Abstract Book

MODERN TRENDS IN HUMAN LEUKEMIA

XVI. Wilsede Meeting / June 18 – 22, 2005

Title picture, artist: Michel Weidemann

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SPEAKER ABSTRACTS

Regulation and Hierarchy of Mesodermal-Derived Stem Cells

Speaker 2:

Identification of a novel hierarchy of endothelial progenitor cells from the blood and blood vessels of human subjects

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Endothelial progenitor cells (EPCs) can be isolated from adult peripheral and umbilical cord blood and expanded exponentially ex vivo. In contrast, human umbilical vein endothelial cells (HUVECs) or human aortic endothelial cells (HAECs) derived from vessel walls are widely considered to be differentiated, mature endothelial cells (ECs). However, similar to adult and cord blood derived EPCs, HUVECs and HAECs derived from vessel walls can be passaged for at least 40 population doublings in vitro. Based on this paradox, we tested whether EPCs reside in HUVECs or HAECs utilizing a novel single cell deposition assay, which discriminates EPCs based on their proliferative and clonogenic potential. We demonstrate that a complete hierarchy of EPCs can be identified in HUVECs and HAECs derived from vessel walls and discriminated by their clonogenic and proliferative potential. This study provides evidence that a diversity of EPCs exists in human vessels and provides a conceptual framework for determining both the origin and function of EPCs in maintaining vessel integrity.

Speaker 3:

Mesenchymal stem cell: Ability to transfer hematopoietic microenvironment, radiosensitivity, self-maintenance potential, sensitivity to cytokines and hormone, precursor hierarchy.

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Bone marrow derived mesenchymal stem cells (MSCs) are adherent cells capable of multilineage differentiation (osteogenic, chondorgenic, adipogenic, etc. when exposed to appropriate in vitro stimuli). MSCs do not express main hematopoietic markers such as CD45. However, markers of MSC (Stro-1, HOP26, SH-2, SB-10, integrin b1 and others) are insufficient for their separation into homogenous population. Here we studied the MSC characteristics in vivo. MSCs are capable to transfer hematopoietic microenvironment. After implantation of femur bone marrow plug under renal capsule of syngeneic recipients the ectopic hematopoietic foci formed. Stromal cells in such foci are derived from donor MSCs while hematopoietic cells have recipient's origin. The size of the foci formed is proportional to the femur equivalent transplanted, which can be used for semi-quantitative determination of MSC number. The radiosensitivity of MSC is characterized by Do = 444 cGy and extrapolation number n = 5.2. The data showed high radioresistance of MSC and their capacity for intracellular Elkind's repair because after neutron irradiation, which made repair impossible, the n was about 1.2. MSCs have high self-renewal capacity – it is possible to transfer ectopic foci more than 9 times without decrease in their size. Explantation of bone marrow plug or 3–30 week-old adherent cell layer of long-term bone marrow culture in irradiated recipients produced foci 2-3-fold bigger that in normal ones. However, secondary transfer of such big foci to normal recipient led to formation of normal size foci. This suggests existence, in hierarchy of mesenchymal cells, of progenitors with decreased proliferative potential that are more mature than MSC. Stromal growth factor produced by irradiated bone cells induces proliferation of more mature than MSC progenitors. In mice treated for 4–17 days with cytokines G-CSF and SCF the content of MSC in bone marrow increased about 2-3-fold. In TNF-/- knockout mice foci formation was blocked. Vise versa, parathyroid hormone stimulated bone formation by more mature mesenchymal progenitors. The system applied provides new possibilities for studying MSC properties and mechanism of their differentiation; which could be helpful for evaluation of clinical significance of MSC transplantation.

Defining and Manipulating the Hematopoietic Stem Cells

Speaker 4:

Segregation of long- and short-term reconstituting hematopoietic stem cells at near homogeneity

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Sustained self-renewal is the defining characteristic of stem cells, and self-renewal in leukemia stem cells is increasingly recognized as a potential target for cure. One approach to understanding the self-renewal process is to isolate the RNA- and protein-level differences between cells that self-renew and cells that do not. For that approach to work, it is necessary to obtain both kinds of cell in nearly pure form. We previously showed that in the RholoKit+Sca1+Lin- (RKSL) fraction of mouse bone marrow, most cells are individually multipotent and capable of multilineage engraftment 4–8 wk after transfer of single cells into irradiated recipient mice. However, of cells initially transplanted, only 20% established grafts enduring to 32 wk and beyond. We now show that the RKSL fraction can be further divided on the basis of expression of surface a2-integrin (CD49b). Both CD49b+ and - cells were again shown to be individually multipotent for generation of both myeloerythroid and lymphoid progeny, and for high frequency generation of 4–8 wk grafts in mice. However, they differed markedly in other biological parameters. While both showed significant delay to onset of cell division in culture commensurate with their initial quiescent state, first mitoses of CD49b- cells cultured with KL/FL/IL7/IL11 were delayed beyond 40 hr, 12 hr beyond the onset of division in CD49b+ cells. Strikingly, macroscopic 12 day spleen colonies were generated from CD49b+ but not CD49b- cells. Of greatest interest, grafts from CD49b+ cells uniformly faded after 8–16 wk, while most early grafts from CD49b- cells persisted to 32 wk and beyond. By quantitative competitive assay, 60% of RKSL49b- cells yielded long term erythroid grafts, an estimate strengthened by assay at limiting dilution where 45% of individual injected cells, and 60% of cells that initially engrafted, generated persisting myeloerythroid grafts. Thus, RKSL49b- and + cells both represent nearly homogeneously pure, quiescent, lymphomyeloerythroid reconstituting cells differing nearly quantally in their capacity to sustain self-renewal into the long term. Analysis of transcript expression in these fractions has begun, and early results will be presented.

Speaker 5:

Tracking hematopoiesis at the single cell level

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Despite intensive research, many long-standing questions of experimental hematology remain unsolved. One major reason is the fact that hematopoiesis is usually followed by analyzing the fate of populations of cells – rather than individual cells – at very few time points of an experiment and without knowing (or quickly loosing) their individual identities. The static picture yielded by this approach makes it impossible to appreciate the dynamic developmental processes leading to the (re)generation of the hematopoietic system from individual hematopoietic stem cells (HSC). Real-time tracking of individual cells in culture, tissues or whole organisms would be an extremely powerful approach to fully understand the developmental complexity of hematopoiesis. However, many of the needed tools are still under development and their application in hematology remains difficult. Here, a computer aided culture and imaging system was developed to follow the fate of individual cells over long periods of time with highest temporal resolution. A new software module was written, which helps to record and display the divisional history, position, properties etc. of individual cells. This system was used to analyze the development of multilineage cobblestone colonies from highly purified individual adult HSC in stroma co-cultures at single cell level over many generations, yielding new insights in the behavior of HSC under conditions closely resembling their physiological environment in the bone marrow. The presented data will improve the understanding of the cellular dynamics of adult and embryonic hematopoietic development.

Speaker 6:

Isolation and characterization of a novel hematopoietic stem cell gene, S76

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The fact that CD45-negative and vascular marker-positive cell population in aorta-gonad-mesonephros (AGM) region of mouse embryos possesses long-term hematopoietic repopulation activity has suggested that hematopoietic stem cells (HSCs) are derived from hemangioblasts, common precursors for blood and vascular cells. However, equivalent repopulation capacity has not been found in ES-derived hemangioblasts which are defined by the expression of Flk1 and SCL/Tal1 and blast-colony forming activity. To obtain an insight for the genetic program that supports the HSC development in vivo, we have compared the mRNA expression between the ES-derived hemangioblasts (Flk1+SCL+ cells from day2.75 embryoid bodies) and HSCs (Lin-Kit+Sca-1+CD34-/low cells from adult mouse bone marrow) by representational differentiation analysis. By this effort, we identified 20 known genes (Shp1, Mpl, Nore1, Cyt28, Sca-2, Egr1, etc) and 10 previously uncharacterized genes, all of which were predominantly or more abundantly expressed in HSCs than in the ES-derived hemangioblasts. We chose S76 gene, one of the novel HSC genes, for further analysis. S76 mRNA was mainly expressed in hematopoietic tissues in adult mice and encodes a membrane protein. The expression level of S76 mRNA was higher in Lin-Kit+Sca-1+CD34-/low bone marrow cells (containing long-term repopulating HSCs) than in Lin-Kit+Sca-1+CD34+ bone marrow cells. When S76 mRNA was suppressed in the HSC primary culture by infecting a retrovirus vector encoding S76-siRNA and PGK promoter-driven EGFP, frequency of myeloid colony-forming cells in the EGFP+ cells was severely reduced. On the other hand, an MC/9 mast cell transfectant expressing S76-siRNA exhibited a higher growth rate in response to IL-3. Therefore, S76 protein could be a novel modulator of cytokine responses in hematopoietic cells. As an ongoing study, we have generated a mouse line in which one of the S76 gene loci is replaced with Venus (GFP derivative) gene. Consistent with the RT-PCR data, approximately 2% of lineage-negative hematopoietic cells in both fetal liver and adult bone marrow were Venus-positive. Further characterization of S76+ cells and phenotypic analysis of S76 null mice would clarify a function of this novel protein.

Speaker 7:

Clonal dominance in murine long-term hematopoiesis triggered by retroviral gene marking

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Replication-defective gammaretroviral vectors have successfully been used for >20 years to track the *in vivo* fate of cell clones in animal and clinical gene marking studies. Recent observations of malignant transformation of engineered cells due to proto-oncogene upregulation after retroviral vector insertion reminded us of the mutageneic potential of those vectors. Here we investigated whether insertional mutagenesis may also influence the contribution of individual cell clones to normal long-term hematopoiesis. Therefore, using ligation-mediated (LM-) PCR we retrieved retroviral insertion sites from hematopoietic cells of mice exhibiting progression to oligoclonal or monoclonal hematopoiesis upon serial bone marrow transplantation. In samples harvested from primary recipients seven months after transplantation of gene-marked cells a number of intriguing hits *(HoxB4, Evi1, Ly78, Ccnd3, Pip5k2a)* was found obviously already reflecting selection for long-term repopulating ability. Even more strikingly, all 29 insertions recovered from clones dominating serially transplanted recipients affected loci with an established or potential role in the self-renewal or survival of hematopoietic stem cells. Quantitative RT-PCR revealed transcriptional dysregulation in all insertion sites analyzed (n=12). Thus our data clearly demonstrate that retroviral integrations themselves may trigger non-malignant clonal expansion in murine long-term hematopoiesis. These findings have major implications for diagnostic gene marking and gene therapy as well as the discovery of genes regulating stem cell turnover.

Speaker 8:

HOXB4 enforces similar fates of ES-cell derived and adult hematopoietic cells

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In the context of somatic gene therapy of the hematopoietic system, transplantation of molecularly defined and, hence, "safe" clones would be highly desirable. However, techniques which allow gene targeting, subsequent *in vitro* selection and clonal expansion are only available for embryonic stem (ES) cells. Previously, it has been shown that *in vitro* differentiated ES-cells engraft when ectopically expressing HOXB4, but it remained unclear whether these cells could fully resemble adult bone marrow function after transplantation[1)]. We here demonstrate that HOXB4 enforces similar fates of *in vitro* differentiated ES-cells (CCE) and adult bone marrow cells (C57Bl/6J). Differentiated ES-cells expressing HOXB4 from a retroviral vector and grown *in vitro* for 20 days, recapitulated the growth and differentiation properties of adult bone marrow cells after transplantation into $Rag2^{(-/-)}\gamma C^{(-/-)}$ and C57Bl/6J recipient mice. Furthermore, we show that the amount of ectopically expressed HOXB4 influences differentiation in both systems in a similar manner. HOXB4 enforced myeloid development over a wide range of expression levels, whereas only high expression levels of HOXB4 were detrimental for erythroid development.

Incompatibility of high levels of HOXB4 expression with erythroid differentiation was also directly demonstrated using a recently described *in vitro* ES-cell differentiation system[2)]. Histological analysis of the "HOXB4-transplanted" mice revealed increased granulopoiesis both in sternal bone marrow and in spleen sections. However, all stages of granulocytic differentiation were present and neither were immature cells detected in the periphery nor was leukemic infiltration detected in other organs. Hence, none of the animals became leukemic during the observation period. In summary, ES-cells should be considered a promising alternative to bone marrow stem cells carrying the potential of safe somatic gene therapy, provided that human ES-cells can be similarly manipulated. Nonetheless, advanced cell therapy will certainly require the expression of HOXB4 in a regulated manner to avoid unwanted effects such as disturbed lineage differentiation.

1) Kyba,M, Perlingero,RC, Daley,GQ (2002) HOXB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell*, **109**, 29–37.
2) Carotta,S, Pilat,S, Mairhofer,A, et al. (2004) Directed differentiation and mass cultivation of pure erythroid progenitors from mouse embryonic stem cells. *Blood*, **104**, 1873–1880.

Speaker 9:

In vivo selection of genetically modified primary human hematopoietic cells using a cell growth switch

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Methods for regulating the growth of transplanted cells have many applications in gene and cell therapy. One such method uses conditional signaling molecules that are activated by artificial ligands called chemical inducers of dimerization (CIDs). Here we examine the response of human cord blood cells to a CID-triggered growth signal, in vivo. $CD34^+$ cells transduced with a lentivirus vector encoding a derivative of the thrombopoietin receptor (F36VMpl) and green fluorescent protein (GFP) were transplanted into immune deficient mice. CID treatment induced a 12–17 fold expansion of GFP^+ erythroid cells in the marrow, and the appearance of a unique subset of human erythroid burst forming units (BFUe) and mixed progenitors (CFU-mix) in the spleen. Linker amplification mediated (LAM)-PCR demonstrated that the CID response was polyclonal, that most CID responsive clones contained more than one transgene copy, and that there was compartmentalization of the CID-responsive BFUe and CFU-mix in the spleen from the CID-responsive erythroblasts in the marrow. Both the CID-responsive marrow erythroblasts and splenic progenitors were detectable for up to 5 weeks following completion of CID, but neither was detectable in secondary transplant recipients. These findings establish CIDs as in vivo growth factors for genetically modified human hematopoietic cells.

Speaker 10:

Gene transfer into hematopoietic CD34+ cells as a therapy for Chronic Granulomatous Disease

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Chronic Granulomatous Disease (CGD) is a primary immunodeficiency characterized by recurrent and life-threatening bacterial and fungal infections. CGD is caused by mutations in any one of four genes encoding for subunits of the phagocytic NADPH oxidase complex. Most patients contain mutations or deletions within the gene coding for gp91phox, the larger subunit of the complex. Based on our preclinical work, two X-CGD patients, 26 and 25 years old, were treated with gene modified cells. G-CSF mobilized CD34+ cells were transduced with a monocistronic gp91phox retroviral vector. The transduction efficiency was 45% for Pat.1 and 40% for Pat. 2. The number of CD34+ cells reinfused was 1.2x10e7 per kg for Pat.1 and 0.9x10e7 per kg for Pat. 2. Before reinfusion of gene transduced cells, patients were treated with liposomal busulphan given i.v. at a dose of 4 mg/kg at two consecutive days, starting at day -3. The treatment was well tolerated and no adverse effects have been observed. Neutrophil counts declined to less than 100 cells per µl at day 15 post reinfusion for both patients and recovered to more than 500 cells per µl at day 30 for Pat. 1 and day 20 for Pat. 2. A significant fraction of gene marked cells has been detected in peripheral blood of both patients since day +21. Similarly, therapeutic relevant levels of NADPH oxidase activity have been observed since day +21. Both patients are well and have been free of severe bacterial and fungal infections since transplantation. Our data suggests that gene therapy is an option for the treatment of CGD, despite the fact that gp91phox corrected cells will not have per se a proliferative advantage over non-transduced cells.

