) over 16d in a two-phase liquid assay (d1-9 EPO, SCF, IGF1; d10-16 EPO, Insulin) or 2) over 21d in a three-phase assay (d1-7 SCF, Flt3-L, TPO, Insulin; d8-14 SCF, EPO, IGF1, Insulin; d15-21 EPO, Insulin). Cell growth and differentiation was evaluated by flow cytometry (staining against CD34, 45, 71, 117 and glycophorin A (GPA)). Cytospin preparations were stained by Pappenheim and neutrale benzidine. RNA of various culture days was islolated and ADAR1 mRNA was quantified by RT-PCR. **Results:** The initial purity of CD34+ cells was always >95%. During the two-phase model absolute cell numbers increased up to 120fold. On d16 >95% expessed GPA and were strongly hemoglobin positive. Cells showed morphological characteristics of normoblasts and >40% were enucleated reticulocytes. In the three-phase model, a better proliferation (up to 800fold) could be observed. Contrary to this abundant proliferation, only a moderate stage of differentiation could be observed. On d21 70% cells were GPA+, most of them normoblasts and reticulocytes, and 30% were non-erythroid. The ADAR1 RT-PCR indicates that human CD34+ cells express ADAR1 in a moderate degree. Most ADAR 1 was expressed during d4-8 of the two-phase model and between d10 and d14 in the three-phase model. **Conclusions:** The two established in vitro models are able to show the different stages of human erythropoiesis including terminal enucleation. Compared with each other, the higher proliferation in the three-phase model is associated with a lower grade of differentiation. The detection of ADAR1 mRNA in the two-phase model shows the highest content between d4 and d8, in the second model between d10 and d14. During this time most erythroid cells were in an early stage (Pro-, basophile and polychromatic erythroblast). Next we want to investigate the influence of ADAR1 on proliferation and enucleation of erythroid progenitors by inhibition of ADAR1 mRNA using an antisense-technique.

SP106

Morphology of mitochondria and c-kit of peripheral collected hemopoietic stem cells

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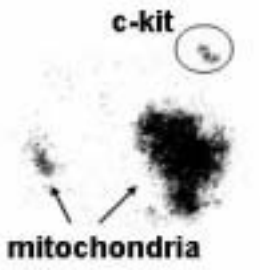
Purpose: Binding of stem cell factor (SCF) to c-kit can initiate intracellular Ca2+ release. Mitochondria are cellular Ca2+ buffer and play an important role in regulation of survival / apoptosis of hemopoietic cells. In excitable cells it has been shown that mitochondria are located on sides of Ca2+ release. In order to get more insight into mitochondrial location and activity and effects of progenitor cell mobilization in hemopoietic progenitor cells, we investigated the mitochondrial network of peripheral collected CD34+ hemopoietic stem cells (HSC) and the distribution of c-kit on the cell surface of these cells. **Methods:** Laser scanning confocal microscopy in combination with different fluorescent probes was used to generate images from HSCs. Mitochondria were stained with mitotracker green. CD117 (c-kit) was imaged with a PE marked monoclonal antibody. Image stacks were generated for individual HSCs. Independent mitochondrial or c-kit clusters were identified, the maxiumum size in the xy-direction (horizontal) and in the z-direction (vertical) was analysed. **Results:** 2 to 3 independent mitochondrial networks can be detected in HSCs. Normally there is a big network with a maximum extension in the xy-direction of 5.2±1.5 µm and 7.5±2.0µm in the z-direction. The smaller networks might contain only one mitochondrium and are 1.6±0.8µm (xy-direction) and 3.3±1.4µm (z-direction) in size. CD-117 is expressed in clusters on HSCs, an average of 4.2±2.4 such c-kit clusters can be detected per cell and are not associated with the mitochondrial networks. The picture shows an (xy-direction) image of 3 mitochondrial networks of a HSC, the circle marks the location of c-kit.



Conclusion: C-kit is expressed in clusters in HSCs and shows no colocalization with the mitochondrial networks of HSCs.

SP2: Immunohematology I – Erythrocyte Antigens and Erythrocyte Antibodies

SP201

The Blood Stocks Management Scheme in the United Kingdom

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In April 2001, the National Blood Service (NBS) of England and North Wales and the hospitals it supplies set up a national system for monitoring the blood supply; the Blood Stocks Management Scheme (BSMS). Data collection centres on a Structured Query Language (SQL) database, in which various data are stored on issues, inventory wastage and shelf life. Data are entered onto the database either through downloads from computer systems or manual data entry via the BSMS website (www.bloodstocks.co.uk). The

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web deployment of this system gives flexibility in terms of multi-user access for the input and extraction of data and information. Using the web, standard reports can be generated automatically from the data in real time allowing tactical use of the information on a day to day basis. Participating hospitals and all blood centres can access real-time graphical displays including wastage, shelf life of stock, and the number of day's worth of stock held. Graphs show the average of individual hospitals and all participants and the data are anonymised. These graphical displays act as the driver for change as hospitals and blood centres can compare their performance to their peers.Yearly inventory practice surveys capture information on issues related to stock management practice, the findings of which can be linked to the submitted stock and wastage data giving a comprehensive view of stock management practice. In April 2004 hospitals and the blood centres in Wales and Northern Ireland joined the scheme. Currently 273/313 hospitals are registered covering 93% of red cell issues. While still in its infancy, the BSMS has generated much information in two years on hospital demand and red cell inventories and red cell wastage in both the NBS and hospitals and has for the first time enabled integration of blood centre and hospital stock management. It has been a valuable resource for inventory monitoring, strategic planning and a driver for change in practice.

SP202

JAHK (Rh53) antigen is caused by a single mutation in exon 3 of THE *RHCe* gene

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Purpose: The JAHK antigen was first described in 1995 as a low prevalence antigen. Family studies reported in 2002 confirmed the association with the Rh blood group system and the Rh number was allocated (RH53). Though the association with the rG phenotype was described, so far the molecular basis has not been known. Serological findings indicated the location of the JAHK antigen on the RhCE protein. **Methods:** The *RHCE* gene of 8 samples from 3 unrelated families was analyzed by exon amplification and direct sequencing. Four of the samples were JAHK+, the remaining 4 were JAHK-. In the first JAHK+ sample the entire *RHCE* gene was sequenced. The remaining samples were analyzed for exon 3.RESULTS: Analysis of the entire *RHCE* gene of one JAHK+ sample showed the expected CcEe specific nucleotide sequence and revealed an additional single nucleotide change in exon 3. This change represented a missense mutation (C365T), which led to an amino acid substitution of serine by leucine at position 122 of the RhCE protein. Of the remaining 7 samples from 2 unrelated families, the three JAHK+ samples showed the identical mutation (C365T) and confirmed the correlation of the Ser122Leu exchange with the JAHK+ phenotype. **Conclusions:** The molecular basis of the JAHK antigen (RH53) is probably defined by a mutation (C365T) in exon 3 of the *RHCE* gene leading to the amino acid substitution Ser122Leu in the RhCE protein. Because the amino acid 122 is located in the transmembrane region of the second loop, the substitution of the polar serine by the nonpolar leucine seems to cause the JAHK antigen by a conformational change of the RhCE protein.

SP203

RHD genotyping for quality control of D negative RBC units

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Purpose: Standard serology is limited in detecting some weak D and chimeric red blood cell (RBC) populations. This limitation may be overcome by genotyping, thus potentially enhancing clinical safety. We evaluated the utility of *RHD* genotyping for the quality control of D negative RBCs. **Methods:** In 2002 and 2003, we examined all 9,930 serologic D negative first time donors in Ulm and, for 5 months in 2003, all 5,115 D negative donors in Linz. Samples were tested in pools of 20 donors by PCR-SSP for *RHD* intron 4 or for *RHD* exons 4, 7 and 10, respectively. The molecular bases of the *RHD* positive samples were resolved. The full length nucleotide sequence of the 10 exons was determined for all new *RHD* alleles. **Results:** 21 *RHD* positive samples (0.21 %) from Ulm, and 18 *RHD* positive samples (0.35 %) from Linz were found among the D negative donors. A total of 9 *RHD* alleles were novel: *RHD*(T201R, F223V, P291R) dubbed weak D type 4.3, weak D type 32, *RHD*(del147), *RHD*(del343), *RHD*(del449), *RHD*(del785), *RHD*(L153P), *RHD*(Y269X) and *RHD*(IVS3+2T>A). 15 samples from Ulm represented 7 known *RHD* alleles, most often *RHDΨ* (n = 5), *RHD*(IVS3+1G>A) (n = 4) and *RHD*(M295I) in *CDe* (n = 2); 7 samples from Linz represented the 3 known alleles *RHD*(IVS3+1G>A) (n = 4), *RHD-CE*(2-9)-*D* (n = 2) and *RHD*(M295I) (n = 1). **Conclusions:** *RHD* genotyping of serologic D negative donors at two different facilities revealed carriers of the *RHD* gene in the range of up to 1:285. Several of the observed *RHD* alleles were shown or known to express weak D. Weak D positive RBC units are likely capable of causing at least secondary anti-D immunization. This adverse clinical outcome can be avoided by only one *RHD* genotyping for each donor. Use of *RHD* genotyping would obviate the need to tightly control the sensitivity of serologic ant-D testing.

SP204

Evidence for recombinations, gene conversions and point mutations in the *Rhesus boxes*

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Purpose: In the common *RHD* negative haplotype, a recombination of upstream and downstream *Rhesus box* resulted the deletion of the *RHD* gene and the formation of a hybrid *Rhesus box*. PCR methods have been developed to detect the presence of the hybrid *Rhesus box*, thus differentiating *RHD* homozygous and heterozygous samples. Recent data indicated that aberrant *Rhesus box* sequences may confound this typing approach. However, a systematic analysis of the variability of the *Rhesus boxes* among *RHD* alleles was lacking. **Methods**: Samples representative of alleles for all four *RHD* clusters (*Ccde*^{s}, *DIII type 4*, *DIVa*, *weak D type 4.0* to *4.2*, *RHDΨ* , *DAU-0* to *DAU-4*, *DVI type 1* and *type 3*, *DIV type 3*, *weak D type 1* and *type 2*) were investigated. DNA segments of more than 6,000 bp representing the upstream and downstream *Rhesus boxes* were sequenced. These DNA segments included the *RHD* deletion site and many adjacent polymorphisms of diagnostic importance. **Results**: The previously described differences between the upstream and the downstream *Rhesus box* were confirmed. Several differences were found to be restricted to a subset of *RHD* alleles. More than 40 additional polymorphisms were detected that were characteristic for a single allele or a group of alleles. Evidence of gene conversions was found in the *downstream Rhesus boxes* of *Ccde*^{s}, *weak D type 4.1*, *type 4.2* and *DAU-0* and in the *upstream Rhesus boxes* of *RHDΨ* , *DAU-1* and *DAU-3.* The gene conversions started generally at the identity region and extended into the 5' or 3' direction. *Rhesus box* sequences of several alleles, e.g. in DIII type IV, were best explained assuming recombinations between different *RH* alleles. **Conclusions:** The genetic variability at the *Rhesus box* is considerable and was formed by point mutations, gene conversions and interallelic recombinations. The accrued data allow a more knowledgeable selection of polymorphism for the discrimination of *upstream* and *downstream Rhesus box*-like sequences. They may well be instrumental for the development of improved methods for the detection of the *hybrid Rhesus box*.

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SP205

Warm autoimmune hemolytic anemia reveals a regulatory role of IgG2 in IgM-IgG immune complexes of normal human plasma

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Purpose: The different physicochemical and sterical properties of IgG subclasses might favour a selective enrichment of IgG subclasses in IgM-IgG immune complexes (IC) under physiological conditions. Such selective enrichment of IgG subclasses in IgM-IgG IC of plasma might differ from the normal IgG subclass distribution in plasma itself, and contribute to the physiological functions of IgM-IgG IC. Systematic studies on the IgG subclass distribution in IgM-IgG IC are lacking. We here report on the IgG subclass distribution in IgM-IgG IC under various conditions. **Methods:** IgM-IgG IC were purified from plasma of 10 healthy blood donors, 10 blood donors exhibiting a common cold, 6 patients with acute cold agglutinin disease (CAD) due to monoclonal B cell proliferation, and 10 patients with warm autoimmune hemolytic anemia (WAIHA) using FPLC-based protocols. Immunoglobulin concentrations and subclasses were determined by ELISA and by radial immune diffusion. Peripheral blood mononuclear cells (PBMNC) were derived by metrizoite-Ficoll centrifugation from blood samples of healthy blood donors. Binding of PBMNC to IgM-IgG IC was analysed under real-time conditions using optical biosensor technology. **Results:** IgG2 is the predominant IgG subclass in IgM-IgG IC under physiological conditions and in a variety of diseases except for WAIHA. Normal PBMNC bind to IgM containing IgM-IgG IC of normal human plasma significantly better than to IgM devoid of IgM-IgG IC ($p < 0.0293$). IgM-IgG IC containing predominantly IgG2 mediate IgM-PBMNC interaction more efficiently than IgM-IgG IC containing IgG3, as shown using IgM-IgG IC derived from plasma of patients with WAIHA ($p < 0.05$). **Conclusion:** IgG2 is the predominant IgG subclass in IgM-IgG IC under physiological conditions. The IgG subclass distribution in IgM-IgG IC is of critical influence for the interaction between the immune complex and peripheral blood cells involved in antigen presentation and processing. The data presented here therefore extend the physiological function of IgG2, which is the protective immune response toward carbohydrate antigens in bacterial infections, and suggest IgG2 dependent regulation mechanisms for immune responses to self-immunoglobulin under physiological conditions in humans.

SP206

DGTI Workshop 2003 for the molecular diagnosis of red blood cell, platelet and granulocyte alloantigens

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Typing of alloantigenic polymorphisms on red blood cells, platelets and granulocytes by molecular methods has been established during the last years and is now used in many laboratories. Currently, a large variety of test protocols and a low grade of standardisation exist. We therefore performed a series of external proficiency controls under the patronage of the German Society of Immunohaematology and Transfusion Medicine (DGTI). In the 2003 workshop a total number of 41 laboratories from 5 countries participated in different categories: red blood cells 31, platelets 36, granulocytes 10. Blood donors were carefully selected according to their ABO, RHD, RHCE, KEL, FY, JK; HPA-1, -2, -3, -5 and HNA-1a, -b, -c alleles. Genomic DNA was isolated from peripheral blood leukocytes or from non-human source for 1 sample by standard procedures. The selection of the typing method was left to participants. Results were reported on standardised questionnaires. The error rates were as follows: Red blood cells: ABO: 20/312 (6.4%), RHD: 28/162 (17.3%), RHCE: 41/648 (6.3%), KEL: 11/240 (4.6%), FY: 15/216 (6.9%), JK: 10/216 (4.6%). Platelets: HPA-1: 17/504 (3.4%), HPA-2: 11/476 (2.3%), HPA-3: 11/462 (2.4%), HPA-5: 15/462 (3.6%). Granulocytes: HNA-1a: 0/120, HNA-1b: 0/120, HNA-1c: 0/48. Samples from donors with the rare alleles R0Har and weak D Type 2 induced the highest number of mistypings. The FY*X was frequently typed as FY*B allele. Non-human DNA in a sample for platelet antigen typing and a sample for red blood cell typing that contained artificial protein contamination did not cause considerable interpretation problems. 24 participants (58,5%) could be certified in all workshop categories and 9 laboratories (37.5%) in more than 80% of the categories in which they had participated. In 8 laboratories (19,5%) serious typing errors occurred (less than 80% certified [6], no category certified [2]). In conclusion, genotyping as a standard technique for the determination of red blood cell, platelet and granulocyte alloantigens has reached a high level of quality. However, compared to former workshops (1998-2002) the frequency of typing errors has increased which parallels an increase of the number of participants. Thus, our analysis clearly demonstrates that high quality genotyping can only be established and maintained by external proficiency testing. Further developments of typing techniques will allow us to include other important alleles in future workshops.