Mechanisms of Differentiation

Speaker 11:
Replication timing and lineage restriction

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Our interest is to understand how patterns of gene expression are established, maintained through cell division, and changed in a progressive and ordered fashion in development. Although we do not yet understand how this is achieved, it is likely that chromatin-based modifications to the genome play a significant role both in maintaining cell identity and in specifying (or restricting) lineage fate.

We have recently described a novel PCR-based approach that allows us to determine the time of DNA replication of genes in various cell types. Late replication is a consistent feature of constitutive heterochromatin (and some facultative heterochromatin), suggesting a direct link between chromatin structure/condensation and delayed DNA replication. Using this PCR-based approach we have begun to compare the chromatin status of key genes that specify lineage fate in cells with different developmental potentials; omnipotent ES cells, multi-potent haematopoietic precursors and unipotent lymphocytes. We show that most genes replicate early in S-phase in ES cells, consistent with a broadly accessible chromatin state. Many of these genes also replicate 'early' in haematopoietic cells at subsequent stages of development, irrespective of whether these genes are expressed. However, a subset of 'master-regulator genes' was found to replicate early in ES cells but consistently later in more differentiated cells. Collectively these data provide novel molecular evidence that lineage restriction operates at the level of declining chromatin accessibility and show that 'chromatin-profiling' can be used to predict the developmental potential of different stem cell populations. The implications of this analysis for tracing lineage fate-maps and understanding cellular plasticity are discussed.

Speaker 12:

Toward guided differentiation of ES cells: A microarray database of intermediates generated during ES cell differentiation

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ES cells are multipotent cells that can undergo unlimited proliferation in vitro without differentiation. As many cell lineages have been generated by in vitro differentiation of ES cells and there are no known limits to the number of cells which can be generated in culture, it is widely accepted that ES cells may serve as a source of materials for cell based therapies. There is also increasing evidence to support the notion that ES cells follow the same path of differentiation as that which occurs in the embryo, suggesting that in vitro ES cell differentiation can serve as a model for studying embryonic development. However, after decades of studying ES cell differentiation we are still very limited in our ability to guide the differentiation process to specific cell types. To overcome our current inabilities we feel that a more systematic approach to the problem is required, where data describing intermediate stages and lineage divergences is collected in a systematic manner and analysed to provide new strategies for steering each divergence point.

For this purpose we have created, and are continuously expanding a database of transcription based on Affymetrix oligonucleotide arrays. This database currently contains over 70 purified samples including more than 16 distinct early intermediates appearing during ES cell differentiation representing all three primary germ layers. In addition, this database contains a similar number of samples derived from embryonic and adult tissues which provide a powerful transcriptional context aiding the meaningful analysis of the differentiation data. In order to handle the data generated in this process we have developed novel analysis systems that allow both analysis and dissemination of the data. This system does not aim to answer narrowly defined questions but rather facilitates the rapid appraisal of results obtained by fuzzy queries by providing both expression and genomic context in a convenient graphical interface. Importantly this system allows the data to be accessed concurrently by a number of researchers specialising in various aspects of ES cell differentiation, thus allowing the biologically relevant information to be extracted.

This database is useful for many different aspects of differentiation including;
1) defining growth factor requirements for distinctive stages,
2) developing novel surface markers that can be used for monitoring and purifying intermediates,
3) identifying novel genes involved in embryogenesis,

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Speaker 13:

Hematopoietic differentiation hotspots

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The conception of the present-day model of hematopoiesis was begun by the work of Professor Ernst Neumann in the nineteenth century when he established that immature blood cells in the bone marrow migrate out into the blood vessels. Here was the birth of the hierarchal model of hematopoiesis. The hierarchical models of hematopoiesis suppose an ordered system in which stem cells and progenitors with specific fixed differentiation potentials exist. Jumping 135 years into the present day, recent data suggests that the stem cell regulation is not based on the classic hierarchical model, but instead more on a functional continuum. Data now exists showing that the potential of marrow stem cells to differentiate changes reversibly with cytokine-induced cell cycle transit. A hematopoietic stem cell is defined as a cell with extensive self-renewal and proliferative potential, coupled with the capacity to differentiate into the progenitors of all the blood cell lineages. Self-renewal refers to the potential to produce daughter cells with identical characteristics. Self-renewal resulting in production of identical stem cells without any new differentiated characteristics has not been established experimentally, but renewal on a cell population basis clearly occurs. The most primitive cells are characterized by absence of lineage markers, low-level expression of a relatively large number of cytokine receptors and relative exclusion (or pumping out) of the dyes rhodamine and Hoechst. Murine experiments have been performed on these lineage^{negative}rhodamine^{low}Hoescht^{low} (LRH) purified marrow stem cells. When exposed to thrombopoietin (TPO), FLT3-ligand and steel factor, they synchronously pass through cell cycle, the capacity of these cells to respond to a differentiation inductive signal (G-CSF, GM-CSF and steel factor) is altered at different points in cell cycle. Megakaryocyte production is specifically focused at mid-S phase; a time at which megakaryocytic active transcription factors (GATTA-1, GATTA-2 and FOG) and receptors for G-CSF, GM-CSF and TPO are up-regulated. These changes are all reversible. Granulocyte differentiation also shows “differentiation hotspots”. These data indicate that marrow hematopoiesis at the stem cell level is regulated on a continuum, not in a hierarchy and indicate that stem cells and progenitors could be the same cell in different functional states.

Speaker 14:

Epigenetic plasticity of hematopoietic cells

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Epigenetic processes involve the establishment of patterns of gene expression via heritable alterations in the chromatin structure of different genes. However, the molecular mechanisms of how this is achieved at the level of individual genes are poorly understood. To identify the order of molecular events taking place during the developmentally controlled activation and silencing of genes, we are examining the regulation of chromatin structure of the macrophage-colony-stimulating factor receptor (csf1 or c-fms) gene, which is expressed in monocytes and macrophages[1].

We previously examined how c-fms chromatin structure is organised in hematopoietic stem cells[1]. We also showed that c-fms is expressed in early lymphoid as well as myeloid progenitor cells, indicating that these cells are epigenetically similar. We have now performed experiments demonstrating which transcription factors are required for the de novo establishment of active chromatin at c-fms and we studied the order of event occurring during active chromatin formation.

To study lineage specification at the chromatin level we previously examined silencing of c-fms during B cell development[2]. We showed that epigenetic silencing of the c-fms locus during B lymphopoiesis occurs in distinct steps and that even mature B cells displayed a partially active chromatin structure. This partially active structure correlated with the ability of c-fms to be re-expressed after the conditional inactivation of the B cell specific transcription factor Pax5. Our experiments indicate that Pax5 has to be present throughout B lymphopoiesis to counteract active chromatin formation and to maintain myeloid-specific genes in a silent state. To gain insight into the molecular mechanism by which Pax5 represses c-fms expression we have now characterised the how Pax5 regulates c-fms chromatin modification and chromatin accessibility. Most importantly, we identified the cis-regulatory elements on the c-fms locus that mediate the Pax5 response, and we analyzed the molecular details of their mode of action. Our results provide important mechanistic insights into how lineage appropriate gene expression programs are established and maintained in the hematopoietic system.

1) Tagoh et al., Genes and Dev. 2002
2) Tagoh et al., EMBO J. 2004
Our work is funded by the Leukaemia Research Fund and the BBSRC.

Speaker 15:

Regulation of hematopoietic stem cells by myeloid transcription factors

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Acute Myelogenous Leukemia (AML) is the most common form of acute leukemia in adults. It is characterized by a block in myeloid differentiation. Myeloid transcription factors, including PU.1 and C/EBPα play critical roles in myeloid lineage differentiation. Disruption of PU.1 function in non-conditional knockouts in mice leads to an early multi-lineage block, and C/EBPα knockout mice demonstrate a block at the earliest stage of granulocyte differentiation and have myeloid blasts in the blood. Introduction of PU.1 or C/EBPα into multipotential precursor cells can restore lineage specific differentiation. Consistent with these phenotypes, mutations and other disruptions of C/EBPα and PU.1 have been found in human AML. However, the embryonic and/or perinatal mortality of both PU.1 and C/EBPα non-conditional knockouts has limited our ability to utilize them to model human AML. In order to further characterize the stage at which myelopoiesis is blocked, and to develop animal models of human AML, we have developed conditional knockouts of PU.1 and C/EBPα. Analyses of non-conditional fetal liver and conditional bone marrow PU.1 knockout hematopoietic cells demonstrate an early block at the hematopoietic stem cell (HSC) level, with a defect in self-renewal. In addition, PU.1 is required later for the differentiation of myeloid progenitors to granulocytes and monocytes. In contrast, non-conditional (fetal liver) and conditional (adult bone marrow) C/EBPα cells are blocked at a later stage, the CMP to GMP (granulocyte/macrophage progenitor) transition. Granulocytic differentiation is not observed in the peripheral blood, spleen, and bone marrow of the conditional C/EBPα knockout mice. However, erythroid and megakaryocyte development are normal. While CMP cells from C/EBPα$^{-/-}$ mice can differentiate to all different lineages in vitro, cells in GM colonies were mainly immature myeloid cells. Loss of C/EBPα function selectively blocks myeloid cell differentiation, but does not inhibit the development of other hematopoietic cell types. In contrast to what was observed for PU.1, C/EBPα$^{-/-}$; stem cells have a competitive advantage over wild type, and C/EBPα$^{-/-}$ blasts persist in the bone marrow and peripheral blood for months. C/EBPα$^{-/-}$ HSC demonstrate upregulation of Bmi1, and we hypothesize that this accounts for the increased self-renewal activity. However, the animals do not develop a malignant leukemia. It is possible that additional abnormalities in addition to loss of C/EBPα function, such as FLT3 mutations and/or upregulation of anti-apoptotic signals, may be required to induce leukemia in the mouse, and this can be tested using our conditional knock-out lines. In contrast to PU.1, C/EBPα is required differentiation from CMP to GMP, but not for later stages of granulocytic differentiation. Therefore, there are significant differences in the function of C/EBPα and PU.1 at both the HSC stage and later in granulocytic development.

Speaker 16:

Cholinergic facilitation of stress-induced myelopoiesis

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Glucocorticoid-initiated granulocytosis following trauma persists long after cortisol levels decrease, through yet unknown modulators of hematopoiesis. An intriguing candidate for such a modulatory role is the AChE-R splice variant of acetylcholinesterase. In brain and muscle a functional glucocorticoid response element (GRE) in the distal promoter of the ACHE gene induces overexpression of AChE-R following trauma. Here, we report that this acetylcholinesterase variant, AChE-R, and its cleavable, cell-penetrating C-terminal peptide ARP_{26}, facilitate granulocytosis. To explore the possibility that circulating and/or blood cells-associated AChE-R regulates sustained granulocytosis following stress, we employed labor and childbirth as the paradigm for acute, transient stress. In intra-partum women, increased AChE-R expression in granulocytes observed by flow cytometry, correlated significantly with their high cortisol levels and elevated AChE activity in plasma. In post-delivery patients, AChE-R-expressing granulocytes increased concomitantly with AChE activity levels which persisted after cortisol had declined. In cultures of $CD34^+$ HPC, cortisol induced AChE-R production whereas ARP_{26} promoted ACHE gene expression and facilitated myelopoiesis, granulocyte expansion and maturation and megakaryocytopoiesis in an antisense-suppressible manner. ARP_{26} for 24 hours facilitated the production of the pro-inflammatory cytokines interleukin (IL)-6, IL-10 and TNFα in adult peripheral blood mononuclear cells and enhanced proliferation of newborn $CD34^+$ progenitors in culture as well as their engraftment in NOD/SCID mice at 3 and 6 weeks post-transplant. Stress-induced AChE-R accumulation and consequent pro-inflammatory reactions thus facilitate prolonged myelopoiesis.

1) Deutsch et al., Experimental Hematology 30: 1153–1161, 2002.
2) Perry et al., Neoplasia, 6: 279-286, 2004
Supported by the Defense Advance Research Project Agency (#N66001-01-C-8015, to H.S.) and the Deutsches Krebsforschungszentrum (DKFZ), the BSF-US-Israel Binational Science Fund (2003028-01, to H.S.) and the Israel Ministry of Science (#CA00097/0174 to VRD)

Speaker 17:

Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders

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Human myeloproliferative disorders form a range of clonal haematological malignant diseases, the main members of which are polycythaemia vera, essential thrombocythaemia, and idiopathic myelofibrosis. The molecular pathogenesis of these disorders is unknown, but tyrosine kinases have been implicated in several related disorders. We investigated the role of the cytoplasmic tyrosine kinase JAK2 in patients with a myeloproliferative disorder. We obtained DNA samples from patients with polycythaemia vera, essential thrombocythaemia, or idiopathic myelofibrosis. The coding exons of JAK2 were bidirectionally sequenced from peripheral-blood granulocytes, T cells, or both. Allele-specific PCR, molecular cytogenetic studies, microsatellite PCR, Affymetrix single nucleotide polymorphism array analyses, and colony assays were undertaken on subgroups of patients. A single point mutation (Val617Phe) was identified in JAK2 in 71 (97%) of 73 patients with polycythaemia vera, 29 (57%) of 51 with essential thrombocythaemia, and eight (50%) of 16 with idiopathic myelofibrosis. The mutation is acquired, is present in a variable proportion of granulocytes, alters a highly conserved valine present in the negative regulatory JH2 domain, and is predicted to dysregulate kinase activity. It was heterozygous in most patients, homozygous in a subset as a result of mitotic recombination, and arose in a multipotent progenitor capable of giving rise to erythroid and myeloid cells. The mutation was present in all erythropoietin-independent erythroid colonies. Our results demonstrate that a single acquired mutation of JAK2 was present in more than half of patients with a myeloproliferative disorder. Its presence in all erythropoietin-independent erythroid colonies demonstrates a link with growth factor hypersensitivity, a key biological feature of these disorders. Identification of the Val617Phe JAK2 mutation lays the foundation for new approaches to the diagnosis, classification, and treatment of myeloproliferative disorders.

The Hematopoietic Stem Cell Niche

Speaker 18:

Key Molecules within the hemopoietic stem cell niche

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The concept of a stem cell “niche” within the bone marrow (BM), first proposed by Schofield (1978), suggested a complex interaction of signals between HSC and their immediate microenvironment that due to the difficulties in identifying HSC in-situ, has remained relatively theoretical. Our data demonstrate a discrete spatial localization of transplanted haemopoietic cells within the BM as a result of specific, hierarchically dependent patterns of migration suggesting the endosteal region as the site for HSC “niches” in adult BM. However the molecular mechanisms responsible for this unique distribution remain unclear. We recently demonstrated that following transplantation and homing to the BM, membrane bound SCF, and the two CD44 ligands hyaluronic acid (HA) and osteopontin all play different but key roles in the lodgment and or regulation of HSC in the endosteal marrow niche. Specifically, membrane bound SCF and osteopontin within the endosteal hemopoietic microenvironment are important in HSC trans-marrow migration to the endosteal region, while HSC synthesise and express HA and the presence of this glycosaminoglycan on HSC is also critical for the spatial distribution of transplanted HSC in vivo. Analysis of HAS 1 and 3 knockout mice identified HAS 3 as the key HA synthase in this component of stem cell engraftment. In addition, we have demonstrated that both HA and Opn have key physiological negative regulatory roles in HSC proliferation and differentiation. Furthermore, we have developed methodology allowing the separate isolation of HSC from the central marrow core and the endosteal region of the BM. Cells isolated from the endosteal region have enhanced proliferative and regenerative potential in vitro and in vivo. Overall, we have identified multiple molecules that have important roles in the lodgement and regulation of HSC within the haemopoietic stem cell “niche” and developed methodology to specifically isolate HSC from this region.

Speaker 19:

Mechanism of human stem cell homing and mobilization in transplanted NOD/SCID mice

Tsvee Lapidot

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Migration of hematopoietic stem cells in vivo is essential for their repopulation potential. We revealed the central roles of the chemokine SDF-1 and its receptor CXCR4 in human CD34+/CD38-/low SRC homing, repopulation and mobilization in transplanted NOD/SCID mice which involve cAMP activation and PKC-z signaling. Activation of the adhesion molecules CD44, LFA-1, VLA-4 and VLA-5 by SDF-1 is essential for human CD34 cell homing and repopulation and blocking CD44 inhibited SDF-1 activation and protrusion formation by these progenitors. Over expression of CXCR4 on human CD34 enriched progenitors increase SDF-1 migration, bone marrow repopulation and proliferation since this chemokine is also a survival factor. Release of progenitors from the bone marrow to the circulation involves signaling by the pan-leukocye antigen CD45 which is also expressed on hematopietic CD34+ progenitors. In addition to MMP2/9 secretion SDF-1 also activated MT1-MMP which is needed for optimal migration, homing and mobilizaion of human CD34+ progenitors. In addition, stress induced recruitment of immature and maturing leukocytes from the bone marrow reservoir during bleeding, injury and viral or bacterial induced inflammation as part of host defense and organ repair also involve SDF-1/CXCR4 interactions. This process is mimicked in clinical stem cell mobilization and include activation of osteoclasts, osteobalsts, secretion of proteolytic enzymes, cytokines and chemokines. Finally, migration, survival, proliferation and invasion of leukemic human progenitors (SLIC) obtained from AML and Pre B ALL patients in transplanted NOD/SCID mice are also dependent on CXCR4 signaling and the proteolytic enzyme elastase is essential for AML SLIC function.

Speaker 20:

Rac and Rho: Antagonistic GTPases regulating hematopoietic engraftment, lymphopoiesis and possibly lymphomagenesis

David Williams, Jose Cancelas, Yi Gu

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Rho GTPases regulate multiple cell functions, including cell shape, migration and adhesion, proliferation and survival. We have reported the key roles that the related Rho GTPases Rac1 and Rac2 play in hematopoiesis (Gu and Filippi et al. Science, 2003), and specific roles these GTPase play in neutrophil biology (Filippi et al., Nature Immunol., 2004). Indeed, mutations in Rac2 have been implicated in a human phagocytic immunodeficiency disease (Williams et al. Blood, 2001). We have now examined the role of the Rho GTPases Rac1 and Rac2 in HSC engraftment and mobilization. Rac1, but not the hematopoietic-specific Rac2, is required for the engraftment phase of hematopoietic reconstitution, since Rac1-/- HSC fail to rescue in vivo hematopoiesis after transplantation, but induced deletion of Rac1 sequences after initial engraftment does not impair steady-state hematopoiesis. Rac1-/- HSC/P show impaired spatial localization to the endosteum but near normal homing to the medullary cavity in vivo. Interaction with the bone marrow microenvironment in vitro is markedly altered. While post-engraftment los of Rac1 alone does not impair hematopoiesis, combined loss of Rac1 and Rac2 leads to ineffective hematopoiesis associated massive mobilization of HSC from the marrow and intense selection for Rac-expressing HSC. This mobilization is reversible by re-expression of Rac1. In addition, a rationally designed, small molecule inhibitor of Rac activation leads to transient mobilization of engraftable HSC/P. Rac proteins thus differentially regulate engraftment and mobilization phenotypes suggesting that these biological processes and steady-state hematopoiesis are biochemically separable and that Rac proteins may be important molecular targets for stem cell modification. We also have examined the role of a second hematopoietic-specific Rho GTPase, RhoH, in hematopoiesis. RhoH appears to act in an antagonistic fashion to Rac (Gu et al. Blood, 2005). RhoH is ‘GTPase- deficient’ and thus is expressed and remains in the active, GTP-bound configuration. Our preliminary studies using overexpression and knock-down of RhoH expression in hematopoietic stem/progenitor cells suggest that altered RhoH expression levels can result in defective growth and actin-based function of these cells. Consistent with these results, initial characterization of RhoH-/- mice generated by us have demonstrated that loss of RhoH leads to an abnormal increase of HSC/P cells in bone marrow and marked defects in thymic T cell development and T cell receptor-mediated peripheral T cell activation and function. Given the lineage-restricted expression of RhoH and previous observation of mutations of the RhoH gene in human lymphomas (Preudhomme et al., Oncogene, 2000; Pasqualucci et al. Nature, 2001), we believe RhoH could be an important molecular target in lymphomas. Rho GTPases appear to be key regulators of hematopoietic stem cell function and lymphoid differentiation.

Cancer Stem Cells and Progression

Speaker 21:
Functional changes in aging stem cells

Gary Van Zant Ph.D.
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Stem cells are required for the replacement of worn-out tissues in many parts of the body. To do so throughout a lifetime, a careful balance must be struck between stem cell differentiation and self-renewal. Since stem cells are necessarily a long-lived population, they are subjected to prolonged exposure to various forms of stress. These include replicative stress and stresses caused by the environment; for example by reactive oxygen species. These stressors increase the risk of genomic instablity and cancer. Some types of leukemias have long been recognized as stem cell diseases, but there is a growing awareness that adult stem cells in other tissues may be the source of a number of solid tumors. Using hematopoietic stem cells as a model, my lab is investigating age-related changes in stem cell functions.

Speaker 22:

Characterization of the leukemia propagating cell in a murine model of CALM/AF10 positive leukemia

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Identifying the leukemic stem cell in acute leukemia is a key step in understanding the pathogenesis of this disease and to develop novel therapeutic strategies aiming at eradicating these leukemia propagating cells. Here we show in a murine bone marrow transplantation model of the t(10;11)(p13;q14) CALM/AF10 (C/A) positive acute myeloid leukemia (AML) that AML can be propagated by a lymphoid determined progenitor with myeloid differenation capacity. When mice were transplanted with BM cells retrovirally engineered to express the C/A fusion, all animals (n=13) died from an acute leukemia (median survival 125 days). Immunophenotyping and histochemistry was compatible with the diagnosis of AML, demonstrating a Gr1 (72%)/Mac1 (84%), MPO and CAE positive myeloid bulk population. Some of the transduced cells co-expressed B220 and Mac1 (27%) or displayed only B220+ (B220+/Mac1-)(5.5%). To determine the frequency of the leukemia repopulating cell (LRC) in the 3 different populations, cell populations were highly purified from primary leukemic mice and injected into cohorts of recipients in a limiting-dilution assay: the frequency of the LRC was more than 380fold higher in the 'B220+/Mac1-' population (1 in 36 cells) than in the 'Mac1+/B220-' bulk population (1 in 13906 cells) and more than 12fold than in the 'B220+/Mac1+' population (1 in 437 cells). When single cells of the B220+/Mac1- population were expanded in vitro, they gave rise to B220+/Mac1+ as well as the Mac1+/ B220- population; the B220+/Mac1- population as well as its B220+/Mac1+ and Mac1+/ B220- progeny were positive for the identical genomic DJ rearrangement at the IgH locus, demonstrating its capacity to differentiate into the myeloid lineage at the single cell level. The B220+/Mac1- population displayed a B220+/CD43+/AA4.1+/HSA+/CD19-/IL-7R+ phenotype, transcribed E2A, EBF, but also MPO and was negative for PAX5 cell. Taken together, this murine leukemia model indicate that AML can derive from a transformed progenitor cell with lymphoid characteristics, which opens the perspective to differentially target leukemic stem cells in a subset of AML patients.

Speaker 23:

JUNB in normal and leukemogenic hematopoiesis

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JunB is a component of the AP-1 (activator protein-1) family of transcription factors that have been implicated in the regulation of a broad range of biological processes. JunB has a strong anti-proliferative ability and pro-apoptotic function, thus emerging as a tumor suppressor that requires inactivation to allow tumor cells to acquire indefinite proliferation and to escape from programmed cell death. While no activating mutations, deletions or amplifications of any of AP-1 genes have been reported so far in human cancers, changes in their expression levels have been linked to the occurrence of various human diseases.

JunB is constitutively expressed in myeloid cells and its expression is modulated by various growth factor signalling cascades regulating proliferation, differentiation and/or survival in the myeloid lineage. Using gene-knockout technology in the mouse, we have previously shown that JunB acts as a tumor suppressor gene in myeloid cells. Mutant mice with age-dependent silencing of junB expression in the hematopoietic system ($junB^{-/-}$Ubi-junB transgenic rescued mice) progressively developed a transplantable myeloproliferative disorder (MPD) with features of human chronic myelogenous leukemia (CML), including progression to blast crisis and death. In addition to increased numbers of granulocytes, we recently found that junB-deficient MPD mice also displayed a specific expansion in two subsets of immature hematopoietic cells: the self-renewing long-term hematopoietic stem cell (LT-HSC) and the committed granulocyte/macrophage progenitor (GMP) populations. By selectively targeting junB deletion in LT-HSC or GMP through the use of conditional gene-knockout approaches, we demonstrated that junB inactivation must take place directly in LT-HSC and not at later stages of myeloid differentiation to induce MPD development. Using transplantation experiments, we also identified the LT-HSC as the population containing the leukemic stem cells (LSC) for the junB-deficient MPD since only junB-deficient LT-HSC and not GMP were capable of transplanting the disease in recipient mice. In addition, these results demonstrated that the HSC defects are inherent to JunB loss, while extrinsic factors, such as increased responsiveness and/or elevated levels of myeloid growth factors, are likely to contribute to the GMP and granulocyte defects. Finally, we showed that raising JunB levels in HSC through lentiviral transduction inevitably leads to stem cell loss.

Together these findings uncovered a novel and critical biological function for JunB in controlling homeostasis of the HSC compartment during normal and leukemogenic hematopoiesis, and demonstrated: 1) that JunB is a negative regulator of stem cell behavior; 2) that loss of JunB at the stem cell level leads to the development of a MPD similar to human CML; and 3) that cells at the HSC level are the only LSC in this mouse model of chronic leukemia, thereby clearly substantiating indirect evidence from the chronic phase of human CML. The BCR-ABL translocation (the oncogene encoded by the Philadelphia chromosome, the hallmark of human CML) is central to the development of CML but it is now clear that additional mutations are required for its leukemic evolution. Since down-regulation of junB expression has been observed in BCR-ABL-expressing CML cells, we propose that inactivation of junB in a subset of HSC could be one of the early pre-leukemic events occurring during the chronic phase of human CML, which confers proliferative and survival advantages to primitive HSC and myeloid progenitor cells, and allows them to gain the additional mutations required for their progression to a more aggressive phenotype. A current challenge is now to determine how JunB interact with the BCR-ABL pathway in mediating CML leukemogenesis in humans. The recent findings that deregulation of genes intimately tied to HSC homeostasis is associated with leukemia is a new and powerful paradigm in cell biology and cancer research.

Speaker 24:

C/EBPs in health and disease

A. Fritz Gombart Ph.D., Sigal Gery Ph.D., Phillip Koeffler M.D.

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CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors that regulate cell growth and differentiation in numerous cell types. To identify novel C/EBP-target genes, we performed transcriptional profiling using inducible NIH 3T3 cell lines expressing one of four members of the C/EBP family. Functional analysis revealed a previously unknown link between C/EBP proteins and circadian clock genes. Our microarray data showed that the expression levels of two core components of the circadian clock network, Per2 and Rev-Erba, are significantly altered by C/EBPs. Recent studies suggested that Per2 behaves as a tumor suppresser gene in mice. We, therefore, focused our additional studies on Per2. Using reporter assays, chromatin immunoprecipitation and conditionally expressing cell lines, we showed that Per2 expression is upregulated by C/EBP-alpha and C/EBP-epsilon. We found that Per2 expression is reduced in lymphoma cell lines and in acute myeloid leukemia (AML) patient samples. In addition, ectopic expression of Per2 in leukemia cell lines led to a significant G2/M growth arrest. These results suggest that Per2 is a downstream C/EBPalpha-target gene involved in AML, and its disruption might be involved in AML initiation and/or progression. Further elucidating the links between circadian rhythms and malignant growth may help open new therapeutic avenues.

In additional studies, low expression of C/EBPalpha mRNA was found in 83% of primary breast cancer samples. Immunohistochemical study further demonstrated either a markedly reduced or undetectable expression of C/EBPalpha protein in 30% of breast cancer specimens. The other 70% of breast cancers had C/EBPa expression in both the cytoplasm and nucleus; in controls, C/EBPalpha was localized to the nucleus in the normal ductal cells. C/EBPalpha expression was associated with estrogen- and prostesterone-receptor negative status. Induction of C/EBPalpha expression in these cell lines resulted in growth inhibition accompanied by G0/G1 cell cycle arrest and reduced anchorage-independent cell growth. C/EBPalpha expression was associated with downregulation of c-myc and up-regulation of p21, PPARgamma and the breast epithelial differentiation marker, maspin. These results suggest that reduced expression of C/EBPalpha may play a role in the development and/or progression of breast cancer.

Further studies explored the fact that both humans and mice lacking active C/EBPepsilon suffer frequent bacterial infections due to functionally defective neutrophils and macrophages (M-phi). We hypothesized that these defects reflected dysregulation of important immune response genes. To test this, gene expression differences of peritoneally-derived neutrophils and M-phi from C/EBPepsilon-/- and wild type mice were determined with DNA microarrays. Of 283 genes, 147 known genes and 21 ESTs were down- and 85 known genes and 31 ESTs were up-regulated in the C/EBP-/- mice. These included genes involved in cell adhesion/chemotaxis, cytoskeletal organization, signal transduction and immune/inflammatory responses. The cytokines CCL4, CXCL2, and IL6, as well as cytokine receptors IL8RB and CSF3R were downregulated. Chromatin immunoprecipitation (ChIP) analysis identified binding of C/EBPepsilon to their promoter regions. Increased expression for lipid metabolism genes APOE, SCARB1, SORL1 and APOC2 in the C/EBPepsilon -/- mice correlated with reduced total cholesterol levels in these mice before and after maintenance on a high fat diet. Also, C/EBP-epsilon-deficient M-phi showed a reduced capacity to accumulate lipids. In summary, dysregulation of numerous, novel C/EBPepsilon target genes impairs innate immune response and possibly other important biological processes mediated by neutrophils and M-phi.

Speaker 25:

Retroviral integration site analysis identifies ICSBP as a collaborating tumor suppressor gene in NUP98-TOP1 induced leukemia

Rhonna M. Gurevich, Patty M. Rosten, Carol Stocking, R. Keith Humphries

Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada and the Heinrich-Pette-Institute, Hamburg, Germany

Retroviral expression of the *NUP98-TOP1 (NT)* fusion gene in murine bone marrow (BM) induces a lethal, transplantable leukemia. However, the long latency (225±56 days) suggests the in vivo acquisition of additional mutations and/or the time required for clonal outgrowth of rare transformed cells arising from the combination of *NT* and a co-operating event triggered by the proviral integration. To test the latter possibility and gain insight into the nature of collaborating genes, we analyzed genomic sites of retroviral integration in *NT*-induced AML. We identified in one mouse, a proviral integration predicted to disrupt expression of *Interferon consensus sequence binding protein (ICSBP)*, a tumor suppressor gene implicated in human leukemia and which induces a CML-like disease in a murine knock-out model. To confirm that *ISCBP* deficiency collaborates with *NT*, we expressed *NT* in *ICSBP-/-* BM. *NT* induces an in vivo proliferative advantage which was markedly accelerated in the *ICSBP-/-* background; at 1 month post-transplant a 44-fold increase in the percentage of transduced cells was observed in the peripheral blood (PB) compared to the initial transduction frequency (vs. 10-fold increase for NT/ICSBP+/+ BM). Moreover, at 1 month, nucleated PB cell counts in NT/ICSBP-/- mice were increased significantly compared to GFP-control/ICSBP-/- mice (54.6±72x106 vs. 3.9±1.6x106 cells/ml; p=.005) and to NT/ ICSBP+/+ mice (9.2±5.0x106 cells/ml, p=.008.). Notably, at this early time point, 75% of PB cells in NT/ICSBP-/- mice expressed myeloid markers (Gr-1/Mac-1) (vs. <40% in NT/ICSBP+/+ mice, p≤.005). Strikingly, when expressed in the *ICSBP-/-* background, *NT* induced disease in one mouse at 33 days, with 4/6 mice succumbing to leukemia by 150 days, significantly earlier than NT/ICSBP+/+ mice from all experiments (p=.03). These results reveal the novel finding of collaboration between the *ICSBP* tumor suppressor gene and *NT* in leukemogenesis and further illustrate the power of retroviral integration site analysis for identifying novel cooperating genes.