SP3: Experimental Hemostaseology

SP301

Perspectives of anticoagulant therapy

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For many years anticoagulant therapy has been very simple. Primary and secondary prevention of arterial thromboembolic disease was managed by platelet inhibition with acetylsalicylic acid. Prophylaxis and treatment of venous thrombosis was done with unfractionated heparin in the acute phase and vitamin K antagonists, i.e. coumarins, for long term therapy. However, side effects and other disadvantages of these compounds have induced development of new drugs. Characteristics of an ideal anticoagulant include fixed dosing, independency of autologous coagulation inhibitors, no interactions with foods and other drugs, variability in the route of administration (i.e. oral, s.c., i.v.), minimal need for monitoring as a result of broad therapeutic range and availability of an immediately reacting antidote. Some of these properties have been realised with introduction of fractionated heparins, danaparoid and the synthetic pentasaccharid fondaparinux (longer half life, less/no monitoring, less heparin-induced thrombocytopenia, fixed dosing in case of fondaparinux). Shortening of polysaccharide chains of heparin resulted in a more selective inhibition of factor Xa, which revealed favourable for many patients. Hence, standard treatment for thromboprophylaxis has changed within the last decade, increasingly replacing unfractionated by low-molecular-weight heparins. Introduction of direct thrombin inhibitors such as hirudins and ximelagatran/melagatran have added further advantageous properties to antithrombotic treatment, allowing inhibition of free and fibrin-bound thrombin independently of antithrombin. However, lack of specific antidotes remains as one problem with all the newer drugs. Conflicting interests will determine future anticoagulant strategies. On the one hand, diversification of the drugs enables individually tailored therapy taking into account various aspects of the patient's circumstances and risk profile. On the other hand, in clinical routine therapeutic concepts are desired to be as simple as possible, at best, fixed dosing, large therapeutic width, no monitoring, variable application forms, and all this at low costs.

SP302

Significance of Molecular Genetic Tests in Blood Coagulation

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Genetic tools as PCR, mutation screening and sequencing nowadays allow routine diagnostics tests of genes in blood coagulation. Either single polymorphisms or complete genes can be analysed within hours to days. In thrombophilic conditions genetic tests allow to assess the hereditary contribution to thromboembolic events for a more safe prediction of risk figures and subsequently better therapeutic decisions on dosages and duration of anticoagulant treatment. In haemophilia A, the underlying gene mutations provide information on the expected clinical course of the patients, espe-

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cially for the severe complication of inhibitor development. In a number of further clinical conditions as bleeding, wound healing and recurrent abortions analysis of the FXIII and fibrinogen genes might be beneficial for achieving a clear diagnosis. Pharmacogenetics represents another fast developing field. Polymorphisms in several genes (CYP2C9, VKORC1, PTSG1, P2Y12) are causing increased sensitivity, increased resistance or other side effects of haemostasis related drugs as Coumarins, ASS or Clopidogrel. In such cases genetic analysis can prevent side effects by adjusting dosages or switching the treatment mode. While phenotypic tests show an actual situation of blood coagulation, genetic tests reveal an individual characteristic that will not change over life. Although this result is expected to be beneficial for the individual with respect to health management it may also bear personal and social implications. Therefore, these tests require a specific and safe management with respect to clear indications, detailed information of the individual before performing the analysis and competent counselling on the results and the related consequences. Keeping this in mind, genetic tests may provide important, complementary information to the classic phenotypic assays.

SP303

Analysis of factor VIII trafficking in living cells using FVIII/EGFP fusion proteins

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Purpose: Yield of FVIII obtained by recombinant expression systems is 2-3 of magnitude lower than that observed with other genes using similar vectors and approaches, which is – at least in part – attributed to its impaired secretion. For the analysis of intracellular trafficking of FVIII protein in living cells, three different FVIII/EGFP fusion proteins exhibiting a FVIII full length protein (FL), a B-domain deleted FVIII (FVIIIdelB) and FVIII with a mutation in the C2-region (FVIIImut2315), have been constructed. The impact of EGFP-fusion on the recombinant expression, procoagulant activity and intracellular processing of the FVIII protein was analyzed. Methods: Fusion proteins of FVIII, FVIIIdelB and FVIIImut2315 along with EGFP have been generated and were further cloned into pcDNA3 expression vector under the control of the CMV-promotor. FVIII expression and activity was assessed in transfected 293T cells and compared to levels of the respective parental FVIII cDNA. FVIII antigen (FVIII:Ag) was determined using an ELISA (American Diagnostics, Greenwich). FVIII activity (FVIII:C) was analyzed using a chromogenic assay (Baxter, Vienna). Expressed fusion proteins were immunoprecipitated and westernblotted using EGFP specific antibodies. Results: Despite the fusion of EGFP to the FVIII protein, the transfection yielded comparable FVIII protein levels for all three variants compared to the non-fused FVIII proteins (8,9 % FVIII/EGFP versus 7,7% FVIII; 13,6 % FVIIIdelB/EGFP versus 17,7 % FVIIIdelB and 8,5 % FVIIImut2315/EGFP versus 8,5 % FVIIImut-2315). Interestingly, the fusion to EGFP seems not to have an impact on the coagulant activity of the factor VIII protein, as assessed by the FVIII:C/FVIII:Ag ratio (0,86 FVIII/EGFP; 0,92 FVIIIdelB/EGFP; 0,83 FVIIImut2315/EGFP). Westernblot analysis shows correct intracellular processing of fusion proteins. Conclusion: Expression of FVIII along with EGFP seems not to alter the expression level, procoagulant activity or intracellular processing. Hence, FVIII/EGFP chimeras appear to be suitable to further analyse the secretion pathway of FVIII in living cells. This work should ultimately allow the identification of factors that currently limit the effective secretion of FVIII in gene therapy and manufacture.

SP304

Screening for hereditary hypercoagulability using the amplification refractory mutation system

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Purpose: A number of genetic factors that contribute to the development of deep vein thrombosis, cardiovascular disease and/or cerebrovascular disease have been characterized during the last decades. Identification of certain hereditary hypercoagulability factors, e.g. factor V Leiden (1691G->A), and prothrombin (20210G->A), is used as a tool for the stratification of patients into groups with different risks for recurrence of thromboembolic disease. Recently, many other hereditary factors, including ACE (intron 16 insertion/deletion), apolipoprotein E variants, factor V HR haplotype (4070 G/A), PAI-1 (-675 5G/4G), thrombomodulin (127G->A), TFPI (536C->T), and tPA (intron h deletion/insertion) were described to influence the risk for thrombotic events. The aim of this study was to design an ARMS-based screening method for the detection of these hereditary hypercoagulability factors. **Methods:** DNA samples were obtained from 196 patients and relatives of patients with thromboembolic diseases. Allele- and gene-specific primers were designed and the samples pre-tested by previously described techniques (AFLP, RFLP, ARMS) were analyzed blinded with respect to their genotypes using the novel ARMS-based method. **Results:** The results of genotyping were concordant in all cases. Most factors were tested for all possible allelic constellations. The only exceptions were thrombomodulin (127G/A) and TFPI (536C/T), where only wildtype homozygous and risk allele heterozygous samples could be tested. Samples homozygous for the risk allele (thrombomodulin: 127G->A; TFPI: 536C->T) were not available in either case. Until now, individuals homozygous for thrombomodulin (127G->A) or TFPI (536C->T) have not been described. **Conclusions:** The approach described here makes it possible to use an ARMS based technique for the screening for hereditary hypercoagulability. Using pre-aliquoted primer mixes, it takes only about 120 minutes to amplify all target hypercoagulability factors, with only 20 minutes of hands-on-work. We emphasize that the combination of genetic hypercoagulability factors tested is not invariably complete and can be varied. Each laboratory might adapt the range of the factors tested according to their specific requirements.

SP305

On the predictive value of laboboratory assays and diagnostic aspects of phospholipid antibodies (PLA)

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Background: Phospholipid antibodies are thought to be involved in the development of venous and arterial thrombosis. Prolongation of phospholipid dependent clotting reactions as well as thrombocytopenia might be associated with this laboratory phenomenon. Althoug described for the first time in 1963 (Bowie et al) its significance as a thrombophilic risk indicator is up to date unknown since data are lacking. We therefore aimed to a.) calculate the risk of thrombotic complications attributed with elevated phospolipid antibodies comparing healty individuals (group I) and patients with venous/arterial thrombotic diseases (group II) and b.) compare the antibody levels within both groups. Patients and Methods: 740 patients with venous (deep venous thrombosis, pulmonary embolism) and arterial (myocardial infarction, stroke, peripheral thromboembolism) thrombotic diseases (group II) were analyzed. Phospholipid antibodies (PLA Ig/-IgM - Asserachrome PA reagent, Diagnostica Stago, Asnières-Sur-Seine; normal range <10/<20 GPL - PLA IgM/PLA IgG), were controlled. These data were referred to those obtained by the control of 208 healthy individuals (group I). Results: APA IgG was found to be elevated in 171 thrombotic patients (18%) [mean: 25.78 +- 6.95] vs. 29 healthy indivudals (3.1%) [mean: 25.09 +- 5.51]. In contrast normal values for APA IgG were related to 569 (60.0%) thrombotic patients (60%) [mean: 11.21 +- 4.27] and 179 healthy people (18.9%) [mean: 11.28 +- 4.32]. The chi-square assay, as well as the Fisher

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assay - comparing group I/II with and without elevated phospholipid antibodies - resulted in high significance ($p < 0.004$). The calculated odds ratio and 95% confidence intervals were defined as 1.855 [1.209/2.846]; In contrary the Mann Whitney assay comparing the level of phospholipid antibodies in group I/II s resulted in no significance (group I: 14.58 +- 7.93; group II: 13.21 +- 6.57). Since only 4.9% of all thrombotic patients demonstrated elevated PLA IgM levels further considerations were omitted at this time. Conclusions: Elevated levels of phospholipid antibodies (PLA IgG) were found in 171/740 thrombotic patients (23.1%) which underline their significance in screening for thrombophilia. The calculated odds ratio implicates an up to threefold risk of thrombosis despite the mean values of both groups did not differ significantly. In a minority of patients PLA IgM was found to be abnormal. Further investigations will have to clarify if there is a clinically based ranking due to different levels of PLA and in association with aPTT prolongation.

SP306

Patient Risk Factors for developing Thromboembolic Complications in Heparin-Induced Thrombocytopenia – a survey of 408 patients

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Background: Immune mediated heparin induced thrombocytopenia (HIT) affects up to 2-3% of patients receiving heparin. Only a subgroup of patients developing HIT also develops thromboembolic complications (TEC). We aimed to identify risk factors for the development of HIT associated TECs. **Methods:** We retrospectively analyzed patients who had been clinically suspected to have HIT and who tested positive in a platelet function assay (HIPA test) in two laboratories. Patient information was obtained by a standardized questionnaire. **Results:** 408 patients were enrolled (58.9% female; age 0-19 years, 1.5%; 20-39, 6.9%; 40-59, 25.4%; 60-79, 57.3%; ≥80, 8.9%). 52.2% were treated in surgical departments, 35.8% in internal medicine, and 12% in other specialities. 227 (55.6%) patients developed 434 TECs (1.92/patient; venous:arterial, 2.4:1). Arterial TECs: limb artery (16.4%) > stroke (6.0%) > aortal thrombosis (3.7%) > myocardial infarction (2.3%). Venous TECs: venous proximal (26.4%) > pulmonary embolism (23.8%) > venous distal (18.1%) > sinus vein thrombosis (1.6%). Age and gender were no isolated risk factors to develop TECs. Orthopedic/trauma surgery was an independent risk factor for HIT associated TECs (OR 5.3; 95% CI 2.67–10.68; $p<0.001$). At the time of clinical diagnosis of HIT, a decrease in platelet counts of ≥ 30% occurred in 94.0% patients; median nadir 41.000/µl. The risk to develop a HIT associated TEC was directly correlated to the relative decrease in platelet counts (>50-70%, OR 3.6, 95%CI 0.95-13.86, p=0.06; >70%, OR 8.1, 95% CI 2.29–28.66; p=0.001). **Conclusion:** patients with HIT in orthopedic/trauma surgery and those with a pronounced decrease of platelet counts are at a high risk to develop HIT associated TECs.

SP4: Safety Aspects of Hemotherapy I – Viruses and Parasites /Pathogen-Inactivation

SP401

Screening for Hepatitis-B-Infection – the Past and the Future

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Hepatitis B-Virus (HBV) Infection is a serious health problem with 350 million persons infected world-wide and 15 million chronic carriers. The first screening assays introduced were ALT, a non-specific liver enzyme elevation as a surrogate test. After the detection of the Austrialia Antigen HBV in the late 60´s, the first specific HBS-Antigen Tests were introduced in the early 70`s. HBS-Antigen Tests were preferred as a screening parameter compared to antibody tests, because they indicate infectivity and antibodies are mostly protective. However, transmission occurred and transfusion-transmitted risks were calculated for Germany at 1:20,000 – 100,000. This exceeds the risk for the other transfusion-transmitted viruses like Hepatitis E and HIV significantly, because of the higher prevalence and potential carrier state, which is HBS-Antigen negative. German transfusion services were the first to introduce NAT testing for HBV in conjective with NAT for HIV and HCV. There was a substantial yield by HBV-NAT with respect to HBS-Antigen negative pre-zero converters as well as HBS-Antigen negative chronic carriers. However, a significant numbers of donors are missed because of the low-level viremia, especially in chronic carriers and the slow wrap-up in the pre-zero conversion window phase. Additional risks may emerge from HBS-Antigen escape mutants that are not covered by the present assays, which can also have mutations for present NAT-screening assays. Moreover, adrenotypes like Hepatitis-G may emerge in the population which also may pose a risk by a highly divergent sequence resulting in reduced sensitivity of HBS-Antigen assays as well as NAT assays. The introduction of Anti-HBC screening would significantly reduce the risk from tail-end carriers which are HBS-Antigen negative and have a viral load below the detection limit of most present NAT assays. However, the rate of those that are HBC-Antibody positive would be very high and therefore effect blood supply and introduced for all donors. Different strategies are considered e.g.: introducing Anti-HBC testing for first time donors only or intoducing Anti-HBC testing on a delayed scale. Present data would not support discontinuation of HBS-Antigen testing or HBV-NAT upon the introduction of Anti-HBC testing. Alternative approaches like vaccination of donors would be very cost intensive and not as efficient as sensitive screening. Only pathogen inactivation has the potential of further reducing the risk of HBV-infection by blood transfusion. However, costs are high and may not be justified in all countries. Whether new screening assays like immuno PCR or the chip technology may bring a breakthrough is still open, because these methods are not yet ready for routine application in blood bank environments.

SP402

In vitro reactivation of CMV infection in peripheral blood mononuclear cells of seropositive blood donors

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Purpose: The infection with Human Cytomegalovirus (CMV) develops a so-called lytic cycle which is followed by a phase of latency characterised by detection of CMV DNA in infected cells in absence of viral replication. CD34+ hematopoietic stem cells, monocytes and endothelial cells have been identified to be places of latency. Reactivation of CMV infection in latently infected patients after stem cell or organ transplantation may lead to a life-threatening CMV disease. CMV-seronegative and leukocyte-reduced blood products are used to prevent transmission of latently CMV-infected donor cells. The aim of this study was to investigate a possible in vitro reactivation of CMV infection in allogenically stimulated cells of latently infected healthy blood donors. **Methods:** Peripheral blood mononuclear cells (PBMC´s) of latently CMV-infected healthy blood donors were cocultivated with PBMC´s of another blood donor who was either also seropositive or seronegative for CMV. Cell cultures were maintained over 90 days, and every 3-4 days they were tested for CMV pp72 immediate-early antigen expression by means of immunoperoxidase staining. Culture supernatants were tested for CMV DNA using a newly developed TaqMan PCR and for CMV pp67 mRNA by NASBA following total nucleic acid isolation by means of NucliSens™ Extractor. Cytokine levels of supernatants were determined for neopterin, interferon-alpha and -gamma as well as for tumor necrosis factor-alpha. **Results:** During the whole period of 90 days, we observed reactivations of CMV infection in every cell culture (n=9). The earliest reactivation was on day 19, the latest occurred on day 72. At the time of reactivation, CMV pp72 antigen was visible in every culture. So far, CMV DNA and pp67 mRNA were detected in supernatants of 2/3 and of 1/3 cultures, respectively. For the cytokine levels determined in culture supernatants, characteristic courses in relation to the reactivation of CMV infection have been observed. **Conclusions:** In long-time mixed PBMC cultures of latently CMV-infected healthy blood donors, reactivation of CMV infection was detected in every culture. These experiments may be a useful tool for further research on the mechanisms of CMV reactivation and on possible inhibitory agents.

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SP403

NAT screening of SARS-CoV improve blood safety - Comparison of two real time quantitative SARS CoV assays

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Background: The new SARS corona virus, described in February 2003, infected a total of 8439 people. Close contact to symptomatic patients appears to be the main route of transmission. However potential transmission by blood transfusions could not be definitely excluded. **Material/Methods:** Two real time SARS PCR assays were assessed for sensitivity, agreement of test results and intra-assay variability. Both assays rely on reverse transcription and amplification of extracted RNA. Dilutions of gamma-irradiated cell culture supernatants of SARS-CoV infected Vero E6 cells, were prepared to determine the precision, linear range and accuracy of the assays. Further we tested 31,151 blood donor samples in mini pools of up to 96 samples. **Results:** The detection limit of the Roche-assay was 3,982.1 copies/mL whereas the Artus-assay detected 37.8 copies/mL. None of 31,151 blood donors were positive for SARS. Two 96 member plasma pools that were each spiked with 100µl plasma of the German index patient or his wife, respectively, were positive. **Conclusion:** Both assays are suitable for quantitative measurement of SARS CoV in high concentrations as expected in sputum samples. The RealArt™ HPA-Coronavirus© assay is also suitable for detecting the SARS virus in low concentrations as found in plasma samples. Therefore screening of blood donations for SARS CoV could potentially prevent transfusion associated transmission.

SP404

Helinx® Technology Inactivates High Titers of SARS-CoV, WNV and Vaccinia in Platelet Concentrates

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Background: Helinx technology, as used in the INTERCEPT Blood System, was developed to address the problem of pathogen contamination, especially bacterial contamination, of platelet concentrates (PC). The technology utilizes amotosalen HCl and UVA illumination, and has been demonstrated to inactivate a wide variety of blood-borne pathogens in platelets, including enveloped and non-enveloped viruses, gram negative and gram positive bacteria, and parasites. Recent investigations evaluated the efficacy of this treatment against viruses of emerging concern in the blood supply: the agent of severe acute respiratory syndrome (SARS-CoV), West Nile virus (WNV) and vaccinia virus (VV). **Methods:** For SARS-CoV and VV studies 30 mL platelet aliquots were prepared from single donor apheresis platelets. The WNV studies utilized full-size (~300 mL) PC units. PC collected in 37% plasma/63% platelet additive solution (PAS III) were inoculated with virus to a final concentration of ~10^6 pfu/mL, then treated with 150 µM amotosalen HCl and 3.0 J/cm^2 UVA. SARS-CoV and WNV were treated with a single 3.0 J/cm^2 UVA treatment, VV was treated with a single 1.0 J/cm^2 and cumulative 2.0 J/cm^2 and 3.0 J/cm^2 UVA treatments. Samples were taken prior to illumination to determine input titer and immediately after illumination to detect residual viable virus. Titers were determined by plaque assay on Vero (WNV and VV) or Vero E6 cells (SARS-CoV). **Results:** Inactivation was expressed as the mean log reduction for replicate experiments. A standard deviation was not calculated for the log reduction of SARS-CoV because only two experiments were performed.

Inactivation of Viruses of Emerging Concern in Platelet Concentrates

Virus	Strain	Number of Replicates	Log Reduction (Mean ±SD)
SARS-CoV	Urbani	2	>6.3
WNV	Lineage 1 (pFLWNV)	4	>6.0 ±0.4
Vaccinia	IHD-W	3	>5.2 ±0.2

Conclusions: SARS-CoV, WNV, and VV in platelet concentrates were inactivated to below the limit of detection by treatment with 150 µM amotosalen HCl and 3.0 J/cm^2 UVA.

SP405

Removal Infectious Prion From Red Cell Concentrates

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BACKGROUND: Prion diseases are fatal neurodegenerative diseases that affect both humans and animals including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob Disease (CJD)in humans. The occurrence of a variant CJD in the United Kingdom together with the recent animal data and the evidence that the distribution of the vCJD may differ from the classical CJD has raised the question of the possibility of transmission of the causative agent by blood transfusion from infected individuals with no clinical symptoms of the disease. In the present study, we evaluated the use of a prototype of a new leukocyte reduction filter for the removal of abnormal prion from RCC. **STUDY DESIGN: In vitro Spiking Studies:** Units of whole blood (450±50) were collected from healthy volunteers into blood bags containing 63mL of CP2D anticoagulant. RCC were prepared from whole blood and then resuspended in AS3 additive solution according to standard blood bank protocol. Ten Percent (10%) scrapie brain homogenates (SBH) in buffered saline was prepared from brains of hamsters infected with 263K hamster-adapted scrapie (PrPsc). The SBH was clarified by ultracentrifugation and about 30mL of the SBH was added into 270mL of RCC. The SBH-contaminated RCC was filtered at room temperature with a prion removal filter (Pall Medical, East Hills, NY). The presence of SBH in the RCC was determined before and after filtration using a Western blot assay with 7G5 prion specific monoclonal antibodies. In addition to the Western blot, bioassay was performed in which different dilutions of aliquot of the pre and post filtration scrapie contaminated RCC were injected through the intracerebral route into scrapie susceptible hamsters. **Endogenous Infectivity Study:** Blood samples were collected into CP2D anticoagulant from scrapie infected hamsters. The blood sample (500mL) was processed into RCC. About 300mL of the RCC in AS3 additive solution were filtered with a prion removal filter (Pall Medical, East Hills, NY). The levels of infectious prion in the RCC before and after filtration were measured with a Western blot assay. **RESULTS:** Preliminary results showed removal of the scrapie prion from full units of RCC to a level well below the limit of detection of the assay at 2 logs. After 300 days, none of the animals injected with filtered PrPsc-contaminated RCC have developed any clinical symptoms of the disease. The concentration of residual leukocytes in all the filtered units of RCC was reduced to less than 1 x 10^6 leukocytes per unit of RCC. **CONCLUSION:** The present results show that prototype of a new leukocyte reduction filter was effective in removing infectious prion from RCC below the limit of detection of Western blot assay and none of the hamsters injected with filtered RCC have developed the disease. The use of this type of filter may help reduce the risk of vCJD through blood transfusion.

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SP406

PCR-Based Documentation of the Photochemical Pathogen Inactivation procedure for Platelet Concentrates

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PURPOSE: The photochemical pathogen inactivation method "INTERCEPT Blood System" for platelets (Baxter Healthcare Corporation), based on the Helinx technology, using 150 µM amotosalen and a 3 Joules/cm^2 UVA treatment has been shown to inactivate a broad spectrum of organism and leukocytes. Inactivation is achieved because covalent adducts between amotosalen and genomic nucleic acids are present and thereby blocking replication. Actually this process can only be monitored indirectly by documentation of the amount of UVA light applicated on the platelet concentrate (PC). Using mitochondrial DNA (mtDNA) we evaluated a polymerase chain reaction (PCR) inhibition assay to monitor the success of photochemical treatment (PCT) directly on its target. **METHODS:** Buffy coats (BC-PC) or single donors platelet concentrates (SD-PC) were prepared according to routine blood bank manufacture procedures and treated with PCT. Platelet mtDNA was prepared using the Qiagen Mini Blood Kit. Using GenBank reference sequences, the PCR amplification DNA targets were designed within a regions on conserved mtDNA to avoid polymorphisms: 16S rRNA, Cytochrome C oxidase I or Cytochrome C oxidase III. PCR amplification conditions were optimized for a Monoplex and Multiplex system reflecting the pathogen inactivation process by its PCR inhibition. **RESULTS:** The PCR inhibition assay includes two primer systems: The first generating a product short enough to be essentially transparent to the PCT process (internal control). Regardless the efficacy of the photoreactive process, this PCR product will always be detected under a defined set of amplification conditions. Amplicons ≤ 300 bp were found to suitable for it. The second generating a product long enough to be affected by PCT. Amplicon sizes between 868 – 1,248 bp gave consistent signal without PCT and complete inhibition of the PCR signal with PCT. **CONCLUSIONS:** PCT induced DNA modifications of platelet mtDNA can be employed for a PCR assay that documents the amotosalen-UVA PCT process. DNA sequences as well as PCR amplification conditions can be selected to provide a direct monitoring of the success of PCT process.

SP5: Collection and Processing of Hematopoetic Stem Cells

SP501

Collection and Processing of Hematopoietic Stem Cells

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In Germany 1407 allogeneic and 2333 autologous hematopoietic stem cell (HSC) transplantations were registered in the year 2003. Only 9 (= 0.4 %) of the autologous and 314 (= 22 %) of the allogeneic grafts were prepared from bone marrow harvests (BM). The remaining transplants were peripheral blood progenitor cells (PBPC) collected by apheresis following G-CSF mobilization. The preferential use of PBPC reflects the advantage of PBPC for the donor and the recipient in comparison to BM. The percentage of unrelated donors was 57 % and is still rising, whereas umbilical cord blood as an alternative source of transplantable HSC is yet to find it's place in the future. Despite a recent EBMT study showing a higher number of severe adverse events in PBPC donors (0.09 %) compared to BM donors (0.04 %), G-CSF mobilization and stem cell collection by apheresis are standardized and safe procedures. No significant difference was seen in the incidence of hematological malignancies following PBPC or BM donation (0.05 % vs. 0.03 % respectively). However, an international collaborative long-term follow-up of HSC donors is necessary to define the precise risks of donation. Further processing of collected stem cells has become routine to improve the outcome of autologous and allogeneic transplantation. For example, immunomagnetic preparation of stem cells like CD34 positive selection is a highly efficient technique to reduce tumour cell contamination of autografts or to deplete T-cells for GvHD-prophylaxis in allogeneic transplantation. Such manipulations of stem cells have to be performed under clean room conditions according to the GMP-guidelines for the prevention of microbial contamination. A purity and homogenicity up to 99 % would allow genetic modification of these cells and opens new therapeutic strategies even for the repair of non-hematopoietic tissue. Monitoring of all steps from the initial G-CSF mobilization to the final HSC transplant should guaranty the safety of the donor and the quality of the stem cell product. These issues are essential for the further development of stem cell collection and processing.

SP502

Side effects of peripheral blood progenitor cell (PBPC) mobilization and collection - long term follow-up of 1125 unrelated donors

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Purpose: Peripheral blood progenitor cells (PBPC) are increasingly used as an alternative source of hematopoietic stem cells for allogeneic transplantation, but information about donor safety and follow up is still limited. **Methods:** 1125 allogeneic unrelated PBPC-donors received G-CSF (lenograstim) subcutaneously at a dose of 7.5µg/kg/d for 5-6 days. Leukaphereses (Cobe Spectra, GAMBRO BCT, Lakewood, Co.) were started on day 5. A baseline investigation was performed 2-4 weeks before leukapheresis and follow-up investigations were carried out 4 weeks, 6 months and 1 year after PBPC collection. **Results:** 4 weeks after leukapheresis, WBC and absolute neutrophile counts (ANC) declined significantly below the baseline values. During follow up, WBC and ANC normalized but did not return completely to the initial values. Lymphocyte counts returned to the initial values after 6 months. In donors undergoing 2 leukaphereses, lymphocyte counts remained significantly below baseline values up to 1 year after PBPC donation. Platelet counts decreased significantly from 245 x 109/L to 235 x 109/L after G-CSF administration, decreased further by about 50% after the first, by about 30% after the second PBSC collection. Five months after stem cell collecton, platelet counts reached baseline values. During G-CSF administration, the majority of donors (91%) complained of bone pain, headache occurred in 43% and flu-like symptoms in 8.5%. During leukapheresis, 80% of the donors had paresthesias, 6% nausea and 14% symptoms of hypotension. No serious short or long term clinical side effects of G-CSF administration or leukapheresis were reported. **Conclusions:** Our analysis of 1125 donors revealed that clinically apparent side effects of G-CSF administration and leukapheresis were mild and resolved rapidly in the majority of donors after discontinuing G-CSF-application. Changes in blood cell counts were minimal but the decrease in ANC and WBC lasted for several months. Monitoring and long-term follow up of all healthy PBPC-donors has to be continued prospectively to guarantee the quality standards of PBPC mobilization and collection.