Speaker 26:

MLL fusion proteins and mixed lineage leukemia: Hox and beyond

Robert Slany, Claudia Bittner, Deniz Zeisig

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Mixed lineage leukemia (MLL) fusion proteins are derived from translocations at 11q23 that occur in aggressive subtypes of leukemia. As a consequence MLL is joined to different unrelated proteins to form oncogenic transcription factors. An inducible derivative of the prototypical MLL fusion MLL-ENL was used to search for downstream target genes in primary hematopoietic cells. 27 genes under control of MLL-ENL were identified. Amongst these Hoxa9 and Meis1 played a crucial role in mediating the transforming activity of MLL-ENL. Since Hoxa9 as well as Meis1 are themselves transcription factors a further screen for Hox/Meis regulated genes was performed. This experiment uncovered a surprising connection linking homeobox genes to known pathways of transformation in the hematopoietic system.

Speaker 28:

Signal integration in EMT and tumor progression: New key players identified by expression profiling

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Most human tumors (carcinomas) are of epithelial origin. Metastases from carcinomas lead to >80% of all cancer deaths. Besides aberrant proliferation, cell cycle control, apoptosis, angiogenesis and lifespan control, cancer progression involves ill-understood cellular and molecular changes leading to local invasion and metastasis. Combined in vitro/in vivo data suggest that diverse types of changes in polarized epithelial cells towards fibroblastoid phenotypes – the most extreme of these changes referrred to epithelial-mesenchymal transition (EMT) – increasingly emerge as crucial and central events during cancer progression and metastasis. Cell models forming tumors/metastasis in vivo and organotypic structures under near-physiological 3D culture conditions in vitro allowed to identify molecular players and signaling pathways (e.g. TGFbeta, MAPK, PI3K, PDGF) which alone or together contribute to distinct types of epithelial plasticity (for instance loss of polarity, scattering and EMT) and are correlated with distinct steps in cancer progression and metastasis. Existence of these diverse epithelial plasticity phenotypes is also strongly suggested by expression profiling of polysome-bound mRNA, yielding a better representation of the proteome than expression profiling employing total mRNA. The talk will focus on describing the biological characterization of candidate genes from this expression profiling screen, concentrating on roles of the PDGF-receptor pathway and ILEI, a novel, secreted protein, for their impact on epithelial plasticity and EMT in vitro, and on tumor formation and metastasis in vivo.

Tolerance and Immunology

Speaker 29:

Myeloablative conditioning using I.V. Busulfex reduces toxicity to the level observed in low intensity conditioning regimens: Dose does matter

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High dose chemotherapy followed by allogeneic stem cell transplantation (alloSCT) is an effective and potentially curative treatment of high risk hematological malignancies. However, standard myeloablative conditioning is with significant transplant related organ toxicities. On the other hand the reduced intensity (RIC) and non myeloablative preparative regimens may be associated with higher relapse rates. There are no prospective studies comparing myeloablative versus RIC preparative regimens or different RIC and NST protocols. Busulfan is a common component of both myeloablative and RIC regimens, however it has erratic and unpredictable bioavailability when administered orally. I.V. Busulfex, has consistent and predictable drug delivery profile resulting in tight control of plasma levels, avoiding over - and under – dosing. We have previously demonstrated (Exp Hematol 13: 428-434, 2003) that I.V. Busulfex – containing regimens allow administration of myeloablative conditioning with a toxicity profile typical of NST protocols. In a subsequent study we compared two of the most popular RIC regimens, Flu/I.V Busulfex (IV FB) Vs Flu/ Melpalan (FM) in 100 pts with hematological malignancies who were not eligible for conventional alloSCT due to advanced age, comorbidities, or extensive prior therapy including prior autoSCT (n=42). The median age was 53 years (up to 66 years). Fifty eight and 42 pts underwent alloSCT from matched siblings and unrelated donors, respectively. In this study we were able to show that IV FB had a relatively low TRM and more favorable outcome in comparison to FM as RIC for alloSCT. Specifically, TRM occurred in 4 (estimated risk 11±6%) and 20 (36±7%) pts within 1-year following IV FB and FM, respectively (p=0.02). Furthermore, the hazard – ratios (HR) for TRM in multivariable Cox regression model for FM was 3.8 (1.3-11.2; p=0.02). Severe mucositis and organ dysfunction were more common after FM. 2 – year OS was 57±11% and 39±7% after IV FB and FM, respectively (p=0.05). Finally, multivariable analysis identified FM (HR 2.2 P=0.03) as a predictive factor for reduced survival. Recently, we compared I.V. Busulfex based myeloablative versus RIC protocols in 91 pts, median age 50 years, up to 70 years, with AML (33 secondary) and MDS who were not eligible for standard conditioning. The donors were HLA-matched siblings (n=45), 1-Ag mismatched relatives (n=6) or matched-unrelated (n=40). Forty two pts were in first or second remission at alloSCT, or untreated with < 10% marrow blasts; 49 were chemo-refractory or with > 10% marrow blasts (active disease). Forty three pts had ablative conditioning with IV Bu (3.2mg/kg x 4d), and cyclophosphamide (Cy). While, 48 pts, considered not eligible for ablative therapy, were given Flu and IV Bu at escalated doses (3.2mg/kg x 2-4d) on successive protocols. IV Bu dose had a significant influence in pts with active disease. OS, TRM and relapse rates were 45%, 22%, and 34% in pts given IV Bu x 4d (with Cy or Flu) compared with 0% (p=0.05), 18% (p=NS), and 82% (p=0.04) after IV Bu x 2-3d, respectively. In contrast, in pts in CR or <10% blasts. There was no difference in OS between pts given different doses of IV Bu. The OS of pts given IV Bu x 4d (with Cy or Flu) was 49%, compared with 51% in pts given Flu and IV Bu x 2-3d. Pts given IV Bu x 4d had higher risk of TRM (19% vs 7%) and lower relapse rate (34% vs 46%), not reaching statistical significance. In conclusion, IV Bu containing regimens are well tolerated with relatively low TRM rates. Dose intensity is not predictive of outcome in chemo-sensitive disease. However, pts with active disease could only be salvaged if given ablative doses of IV Bu. Pts considered not eligible for ablative conditioning tolerated Flu and ablative doses of IV Bu relatively well, and could be salvaged with this regimen even when treated for active disease. Randomized studies will be needed to further determine the best dose-intensity in each setting.

Speaker 30:

Haploidentical bone marrow transplantation under reduced intensity conditioning

Professor Yair Reisner

Weizmann Institute of Science, Rehovot, Israel

Studies in mice and humans demonstrate that transplantation of hematopoietic progenitors in numbers larger than commonly used ("megadose" transplants) overcomes major genetic barriers. In vitro studies suggest that veto cells, within the population of hematopoietic progenitors, facilitate this favorable outcome. This tolerizing activity, mediated by apoptosis, can be blocked by anti-TNF. Considering that the number of human CD34 cells which can be harvested is limited, the availability of other types of veto cells or immuneregulatory cells is crucial for further application of allogeneic stem cell transplantation under reduced intensity conditioning. Perhaps the most potent veto cell is the CD8+ CTL. However, this cell is also associated with marked GVHD. GVHD can be separated from the veto activity by generating anti-third party CTLs under IL2 deprivation. Under such selective pressure only the stimulated clones which make IL2 can survive, while anti host clones die. In vivo studies show that such anti-third party veto CTLs can be used safely for tolerance induction without GVHD. Very recently we demonstrated the potential synergism between the effect of Rapamycin, which interferes with IL2 R signalling, and the veto activity of anti 3rd party CTLs which operate via Fas-FasL mechanism. Moreover, we found that CD4+CD25+ T cells (regulatory cells), could also contribute to the enhancement of BM allografting by veto CTL and Rapamycin. This synergism might prove effective if tested in recipients of HLA matched or even mismatched mega dose CD34 stem cell transplants, for whom lethal conditioning is unacceptable.

Speaker 31:

Minor Histocompatibility antigens: they immunize and tolerize

E. Goulmy

LUMC, Leiden, The Netherlands

Our clinical related work is centered on the allo immune responses that are induced by minor H antigens in the non-physiological settings of Stem Cell Transplantation (SCT) and solid organ transplantation and in the physiological situation of pregnancy.

Pregnancy leads to a mutual flow of cells between mother and child. Low number of allogeneic cells persists in both mother and child; a situation called micro-chimerism. The exposure to the either foreign maternal or to foreign paternal minor H antigens during pregnancy drives the generation of minor H antigen specific T cells in mutual direction. Both the T cells and microchimerism can be detected in the blood of healthy females who have been pregnant and in cord blood samples. We study the relevance of the minor H antigen immune responses and their immune regulation in relation to subsequent Stem Cell (SC) or Cord Blood (CB) transplantation and solid organ transplantation.

In the latter setting of kidney transplantation, recipients of HLA matched minor H antigen HA-1 mismatched renal transplants have been studied. One of the patients had discontinued immunosuppression over 30 years ago while sustaining normal kidney function. HA-1 specific T regulator cells were present in the latter long-term kidney allografted recipient. These cells coincided with HA-1 specific CTLs and HA-1 microchimerism. Thus, minor H antigens may play a crucial role in the lifelong tolerance to an allograft.

Relevant references:

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J. Cai, J. Lee, E. Jankowska-Gan, R. Derks, J. Pool, T. Mutis, E. Goulmy, W.J. Burlingham. Minor H antigen HA-1-specific regulator and effector CD8+ T cells, and HA-1 microchimerism, in allograft tolerance. J. Exp. Med. 2004, 199 (7), 1017–23.

Speaker 32:

MSCs in Immunotherapy

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Mesenchymal stem cells (MSCs) may be derived from adult bone marrow, fat and several fetal tissues. In vitro, MSCs can be expanded and have the capacity to differentiate into several mesenchymal tissues, such as bone, cartilage and fat. Human MSC are not inherently immunogenic in an allogeneic system in vitro, nor are they rejected in vivo according to preliminary results. This makes them candidates for cellular therapy in an allogeneic setting. MSC have immunomodulatory effects; they inhibit T-cell proliferation in mixed lymphocyte cultures, prolong skin allografts survival in baboons and may decrease graft versus host disease (GvHD) when co-transplanted with hematopoietic cells. MSC induce their immunosuppressive effect, at least in part via a soluble factor. Several candidates have been suggested including prostaglandin E2, indoleamine 2, 3-deoxygenase – mediated tryptophane depletion and hepatocyte growth factor in combination with transforming growth factor (TGF). However, contradictory data exist that may be due to differences in the cells and system tested. A major problem has been that it has been difficult to identify and isolate MSCs after transplantation in vivo. However, MSCs seem to enhance hematopoietic engraftment in recipients of autologous and allogeneic grafts. Recently, they were found to reverse grade IV acute GvHD of the gut and liver. No tolerance was induced. Thus, in allogeneic stem cell transplantation, MSCs may be used for haematopoiesis and graft enhancement, as GvHD prophylaxis, and for the treatment of severe acute GvHD. Control studies are warranted to fully evaluate the possibilities of using human MSC as immunomodulators.

Immunotherapy and Novel Therapeutic Strategies

Speaker 33:

Tumor stroma: The *Achilles heel* of the tumor

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It is established that most experimental and probably many human tumors express antigens against which an immune response can be induced. Immunity to transplanted tumors usually relies on T cells, often both $CD4^+$ and $CD8^+$. Their activation requires antigen cross-presentation by host antigen presenting cell (APC) in draining lymph nodes. Following activation T cells migrate to the tumor site and, when they arrive in time, can reject the tumor. In some tumor models $CD4^+$, in other $CD8^+$ T cells are the main effectors. Both cell types reject tumors by a similar mechanism. Most importantly, T cells have to express IFNγ that acts on non-bone marrow derived tumor stroma cells resulting in angiostasis and inhibition of rapid tumor burden. This may allow residual tumor cell elimination by other mechanisms such as direct killing, yet in several models perforin expression by T cells is not necessary. Together, the primary target during tumor rejection is the stroma that is essential for solid tumors and appears to be the most vulnerable component. IFNγ producing T cells inhibit the establishment of the stroma, e.g. vascularization, but usually fail against established tumors which could explain why therapeutic vaccination is so notoriously unsuccessful, simply because the T cells come too late. These findings will be discussed in relation to cancer immunosurveillance.

Speaker 34:

Functional analysis of TCR-transduced MHC class I-restricted helper T cells and their role in tumor protection

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Adoptive transfer of antigen-specific CD4+ and CD8+ T cells is one of the most efficient forms of cancer immunotherapy. However, the isolation of antigen-specific CD4+ T cells is limited since only few tumor-associated 'helper epitopes' are identified. The generation of tumor antigen-specific CD4+ helper T cells is therefore limited by the paucity of known MHC class II-binding tumor epitopes and by the lack of expression of MHC class II molecules on most tumor cells. In contrast a large number of CD8+ T cell-recognised tumor epitopes presented by MHC class I molecules has been identified, and in many cases CTL clones against such epitopes have been isolated in the past. In this study we explored in a murine model system if the TCR genes of 'tumor-specific' CTL can be transferred to CD4+ T cells to generate MHC class I-restricted, 'tumor-specific' T helper cells. We show that the TCR specific for an H2-Db-presented epitope of influenza nucleoprotein (NP) can be used to produce H2-Db-restricted, NP-specific CD4+ T cells. Adoptive transfer of CD4+ T cells producing high levels of IL-2 and low levels of IFNg provides 'help' for CTL-mediated rejection of NP-expressing EL4 tumors, while adoptive transfer of helper T cells producing high levels of IFNg was ineffective. The data demonstrate that co-transfer of TCR-transduced CD4+ and CD8+ T cells specific for the same epitope can result in long-term tumor protection. Further experiments will be performed to study the mechanism by which TCR-transduced CD4+ T cells enhance the anti-tumor activity of TCR-transduced CTL.

Speaker 35:

Suppressor T cells: answered and unanswered questions

Harald von Boehmer

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Suppressor T cells consist of Foxp3-expressing cells that may or may not express the CD25 surface marker. These cells are able to suppress a variety of T cell effector functions in vivo that enables them to prevent as well as revert unwanted immunity. While we have clues about the antigen-induced generation of these cells in primary and secondary lymphoid organs, our information on the mode of their action is less complete. It will be discussed that suppression can affect different T effector functions by different mechanisms through interference with different transcription factors that depend on TCR signaling. The likelihood will also be discussed that the antigen specificity of suppressor T cells can be exploited to replace general immuno-suppression with its harmful side effects in the near future. It seems equally likely that interference with suppression will result in more efficient wanted immunity against tumors and/or infectious organisms.