SP503

Higher CD34+ Cell counts in poor mobilizers through every-other-day HPC-collection

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Purpose: Collecting satisfying autologous transplants is often hard in poor mobilizing patients. They will undergo repeated aphereses and be exposed to excessive amounts of DMSO when receiving transplants with high nucleated cell but low hematopoetic progenitor cell (HPC) counts. To evaluate a different apheresis strategy, the CD34+ cell precounts of an every-day harvest schedule were compared to those of an every-other-day collection regimen within the same patients. **Methods:** Thirteen patients suffering of advanced stages of hematologic malignancies had been treated with standard chemotherapy followed by G-CSF 5-10mcg/kg/die. For each patient the circulating HPC counts on every-day collections were compared to those following a one or two-day harvest free interval. 5 out of these 13 patients returned to an every-day apheresis rhythm for logistical reasons. Peripheral HPC counts were evaluated by dual-platform analysis on a BD FACSort-analyser using

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the CELLQuest software and the analyzer Abbott CellDyn 3200. **Results:** Circulating CD34+ cell counts showed an average of 9.8 +/- 4.8 c/mcl before collection on day one and 9.5 +/- 5.3 on day two before the following second collection. After a one or two day harvest free interval a statistically significant increase to 15.2 +/- 6.9 circulating CD34+ c/mcl was observed (Wilcoxon-rank $p<0.01$). For logistical reasons 5 of these patients returned to an every-day apheresis schedule showing 6.5 +/- 2.9 CD34+ c/mcl before collection interval, 13.5 +/- 6.7 CD34+ c/mcl after the one-day collection interval (Wilcoxon-rank $p<0.05$) and a significant drop to 8.5 +/- 4.0 CD34+ c/mcl on the following day after an every-day apheresis schedule was resumed (Wilcoxon-rank $p<0.05$). **Conclusions:** There seems to be a slow release of CD34+ HPC in poorly mobilizing patients and an accumulation of HPC in the peripheral blood. An every-other-day harvest schedule leads to significantly higher CD34+ cell precounts and improved collection conditions. This benefit is not a consequence of prolonged mobilization since a return to an every-day apheresis strategy is paralleled by a drop in circulating HPC.

SP504

Platelet derived microparticles (PMP) influence migration behaviour of hematopoietic progenitor cells (HPC)

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Purpose: We wished to investigate if small particles derived from platelet membranes (PMP) which are present in blood may influence migration of HPC. **Methods:** PMP were prepared from either random donor pooled platelet concentrates (PPC) after activation with PAR-1 agonistic peptide SFLLRN (TRAP), or from anticoagulated rat blood by two different centrifugation methods. PPCs were centrifuged at different centrifugation speeds at 800, 2000 and 5000g by pelleting PMPs from 2000g supernatants at 5000g, or by repeated (3x) centrifugation of PPC at 1125g from platelet rich plasma (PRP). Rat PMPs were prepared from PRP of anticoagulated blood by a 4500g pelleting step. The quality of the PMPs was assessed by flow cytometric analysis using anti-human CD41 and anti-human CD61 monoclonal antibodies. Stromal Derived Factor 1 (SDF-1) induced chemotaxis was elicited in FDCP-mix murine hematopoietic progenitor cells in transwells through 5µm pore membranes. **Results*:*** Thrombocytes were quantitatively depleted and PMP were enriched up to 15 fold in the 5000g pellet. Flow cytometric analysis separated the typical scatter profiles of large thrombocytes and small PMPs. Like the thrombocytes, PMPs were highly positive for CD41 and CD61. Rat PMPs bound to the cytoplasmic membranes of FDCP-mix cells but not to human CD34 positive cells, showing high species specificity. SDF-1-induced migration of FDCP-mix cells was reduced ca 10-fold in the presence of PMPs, regardless whether the PMPs were in the upper or lower compartment of the transwell. **Conclusions:** We demonstrate a method to purify PMPs to high homogenicity. The membrane microparticles can bind to hematopoietic progenitor cells (HPC) and inhibit their SDF-1 induced migration. PMPs may therefore be part of a physiological mechanism which protects circulating hematopoietic stem cells from entrapment into activated coagulation pathways.

SP505

Whole blood derived stem cell support in patients with small cell lung cancer under intensified chemotherapy: A laboratory analysis

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PURPOSE: Intensified chemotherapy in combination with autologous stem cell support represents a new treatment option in small cell lung cancer (SCLC). A prospective randomized study (standard [ST] versus intensified chemotherapy arm with stem cell support [IT]) has been performed with a particular focus on the effectiveness of autologous whole blood as a cost efficient stem cell source. **METHOD:** 6 cycles of chemotherapy (ICE scheme) were given either in 2 weeks (IT) or in 4 weeks (ST) intervals. Stem cells were mobilised through administration of G-CSF on days 4-14 of each IT cycle. 750 ml of whole blood were collected by venesection on day 1, cycle 2-6 (or day 14, cycle 1-5, see below). The collected blood product was stored for 3 days at 4°C and retransfused after chemotherapy. Stem cells were quantified by counting CD34+ cells via FACS analysis. **RESULTS:** A total of 83 patients were enrolled in the study (IT n=41, ST n=42). In the IT arm 17/41 patients (41%) received all 6 cycles of chemotherapy with 5 venesections. On day 1, cycle 2 (after first G-CSF mobilisation period), CD34+ counts in the patients of the IT arm varied interindividually over a large range: 3 - 719 CD34+ cells/µl (median: 150). In all patients individual CD34+-mobilisation capacity reached its maximum in either the 2nd or 3rd cycle. Initially, CD34+ cell counts during mobilisation reached a maximum on day 14 of the previous cycle. Therefore the time of venesection was set one day earlier. Yet, this change of therapy protocol did not result in a further improved CD34+ cell content in the whole blood unit since 31/78 (40%) venesections had to be delayed for at least one day because of hematotoxicity. The threshold of 2×10^6 CD34+ cells/kg body weight per retransfusion was reached in 17/69 (25%) venesections on day 1, cycle 2-6, and in 5/26 (19%) on day 14, cycle 1-5. **CONCLUSIONS:** Autologous whole blood donation as a simple, well tolerated and cost-saving method seems to provide a substantial opportunity to intensify chemotherapy in a relevant number of patients with SCLC. However, a final evaluation of this approach, particularly in comparison with stem cell apheresis or G-CSF administration alone, needs further investigation.

SP506

–80 °C or Below –170 °C: Which Temperature Do We Need for Long-Term Storage of Human Peripheral Blood Progenitor Cells (PBPC)?

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Background: Autologous PBPCs for therapeutic use are stored either at -80˚C in mechanical refrigerators or at <-170˚C directly in or over liquid nitrogen. Usually, in clinical situations, storage time varies from several weeks to months. The aim of this study was to investigate the above-mentioned storage temperatures with regard to WBC recovery, membrane integrity and colony forming potential (CFU) in semisolid culture assays after long-term storage of 1 to 2 years. **Materials and Methods:** Forty-eight frozen PBPC concentrates containing 10% (v/v) DMSO from 24 patients (2 identical 100 ml units from each patient, stored for approximately 1 year over liquid nitrogen) were divided into two groups: In **group 1** storage over liquid nitrogen at <-170˚C was continued for another 1 to 2 years. In **group 2**, however, the units were transferred into a -80˚C refrigerator and stored for the same period as the corresponding unit in group 1. The paired samples were thawed together and analyzed in parallel. **Results:** Numerical WBC recovery in both groups did not differ significantly: $7.5 \pm 6.2 \times 10^8$/kg b.w. (group 1) vs. $8.4 \pm 8.9 \times 10^8$/kg b.w. (group 2). However, membrane integrity (in terms of trypan blue exclusion) differed significantly (Wilcoxon test, $p<0.05$): $81.4 \pm 10.2\%$ (group 1) vs. $52.7 \pm 18.2\%$ (group 2). Also there was a significant difference in the colony forming potential (CFU-GM + CFU-Mix + BFU-E): $8.6 \pm 9.5 \times 10^5$CFU/kg b.w. (group 1) vs. $0.2 \pm 0.3 \times 10^5$ CFU/kg b.w. (group 2). **Conclusions:** Long-term storage of PBPCs in mechanical refrigerators at -80˚C leads to a significant loss of cell membrane integrity and clonogenic potential compared to storage in the vapor phase over liquid nitrogen (-170˚C). For long term cryopreservation of PBPC (i.e., >1 year), storage should be performed in the vapor phase over liquid nitrogen.

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SP507

Analysis of particle numbers and microbial contaminations in cleanroom environments during peripheral blood stem cell processing

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Purpose: Manipulation of cellular therapeutics carried out in an open system has to be performed in a class A environment and a surrounding class B environment. According to the new Annex 1 of the EU guide to good manufacturing practice (GMP) for medicinal products, environmental monitoring should consist of a continuous particle measurement and of an appropriate microbiological control. We examined both parameters by random sampling during processing of 106 consecutive peripheral blood stem cell grafts. **Methods:** During processing of 106 peripheral blood stem cell grafts, particles ≥ 5µm and ≥ 0.5µm were measured concomitantly by analysis of 283L of air in cleanroom area A and B. In addition, microbiological monitoring was performed for each batch by sampling 1,000L of air in area A as well as in area B during production, by analysis of settling plates after exposition in area A, and by analysis of glove prints of all fingers of both hands at the end of production. **Results:** In B areas, particle numbers remained far below the threshold levels with a median number of 7347 (≥ 0.5µm, range: 239 - 146285) and of 39 (≥ 5µm, range: 0 – 636). In A areas, the number of particles ≥ 0.5µm (median: 28, range: 0 – 460) were all below the limit value. The median number of particles ≥ 5µm was 0 but with a range from 0 – 32. In 51 of the 106 productions, the allowed amount of particles ≥ 5µm was exceeded but in all these batches, air samples as well as glove prints were sterile. In 2 of the 51 batches 0.5 CFUs were detected on the settle plates. Additionally, a sample of each product was tested for sterility and all 106 batches were sterile and could be released. **Conclusions:** According to the new Annex 1 of the guide to GMP for medicinal products, continuous monitoring of particles and concomitant measurement of microbiological contaminantes for grade A zones are demanded. Our random analyses demonstrate that particles are present above threshold-levels in the grade A zone. These particles seem to be the consequence of the regular application of sterile disinfection spray as microbiological contaminants could not be detected. Such monitoring proves to be a useful tool for trend analyses and for monitoring and continuos validation of critical processing steps, but the decisive criterion for the graft release is the sterility of the product.

SP6: Platelet Immunology

SP601

The Formation of Platelet-Leukocyte Aggregates Varies During the Menstrual Cycle

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Objective: Particular phases during the menstrual cycle may constitute an increased susceptibility for thromboembolic events and chronic inflammatory diseases due to hormonal influences on platelets. **Methods and Results:** We measured platelet-leukocyte interaction by the determination of platelet-leukocyte aggregates (PLA), platelet P-selectin expression, and PAC-1 binding in 20 healthy women during their menstrual cycle by flow cytometry. The number of platelet-granulocyte aggregates (PGA) and platelet-monocyte aggregates (PMA) was higher on day 14 compared to day one of the menstrual cycle (p=0.016, p=0.023, respectively). Likewise, PAC-1 binding increased from day 7 to day 14 (p=0.029). Platelet P-selectin did not change, however. The course of PLA formation expression during the menstrual cycle followed the course of estrogen levels, strongly suggesting direct effects of estrogen on platelet-leukocyte interaction. **Conclusions:** Our findings corroborate the clinical observations that the susceptibility to thromboembolic events and chronic inflammatory diseases vary during the menstrual cycle.

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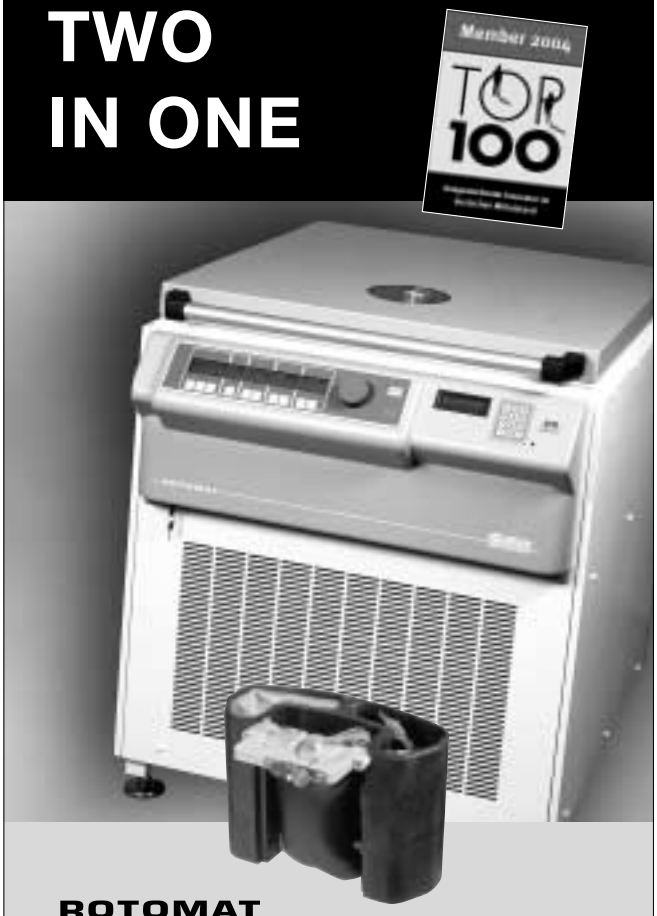

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SP602

Platelet collagen receptors: GPVI as a new antithrombotic target

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At sites of vascular injury, platelets come into contact with subendothelial collagen which triggers their activation and the formation of a hemostatic plug. Besides GPIb and alpha IIb beta 3 integrin which indirectly interact with collagen via von Willebrand factor (vWf), several collagen receptors have been identified on platelets, most notably alpha 2 beta 1 integrin and the Ig superfamily member GPVI. Within the last few years, major advances have been made in understanding platelet-collagen interactions including the molecular cloning of GPVI, the generation of mouse strains lacking individual collagen receptors, and the development of collagen receptor-specific antibodies and synthetic peptides. For a long time, alpha 2 beta 1 was thought to be essential for platelet adhesion to subendothelial collagens, facilitating subsequent interactions with the activating platelet collagen receptor, GPVI. However, studies on mouse platelets lacking either GPVI or alpha 2 beta 1 revealed that GPVI, but not alpha 2 beta 1, is the central platelet collagen receptor. While GPVI-deficient platelets do not aggregate in response to collagen, aggregation of alpha 2 beta 1-deficient platelets is delayed but not reduced. Furthermore, it is now recognized that GPVI is essential for adhesion to collagen under both static and high shear flow conditions, as it mediates the activation of integrins which is a strict prerequisite for firm adhesion and thrombus growth. These in vitro findings were confirmed in a model of arterial thrombosis, where platelet adhesion and thrombus formation on the injured vessel wall in the carotid artery was virtually abolished in GPVI-deficient mice, whereas no effect of alpha 2 beta1-deficiency on thrombus formation was observed in this model. These developments have lead to revision of the original "two-site, two-step" model which now places GPVI in a central position in the complex processes of platelet tethering, activation, adhesion, aggregation, degranulation, and procoagulant activity on collagen. Studies in mice have shown that the receptor can be irreversibly down-regulated on circulating platelets in vivo by injection of anti-GPVI antibodies resulting in abolished collagen responses of the cells. Such GPVI-depleted mice are profoundly protected from arterial thrombosis but do not display major bleeding. Similar mechanisms of GPVI down-regulation appear to exist in humans, making GPVI a promising target for new therapeutic strategies to prevent and treat ischemic cardiovascular diseases.