Speaker 36:

In vitro generation and adoptive transfer of antigen-specific T cells in cancer patients

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The adoptive transfer of in vitro induced and expanded tumor antigen-specific cytotoxic T lymphocytes (CTL) provides a promising approach to the immunotherpy of cancer. We have previously shown that tumor-reactive CTL can be generated from HLA-A2.1+ melanoma patients by 4 rounds of in vitro stimulation of purified CD8+ T cells with autologous dendritic cells (DC) pulsed with HLA-A2 binding peptides. Based on these results we have initiated a pilot study of adoptive T cell therapy in advanced melanoma patients demonstrating that in vitro generated peptide-specific CTL survive intact in vivo for several weeks and localize preferentially to tumor (Meidenbauer et al., J. Immunol., 2003). Clinical results of a phase I study of 12 HLA-A2+ melanoma patients that received at least three i.v. infusions of Melan-A peptide-specific CTL i.v. at 2-week intervals demonstrated that the adoptive transfer of antigen-specific T cells in melanoma patients is capable of inducing clinical and systemic tumor-specific immune responses without provoking major side effects. Furthermore, 111In labeling of Melan-A-specific CTL demonstrated localization of transferred CTL to metastatic sites as early as 48h after injection in 3 out of 3 patients.

The current standard approach to generating antigen-specific CTL entails generating monocyte-derived DC for expansion of CTL. This step is both time consuming and expensive. Variability seen with both the quantity and quality of DC obtained, which presumably relates to underlying disease and patient pretreatment, also significantly impacts on the viability of DC-based ex vivo therapeutics. For these reasons, use of DC has been a limiting step in ex vivo expansion of T cells. To address this issue, artificial Antigen-Presenting Cells (aAPC) were made by coupling soluble HLA-Ig and anti-CD28 to beads. First data show that aAPC can be used to induce and expand antigen-specific CTL (Oelke et al., Nat. Med. 2003). In these preliminary experiments, peptide-pulsed aAPC were used to replace peptide-pulsed DC during the induction of antigen-specific CTL. Data demonstrate that aAPC-mediated expansion compared favorably to the “gold standard” DC-mediated CTL induction and expansion.

Finally, recent data on extremely high frequencies of tumor-specific CTL in cancer patients that exhibit competent T-cell effector functions, but fail to lyse the autologous tumor cells will be presented. These findings have profound implications for cancer immunotherapy, and may provide one explanation why malignant tumors do not respond to specific immunotherapeutic regimens despite the induction of a strong tumor-specific T-cell response.

Speaker 37:

DC-SIGN and escape from immunosurveillance

Angel L. Corbi

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Dendritic Cell-Specific ICAM-3 Grabbing Nonintegrin (DC-SIGN) is a type II membrane cell surface C-type lectin expressed on myeloid dendritic cells and certain tissue macrophages. Structurally, the DC-SIGN carbohydrate-binding domain is followed by a \“neck\” region with eight 23-amino acids repeats, a transmembrane region and a short cytoplasmic tail with recycling and internalization motifs. DC-SIGN mediates antigen capture for processing and presentation, and participates in dendritic cell intercellular interactions with naive T lymphocytes or endothelial cells. In their strategy to evade immunosurveillance, numerous pathogenic microorganisms, including HIV, HCV and Mycobacterium, bind to DC-SIGN in order to gain access to dendritic cells. Structure/function studies on DC-SIGN and its naturally-ocurring isoforms indicate that the length of the “neck” domain is a major determinant for pathogen recognition. DC-SIGN-interacting pathogens have been hypothesized to modulate dendritic cell maturation by interferring with intracellular signaling from Toll-like Receptor molecules. To dissect the molecular basis of such an interference, we evaluated the intracellular signaling ability of DC-SIGN. Engagement of DC-SIGN by specific antibodies or pathogenic ligands does not promote dendritic cell maturation but triggers intracellular signals compatible with a diminished pro-Th1 polarizing ability and increased production of IL-10. In agreement with its signaling capability, a relevant fraction of DC-SIGN molecules partition within lipid raft-enriched membrane fractions both in dendritic and DC-SIGN transfected cells. Results will be presented that indicate that DC-SIGN-triggered intracellular signals modulate dendritic cell maturation in a manner that might contribute to pathogen escape from immunosurveillance.

Speaker 38:

Lentiviral vectors encoding antigens transduce dendritic cells in vivo and initiate CD4 and CD8 immune responses

Mary Collins, Helen Rowe, Luciene Lopes, Ranbir Bailey, Yasuhiro Ikeda, Benjamin Chain.

University College London

Lentiviral vectors based on human immuno-deficiency virus type 1 (HIV-1) transduce both myeloid and plasmacytoid dendritic cells in spleen or draining lymph nodes after intravenous or subcutaneous injection. These transduced dendritic cells can persist for several months after vector injection. Using vectors encoding ovalbumin, or the tumour antigen NY-ESO-1 we have shown that lentiviral vector injection initiates potent antigen-specific CD4 and CD8 T cell responses, which can be boosted by antigen-vaccinia injection. We are currently developing lentiviral vectors with uptake and antigen expression targeted to dendritic cells.

Speaker 39:

Could we improve the effectiveness of antitumor vaccines?

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The idea to establish an effective immune response against endogenously developed tumor cells is not novel. Last years viewpoints on "self" and "non-self" recognition and primary induction of the immune response have changed. The role of lymphocytes switched on a key role of DC in "non-self" recognition and induction of both innate and adaptive responses. Moreover, innate response was suggested as an essential starting point in induction of successful acquired response. Modern cancer vaccines do not have presentation of "non-self" due to their origin, therefore lacking the effectiveness in the induction of immune response. Thus, to make cancer vaccine more effective we have to present tumor antigens together with the molecules that can potentially activate downstream "non-self" recognition events. Our strategy is based on the utilization of a member of a novel family of pathogen molecular pattern recognition molecules (Gene Ther. 2004;1:18-25): Tag7/ PGRP-S. This protein is able to induce lymphocytes chemotaxis *in vitro* and *in vivo* and induce DCs maturation via CD80, CD86 co-stimulatory molecules expression The use of genetically modified tumor cells by the tag7/PGRP-S expressing construct as a vaccine led to the establishment of long-lasting antitumor protective immunity (J Gene Med. 2004;6(7):798-8). Multicenter phase I/II clinical trial of the autologous tag7-modified anti-tumor vaccine demonstrated the effectiveness of the approach chosen (Ann Oncol. 2005;16(1):162-8).

Speaker 40:

Mesenchymal stem cells as tumor stromal elements and potential antitumor therapy: Tropism and efficacy

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We have previously demonstrated that bone marrow-derived mesenchymal stem-cells (MSC) integrate into solid tumors as stromal elements following intravenous injection (JNCI 96(21):1593, 2004, Cancer Res 62:3603, 2002). These finding suggests the development of novel anti-cancer therapies based on the local production of biological agents by gene-manipulated MSC. However, no direct evidence has demonstrated this migration and incorporation into the tumor microenvironment. To this end, we utilized noninvasive imaging of migrating MSC in animal models.

MSC were labeled by a fiber modified Ad vector expressing firefly luciferase (AdLux-F/RGD) and these MSC-Lux were injected into normal (healthy) SCID mice or mice bearing established metastatic breast or ovarian tumors. Biodistributed MSC-Lux were imaged utilizing the Xenogen IVIS detection system. In normal mice, human MSC (hMSC) migrated to the lungs where they remained resident for 7–10 days. In animals bearing established metastatic lung tumors, IV injected hMSC again migrated to the lungs. However, in contrast to control mice, the Lux signal remained strong over a 15 day period with only a slight decrease over the first 10 days. After IP injection, hMSC-LUX were detected in the peritoneum, and after 7 days, no hMSC-LUX was detected in normal animals, while strong punctate regions of LUX-activity were observed in ovarian tumors. In contrast to SCID mice injected with hMSC, healthy Balb/C mice injected with Balb/C derived MSC-LUX initially migrated to the lungs and within 2.5 hrs had exited the lungs to remain liver and spleen resident for 5–7 days.

We then examined whether hMSC producing interferon-beta (IFNβ-MSC) can inhibit the growth of metastatic tumors in the lungs of SCID mice. When injected IV (4 doses of 10E6 MSC/week) into SCID mice bearing pulmonary metastases of carcinomas or melanomas, tumor growth was significantly inhibited as compared to untreated or vector-control MSC controls ($p = 0.007$), while recombinant IFNβ protein (50,000 IU qod) was ineffective ($p=0.14$). IV injected IFNβ-MSC prolonged the survival of mice bearing metastatic breast carcinomas ($p=0.001$). Intraperitoneal (IP) injections of IFN-MSC into mice carrying ovarian carcinomas resulted in doubling of survival in SKOV-3, and cures in 70% of mice carrying OVAR-3 tumors.

These data suggest that systemically administered gene-modified MSC selectively engraft into the tumor microenvironment and remain resident as part of the tumor architecture. MSC-expressing IFN-β inhibit the growth of metastatic breast cancer, and ovarian cancers in vivo and prolong the survival of mice bearing established tumors. Clinical trials are in preparation.

POSTER ABSTRACTS

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Leukemia (Biology, Diagnosis, Treatment)

Poster 1:

Chemotherapy-Induced T-MDS/AML-Associated Genetic Aberrations

Jörg Bäsecke[1], Martina Podleschny[1], Julia Goldstein[1], Claudia Riechel[1], Detlef Haase[1], Bertram Glass[1], Volker Viereck[2], Lorenz Trümper[1], Frank Griesinger[1]

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Hematological neoplasias, caused by chemo- and radiotherapy, are a significant risk for patients, who are cured from their primary cancer. The cumulative incidence of therapy related myelodysplastic syndromes and acute myeloid leukemias (t-MDS/AML) ranges between 5–15% in non-myeloablative and myeloablative treatment protocols respectively. t-MDS/AML share characteristic genetic aberrations which include translocations (e.g. involving 11q23, MLL and t(9;22), inv 16, t(8;21), t(3;21), t(15;17) and cytogenetic aberrations (e.g. 5q-, 7q-). We have investigated if these genetic aberrations are induced in patients who underwent chemotherapy. We also wanted to determine if t-MDS/AML early recognition can be performed by this screening. We enrolled patients with Non Hodgkin Lymphoma, either treated by conventional or high dose chemotherapy, included in the MegaChoep protocol of the German High Grade Lymphoma Study Group. Samples were taken as fresh peripheral blood stem cells (PBSCT), bone marrow aspirates (BM) or peripheral blood (PB) after informed consent according to the convention of Helsinki. PBSC of healthy adult donors patients who underwent chemotherapy were used as control. Blood samples were submitted to RT- or Real-Time PCR (sensitivity 10-4 to 10-5) of t(9;22), inv 16, t(8;21), t(15;17) and MLL-partial tandem duplications (MLL-PTD). We also applied an inverse PCR for the detection of MLL-instabilities in the t-AML associated break-point cluster region. RNA preparation, reverse transcription, and PCR were performed in separate laboratories. Cytogenetic analysis was performed by FISH (probes EGR1-del5, p53, cep7-del 7, MLL and cep8-tris 8). The results of the ongoing study are as follows. No translocations were observed in PBSC samples of healthy donors (22 samples). 97 samples (76 peripheral blood or bone marrow, 21 PBSC),of patients, who underwent dose intensified and conventional chemotherapy have been investigated so far. In 11,7% the translocation t(15;17) was detected. All positive samples were PBSC. The incidences of the inv 16, the translocations t(8;21) and t(9;22) were 6,3%, 5,0% and 2,5%, respectively. Results were confirmed by cycle sequencing. None of the samples were positive in the MLL-PTD primary PCR, as would have been comparable to MLL-PTD positive AML. In the adult cohort trans-locations were only observed in patients with dose intensified treatment. The incidence of these translocations in cord blood ranges between 1,7 and 5,6 % and may reflect an inherent genetic instability in fetal hematopoiesis which is not related to treatment. The inverse MLL PCR exhibited genomic instability in the MLL BCR (aberrant intra-genetic insertion) of patients, who did receive chemotherapy but, most interestingly, also in healthy adults. In FISH analysis two of 24 patient PBSC samples (8,3%) exhibited aberrations in the p53 locus, which were classified as abnormal but still non-clonal. Our results confirm the existence of AML-associated translocations in adult patients who underwent dose intensified chemotherapy at frequencies which exceed the incidence of t-AML. Since the adult control groups were PCR negative, positive PCR-results of the group with dose intensified chemotherapy may be informative of a therapy induced genetic instability. Due to the high frequency of these aberrations, compared to the expected incidence of t-MDS/t-AML, some of them may be transient. Instabilities of the MLL gene in the t-AML associated BCR, which have only been expected post-therapy, seem also to be present in normal hematopoiesis. Theses are currently further characterized to identify if chemotherapy associated patterns exist. Positive patients are currently observed and sequentially investigated to determine if they are at an increased risk of t-MDS/t-AML.

Poster 2:

T-cell clonality detection in the patients with chronic myeloid leukemia.

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Background: Chronic myeloid leukemia (CML) is a malignant myeloproliferative disorder originating from a pluripotent hematopoietic stem cell that acquires a Philadelphia (Ph) chromosome encoding the BCR-ABL oncogenic fusion protein. The clinical significance of clonal T cell receptors (TCR) expansion is still unclear for the patients at different phases of the disease. Present study was aimed to evaluate the presence of T-cell clonality (TCR-gamma) in CML patients.

Methods: We observed a group of eight patients with CML. All the patients obtained standard therapy with Glivec. Four patients with proven -cells ALL and four healthy donors served as appropriate positive and negative controls. Genomic DNA was isolated from peripheral blood leukocytes (PBL) or bone marrow cells by “DNA Blood Mini Kit” (QIAGEN, Germany). A segment of TCR-gamma gene has been chosen as a target for -clonality assays. PCR protocol was designed using consensus primer sets. Molecular clonality was determined by heteroduplex mobility analysis (HMA) in polyacrylamide gel.

Results: -cell clonality has not been revealed in DNA samples from healthy donors. T-cell clonality was observed in all studied patients with -cell ALL and correlated with clinical features and immunological markers of malignancy. Meanwhile, TCR-monoclonality was detected in three of eight cases with CML, whereas polyclonal patterns were found in five other cases. Interestingly, all the patients with T-clonality have shown complete cytogenetic response by the time of analysis.

Conclusions: We suggest that the presence of -cell clonality in patients with CML may give new insights into pathogenesis of CML, i.e., probable involvement of T cell lineage in leukemia progression, or development of cellular clones with potential anti-leukemic activity. This finding deserves ongoing studies, in order to define clinical significance of T-clonality in CML.

Poster 3:

Identification of genes in human AML stem cells and evaluation of their role in leukaemogenesis

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Haematopoietic stem cells are a rare population in the haematopoietic system capable of self-renewal and/or differentiation into myeloid and lymphoid lineages. Using the NOD/SCID repopulation assay, based on the ability of HSC to engraft recipients for long time period after transplantation, the CD34+CD38- cell population was identified as highly enriched for multilineage repopulating cells, termed SCID-repopulating cells (SRC).
Acute myeloid leukaemia (AML) is a clonal disorder characterized by an accumulation of immature blasts that fail to differentiate into functional granulocytes or monocytes. Those blasts have limited proliferative capacity; a sub-population with stem cell properties must therefore exist to maintain the leukaemic clone. Such cells have been identified using the NOD/SCID repopulation assay: SCID-Leukemia Initiating Cells (SL-IC) purified from AML patient based on CD34+CD38- expression initiate AML in NOD/SCID mice, giving rise to a myeloid only engraftment. Thus, SL-IC differ from SRC only by the quality of their engraftment.
Comparing gene profiles between normal and leukaemic stem cells would help to identify molecular properties that make LSC different from HSC. Gene silencing of selected genes using RNA interference would allow a further characterization of their role in leukaemia and the identification of novel therapeutic targets.