SP603

The 807C/T polymorphism in the α-subunit of integrin α2β1 modulates platelet adhesion onto immobilized collagen under arterial flow conditions

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Purpose: Adhesion of platelets onto collagen is a crucial step in arterial thrombogenesis. After initial reduction of platelet velocity modulated through the contact between GPIb and immobilized von Willebrand factor subsequent contact between platelets and collagen is dependent on integrin α2β1. Recently, a significant role of the 807C/T polymorphism in the α-subunit of α2β1 has been suggested in the formation of platelet thrombi under arterial flow conditions. In this study, we evaluated 1) the influence of the 807C/T polymorphism of α2β1 on platelet adhesion onto immobilized collagen and 2) the synergism with β polymorphism (HPA-1) of integrin αIIbβ3 on consecutive platelet adhesion using an in vitro system simulating arterial flow. **Methods:** Anticoagulated blood was obtained from 44 blood donors previously genotyped for examined polymorphisms. Platelets in whole blood were labelled with the fluorescence dye and platelet adhesion onto collagen-coated glass cover slips was assessed in a rectangular chamber (shear rates of 50 s^{-1}, 500 s^{-1}, and 1500 s^{-1}). Allotment of the β-polymorphism of integrin αIIbβ3 onto platelet adhesion rate and subsequent thrombus growth was tested in experiments with blood preincubated with Abciximab. A laser-scan microscope was used for visualization and quantitation of platelet adhesion. **Results:** During perfusion, platelet adhesion onto collagen linearly increased with exposition time and shear rate. Platelets with the 807TT genotype exhibited significantly higher adhesion under arterial shear rates compared to 807CC platelets (18,800±10,200 vs. 7,600±7,300, $p<0.05$). Adhesion activity of platelets with combined 807TT and HPA-1b/1b genotypes was approximately 20% higher compared to 807CC/HPA-1a/1a platelets. Coincubation with Abciximab reduced the platelet adhesion rate within 5 min of perfusion by 37% (5 ± 2.2 vs. 3.16 ± 2). **Conclusions**: We provide experimental evidence that platelet adhesion onto collagen under arterial flow conditions is modulated by the 807C/T polymorphism of integrin α2β1. Thus, platelets with the 807TT genotype exhibit significantly higher adhesion rates compared to platelets with the 807CC genotype. After initial platelet adhesion onto immobilized collagen subsequent adhesion of platelets are enforced by the HPA-1 polymorphism. Our data support the contention that genetically determined variants of platelet integrins are risk for arterial thrombogenesis and thus confirm the hypothesis derived from clinical association studies.

SP604

Alloantigenic polymorphisms of the beta terminal domain impair the function of platelet alphallbbeta3 integrin

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The HPA-1a/1b dimorphism (Leu33Pro) located in the PSI domain of platelet beta3 integrin has been discussed as a genetic risk factor for cardiovascular disease. Recent studies showed that the Pro33 isoform (HPA-1b) could enhance adhesive functions and also modulates cell migration into extracellular matrix proteins. However, the role of the PSI domain in integrin activation is not yet clear. In contrast, crystal structure analysis strongly indicated that the beta terminal domain (beta TD) plays an important role in the regulation of alphaIIbbeta3 integrin states (active-inactive state). Mutations in this region lead to platelet functional defects in patients with Glanzmann´s thrombasthenia. We, therefore, analysed the functional impact of point mutations located in the beta TD, which are responsible for the formation of platelet alloantigens (HPA-14, -11, and -8). Full-length beta3 cDNA constructs encoding for the respective mutations (dLys611, His633, Cys636) were generated to establish stable CHO cell lines expressing allele-specific alphaIIbbeta3 isoforms. Conformational changes leading to fibrinogen binding of alphaIIbbeta3 were measured both in inactive and active state after treatment with dithiotreitol (DTT). After DTT treatment, the cell lines expressing Cys636 and His633 showed no/weak binding to monoclonal antibody PAC-1 and do not form cell aggregates in the presence of fibrinogen. In contrast, deletion of Lys611 did not impair both PAC-1 and fibrinogen binding. The consequence of these mutations in outside-in signalling was confirmed by phosphorylation analysis of focal adhesion kinase. In conclusions, these observations indicated that alloantigenic point mutations located in the beta TD of beta3 integrin can impair platelet function. These findings allow new insights in understanding of the impact of platelet alloantigens in cardiovascular and immune mediated diseases.

SP605

Hereditary Thrombocytopenia Due To Mutations in the Non-Muscle Myosin 9 Gen, Approaching Gen-Mutation Function Analysis

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Purpose: Dominantly inherited thrombocytopenias are a group of platelet disorders bridging the black box between megakaryocytopoiesis and platelet production. Recently we and others identified the non-muscular myosin 9 (MYH9) gene on chromosome 22 as the most frequently underlying mutation. As the gene product, non-muscular myosin type IIA is also expressed in the cochlea, the eye lens and in the tubular system of the kidneys, some patients are affected by deafness, interstitial nephritis and cataract. We now identified several unrelated families with different mutations of the MYH9

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gene which allows gene-function analysis. **Methods:** Patients were identified by chronic thrombocytopenia, giant platelets, and positive family history. MYH9 mutated protein, characteristically clustering in leukocytes, were demonstrated by a newly developed immunohistochemistry staining technique. Electronmicroscopy was performed by standard methods. DNA was isolated from EDTA blood and the MYH9 gene was screened for mutations by direct sequencing and RFLP for SNPs. **Results:** We identified 31 individuals resembling 7 different mutations in exon 1 (Y11X; S96L), exon 10 (K371N), 16 (R702C; R702H), 25 (R1165L), 30 (D1424N; D1424H), 38 (E1841K), 40 (R1933X). Clinically these patients presented with various degrees of bleeding symptoms, impaired hearing, renal function and cataract. Electronmicroscopy revealed different ultrastructural features of leukocyte inclusion bodies. As less these clusters are organized, as more pronounced were the clinical syndromes. Correlation of mutation and phenotype indicates that mutations in the globular head of the motoprotein at exon 9-11 cause cataract, renal impairment, and deafness, mutations in exons 25-32 result in a mild phenotype, mutations around exon 38 result in thrombocytopenia only, whereas mutations in exon 40 affecting the anchoring part of the molecule can again be associated with a more severe phenotype. **Conclusion:** We provide a feasible method for identifying mutated non-muscular myosin type IIa and to characterize the underlying mutation in MYH9 which may allow to predict the likelihood how the syndrome manifests clinically.

SP606

A Novel method for simultaneous analysis of specific platelet antibodies (SASPA) by flow cytometry

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Institute of Transfusion Medicine and Immunology, Faculty of Clinical Medicine Mannheim, University of Heidelberg, German Red Cross Blood Service of Baden-Württemberg – Hessen, Germany

PURPOSE: Platelet antibodies cause several clinical thrombocytopenic disorders by eliminating platelets from circulation in diseases such as autoimmune thrombocytopenia, alloimmune thrombocytopenia, posttransfusion purpura, drug-induced thrombocytopenia and refractoriness to platelet transfusion. The detection and differentiation of these antibodies is of high clinical interest for adequate treatment of patients with the above-mentioned disorders. The monoclonal antibody-specific immobilization of platelet antigen (MAIPA) has been mostly used as a standard assay to detect these antibodies. Here we describe a newly developed method SASPA which is able to detect simultaneously specific platelet IgG- and IgM-antibodies by the use of flow cytometry. **METHODS:** Bead populations with distinct fluorescence intensities, which can be resolved in the fourth channel on the flow cytometer, were coated with rat monoclonal antibodies specific for mouse heavy chain isotypes (IgG1, IgG2a and IgG2b). Typed platelets were incubated with human sera and a mix of 3 distinct mouse monoclonal antibodies against specific platelet (for example GP Ib/IX, IIb/IIIa, HLA).After cell lysis and incubation of lysate and beads, goat antibodies against human IgG and IgM were simultaneously added to the bead-lysate-complexes. Sera containing a known platelet-reactive antibody were used to test the specifity and sensitivity of this assay. MAIPA was performed in parallel as a standard test. **RESULTS:** The SASPA assay was able to detect simultaneously platelet-specific antibodies for different GP without cross reaction. In serial dilution tests, the SASPA assay detected the antibodies at comparable dilutions as MAIPA. With this method 6 distinct specific antibodies could be simultaneously determined within 3-4 hours. **CONCLUSIONS:** The SASPA assay permits simple, precise and rapid detection of specific platelet antibodies. Distinct antibodies can be simultaneously determined. Less platelets are necessary to perform this assay. Accordingly, it is of high clinical interest to use the SASPA-assay to detect platelet-associated antibodies in patients with autoimmune thrombocytopenia. This method will potentially open the way to investigations on additional specific antibodies.

Wednesday, September 22, 2004: Poster Sessions

PS1: Transplantation and Stem Cell Collection, Cell Biology

PS101

Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Cord Blood or Adipose Tissue

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Purpose: Mesenchymal stem cells (MSC) from bone marrow (BM) give rise to multiple tissue types and therefore are a source for providing progenitor cells for tissue engineering applications. Alternative sources for MSC are Umbilical Cord Blood (UCB), which can be attained by a less invasive method, or Adipose Tissue which is relatively abundant compared to BM. We investigated whether alternative sources to BM to isolate MSC exist, which additionally might also contain cells possessing more naive stem cell characteristics . To compare the three populations, we isolated and characterized MSC from BM, UCB and Adipose Tissue in respect of their morphology, multiple differentiation capacity, immuno phenotype and growth characteristics. **Methods:** MSC from UCB, BM and UCB were isolated and subcultivated according to established procedures. Differentiation assays were performed according to standard differentiation protocols. Immuno phenotype was evaluated by flow cytometry. **Results:** In all of the processed BM and Adipose Tissue samples, but only in 63% of all processed UCB units, cells of a fibroblastoid morphology could be isolated. In contrast to BM or adipose tissue UCB contained far less fibroblastoid cells but UCB MSC revealed a higher expansion potential than BM or Adipose Tissue MSC. MSC from all 3 tissues had the capacity to differentiate along the osteogenic, chondrogenic and adipogenic lineages whereby the adipogenic differentiation capacity was much weaker for UCB MCS compared to BM or Adipose Tissue MSC. MSC of all 3 tissues expressed no hematopoietic surface proteins and HLA II. But they all expressed typical MSC surface proteins, whereby differences in the intensity of expression or in the percentage of positive cells could be observed. **Conclusions:** Our results indicate that Adipose Tissue MSC seem to represent a more naive stem cell population than BM derived MSC. However UCB MSC seem to represent the most primitive stem cell population of the 3 tissues investigated. Adipose Tissue but also UCB, which contains a relative low MSC precursor frequency, could be an alternative source for future MSC-based therapeutic applications.

PS102

Exposure to shear stress induces a switch in gene expression pattern in Mesenchymal Stem Cells (MSC).

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Purpose: Culture expanded MSC have been shown to enhance engraftment when transplanted in conjunction with hematopoietic stem cells by the intravenous route. However, it is not known which functional and phenotypical changes occur in these cells in the circulation. **Methods:** We exposed MSC to continuous shear in stirred glass flasks. Control cultures were kept in static culture. Cell surface marker of adherent cells and MSC under shear

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conditions were analyzed by flow cytometry. RNA was isolated from the cells using Trisolv (Invitrogen, Germany). Mircoarray analysis by cDNA oligonucleotide hybridisation was performed using HG-U95-AV2 microchips from Affimetrix. **Results:** MSC viability after 12 to 24h under continuous flow conditions was over 90%. Flow cytometric analysis showed no significant changes in expression of CD29, CD44, CD62L, CD62P, CD106, CD73, CD90, CD105, CD117, CD14, CD34, CD45, and CD133. Comparison of gene expression profiles of MSC disclosed a more than 10-fold transcriptional stimulation of 51 genes in cells under continuous shear. In comparison, only for 14 genes we found more than 10-fold transcriptional repression in cells under continuous flow. Analysis of all genes upregulated > 2-fold revealed a genotypic signature of their multilineage differentiation capacity. Furthermore, in stirred cultures, different cytokine, chemokine and G-protein coupled receptors were upregulated. **Conclusion:** The results of the microarrays are preliminary, nevertheless they show variations in different receptor profiles which point to the nature and the regulation of potential homing receptors of MSC.

PS103

Stroke Does Not Increase Circulating CD34-Positive Stem Cells

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Purpose: Stroke has a great socio-economic impact. Despite this the therapeutic influence on outcome is limited. There is an increasing evidence that hematopoetic stem cells (HPC) may be involved in regeneration after an ischemic event. Our objective was to determine whether patients with an ischemic cerebral event have elevated CD34+ HPC in peripheral blood which would support the hypothesis of an autologous repair processes. **Methods:** The total leukocyte and the CD34+ cell count in the peripheral blood were measured in 19 stroke patients at time of admission and compared to 10 healthy blood donors as control group. Furthermore the time course of these parameters was analyzed 12 hrs, 1 day, 3 days and 7 days after admission. CD34+ cell counts were assessed with FACSort according ISHAGE protocol using a dual platform method. The total leukocyte count was evaluated by a Coulter MDII analyzer. **Results:** The 10 healthy blood donors had a mean leukocyte count of 6.1 +/- 1.4 G/l with 0.033 +/- 0.028% CD34+ cells. The CD34+ cell count was 1.85 +/- 1.45/mcl. The 19 patients showed a mean leukocyte count of 6.7 +/- 3.0 G/l with 0.034 +/- 0.024% CD34+ cells i.e. 2.11 +/- 1.51/mcl CD34+ cells at the time of admission. There was no significant difference in any of these parameters between both populations (t-Test).

MEAN VALUES OF THE PATIENTS WERE:

TIME	LEUKOCYTE	CD34+%	CD34+/MCL
ADMISSION	6,7 G/L	0,034	2,11
12 HRS	7.8 G/L (P=0.20)	0.028 (P=0.87)	1.96 (P=0.66)
1 DAY	8.0 G/L (P=0.06)	0.022 (P<0.05)	1.68 (P=0.42)
3 DAYS	7.1 G/L (P=0.32)	0.026 (P=0.51)	1.48 (P=0.29)
7 DAYS	7.3 G/L (P=0.37)	0.030 (P=0.23)	2.21 (P=0.68)

(T-TEST, VALUES COMPARED TO THE RESULTS AT ADMISSION)

Conclusions: There was no significant difference in the leukocyte count or the CD34+ cell number between stroke patients at the time of admission and the control group. Leukocyte counts of the patients peaked 24 hrs after admission potentially explained by an acute phase reaction. However the absolute CD34+ cell counts remained unchanged. So far we have found no evidence of an increase of circulating CD34+ stem cells in stroke patients that would indicate an involvement in the repair process. However, it still remains the possibility that the circulating CD34+ cells home to the site of tissue damage despite a lack in increase of numbers. Further studies analyzing the expressing of factors involved in emigration from circulation and homing of CD34 cells are warranted.