Poster 4:

The Role of RUNX1 Mutations in Acute Myeloid Leukemia

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The RUNX1 gene encodes an alpha subunit of the core-binding factor (CBF), an important heterodimeric transcription factor in hematopoietic ontogeny and development. RUNX1 and CBFbeta are one of the most frequently disrupted genes in acute leukemia. In addition to their involvement in translocations/ inversion, the RUNX1 gene is often mutated in acute myelogenous leukemia (AML). Interestingly, in addition to complete loss-of-function mutations, many of the mutations involve missense point mutations within the Runt domain that directly contact DNA. In vitro assays have demonstrated that these Runx1 mutants have a dominant negative (DN) activity, presumably due to their ability to bind and sequester CBFbeta but inability to bind DNA. Somewhat puzzling, no direct correlation between the type of mutation and their monoallelic or biallelic incidence has been discerned. Although this has lead to the hypothesis that loss of one allele (haplo-insufficiency) is sufficient for loss of tumor suppressor activity, the relative high incidence of DN mutations proteins suggests a more complex scenario. We thus sought to determine if expression of these DN mutants resulted in a similar phenotype as the loss of Runx1, or if a gain-of-function could be attributed to these mutations. To investigate the consequence of RUNX1 mutations on normal hematopoiesis, we used the previously established retroviral transduction / transplantation model to express mutant RUNX1 in murine bone marrow (BM) cells. Additionally, we took advantage of a conditional knock-out mice, to evaluate the differences between the total loss of Runx1 and the expression of RUNX1 mutants. While the expression of the DN RUNX1 mutants lead to an increased self-renewal of wt murine BM, expression of the Cre recombinase in the BM of floxed Runx1 mice showed a less dramatic effect on self-renewal, as assessed in replating assays. Significantly, colonies derived from CFUs expressing DN-RUNX1 mutants were composed predominately of granulocytic and monocytic cells, even after multiple replatings (>7), whereas residual colonies from Runx1fl/- BM receiving CRE were restricted to mast cells, verifying that expression of the DN-RUNX1 mutants imparted increased replating capacity (\“self-renewal\”) to early GM progenitors without disrupting terminal myeloid differentiation. To investigate the effects of the expression of mutant RUNX1 on hematopoiesis in vivo, we transplanted BM cells expressing mutant RUNX1 and analyzed peripheral blood, spleen and bone marrow after six months. Long-term repopulating HSC expressing DN-RUNX1 mutants were able to contribute to both myeloid and lymphoid compartments, although lower levels of mature B-cell was observed. Consistent with the segregation of RUNX1 mutations in familial platelet disorder with predisposition to AML (FDP-AML), abnormalities in megakaryocyte development was also observed in these mice. No other consistent perturbations in the myeloid compartment were observed during this time period. Thus in contrast to translocations involving RUNX1, DN-RUNX1 mutants do not impair myeloid differentiation. Our data also clearly demonstrate that DN-RUNX1 mutants do not simply mimic loss of RUNX1 function. The molecular mechanism for this gain-of-function is currently under investigation.

Poster 5:

A Non-Restrictive Function of the p53 Tumor Suppressor that Maintains Genetic Stability through the Enforcement of Antioxidant Defense

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It is widely accepted that the p53 tumor suppressor restricts abnormal cells by induction of cell cycle arrest, or by triggering apoptosis. We show that in addition p53 protects the genome from oxidation by reactive oxygen species (ROS), a major cause for DNA damage and genetic instability. In the absence of severe stresses relatively low levels of p53 are sufficient for up-regulation of several antioxidant genes, which is associated with a decrease in intracellular ROS. In contrast, the previously described pro-oxidant function of p53 is associated with the induction of apoptosis and the release of mitochondrial ROS following grave stresses. Down-regulation of p53 results in excessive oxidation of DNA, increased mutation rate, and karyotype instability, which are prevented by incubation with antioxidant N-acetylcysteine (NAC). Dietary supplementation with NAC prevents from frequent lymphomas characteristic to p53 knockout mice, and reduces growth rate of xenografts from A549 cells with p53 inhibited by RNAi. Our results provide novel paradigm for a non-restrictive tumor suppressor function of p53 and highlight potential importance of antioxidants in prophylactics and treatment of cancer.

Poster 6:

Newly identified kit ITD in childhood AML induces ligand independent growth and is responsive to a synergistic effect of imatinib and rapamycin

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c-kit is a member of the class III family of receptor tyrosine kinases (RTK) and is a known proto-oncogene structurally related to the receptors for PDGF and FLT-3 ligand. Activating mutations of c-kit lead to ligand independent growth. In childhood AML c-kit mutations were identified in the extracellular domain (exon 8) and within the kinase domain with a relative frequency of 4% and 7%, respectively. In humans so called internal tandem duplications (ITD) of exon 11 which encodes the juxta-membrane domain are well known constitutively activating mutations in gastroin-testinal stromal tumors (GIST).

Genomic DNA and cDNA from 60 children with AML were screened by PCR for mutations of the juxtamembrane domain. A complex ITD involving exon 11 and exon 12 was identified with a relative frequency of 7% (4/60). The mutation is in frame and expressed on the surface. We characterized the oncogenic potential and signaling properties of this novel c-kit mutation constructing a chimeric molecule that consisted of the murine c-kit backbone and a 102-base pair human c-kit insert. The c-kit isoform containing the ITD mutation (kit-ITD) induced factor-independent growth when introduced in IL-3 dependently growing Ba/F3 cells whereas Ba/F3 cells expressing wild-type (WT) kit required either IL-3 or kit ligand (KL). kit-ITD exhibited constitutive autophosphorylation of the mature and immature form of the receptor. Analysis of the involved signal transduction pathways revealed that kit-ITD in the absence of KL activated the phosphoinositide 3-kinase (PI3K)/AKT pathway as well as STAT5 but only slightly activated the MAP kinase Erk. Incubation of kit-ITD expressing Ba/F3 cells with either the ABL kinase inhibitor imatinib (STI571) or the mTOR inhibitor rapamycin led to complete inhibition of growth with IC50 values at the nanomolar level. Combinations of imatinib below IC10 and rapamycin with constant ratios of 1000:1 showed a very strong synergistic inhibition over a wide dose range.

Taken together, we identified a novel ITD mutation of the juxtamembrane domain of the RTK c-kit with a preliminary frequency of 7% in childhood AML. kit-ITD induces factor-independent growth and leukemogenesis of Ba/F3 cells that is predominantly mediated by the PI3K/AKT pathway. Cells expressing kit-ITD are exquisitely sensitive to a synergistic effect of imatinib and rapamycin.

These findings can have immediate therapeutic implications for a subset of patients with childhood AML.

Poster 7:

A new method for computerized quantitation of ZAP-70 expression in chronic lymphocytic leukemia cells.

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ZAP-70, normally expressed in T and NK cells, correlates with disease progression and shorter survival in chronic lymphocytic leukemia (CLL) when highly expressed in the leukemic B-cells. While several flow cytometry assays have been described which demonstrate a strong correlation between the expression of ZAP-70 in CLL cells and clinical outcome, different staining and gating methods have been employed, making standardization of the assay between different laboratories difficult. We describe a quantitative method of data analysis, using the fluorescence intensity unit allowing comparison of measurements over time and across platforms. ZAP-70 expression was assessed in B-CLL cells using molecules of equivalent soluble fluorochrome (MESF) units defined by fluorescent calibration beads. Using this method a low and high ZAP-70 MESF subgroups were defined (using a cut-off of 47,000 MESF units), in which the low ZAP-70 MESF B-CLL were associated with a stable disease while the high ZAP-70 MESF B-CLL had an early progressive disease ($p=0.0005$). In addition, this analysis enables quantitation not only of high ZAP-70 expression, with clinically relevant predicted outcome, but can also quantifies medium and low levels of ZAP-70 expression with clinical relevance not yet known. We conclude that this quantitative assay method is highly specific, very accurate, provides higher resolution and more information. The method can easily be standardized in any routine flow cytometry laboratory, thereby improving reproducibility and reliability of ZAP-70 analysis in B-CLL cells.

Poster 8:

Identifying Genes Responsible for Apoptosis-Resistance in Hematopoietic Malignancies Expressing wt p53 by Means of Retroviral Insertional Mutagenesis

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High grade Non-Hodgkin's Lymphomas (NHL) respond well to classical chemo- or radiation therapies, a fact that correlates well with the observation that wild-type p53 expression is detected in 80 to 95% of tumors, in striking contrast to carcinomas. However, despite the initial good response, long-term survival rates are generally poor in NHL patients, due to the outgrowth of therapy resistant clones and subsequent relapse of the disease. Interestingly, the wild-type p53 status is often maintained in the secondary disease. Defining the mechanisms by which these tumors escape apoptosis while maintaining an intact p53 gene is an important endeavor towards the development of effective therapies.

Our goal is to define critical mutations in radiation-resistant clones by analysis of integration sites unique to these tumors. For this work, a mouse model was chosen, in which a lymphoid tumor is induced by Moloney murine leukemia virus (MoMLV) infection. The mouse strain carries an activated human c-myc oncogene under control of the Igλ; enhancer, which provides the “first hit” in tumorigenesis. The developing tumors show a phenotype similar to Burkitt's lymphoma and the majority (approx. 80%) express wt p53, consistent with the findings in Burkitt's lymphoma. Significantly, γ irradiation triggers apoptosis in these cells, but cells that escape apoptosis can be detected in transplanted animals at an incidence of $<10^{-4}$. Viral integration sites in ten independent tumor sets were isolated, sequenced and their chromosomal location mapped using available data bases. Moreover, 35 independent tumors were screened for already known common integration sites. Significantly, integrations upstream of the bmi-1 locus were observed in circa 35% of the independent tumors. Integrations in this locus were observed in both, radiation-sensitive and radiation-resistant tumors, supporting the hypothesis that inhibition of the ARF pathway (in this case by Bmi-1) abrogates p53-mediated apoptosis in response to the myc-expression, but not to genotoxic stimulation. A number of other loci were identified that were shared by both radiation resistant and sensitive tumors and previously shown to cooperate with myc-induced tumorigenesis. However, we also identified two loci, at which integration correlated with the acquisition of radiation resistance. One locus was found in two independently derived tumor-sets and mapped to the 1st intron/promoter region of the gene encoding Bcl-X, an important antiapoptotic member of the Bcl-2 family. Quantitative RealTime RT-PCR has confirmed that this gene is overexpressed in these tumors, thus providing a satisfying “proof-of-principle” to our approach. Another integration, also found in two independently derived tumors, mapped downstream of the gene encoding GADD45β, and high levels of expression were observed in γ irradiation-resistant tumors. These results support the recent hypothesis that GADD45β can either induce or block apoptosis, depending on the cellular environment. Demonstrating the importance of this pathway in blocking apoptosis in B-cell malignancies will provide new targets for therapeutic development. These results show that our approach is a powerful tool to identify genes that are responsible for apoptosis resistance in B-cell malignancies.

Poster 9:

Expression of the oncoprotein MN1 in acute and chronic leukemia and normal hematopoiesis

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Recently, we have identified a gene expression profile associated with drug-resistant AML which is reminiscent to hematopoietic stem/progenitor cell-expression profiles. One of the overexpressed genes of this putative stem-cell signature in acute myeloid leukemia (AML) is meningioma-1 (MN1), which has been recently identified as a transcription coactivator of the retinoic acid receptor. Fusion of MN1 to TEL (ETV6) has been found in AML and MDS with t(12;22)(p13;q11), and the fusion protein has shown transforming activity in NIH3T3 cells. However, overexpression of MN1 has not been reported previously in AML. We quantified the expression of MN1 in various hematologic malignancies and normal hematopoietic cells by real-time RT-PCR on a LightCycler using QuantiTect SYBR Green, and associated MN1 expression with characteristics of AML samples. First, AML samples were divided into two groups according to the MN1 expression above or below the median MN1 expression. No significant differences for age, sex, ECOG, FAB subtype, white blood cell count at diagnosis, blast percentage at diagnosis, type of specimen (BM or PB), FLT3 mutation status, and MLL PTD status were found in these groups, although there was a trend for a higher frequency of adverse risk cytogenetics in the group with high MN1 expression. However, 76.5% (13/17) of patients with MN1 expression below the median expression achieved a good response (GR) to the first cycle of induction treatment, compared to 31.3% (5/16)of patients with MN1 expression above the median expression (p=0.009 by chi-square test). In addition, 100% (17/17) of patients with MN1 expression below the median compared to 68.8% (11/16) of patients with MN1 expression above the median achieved complete remission (CR) after 2 cycles of induction treatment (p=0.012 by chi-square test). Mean expression in PB MNC of healthy controls (n=6) was 20-fold and 137-fold lower than the mean expression in AML samples with GR and resistant disease (RD), respectively. Mean expression in CML (n=11) and CLL (n=12) samples was comparable to the background expression in normal PB MNC. MN1 expression in ALL (n=13) and CML blast crisis samples (n=2) was in most samples at the background level of PB MNC. However, in three ALL samples it was similar to the mean expression of poor response- and even RD-AML samples. One of these ALL samples was taken at relapse, one patient relapsed five months after diagnosis and the other one presented with a complex karyotype. MN1 expression was 77-fold higher in CD34+ cells (n=2) compared to PB MNCs. In conclusion, MN1 overexpression seems to be restricted to acute leukemias and normal hematopoietic progenitor cells and was significantly associated with poor response and resistant disease in AML. Thus, a more detailed analysis of MN1 overexpression in acute leukemia and hematopoietic stem and progenitor cells and its potential to predict drug resistance seems warranted.

Poster 10:

Extracellular KIT receptor mutants, commonly found in core binding factor AML, are constitutively active and respond to imatinib mesylate

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Multiple genetic alterations are required to induce acute myelogenous leukemia (AML). Mutations in the extracellular domain of the KIT receptor are almost exclusively found in AML patients carrying translocations or inversions affecting members of the core binding factor (CBF) gene family and correlate with a high risk of relapse. We demonstrate that these complex insertion and deletion mutations lead to constitutive activation of the KIT receptor, which induces factor-independent growth of IL3-dependent cells. Mutation of the evolutionary conserved D419 within the extracellular domain was sufficient to activate the KIT receptor, although high expression levels were required. Dose-dependent growth inhibition and apoptosis was observed using the receptor tyrosine kinase (RTK) inhibitor imatinib mesylate, and strictly correlated with dephosphorylation of AKT but not STAT3. Our data show that the addition of imatinib or other RTK inhibitors to conventional chemotherapy might be a new therapeutic option for CBF-AML expressing mutant KIT.

Poster 11:

Reduced proliferation and GM-CSF-dependent colony formation of CD34+ cells from patients with juvenile myelomonocytic leukemia (JMML) after expression of the Inositol-5'-Phosphatase SHIP-1

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The Inositol-5'-Phosphatase SHIP-1 is a negative regulator of signal transduction in hematopoietic cells. SHIP-1-deficient mice develop a myeloproliferative disease characterized by an increased sensitivity of myeloid cells for GM-CSF and IL-3. We have recently demonstrated that restoration of SHIP-1 expression in a SHIP-1-deficient human leukemia cell line leads to reduced proliferation of the cells. Here we report the effects of SHIP-1 on the proliferation and colony formation of primary leukemia cells from two patients with juvenile myelomonocytic leukemia (JMML) which is characterized by a hypersensitivity for GM-CSF. Leukemia cells were obtained from patients blood and the CD34+ cells were purified and transduced with a retroviral coexpression vector containing SHIP and EGFP. The EGFP positive cells were sorted and analysed either in liquid culture or in colony assays supplemented with GM-CSF. After expression of SHIP-1 we observed a reduction of GM-CSF-dependent colony growth of approximately 50% in both JMML patients, analyzed, whereas an enzymatic inactive form of SHIP had no effect. These data indicate that SHIP-1 is able to reduce the proliferation of primary human leukemia cells and suggest that expression of SHIP in leukemia cells may have a therapeutic value.

Poster 12:

CDDO enhances CEBPa function by a translational mechanism

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The triterpenoid CDDO is a novel anticancer drug which induces apoptosis of a wide variety of tumor cells in vitro and in vivo and leads to differentiation of hematopoietic progenitor cells. Here, we studied the effect of CDDO on CCAAT enhancer binding protein alpha (CEBPα), a transcription factor which is critical for granulocytic differentiation. When HL60 myeloblastic cells were cultured in the presence of 0.01 to 1 µM CDDO, CDDO dose-dependently decreased the number of cells in culture. In parallel, the ratio of granulocytic cells increased, as assessed by morphology, NBT assay, and flow cytometry. Northern blotting showed an increase of GCSFR and a decrease of c-myc mRNA. Phagocytosis of FITC-labeled E. coli bacteria by these cells was enhanced by CDDO. CEBPα protein was increased within 24 hours of treatment, and gel shift assays showed enhanced DNA binding to a GCSFR promoter probe. Conversely, CEPBA mRNA was decreased, suggesting a translational mechanism of CEBPα protein induction. Interestingly, the two CEBPα protein isoforms, transcriptionally active p42 and inhibitory p30, were differentially regulated. In the presence of CDDO, the ratio of p42/p30 increased 3-fold. Since eukaryotic translation initiation factors have been described to alter the CEBPα protein isoform ratio, we studied the effects of CDDO on eIF2α and eIF4E activities. CDDO increased their activities by increasing phosphorylation of eIF4E and decreasing phosphorylation of eIF2α within 5 hours of treatment, which correlated with an increase of p42 CEBPα protein levels. These effects were not seen in the presence of the translation inhibitor cycloheximide. 2-AP, an inhibitor of the eIF2α kinase, PKR, increased the p42/p30 ratio in a similar fashion as CDDO. In addition, CDDO induced apoptosis, cell cycle arrest, and differentiation in primary blasts from patients with AML. This was accompanied by enhanced expression of p42 CEBPα protein and dephosphorylation of eIF2α.
In conclusion, CDDO induces granulocytic differentiation by shifting translation towards p42 CEBPα protein. Since CEBPα function is impaired in many patients with AML, CDDO may provide a novel treatment approach for this disease.

Poster 13:

Endostatin cDNA gene transfer mediated tumor control, as model for bioactivity analysis of polypeptide antiangiogenic factors.

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Formation of new blood vessels is one of the key moments in tumor development, providing two main processes in tumors: tumor metabolism and a path for tumor dissemination. At present time broad rage of factors, which involved in regulation of neoangiogenesis were discovered and characterized. They are divided on promoters: VEGF, bFGF, EGF, angiogenin, which induce new vessels formation, and inhibitors: endostatin, angiostatin, TNP-470, thrombospondin-1, tumstatin and others, which prevent development of new vessels. Most of them are proteins or products of protein enzymatic cleavage. In large set of in vitro and in vivo experimental models biologic activity of different angiogenesis factors was demonstrated. Some of inhibitory molecules are tested in pre-clinical and clinical trials. Unfortunately, brilliant experimental results of in vitro and in vivo testing of angiogenesis inhibitors activity did not find reflection in results of clinical trials. Thus, traditional tests of angigenic activity (CAM assay, Matrigel plugs etc.) reflect no strong tumor inhibition properties, but demonstrate well angiogenesis inhibition only. Moreover, second important problem in investigation of angiogenesis inhibitors is biotechnological difficulties with production of large quantity of functional therapeutic proteins. We propose experimental model in which liver functions as bioreactor and produce recombinant proteins after hydrodynamic based transfection. We constructed plasmid, which contain cDNA of endostatin and secretory signal of human preproinsulin. In preliminary Matrigel plugs experiments we demonstrate that secreted endostatin produced by transfected HEK293 cells abolished bFGF indused vascularization. Utilizing two in vivo mouse tumor models: M3 melanoma and c26 colon carcinoma, we have demonstrated that injection of plasmid, encoding cDNA of endostatin, led to significant tumor growth inhibition in comparison with mock-vector. Survival of animals cured with endostatin expressing plasmid was also increased. Thus, introducing cDNA of interest in expression construct we can analyze possible tumor suppression activity of most natural angiogenic inhibitors in real tumor formation experiments avoiding difficult steps of recombinant protein isolation and purification. Microencapsulated cells secreted such proteins could be used for tumor therapy.

Poster 14:

The hematopoiesis-supporting function

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The long-term bone marrow culture system is the most frequently used in vitro model for hematopoiesis. In this system, the adherent layer, composed of stroma cells of non-hematopoietic (fibroblasts, adipocytes, endothelial cells) or hematopoietic origin (macrophages) and their products (adhesion molecules, cytokines, extracellular matrix), constitutes the hematopoietic microenvironment which orchestrates the proliferation, differentiation and maturation of hematopoietic stem cells and their progeny.

To investigate stroma function in hematological malignancies, we established long-term bone marrow cultures from 21 untreated patients with acute myeloid leukemia (AML), 6 untreated patients with myelodysplastic syndromes (MDS), 9 untreated patients with multiple myeloma and 28 controls, and measured the hematopoiesis-supporting ability of the stroma by determining the number of colony-forming cells in the adherent layer and the supernatant after 5 weeks' of culture (W5 CFC). In order to study stroma function without the confounding effects of endogenous benign or malignant hematopoietic cells the cultures were either irradiated or stroma fibroblasts and macrophages were purified using immunomagnetic techniques. While stroma fibroblasts could be used directly as a support layer for co-cultured CD34+ indicator cells, purified stroma macrophages were unable to form a confluent adherent layer and had to be analysed in conjunction with irradiated murine M2-10B4 cells.

In contrast to purified fibroblasts from healthy or multiple myeloma bone marrow, fibroblasts from AML and MDS marrow showed a significantly reduced capacity to support blood cell formation. The hematopoiesis-supporting ability of stroma macrophages from AML marrow was markedly enhanced in comparison to macrophages from MDS, multiple myeloma or control marrow. Although the results were statistically significant, considerable overlap was observed in the range of activities of stroma cells from controls and patients, suggesting that the occurrence of functionally abnormal cells was limited to a subfraction of AML and MDS patients. Longitudinal studies showed that chemotherapy of patients was followed by a deterioration of stroma function.

The function of stroma fibroblasts and macrophages is markedly perturbed in a substantial proportion of patients with myeloid malignancies. The major questions arising from these observations concern the clonal origin of the abnormal cells and the molecular basis of their perturbed function.

Poster 15:

Leukemias following retroviral gene transfer of multidrug resistance 1 (MDR1) are driven by insertional mutagenesis

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Recent case reports have suggested that proto-oncogene activation by replication defective retroviral vectors may trigger leukemia in mice and men. In a previous study leukemic complications in mice have been demonstrated after high-copy (>10 copies per cell) retroviral gene transfer of MDR1 (Bunting et al., 1998, 2000). However, no such complications were observed in other studies after low dose gene transfer of MDR1 to mice, dogs, non-human primates and human subjects, leading to the suggestion that insertional mutagenesis by the retroviral vector is involved in leukemia development. In our experiments we show that MDR1-induced leukemias were associated with high gene transfer rates: 7 of 14 animals transplanted with bone marrow cells after high dose transduction by a retroviral vector transferring MDR1 developed leukemias, while all low dose animals stayed healthy (31 mice). In a high dose control group of mice which received bone marrow cells transduced by a retroviral vector containing a fluorescent protein (dsRED2) one mouse developed leukemia, but none of the low dose control animals (dsRED2 and htCD34). Leukemias were heterogeneous in their phenotype and latency. Genetic analysis revealed that leukemias were mostly monoclonal and contained 6–12 vector insertions. Analysis of leukemic clones by spectral karyotyping revealed secondary chromosomal instability in two of 6 leukemias examined. Analysis of the insertion sites by LM and LAM-PCR in leukemic clones showed an over-representation of insertions close to proto-oncogenes and signaling genes many of which have previously been implicated in murine and human leukemias, such as Hivep1, Csfr3, Mllt3, Fli1, Foxo3, HoxA7, Brd2, Bcl11a, Sema4b, Ski, Evi1. In addition, in leukemic mice these hits were more often found within 5 kb upstream or downstream of the transcriptions start site compared to the random distribution of retroviral insertions in unselected cell populations (Wu et al., 2003) or retrovirally transduced bone marrow of healthy mice (this study and Kustikova et al., 2005). However, in every case of leukemic development, more than one insertion close to signaling or proto-oncogenes was discovered indicating that combinatorial insertional mutagenesis is required for tumor induction.

Poster 16:

The tyrosine kinase pp60src is highly expressed and phosphorylated in cell lines and clinical samples of human AML without evidence for underlying mutations

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The tyrosine kinase pp60src is involved in several signal transduction pathways such as signaling of hematopoietic growth factors and cytokines. The viral form v-src was the first oncogene described and mutations of regulatory tyrosine residues in cellular src (c-src) have been linked to malignant transformation. However, no mutations in the gene of c-src have been described in leukemia so far, although few data of src mutations in solid tumors have been reported. The current study was undertaken to examine the expression of src in acute myeloid leukemia (AML) and to investigate whether mutations in src might play a role in this disease.
Blood and bone marrow specimen of patients with newly diagnosed or recurrent AML treated at our institution were sampled. AML cell lines or CD34 positive cells of healthy donors served as positive and negative controls, respectively. RNA was isolated, and RT-PCR using 4 different primer pairs spanning the coding region of c-src was performed. Mutational analyses was performed using the SSCP/ Heteroduplex technique. PCR fragments with suspicious performance on silver stained polyacrylamid gels were bi-directionally sequenced and analyzed for mutations. Protein expression and phosphorylation was studied after protein extraction and Western blot analyses using src and phospho-src specific antibodies. In 60 out of 62 specimen of primary AML expression of c-src mRNA was detected by RT-PCR. Western blot analyses confirmed strong expression of src on the protein level and revealed a robust activation of the protein as determined by tyrosine phosphorylation. Mutational analyses as performed by SSCP/Heteroduplex screening and bi-directionally sequencing of fragments with suspicious running characteristics revealed wild-type sequence in all cell lines and 60 clinical samples.
In summary, pp60src is highly expressed and activated in cell lines and clinical samples of human AML without evidence for underlying mutations in the coding sequence causing constitutive activation. These data suggest that pp60src may play a crucial role in AML and should be further studied as a new target for molecular therapy.

Poster 17:

Wnt/beta-Catenin signalling in acute myeloid leukemia

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The Wnt signalling pathway is evolutionary highly conserved and serves important functions in cell fate decisions during embryonal development and in the adult organism. Recently, the Wnt signalling pathway has also been implicated in self-renewal and proliferation of hematopoietic stem cells. Deregulation of its activity has been associated with a variety of human cancers and many molecules downstream of Wnt act as either tumor suppressors or proto-oncogenes. The extracellular Wnt proteins mediate their signalling through binding to Frizzled receptors and the associated LDL receptor related proteins 5 or 6 (LRP-5/6) resulting in the activation of an intracellular cascade that regulates the stability of the transcriptional coactivator beta-Catenin. In the absence of Wnt ligand, beta-Catenin is phosphorylated and thus degraded. Upon Wnt binding to Frizzled and LRP-5/6 beta-Catenin is no longer phosphorylated and unphosphorylated beta-Catenin accumulates in the cytoplasm. Thus it translocates into the nucleus and serves as a transcriptional coactivator for TCF/LEF transcription factors. Amongst the TCF/LEF target genes are known oncogenes like c-myc or cyclin D1 that are thought to mediate the oncogenic function of inappropriate Wnt signal activation.
The type III receptor tyrosine kinase (RTK) Flt3 plays an important role in survival and proliferation of hematopoietic progenitor cells and of CD14+ monocytes. Somatic mutations of Flt3 consisting of internal tandem duplications (ITD) occur in 30% of patients with acute myeloid leukemia (AML), and are associated with a poor prognosis. Recently we showed, that Frizzled-4, a receptor for Wnt ligand, and beta-Catenin are upregulated in Flt3-ITD transfected cells. In the present study we describe that mRNA levels of Frizzled-4 are increased in AML patients compared to normal bone marrow. Interestingly, we could not observe any significant differences between ITD positive and negative AML patients implicating additional mechanism for Frizzled-4 regulation in AML. Tissue array experiments in a total of 78 AML patients revealed that Frizzled-4 is widely expressed (75%) with a high frequency in FAB M3 and M4 subtype class. Prompted by this observation, we then show that Wnt3a can induce increased beta-Catenin stability in stably Frizzled-4 transfected murine 32D cells. This potent Wnt activation is dkk-1 sensitive, implicating a specific Wnt-ligand/ Frizzled-receptor interaction. These findings indicate new mechanisms for the involvement of Wnt signalling by its receptor in the pathogenesis of AML.

Poster 18:

Histone H3 Acetylation and Lysine 4 methylation are associated with transcriptional activity of the human cyclin A1 promoter in Acute Myeloid Leukemia

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Cyclin A1 is an alternative A-type cyclin that is highly expressed in acute myeloid leukemia. Its overexpression can be induced by PML-RARalpha and high cyclin A1 levels contribute to leukemogenesis. On the other hand, cyclin A1 expression is silenced in several solid human tumors. Here, we analyzed the chromatin modifications of the human cyclin A1 promoter that lead to induction or repression of the cyclin A1 gene.

Cyclin A1 was highly expressed in several myeloid leukemia cell lines whereas HeLa cells did not express cyclin A1. In these cells cyclin A1 expression was induced by treatment with histone deacetylase inhibitors such as Trichostatin A. Since these findings indicated that chromatin modifications might play a major role in cyclin A1 regulation, we performed chromatin-IP (ChIP) experiments and analyzed the chromatin structure in the 5000 bp surrounding the cyclin A1 transcriptional start site. Analyses were quantitatively performed using real-time PCR. Chromatin structure as assessed by analyses of Histone H3 acetylation and Histone H3 dimethylation at Lysine 4 indicated a rather repressed chromatin configuration between -5000 to -1000 bp relative to the transcriptional start site. In contrast, increased histone H3 acetylation and lysine 4 dimethylation were observed around the transcriptional start site in U937 leukemia cells. Similar findings were obtained for other leukemia cell lines that express cyclin A1. In HeLa cells that lack cyclin A1 expression, no increased Histone H3 acetylation and lysine 4 methylation was detected. However, exposure of HeLa cells to Trichostatin A significantly increased histone H3 acetylation at the transcriptional start site. These findings indicate that the cyclin A1 gene is tightly controlled by histone acetylation and methylation.

In conclusion, high expression of cyclin A1 in acute myeloid leukemia is associated with specific alterations in the chromatin structure at the transcriptional start site. These findings might help in developing approaches to inhibit expression of this oncogene in leukemia.