PS104

Vertebral Body Bone Marrow is an Excellent Source for Mesenchymal Stem Cells

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Purpose: Mesenchymal stem cells (MSC) from donors can suppress alloimmune responses, promote the engraftment of hematopoietic stem cells, and may help in donor-specific tolerance induction in solid organ transplantation. **Methods:** Cells from cadaveric vertebral bone marrow (V-BM), aspirated iliac crest (IC-)BM, and peripheral blood progenitor cells (PBPC) were compared. Cells were characterized by flow cytometry and colony assays. MSC generated from V-BM were assayed for differentiation capacity and immunomodulatory function. **Results:** A median 5.7 10^8 nucleated cells (NC) were recovered per vertebral body. The mesenchymal progenitor (CFU-F) frequency in V-BM (11.6 per 10^5 NC, range: 6.0–20.0) was considerably higher than in IC-BM (1.4 per 10^5 NC, range: 0.4–2.6) and PBPC (not detectable). MSC generated from V-BM had the typical MSC phenotype ($CD105^{pos}CD73^{pos}CD45^{neg}CD34^{neg}$), displayed multilineage differentiation potential, and suppressed alloreactivity in mixed lymphocyte reactions. **Conclusions:** Vertebral BM may be an excellent source for MSC co-transplantation approaches.

PS105

A flow cytometric assay for determination of anti-Kell antibodies on peripheral blood stem cells

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Purpose: It has been demonstrated that anti-Kell antibodies induce an inhibition of erythroid and megakaryocytic progenitor cells in fetal alloimmune anemia (Vaughan et al., N Engl J Med. 1998; 338: 798ff). Therefore, we investigated the capability of anti-Kell antibodies to bind to peripheral blood stem cells (PBSCs) and evaluated a flow cytometric assay to quantify the amount of anti-Kell bound on progenitor cells. **Method:** Kell-positive PBSCs (n = 2) of blood group 0, anti-Kell sera (n = 15) and defined isoagglutinins or AB serum as negative controls (n = 4) were used in this study. Cryopreserved PBSCs were thawed, washed and incubated with the different sera for 60 minutes at 37°C. After repeated washings, the samples were incubated for 20 minutes using a secondary antibody (fluorescein isothiocianate (FITC) goat anti-human IgG) and a phycoerythrin-(PE)conjugated CD34-antibody for detection of PBSCs. The mean FITC-fluorescence intensity (MFI) of CD34-expressing progenitor cells was measured for all samples. **Results:** For the first PBSC population, the anti-Kell antibodies demonstrated a MFI of 1144 (± 1026; range: 44 – 3172) vs. a MFI of 75 (± 14; range: 75 - 116) for the control sera ($p < 0.01$). The second PBSC population showed a MFI of 2904 (± 1074; range: 1654 – 5335) for the anti-Kell antibodies and a MFI of 1894 (± 343; range: 1657 – 2404) for the control group ($p < 0.05$). First investigations on multipotent PBSCs also demonstrated a binding of anti-Kell antibodies to those progenitor cells (CD34+/CD133+cells) and an enhanced rate of adherence and phagocytosis of anti-Kell sensitized PBSCs by monocytes. **Conclusion:** The flow cytometric crossmatch assay can properly be used to determine the rate of bound anti-Kell antibodies on PBSCs. Obviously, different PBSC populations strongly diverge in the expression of Kell-antigens depending on the grade of differentiation. Further functional investigations with anti-Kell sensitized PBSCs are currently performed.

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PS106

Quality of long-term cryopreserved cord blood units stored in different liquid nitrogen phases

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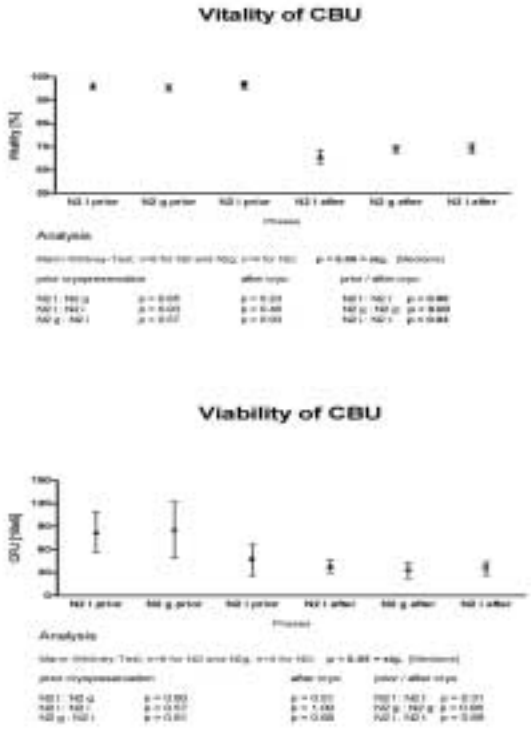
Purpose: Long term storage of umbilical cord blood stem cells in liquid nitrogen (LN) is increasingly performed in many facilities. Little is know about the effect of storage conditions (vapor versus liquid phase versus intermitted changes in between) of LN on the quality of cord blood cells. The aim of our study was to analyze the most important qualilty parameters (vitality and viability) prior to and after cryopreservation for a minimum period of 995 days (1293 d max.). **Methods:** 20 cryopreserved CBU (= cord blood units) were divided into 3 groups: 1) stored in liquid phase (N2l ; n=8), 2) stored in vapor phase (N2g; n=8) and 3) changing gas and liquid phase, depending on the level of nitrogen in the tank (N2i; n=4). Vitality was tested by Acrinidine orange, viability was tested by colony-forming-assay (CFU-GM). All experiments were performed immediately after thawing without washing out DMSO. Statistical-analysis for each group was performed with a Mann-Whitney-Test. **Results:** The following figures show our results:



The vitality and viability was comparable in between the 3 groups before as well as after cryopreservation. Regarding each group separately there was a significant decrease comparing prior to and after cryopreservation in the vitality but in contrast not in the viability. **Conclusions:** Vitality and viability of stem cells from cord blood are not affected by the phase of liquid nitrogen in which they are stored. The data could be further confirmed by investigation of additional parameters.

PS107

Combined treatment of patients undergoing coronary artery bypass grafting (CABG) through intramyocardial injection of CD133+-cells following transmyocardial laser revascularisation (TMLR)

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To restore tissue viability in ischemic myocardium which cannot be efficiently treated by coronary artery bypass grafting alone, transmyocardial LASER revascularization (TMLR) and, more recently, infusion or implantation of autologous bone marrow-derived progenitor/stem cells have been performed in patients. Here, we report on the combined treatment of TMLR and intramyocardial injection of CD133-enriched progenitor/stem cells in five patients to rescue ischemic myocardium at the border of the infarction zone or in hibernating myocardium. Through an interdisciplinary approach, an innovative method for the intraoperative isolation of $CD133^+$-stem cells within less than 3 hours has been established. Before surgery, approx. 200 – 240 ml bone marrow was harvested from the posterior iliac crest and processed using the automated cell selection device CliniMACS. Following standard CABG surgery, LASER channels were shot in predefined areas within hibernating myocardium. Subsequently, autologous $CD133^+$-stem cells (7-10 x 10^6 cells; purity 90-97%), intraoperatively isolated under GMP conditions, were injected in a defined constellation around the LASER channels ($500x10^3$ cells/channel). Improvement of the cardiac function, as assessed by the ejection fraction (TTE) and improvement of cardiac wall motion (ECG-triggered MRI), could be documented 3 months postoperatively. The synergistic angiogenic effect of TMLR in combination with stem cell implantation might be a new avenue for effective treatment of ischemic myocardium. The intraoperative isolation of progenitor/stem cells allows this technique to be applied to patients scheduled for elective and for emergency revascularisation procedures.

PS108

Long-term Safety, Feasibility and Efficacy of Allogeneic Peripheral Blood Stem Cell Apheresis in Healthy Donors After rHuG-CSF Mobilisation with Either Filgrastim or Lenograstim

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Background: Peripheral Blood Stem Cell (PBSC) apheresis in healthy donors has become the main source of hematopoietic stem cells for allogeneic transplantation in Germany (b). However, long-term safety of rHuG-CSF for mobilisation of PBSC in healthy allogeneic donors is not yet fully explored. Therefore, we initiated a monocenter long-term safety study in 2002 to address these issues. **Material & Methods:** Allogeneic healthy donors mainly from our Frankfurt donor registry (n = 46,600 allogeneic donors as of April 2004) are included into the long-term safety study after written informed consent following information, collection of medical history and physical examination by the apheresis physician. The study was approved by the local Ethics Committee. All donors will be followed up to 5 years after their donation. **Results:** As of today (April 19, 2004), 25 donors are included into the monocenter study in Frankfurt (6 female; 19 male). All data are shown as median (minimum – maximum). Median age is 36 yrs. (21 – 52) ; median weight is 80 kg (53 – 110). Three out of 25 donors (12%) reported a slight splenodynia after mobilisation. In 5 out of 25 donors (20%), marginal splenomegaly could be demonstrated by ultrasound after mobilisation. Combined, 7 out of 25 donors (28%) showed signs of splenomegaly at the end of rHuG-CSF mobilisation of PBSC either clinically and/or sonographically. In none of the cases, mobilisation of PBSC had to be stopped due to these signs. All signs disappeared within at least 4 weeks. No severe or serious adverse event was seen. The prescribed amount of CD34+ stem cells was harvested in one apheresis in 16 out of 25 donors (64%), while in 9 out of 25 donors (36%), two sessions were necessary. **Conclusions:** PBSC

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apheresis in healthy donors is a feasible and safe procedure. However, a significant percentage of donors presented with marginal splenomegaly, which requires long-term control of the donors.

([a] both authors contributed equally; b: ZKRD: Annual report 2001/2002)

PS109

GMP-compliant processing of small volume bone marrow samples for tissue repair using the Sepax S-100

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Background: Hematopoietic and mesenchymal stem cells are an attractive cell population that is currently elaborated in many clinical trials for the ability to restore damaged tissue in diseases, such as e.g. acute myocardial infarction. However, since the amount of stem cells that is needed for a therapeutic effect is considerably lower to that which is needed for a hematopoietic reconstitution, the bone marrow volume that needs to be processed in many cases does not exceed 50 ml. Here we have analyzed a new cell separation system (Sepax S-100, Biosafe, Switzerland) allowing the GMP-compliant and fully automated processing of cellular products, in this particular case 50 ml bone marrow samples. **Methods:** Two bone marrow samples were diluted 1:1 with isotonic NaCL solution and subsequently divided into two equivalent parts. While one part of the bone marrow was processed using a classical manual Ficoll-separation, the other part was separated using the Sepax S-100 and a closed system separation kit (Biosafe, Switzerland). The resulting cell preparations were analyzed for the recovery of WBC and CD34-positive cells as well as the depletion of granulocytes and red blood cells. In addition, an undiluted bone marrow (70 ml) was processed entirely with the Sepax-separation system. To assess the capacity for revascularization, the different cell preparations were tested in a murine hint limb ischemia model. **Results:** The Sepax system yielded cell preparations with a final volume of 50 ml. The mean recovery of CD 34-positive cells was 88% (SD 14%), compared to 45 % (SD 17%) with the manual Ficoll-processing. While the depletion of RBC´s proved to be efficient with both methods (99% with Sepax; 100 % with manual Ficoll-processing) the depletion of granulocytes was higher using the Sepax method (65% SD 16% versus 38% SD 1%), respectively. Moreover, the total nuclear cell (TNC) recovery was 29% (SD 19%) using Sepax and 23% after manual processing. The hint limb ischemia model indicates that the cell population mediating tissue repair is equally well preserved after processing with the Sepax S-100. **Conclusion:** The Sepax S-100 offers a simple and automated processing, especially suitable for bone marrow samples that would be too small for conventional separation methods used in hematopoietic graft processing and where previously only non GMP-compliant "hands on" methods have been available. Interestingly, the recovery of CD 34+ stem cells was significantly higher using the automated Sepax system. However, the ability of the Sepax S-100 to preserve the tissue repair cell fraction should be confirmed in further experiments involving in vivo models.

PS110

Optimization of the preparation and yield of human umbilical blood cells

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Purpose: Human umbilical cord blood is a rich source of hematopoietic stem cells that can not only be used for research but also for allogeneic stem cell transplantation to reconstitute hematopoiesis following myeloablative therapy. However, the major limit for the success of the allogeneic stem cell transplantation using human cord blood cells is represented by the low cellular yield of the stem cell population. Therefore, there is a great interest to develop methods and techniques for optimizing the yield of viable cells after preparation and selection procedures. Methods: 35 human cord blood samples were collected using aseptic technique from umbilical veins immediately after placenta delivery. The samples were prepared according to two protocols. 20 samples were centrifuged by 400 g for 30 minutes without acceleration and thereafter selected using the AutoMacs technology. In this first protocol we used Biocoll seperation solution (Biochrom AG) and a separation media using a normal 50 ml tube (Sarstedt AG). In a second protocol a centrifugation by 1000 g for 10 minutes without acceleration was performed using Leukosep tube, which includes porpous filter disc (a fixed plate to avoid mixing of supernatant and cell mass after centrifugation). Results: Recovery of CD34 cells was increased from 23,5 % in protocol 1 to 34 % in protocol 2. The viability of the CD34-positive cells prepared by protocol 2 was 89,2 %. By using the second protocol all stem cells were processed and isolated without any difficulties, whereas in protocol 1 in 15 % of cases the selection failed. All samples were free of microbial contamination after the preparation and selection procedures. Conclusions: Simply modifying the preparation and centrifugation techniques leads to better preparation results and improves the yield of stem cells from human cord blood. However, next to the separation technique, other variables like volume, collection time (pre or post delivery), duration of the collection, clots and apoptosis are also critical to the yield of the CD34+ cells and must be considered and optimized. In addition, not only optimization is required but also the standardization of collection, preparation and isolation procedures to obtain maximal recovery in research and in clinical use.

PS111

Recommendations for optimized settings of the Amicus Crescendo cell separator for the collection of CD34+ progenitor cells

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Purpose: CD34+ peripheral blood stem cells for autologous transplantation purposes are collected by leukapheresis procedures on automated cell separators. In this study, the influence of patients´ white blood cell counts, flow rate and cycle volume settings on collection efficiency of the Amicus Crescendo cell separator (Baxter, Unterschleißheim, Germany) was investigated. **Methods:** 146 stem cell collections with Amicus cell separators were performed in 56 patients either with cycle volume presets recommended by the manufacturer or modified settings in order to identify its impact on collection efficiency (CE). **Results:** Using standard settings with a cycle volume of 1400 ml, CE significantly decreases when patients´ peripheral blood pre-apheresis leucocytes are between 25,000 and 35,000/µl. CE can be improved if cycle volume is reduced to 1000 ml in these patients. If WBC concentrations exceed 55,000/µl pre-apheresis, CE again significantly decreases despite of the reduced cycle volume. Additionally, high flow rates > 60 ml/min significantly reduce CE. **Conclusions:** Collection efficiency of the AMICUS cell separator is strongly influenced by the patients´ peripheral WBC count and the flow rate of the device. We found that a reduced cycle volume of 1000 ml improves the outcome of CD34+ stem cell collections in patients with WBC counts between 25,000 and 35,000/µl and recommend a maximum flow rate of 60 ml/min in general. If further modifications of device settings may improve CE in patients with WBC counts > 55,000/µl have to be investigated in future studies.

PS112

AB0-incompatible bone marrow transplantation: a simple method to remove incompatible donor red blood cells by "washing" with recipient-compatible red blood cells in a closed system

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Purpose: Major AB0-blood group incompatibility affects up to 20% of cases of allogeneic bone marrow transplantation. Various open and semi-closed methods are reported that result in a sufficient depletion of red cells at the expense of the hematopoietic progenitor cells. We therefore report a case of red cell removal in a closed system by "washing" with recipient-compatible red blood cells and consecutive successful bone marrow transplantation in an

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AB0-incompatible setting. **Case Report:** A 10-year-old boy with blood group 0 Rh pos and the diagnosis of a Ph^+ ALL was treated with TBI (12 Gy) and VP-16 (60 mg/kg). A total of 1890ml bone marrow were harvested from the HLA-identical, 16-year-old brother with the blood group A Rh pos. The bone marrow was anticoagulated with heparin and 8% ACD-A. Further processing was performed in a top and bottom whole blood donation system. All tubing connections were established by a sterile connection device. In detail, the CPD-A was removed from each primary bag and the marrow was distributed in 4 bag-systems. The marrow was centrifuged with 4,000 g for 12 minutes and the buffy coat layers were harvested. Starting with 7.74×10^{12} red cells and 4.17×10^{8} white blood cells/kg (recipient body weight) the first centrifugation step resulted in a 74.8% reduction of the red cells and a loss of 16.8% of the white cells. The marrow was then diluted with 3 units of compatible group 0 Rh pos packed red blood cells, which had previously been irradiated with 30 Gy. Using new bag-systems, the marrow was centrifuged a second time and again the buffy coats were harvested. At the end of the procedure, 97.3% of the red cells with blood group A were depleted resulting in a remaining volume of 17.9 ml incompatible red cells. A total of 2.99×10^{8} white blood cells/kg, representing 71.6% of the initial cell number, with 3.69×10^{6} CD34+ cells/kg were infused without clinical signs of hemolysis. On day +10 after transplant, the WBC count exceeded the first time 500/µL. At the same day, a peripheral blood sample was analyzed with PCR for different VNTR loci for chimerism. The analysis revealed a mixed donor chimerism with 87.7% donor cells. **Conclusions:** Red cell depletion of the bone marrow graft in a major AB0-incompatible constellation can successfully be performed in a closed system. "Washing" with recipient-compatible red cells leads to a sufficient depletion of AB0-incompatible red cells and a very good recovery of the white cells.

PS113

Comparison of total nucleated cell measurements of umbilical cord blood samples using two haematology analyzers

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Purpose: The total nucleated cell (TNC) content of umbilical cord blood (UCB) units serves as a measure for determining suitability for transplantation. Hence, it is important that TNC measurements be performed in an accurate manner. TNC content is evaluated routinely by haematology analyzers (HA) using white blood cell (WBC) count channels. The objective of the study was to compare TNC content utilizing two different HA's, one utilizing an impedance channel and the other using only optical channel. **Methods:** The haematology analyzers utilized in this study used two different modes of operation for lysis, regular mode (RM) and extended lysis mode (ELM). Cell-Dyn 3200 (CD3.2) utilizes optical technology for WBC measurements, WBC optical count (WOC), and nuclear optical count (NOC), whereas the Cell-Dyn 3700 (CD3.7) utilizes both the impedance (WIC) and optical technology (WOC) for WBC measurements. NC content was determined with 17 identical samples using CD3.2 in one laboratory and CD3.7 in the other laboratory. Cord blood samples processed to concentrate nucleated cells by either of the laboratories were sent by overnight courier and assays were performed on the same day by both laboratories. **Results:** For CD3.7, the WOC values were consistently lower than the WIC using the regular mode, but showed no significant differences ($p>0.05$). The WIC and WOC values were comparable on using the extended lysis and the regular lysis modes. For CD3.2, WOC values using RM and NOC values using ELM showed no significant differences ($p>0.05$) even though the WOC measurement was lower than the NOC values for most samples. The best comparison of NC measurement between the two HA could be achieved when comparing CD3.7-WIC with CD3.2-NOC values. The results were equivalent ($p>0.05$) and 12 of 17 samples had equal to or less than 10% difference (mean 9.5%). **Conclusion:** TNC measurements of umbilical cord blood samples were essentially identical using the WIC channel of the Cell-Dyn 3700 and the NOC channel of the Cell-Dyn 3200.

PS114

A multicenter trial to investigate the predictability OF the yield of collected $cd34^+$ cells by two leukapheresis programs

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Purpose: In a prospective multicenter trial we investigated the exactness of the prediction tool for peripheral blood progenitor cell (PBPC) collection implemented into the leukapheresis programs MNC and RV-PBSC of the Fresenius cell separator COM.TEC®. Based on the preapheresis $CD34^+$ cell count, the machine calculates the total yield of $CD34^+$ cells collected during the procedure. **Methods:** A total of 166 donors (128 patients with malignancies, 38 healthy individuals) underwent 203 harvests either with the MNC or RV-PBSC program in three clinical centers. **Results:** In total, 112 collections were performed in 91 donors using the MNC program and 91 harvests in 75 donors using RV-PBSC. The median $CD34^+$ cell collection efficiency (CD34-CE) was significantly higher in MNC than in RV-PBSC program ($p<0.0001$): 68% (31-205) vs. 44% (11-100). On the other hand, the median preapheresis count of circulating $CD34^+$ cells was significantly lower in donors undergoing the MNC program than in donors harvested by RV-PBSC ($p=0.0261$): 51/µl (6-834) vs. 64/µl (12-813). Therefore, the difference between both programs concerning the $CD34^+$ cell yield harvested per apheresis was not statistically significant ($p=0.3970$): 384×10^{6} $CD34^+$ cells (22-3281) vs. 262×10^{6} $CD34^+$ cells (30-2521). There was an excellent correlation in both programs between the $CD34^+$ cell count before apheresis and the number of $CD34^+$ cells collected, MNC: $r=0.947$ ($p<0.0001$), RV-PBSC: $r=0.904$ ($p<0.0001$). In median, the predicted $CD34^+$ cell values were higher than the really harvested $CD34^+$ cell yields. The ratio of collected and software-predicted yield of $CD34^+$ cells was significantly higher ($p<0.0001$) for MNC with 85% (31-176) than for the RV-PBSC program with 59% (22-110). There was a significant difference between both programs in the median concentrate volume ($p<0.0001$): 383 ml (204-586) for the MNC program vs. 148 ml (95-250) for RV-PBSC. Concentrates harvested by the RV-PBSC program had a significantly higher percentage of mononuclear cells and a lower red blood cell content than those obtained by MNC ($p<0.0001$; $p<0.0001$): 86% (43-99) vs. 56% (25-95) and 12.5 ml (3.8-47.7) vs. 24.9 ml (4.7-60.5), respectively. **Conclusions:** The higher CD34-CE and the more exact prediction of the $CD34^+$ cells collected make the MNC program a safe and convenient leukapheresis procedure. However, when a PBPC concentrate with a low erythrocyte content is required (AB0-incompatible allografts), the RV-PBSC program should be preferred.

PS115

Hygienical Monitoring of Bone Marrow Harvesting in a Class B/C Unit

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Purpose: Although harvesting of bone marrow is introduced for allogeneic stem cell transplantation for many years it is still a demanding task to gain a product of high quality and safety. Thus, a hygienical monitoring programme is practised in the ITZ for each product. We evaluated our data with special interest on a connection between positive monitoring results and contaminated products. Methods: Between 2001 and 2004 66 bone marrow transplants were collected from unrelated donors. The collection site was an orthopedic surgery theatre. At each time of production microbiological qualification including use of sedimentation plates(SP) and air sampling(AS) for airborne germs, contact plates(CP) for microbial contamination of the operators´ gloves and air particle count(PC) was performed. The results were evaluated due to appendix 1 of the EU-GMP Guideline for manufacturing sterile pharmaceuticals. Furthermore product samples were drawn and tested on bacterial contamination. Results: In 31,8% (SP) resp. 87,8% (AS) we found positive results for airborne microorganisms which contained coagulase-neg. staphylococci(cns), micrococci(m), spore-forming agents(sfa), staph. epidermidis(se), corynebacteriae, yeast and mold. In 40,9% the CP were positive (cns, se, staph. aureus(sa), m, sfa, mold). AP counts were

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classified in 74,2% class B and in 10,6% class C. The overall classification was 35% class B and 65% class C. 90,9% products showed no bacterial contamination and 6 of them were contaminated with 7 various agents. 4 products had cns which also were found in SP,AS and/or CP. One product had sa also found in AS and one had sa without evidence of origin. One recipient developed CMV-related sepsis and one had gram-pos. sepsis after trasnsplantation with a sa-pos. product. Both recovered soon. Four recipients showed no infection. Conclusion: Presence of skin bacteria and air-borne germs in an operating room during bone marrow harvesting by multiple punctions is not avoidable. Factors which minimize contamination of bone marrow products are efficient skin disinfection, accurate product manipulation, small number of attending persons, prevention of unnecessary movement of persons, doors and equipment.

PS2: Hemostaseology and Platelet-Immunology

PS201

Molecular pathology of combined deficiency of vitamin K-dependent coagulation factors (VKCFD) type 1: Novel and recurrent mutations in the gamma-glutamyl carboxylase gene (GGCX) in three patients

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Purpose: Hereditary combined deficiency of the vitamin K-dependent coagulation factors II, VII, IX, X, protein C, S and Z (VKCFD) is a very rare autosomal recessive inherited bleeding disorder with at least two subtypes. VKCFD1 is defined by defective gamma- glutamyl carboxylase activity and VKCFD2 results from functional deficiency of vitamin K epoxide reductase. The clinical presentation is variable with respect to the residual activities of the affected proteins and the response to oral substitution of vitamin K. **Methods:** Patients from three families with VKCFD1 were identified during either bleeding or pre-surgery coagulation testing. All 15 exons and flanking intronic regions of GGCX were sequenced on an automated sequencing system (ABI Prism 310). Haplotypes were constructed after analysing four intragenic polymorphisms and five mikrosatellite markers surrounding GGCX. **Results:** Patient A was found to have vitamin K-dependent coagulation factor activities ranging from 20-40 % of normal. High dose of Vitamin K led to subnormal restoration of the factor levels to 50-60%. Family studies confirmed the indexpatient to be compound heterozygous for two missense mutations (p.His404Pro; p.Arg485Pro). As the p.Arg485Pro mutation has been reported recently in an unrelated patient the hypothesis of a founder effect has been confirmed by an identical haplotype for both patients. Patient B had a more severe phenotype with residual activities of less than 15 % of the corresponding coagulation factors, vitamin K administration did not improve factor activities. However, when Vitamin K was given together with fresh frozen plasma the half live of vitamin K dependent coagulation factors was significantly prolonged. Mutation analysis revealed a homozygous missense mutation (p.Arg204Cys) which may affect the vitamin K-binding site. In Patient C with severe VKCFD we found a homozygous missense mutation (p.Gln328Lys). As Gln328 has been predicted to be located in a cytoplasmatic loop a direct effect on the essential regions (propeptide-, glutamate- or vitamin K-binding site) is unlikely. However, Gln328 is highly conserved in most species and may be critical for maintaining the structure of a catalytically active enzyme. **Conclusions:** Mutation analysis in patients with rare VKCFD1 demonstrated a great variety of underlying mutations. Some of these mutations might be helpful in identifying new functional epitopes of the gamma- glutamyl carboxylase.

PS202

A novel mutation (p.Asp36Tyr) in the vitamin K epoxide reductase complex subunit 1 gene (VKORC1) causes increased Phenprocoumon requirement

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Purpose: Coumarin derivates target blood coagulation by inhibiting vitamin K epoxide reductase (VKOR). Coumarin resistance may result from pharmacological, nutritional or genetical reasons. Recently, we have identified the vitamin K epoxide reductase complex subunit 1 gene (VKORC1) using linkage analysis from three different species. Mutations of this particular gene occurred in four families with hereditary warfarin resistance and in numerous rat strains with resistance to warfarin-like poisons. **Methods:** All 3 exons and flanking intronic regions of VKORC1 were sequenced on an automated sequencing system (ABI Prism 310). **Results:** A 40-year-old patient of Caucasian descent required 40-50 mg phenprocoumon per week to maintain an international normalized ratio (INR) between 2.0 and 3.0, whereas the normal range of weekly phenprocoumon dose is 10-40 mg. After exclusion of acquired reasons for an increased coumarin requirement like high dietary intake of vitamin K or comedication with certain drugs genetic analysis of VKORC1 revealed a heterozygous missense mutation (p.Asp36Tyr). Previously desribed mutations in VKORC1 in four patients led to variable degrees of coumarin resistance. Two of these patients exhibited complete resistance to warfarin while two required 3 to 10 times more warfarin than normal (10-60 mg per week). Here we report on the first case of mutations in VKORC1 leading to increased coumarin requirement of a moderate extend with a phenprocoumon dose close to the upper normal range. Mutations in VKORC1 leading to coumarin resistance are scattered throughout the gene thus indicating that not only one specific epitope seems to be critical for coumarin binding. **Conclusions:** Mutation in VKORC1 may lead to coumarin resistance of highly variable extend. Mutation analysis in patients with increased coumarin requirement might be helpful understand the molecular mechanisms of this phenotype in some patients. Moreover these data may contribute in identifying further functional epitopes of VKORC1 which contribute to coumarin-resistance.

PS203

Spectrum of Mutations in 13 Patients with congenital factor XIII Deficiency

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Introduction: Mutations of the Factor XIII A-subunit gene (F13A) have been found in patients with XIIIA deficiency, a rare autosomal recessive bleeding disorder. F13A gene is located on chromosome 6p24-25 and contains 15 exons. The corresponding protein stabilizes the fibrin clot and increasing its resistance to fibrinolysis. **Materials and Methods:** The F13A gene of 13 patients (5 Turkish, 4 German, single patients from France, Grait Britain, Spain and Lithuania) was analysed on denaturing high performance liquid chromatography (DHPLC) and sequencing. The three – dimensional structure of the mutant amino acides were analysed by a molecular protein model based on the X-ray sructure of FXIIIA. **Results and Discussion:** A total of thirteen different mutations in 13 unrelated patients comprising 6 missense, 3 splice site, 3 small deletions and one small insertion could be elucidated (see table 1). Splice acceptor site mutation in intron 5 (IVS5–1 G>A) was found in 3 families originated from Germany, U.K. and Turkey. Haplotype analysis (see table 2) revealed an ancient founder effect, that likely has occurred some thousand years ago since it was spread at least in central and southern Europe as well as in Asia (Turkey). Six mutations were described for the first time. The protein modelling of novel missense mutation in exon 5 (G215R) demonstrated that R215 is located at the interface of two beta strands and the barrel 1 and barrel 2 domains. Substitution of the

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small Gly by the large Arg obviously affects the three dimensional configuration substantially. Two novel splice site mutations (IVS12+1 G>A, IVS14-2A>G) cause splicing error of exon 6 and exon 15 respectively, while two novel small deletions (c.748delC, c.1475-1476delGA) result in premature termination of FXIIIA protein. A novel 9bp del in exon 5 (c.617-625del) results in frame deletion of amino acids V206, L207 and N208.

Conclusions:

- DHPLC has proven to be a fast and highly sensitive method for the mutation analysis of the F13A gene.
- The identified novel mutations will help better to understand the functionally important sites of the FXIIIA protein.
- IVS5-1 G>A mutation seems to be the most common F13A gene defect in FXIIIA deficient patients, due to an ancient founder effect.

PS204

Molecular genetic analysis in patients with inherited thrombophilia and Antithrombin, Protein C or Protein S deficiency

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Purpose: Thrombophilia is a disorder associated with an increased tendency to either inherited or acquired venous thrombosis. The three classic single-gene disorders causing inherited thromboembolic disease affect the coagulation inhibitors- antithrombin (AT), protein C (PC), and protein S (PS), each being responsible for roughly 5% of familial thromboses. Studies of the molecular basis of hereditary AT, PC and PS deficiencies showed a great variety of mutations occurring throughout the genes. The heterogeneity of the mutations hampered rapid genetic analysis in affected patients. **Methods:** Twelve families - 2 with protein C, 1 with protein S, and 9 with antithrombin deficiency, have been examined. Denaturing High Performance Liquid Chromatography (DHPLC) followed by direct sequencing of the fragments, which showed abnormal pattern was used as a technical approach for mutation identification. **Results:** By this approach we identified 9 mutations, 2 in PC, 1 in PS and 6 in AT, which have been found by examination of patients from eleven families with clinically expressed deficiencies of PC, PS and AT. In AT deficiency we found four missense mutations (S116P, L99F, K114E, S162R) and one stop mutation (W49Stop). All four missense mutations affected the binding site of heparin. In three of the families with AT deficiency no mutation has been found. The mutations found in PC and PS deficiencies patients where a 4 bp insertion (CCTG) and 1 missense mutation (R230S) in exon 9 of the PC gene and an 1 bp (T) deletion in exon 15 of the PS gene. Both, the deletion and the insertion led to a frame shift with a subsequent stop codon and therefore should be regarded as causative for the phenotype. **Conclusions:** We conclude that the strategy of using DHPLC as a mutation screening method followed by direct sequencing of a single fragment with abnormal pattern represents rapid and reliable approach for the mutation analysis for AT , PC and PS genes.

PS205

Efficacy and safety of recombinant activated factor VII (NovoSeven®) in trauma and surgery patients with massive bleeding – a multicenter analysis

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Purpose: The management of massive bleeding is a major clinical challenge. Our objective was to evaluate the efficacy and safety of rFVIIa (NovoSeven®, Novo Nordisk A/S; Bagsvaerd, Denmark) in trauma and surgery patients with massive bleeding. **Method:** Forty-five patients (36 trauma, 9 surgery), aged 16–78 years, with severe massive haemorrhage requiring ≥16 transfusion units of packed red blood cells received rFVIIa (median dosage 90 µg/kg). Post-rFVIIa blood loss and transfusion requirements were assessed. Mortality was retrospectively compared with predicted outcomes using the TRauma Injury Severity Score (TRISS) and Physiological and Operative Severity Score for enUmeration of Mortality and morbidity (POSSUM) systems. **Results:** Blood loss was markedly reduced in 40/43 (93%) patients and transfusion requirements (units of PRBC, FFP, thrombocyte concentrates) decreased after rFVIIa administration. Observed mortality was 11/35 (31.4%) in trauma patients *versus* a mean TRISS prediction of 58.4%. POSSUM analysis of trauma patients predicted 22 deaths (62%). In surgical patients, mortality was 3/9 (33.3%) *versus* a POSSUM prediction of 44%. Mortality for all patients was 14/44 (31.8%) *versus* combined POSSUM prediction of 26 deaths (59%). **Conclusion:** Mortality was greatly reduced in this group of trauma and surgical patients who had massive haemorrhage when compared with predictions using the TRISS or POSSUM scoring systems. This may be associated with the use of rFVIIa.

PS206

C677T MTHFR variants and hyperhomocysteinemia – causal or casual thrombophilic risk variables?

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Background: To date hyperhomocysteinemia (HH) is considered to be of significance in thrombotic vascular diseases which might predominantly be correlated with atherosclerotic lesions (physician´s health study, framingham study). However defects of genes encoding enzymes which are involved in the homocysteine metabolism as e.g. C677T MTHFR are described to be associated with vascular complications. Beyond it the variant of MTHFR is also rather common in the healthy population so that its coincidence with HH has to be critically revealed. **Patients and Methods:** Homocysteine (normal range: < 15 µmol/l) in EDTA-whole blood using "HPLC" was controlled in 98 mutant carriers of MTHFR - 65 thrombotic patients (group I; mean age: 44,8 ± 5,9 years; 35 females, 30 males) with a.) deep venous thrombosis and/or pulmonary embolism (n = 55), b.) myocardial infarction/stroke (n = 10) and 33 asymptomatic controls (group II; mean age: 39,1 ± 15,8 years; 19 females, 18 males). **Results:** In 42% (group I - n = 27/65 patients) of the mutant carriers of MTHFR HH (22.8 ± 7.29 µmol/l) was diagnosed, whereas in 59% of this group (37/65 patients) normal homocysteine levels were registered (11.45 ± 2.27 µmol/l). The asymptomatic individuals (n = 33) demonstrated no abnormal, or minimally elevated homocysteine concentrations (15.95 ± 6.50 µmol/l). Recurrent thrombotic complications were not associated with higher homocysteine levels than single events. The highest serum levels (20 µmol/l (mean) were found to be at patients with arterial vascular complications. At least the severity of the MTHFR defect did not significantly influence the amount of homocysteine measured in serum either in patients with venous or arterial thrombotic complications. **Conclusions:** Hyperhomocysteinemia may be interpreted as an independent thrombophilic risk variable especially in arterial vascular diseases. In a minority this phe-

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nomenon was attributed with defects of the MTHFR gene - only 40% of thrombotic patients demonstrated the coincidence with the MTHFR variant. In healthy mutant controls hyperhomocysteinemia may also rarely occur which underlines our hypothesis as well as the fact that the gene defect of this enzyme (MTHFR) was also found in a large amount of healthy blood donors (186/323) (unpublished own data). This prevalence in healthy individuals (58%) which is comparable to that in thrombophilic patients (59%) throws doubt on its relevance in the broad field of thrombophilia. Thus we suggest to prospectively modify the diet or the oral intake of vitamins - since indicated - but not to consider furthermore the evidence of the C677T (MTHFR-) defect as a thrombophilic risk variable.

PS207

On the interaction of the defected C677T "MTHFR" gene and hyperhomocysteinemia on platelet function in healthy individuals and patients with single and recurrent thrombotic vascular diseases

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Background: Mutations of the gene coding for the enzyme methylentetrahydrofolate reductase are thought to be associated with hyperhomocysteiemia. Its influence on platelet function is also questionable as well as the role of platelets especially in venous vascular diseases is rather unclear. **Patients and Methods:** Platelet count (PC), Mean platelet volume (MPV), as well as platelet function [platelet adhesion acc. to Hellem (PA) (Niessner 1972, Hellem 1970), platelet aggregation (PAT III) (Breddin 1975)] were evaluated in 338 patients with thrombotic complications (n = 261/n = 56/n = 21) (group I; n = 338) and 95 healthy controls (group II). Homocysteine in serum (normal: < 15 µmol/l) was controlled in 116 thrombotic patients and 53 healthy individuals as well as in 98 mutant carriers of MTHFR (65 patients, 33 healthy individuals) using EDTA-whole blood for "HPLC". Platelet rich plasma was obtained by centrifugation at 180g (1000 U./min. ; Rotixa/Ks, Hettich). PA (normal value: < 38%) was immediately recorded using EDTA-whole blood while PAT III (normal: angle $\alpha < 40^{o}$) was evaluated 30 min. after blood withdrawal. For statistical analysis the Chi-Square Assay was performed. Odds ratios and 95% confidence intervals were also calculated. **Results:** PAT III significantly differed between group I/II ($p<0.05$) whereas PC, MPV, and PA did not demonstrate significant differences. Recurrent/multiple thrombotic complications as well as severe (homozygous) variants of the MTHFR gene were not correlated with higher homocysteine levels than single events, or heterozygosity. 37/65 patients (59%) carrying the MTHFR defect as well as all healthy mutant carriers of MTHFR (n = 33) demonstrated normal homocysteine levels (11.5 ± 2.3 µmol/l, resp. 15.9 ± 6.5 µmol/l). The calculated odds ratios for hyperaggregation of platelets (1), increased platelet counts (2), - mean platelet volume (3), - platelet adhesion (4) and hyperhomocysteinemia (5) were found to be as follows: 2.336 [1.055 to 5.170] (1); 1.584 [0.532 to 4.717] (2); 0.921 [0.421 to 2.015] (3); 0.778 [0.394 to 1.535] (4), and 1.932 [0.919 to 4.091] (5). **Conclusions:** PAT III is a reliable platelet function assay, able to discriminate venous thrombotic patients from healthy controls which is in contrast with PA, PC, and MPV. Homozygous mutant carriers of MTHFR do not necessarily result in higher homocysteine concentrations than heterozygous variants which is independent from the presence of thrombotic diseases. Hyperhomocysteinemia is not strongly associated with hyperaggregability of platelets. PAT III, and homocysteine are - in contrast to PA, MPV, and PC - advantageous for the screening of thrombophilia.

PS208

Is the platelet count and platelet size of significance in patients with thrombotic diseases?

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Background: Controversy exists about the role of platelet size in arterial thrombotic diseases whereas there is a lack of data in venous disorders. As demonstrated in previous examinations hyperactivation of platelets (increased PAT III values) might contribute to venous thrombotic complications and lead up to a twofold risk of thrombosis (DGTI 2003). We aimed to a.) evaluate the role of platelet count, and platelet size in patients with spontaneous thrombotic vascular diseases compared with healthy individuals and b.) calculate the odds ratio. **Patients and Methods:** Platelet count, and platelet size were analyzed in 338 thrombotic patients and 100 healthy controls. Measurements of platelet count and platelet size (using EDTA whole blood) were performed using an automatic cell counting system (Sysmex). Platelet counts ranging from 150000/µl to 350000/µl and platelet size between 8 and 13 fl referred to be normal. 261 patients (60.3%) underwent venous thrombotic complications whereas 56 patients demonstrated arterial thrombotic events. No attributable clinical explanation could be verified in 207/338 cases (61.2%). In 131 cases thrombosis was clinically apparant. For statistical analysis "SPSS" (Statistical Package for the Social Sciences) was used. The odds ratios and 95% confidence intervals were calculated as well as the chi square assay was used to compare groups. **Results:** Platelet counts did not significantly differ between thrombotic patients and healthy individuals. Increased platelet counts (> 350000/µl) were found in 22/331 patients (6.65%) and 4/93 (4.30%) controls ($p>0.05$, χ2-assay). Abnormal platelet size was registered in 30/334 patients (8.98%), and 9/84 (9.68%) healthy individuals ($p > 0.05$, χ2-assay). The corresponding odds ratios were calculated as follows: platelet count: or = 1.58 [0.53, 4.72]; platelet size: or = 0.92 [0.42, 2.02]. Comparing spontaneous thrombotic events and healthy controls platelet count and platelet size also did not differ significantly ($p>0.05$, χ2-assay). The odds ratios were calculated as 1.02 [0.31, 3.41] (thrombocytosis), respective 1.06 [0.47, 2.41] macrothrombocytaemia) **Conclusions:** Platelet count as well as platelet size do not take influence on the development of venous/arterial thrombotic complications. Comparing cases (patients, controls) with abnormal platelet counts (> 350000/µl) or size (> 13 fl) the level of significance could not be reached using the chi-square assay. But it should be taken into mind that the cut for diagnosing „thrombocytosis" was set to be rather low. Calculating the odds ratios, an up to threefold, or even fivefold thrombotic risk might be attributed to ″macrothrombocythaemia″, and/or increased platelet counts (individually). Since about 60% of the patients analyzed suffered from venous problems an increase of platelet count and platelet size might also be of signficance in rare cases of venous thrombotic vascular diseases. .

PS209

Platelet function and vascular risk factors in healthy individuals and patients with venous thrombotic vascular diseases

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Background: As demonstrated in own previous examinations hyperactivation of platelets might also contribute to venous thrombotic complications and lead to an even twofold risk of thrombosis. We therefore aimed to find out the influence of conventional vascular risk factors on spontaneous platelet aggregation (PAT III) in clinically inapparent individuals and patients with venous vascular thrombotic vascular diseases**. Patients and Methods:** PAT III was analyzed 30 minutes after venopuncture in 95 healthy people and 261 patients with venous thrombotic diseases. Platelet rich plasma using citrated blood was obtained by centrifugation at 180g (1000 U./min.; Rotixa/Ks, Hettich). PAT III (normal: angle $\alpha < 40^{o}$) was evaluated 30 min. after venopuncture in a clinically "steady state" with no signs for an increased thrombin generation (d-dimer concentrations < 0.5 mg/l; Nycocard/Immuno-Baxter; TAT complexes < 3.5 µg/ml; Enzygnost/Dade Behring). Hyperaggregation of platelets was only diagnosed since reproducible. The influence of nicotine abuse, hypertension, obesity, hyperlipidemia and age above 60 years on platelet function was controlled on either healthy individuals (group I) as well as patients with venous vascular diseases (group II). For statistical analysis the Chi-Square Assay was performed. Odds ratios and 95% confidence intervals were also calculated. **Results:** Neither nicotine abuse, nor hypertension (> 140/80 mm Hg), hyperlipidemia (> 200 mg/dl), obesity (Body mass index > 27.8 kg/m2 (males); > 27.3 kg/m2 (females)), nor the age above 60 years was associated with increased PAT III values (angle $\alpha > 40^{o}$). Comparision between groups demonstrated no significant effect on an increase of PAT III by these conventional vascular risk factors ($p > 0.05$; chi-square assay). Nevertheless an increase of PAT III values were either objected in patients with clinically apparent or spontaneous venous thrombosis as prescribed by own data. **Conclusions:** Conventional vascular

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