Hematopoietic Stem Cells – Biology and "Plasticity"

Poster 19:

Maintenance and Differentiation of Primitive Hematopoietic Progenitor Cells in "Stroma-Non-Contact" Culture in vitro

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Among different approaches of cultivation primitive human hematopoietic progenitors three culture systems: "stroma-contact" described by M.Dexter (1983), "stroma-free" supplemented with cytokines (J. Brandt e.a., 1990) and "stroma-non-contact" in transwells (C.Verfaillie, 1993) are known. During present studies original patented model of gel diffusion capsules (DC) was implemented. AC133+ cells derived from human umbilical cord blood were injected in the inner cavity of diffusion capsule. They were plunged into the 6-well flasks with feeder layer and DMEM culture media with 15% FCS and were incubated with change of the supernatant over 48 hours in absolute humidity at 370C and 5% CO2 for the duration of two weeks. Feeder layers were prepared from 4–6 weeks old human embryos (FLh). Influence of feeder layers from different embryo tissues and feeder layer condition media was investigated and proliferative, differentiative and clonogenic activity of cultivated cells was investigated. Side by side effect of co-cultured feeder cells (FCh) was studied. Influence of the condition media (FL-CMh) was also determined. Culture was at first supplemented with LIF and later with IL-3 and erythropoetin. Estimation of functional properties of AC133+ suspension cell cultures was performed in secondary subculture with semisolid agar. After 14 days clonogenic activity was determined by direct and indirect analysis and quantification of the colony forming units. Multipotential CFU-MIX (CFU-GEMM) and unipotential progenitor cells CFU-GM, BFU-E and CFU-E were observed and analysed using morphological and cytochemical methods. Our data indicated ex vivo expansion and myeloid differentiation of cord blood AC 133+ cells in the presence of FCh or FL-CMh feeder layer supported culture models. Prolonged support of primitive haematopoietic cells (undifferentiated cells such as promyelocytes, myelocytes and metamyelocytes) and their clonogenic capacity and functional characteristics in feeder layer positive cultures, indicates that diffusible factors are sufficient and that direct cell-to-cell contacts may not be exclusively required for successful long term in vitro haematopoiesis.

Poster 20:

Human hematopoietic progenitor cells with side population (SP) characteristics are distinct from stem cells with high aldehyde dehydrogenase activity

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Among the heterogeneous compartment of human hematopoietic progenitor cells CD34-neg lin-neg cells resemble an early and multipotent stem cell type with lympho-hematopoietic reconstitution capacity. Due to their inherent biological function of high ABCG2 transporter activity these cells are enriched within a side population (SP) fraction of Hoechst dye 33342 stained cells. High activity of aldehyde dehydrogenase (ALDHhi) is another attribute of stem cells that is associated with human hematopoietic repopulating cell capacity. ALDHhi cells are associated with a CD34-pos CD38-neg CD133-pos immunophenotype, an antigen pattern that is typically co-expressed on primitive HSC.

We were interested to investigate whether ABCG2 activity, which determines SP phenotype, and aldehyde dehydrogenase activity resemble an equal unique stem cell feature. Therefore we performed a combined staining method for ALDH expression and SP phenotype employing unmanipulated human bone marrow. Upon flow cytometric analysis both stem cell populations appeared clearly distinctive with only little overlap between the SP fraction and the population of ALDH highly expressing cells. To corroborate these findings real-time PCR analysis for ABCG2 gene expression was performed on sorted SP and ALDHhi cells. Expression of ABCG2 proved to be high in the SP fraction, but low within the ALDHhi cell fraction. However, a moderate enhanced ABCG2 cDNA expression was detectable in ALDHhi cells compared to the total cell fraction.

We conclude that ABCG2-transporter activity and aldehyde dehydrogenase activity in human haematopoietic progenitor cells are not closely associated although they are both features of primitive stem cells and have similar detoxifying functions. From our data we propose a model of stem cell development with ABCG2 expression of lineage negative cells as the earliest feature of primitive stem cells, while ALDH activity develops potentially later, probably together with AC133 and CD34 expression.

Poster 21:

Mimicking The Niche – Hematopoietic Stem Cell Interaction With Extracellular Matrix Components Under Spatial Constraints

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Although a lot of details of the hematopoietic stem cells (HSC) niche is already known, the complexity of the interaction of HSC within the niche micro-environment is still not fully understood. While many projects focus on stroma cells as model niches, in our approach the HSC fate is investigated within a defined micrometer sized niche geometry of an artificial scaffold in combination with coatings of specific extracellular matrix (ECM) components. Artificial scaffolds are silicone-based moulds from structured silicon wafers with cavities of 10 to 100µm diameter and 10µm depth. The analysis is focused on the influence of niche size as well as ECM interactions including tropocollagen I, fibronectin, heparin, hyaluronic acid and co-fibrils of collagen I /heparin and collagen I /hyaluronic acid.
In preliminary adhesion studies on planar substrates – without geometric influence – HSC-ECM interaction was investigated by reflection interference contrast microscopy to detect cell contact areas to ECM coated substrates. CD133+ HSC from peripheral blood were grown on covalently bound ECM for 24h after fresh isolation or after 6d of cultivation to clarify influences of G-CSF mobilization. CellGro medium supplemented with low cytokine concentration of SCF, FL3 and TPO (10ng/ml each) was used to prevent high cell proliferation and differentiation. Covalent ECM immobilisation was enabled by poly(ethylene -alt- maleic anhydride) copolymer pre-coated glass slides. In result, strong attachment of HSC to fibronectin with distinct adhesion areas was observed. In contrast to tropocollagen I and hyaluronic acid, heparin and co-fibrils also induced adhesion, however, without a pronounced attachment area. In further experiments the attachment of HSC to fibronectin was analysed in detail. Reflection interference contrast and confocal laser scanning microscopy were used in parallel to clarify involvement and localization of beta1- and alpha5-integrin in HSC adhesion. It was found that blocking the alpha5-integrin inhibited HSC adhesion on fibronectin.
Ongoing experiments aim towards combinatorial influences of spatial constraints and ECM coatings on HSC homing, division, and differentiation.

Poster 22:

Inhibition of the Rho GTPase, RhoA, increases the engraftment potential of hematopoietic stem cells

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Ras-related Rho GTPases regulate actin cytoskeletal organization, adhesion, gene transcription and cell cycle progression. Our lab has previously implicated Rac family of Rho GTPases as critical to HSC (hematopoietic stem cells) engraftment and mobilization (Gu Y et al., Science 2003). In these studies, absence of Rac1 is associated with markedly reduced engraftment of HSC. Using retroviral gene transfer of dominant negative mutants (DNM) of Rho GTPases (RhoAN19 and Cdc42N17), we have further dissected the roles of the related RhoGTPases, Rho A and Cdc42, in stem cell function. Inhibition of RhoA activity after transduction with RhoAN19 expressing retrovirus led to significantly increased stem cell engraftment (84.1 ± 3.8%) compared with control (44.5 ± 28.2%) or Cdc42N17-transduced HSC (34.9 ± 19.9%) measured 4 months post transplant. As expected, expression of DNM of Rac was associated with decreased engraftment (2.9 ± 0.7%). Enhanced engraftment was also seen in secondary recipients of RhoAN19-transduced stem cells (58.81 ± 14.29% vs. 10.92 ± 2.57% in controls and 4.88 ± 1.51% in Rac2D57N and 8.98 ± 4.51% in Cdc42N17-transduced HSC). Expression of DNM RhoA was not associated with any evidence of myeloproliferative disease in serially transplanted mice followed for 16 months from the initial transplant (three independent experiment, n=12). To determine the potential mechanism of increased engraftment in RhoAN19 transduced cells, we investigated cell cycle progression and proliferation of HPC in vitro. Expression of RhoAN19 increased entrance of cKit+ transduced mononuclear bone marrow into cell cycle (S,G2,M - 43.95% vs. 38.41%, RhoAN19 vs. control, n=4, p=0.027) and increased proliferation as measured by thymidine uptake (counts: 14,600 ± 1816 vs. 8,409 ± 1301, RhoAN19 vs. control, two independent experiments, n=12, $p < 0.01$). Immunoblot analysis of ckit+ bone marrow cells also demonstrate increased expression of cyclin D1 and decreased levels of p21 in DNM RhoA-expressing cells. These data thus suggest that, Rac and RhoA, two related GTPases play distinct and apparently opposite roles in HSC biology. In this context, we are currently evaluating whether transient inhibition of Rho via pharmacologic inhibitors improves HSC engraftment following bone marrow transplantation.

Poster 23:

Studying the origin of adult hematopoietic stem cells with a temporally controlled genetic marking strategy

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The engraftment ability of embryonic hematopoietic progenitor cells in adult recipients implies the emergence of adult hematopoietic stem cells (HSCs) during early ontogeny. Multiple anatomic sites such as the yolk sac, the aorto-gonads-mesonephros (AGM) region, the fetal liver (FL) and the bone marrow harbor hematopoietic progenitors at different developmental stages. This chronologic appearance of HSCs at different localizations has lead to the assumption that each site is colonized by the same pool of HSCs. However, the independent de novo generation of HSCs at each site cannot be excluded. Possibly, de novo generated HSC displace their stem cell predecessors at different sites during development. In order to study the origin of adult HSCs in a straight in vivo model we created a transgenic system, which allows temporally controlled, irreversible genetic marking of HSCs in vivo. This was achieved by guiding the expression of the tamoxifen-inducible Cre-ER[T] recombinase with the stem cell enhancer of SCL (stem cell leukemia) locus to HSCs. To assess the functionality of this approach transgenic mice (HSC-SCL-Cre-ER[T]) were crossed with Cre reporter mice, which express EYFP upon Cre-mediated recombination (R26R-EYFP). Flowcytometric analysis revealed tamoxifen-dependent recombination occurring in the vast majority of adult long-term repopulating HSCs (c-kit+Sca-1+lineage-Flk2-) of HSC-SCL-Cre-ER[T]; R26R-EYFP double transgenic mice while the proportion of targeted cells within a more mature progenitor population (c-kit+Sca-1+lineage-) was significantly lower. We confirmed these data by transplanting HSC-SCL-Cre-ER[T];R26R-EYFP bone marrow of tamoxifen-treated mice into lethally irradiated recipients. Six months after transplantation the majority of peripheral blood granulocytes and lymphocytes were derived from EYFP-marked HSCs. We also demonstrated that maternal tamoxifen injections lead to the marking of fetal liver HSC in utero. Therefore, the HSC-SCL-Cre-ER[T] transgenic line is capable of temporally controlled HSC marking in vivo.
Subsequently, we used the HSC-SCL-Cre-ER[T];R26R-EYFP transgenic system to mark HSCs on days 10.5 and 11.5 of embryonic development by maternal tamoxifen injections. At this developmental stage HSCs are mainly located within the AGM and the FL. When these mice were analyzed at 5 months of age we could find significant contribution of embryonicly marked HSCs to adult hematopoiesis. In a second experimental set-up day 14.5 FL HSCs, marked in the same manner, were transplanted into irradiated adult recipients. Interestingly, the proportion of marked cells in the transplanted HSC pool after 5 months was similar to the proportion of marked cells in the vivo remaining HSC pool. These data demonstrate the existence of a lineage relationship between embryonic and adult HSCs in a direct manner. Additionally, these data imply that the de novo generation of HSCs is completed in the midgestation embryo.

Poster 24:

In vivo selection of transduced hematopoietic stem cells and little evidence of their conversion into hepatocytes in vivo.

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We transferred a drug resistance gene to hematopoietic stem cells (HSCs) in adult mouse bone marrow using a FMEV-type retroviral vector containing the polycythemic strain of spleen focus-forming virus long terminal repeats and the mouse embryonic stem cell virus leader sequences. Mice transplanted with the vector-transduced cells showed resistance to a drug. Successful selection of transduced cells in vivo indicated that this type of vector expresses a drug-resistance gene in HSCs highly enough to protect them from a drug in vivo. Next, we examined whether the transduced HSCs trans-differentiate into hepatocytes following in vivo selection. In the liver of recipient mice, we observed efficient transgene expression in many small cells along sinusoid and a small number of large cells near the periportal area. The former did not react with anti-albumin antibody, while the latter did. Although the large cells was positive for expression of both albumin and a transgene, these cells also expressed a recipient marker gene, suggesting fusion of donor hematopoietic cells with recipient hepatocytes.

Poster 25:

Multiple Low Dose G-CSF Treatments Alter Hematopoiesis in Mice

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In clinical practice G-CSF is widely used for mobilization of peripheral blood stem cells for transplantation purposes, leukemia priming before treatment, shortening neutropenia period after aggressive chemotherapy. Rather often the same person undergoes several courses of G-CSF, which makes it important to thoroughly study the long-term consequences of G-CSF treatment. In mice four-day therapy with low doses (25µg/kg) of G-CSF, known to be insufficient for mobilization of peripheral blood stem cells, leads to 2-fold decrease of bone marrow primitive hematopoietic stem cells (P-HSC) at the end of the treatment, while the CFU-S number in the bone marrow doesn't change. At the same time the number of peripheral blood CFU-S doubles whereas leukocyte count and the proportion of granulocytes remain unchanged. The aim of this study was to reveal whether the decrease of P-HSC after G-CSF treatment is hematologically relevant.

Female mice CBF1 (CBA/Lac x C57Bl/6) F1 and DBF1 (DBA/2 x Balb/c) F1 were injected with G-CSF (25 µg/kg) for 4 days once a month with blood cell count and cytology measured before and after the course. A month after the end of 7th course (7 months since the beginning of the experiment) the bone marrow was obtained from anesthetized mice and then tested for the CFU-C and the LTC-IC frequency. There were no significant changes in blood cell count for the whole period of observation. CFU-C (7 days) concentration in the bone marrow didn't differ among control and experimental mice either, while the concentration of CFU-C counted on the 14th and 21st day after the plating, representing more immature hematopoietic precursors, increased more then 2-fold in the bone marrow of G-CSF treated mice while LTC-IC frequency decreased 2-fold in the latter group. Obviously hematopoietic precursors on various stages of maturation respond to the G-CSF treatment with pharmacological doses in different ways. As the dose of G-CSF used was not sufficient for mobilization, decrease in LTC-IC frequency in the bone marrow cannot be explained by the recruitment of these precursors into the peripheral blood. Increase in frequency of more mature hematopoietic precursors suggests stimulation of differentiation of LTC-IC as a probable cause of the effect. Hematopoiesis occurred does not seem to be effective as neither frequency of the mature bone marrow myeloid precursors nor leukocyte count in peripheral blood increased.

During 7 months of experiment overall survival of G-CSF treated mice was 50% versus 100% in untreated group. Two out of ten CBF1 mice died during second course of G-CSF injection. One out of forty CBF1 mice in the next experiment developed myelomonoproliferative disease one month after first course of G-CSF. Two out of ten CBF1 mice developed different myeloproliferative disorders (defined on classification proposed in Blood, 2002; 100:238-245). MPD-like myeloid leukemia with histiocytic sarcoma occurred in one of these mice after 4th course of G-CSF treatment. Bone marrow and liver cells of the mouse are retransplantable, recipients become moribund within 21–32 days since cells injection. No chromosome aberrations were revealed. All tests for probable retransplantation of leukemia by viruses were negative. Another mouse developed myeloid leukemia without maturation after 7,5 month since the beginning of the experiment. Stable decrease of early hematopoietic precursors' frequency and the development of inducible leukemias suggest the occurrence of serious alterations in the hematopoietic system under the G-CSF treatment.

Poster 26:

Effect of parathyroid hormone (PTH) on hematopoietic and stromal precursor cells

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Parathyroid hormone (PTH) is used in clinical trails for treatment of postmenopausal women with osteoporosis. PTH stimulates and activates osteoblasts. The latter are suggested to participate in formation of hematopoietic “niche” needed for regulation of hematopoietic stem cells’ fate. An influence of PTH on hematopoiesis was investigated. Mice were injected i.p. with PTH 80 mkg/kg 5 days/week for 4 weeks. Two weeks later concentration of different hematopoietic precursors was analyzed. PTH-induced alterations varied for hematopoietic precursors of different maturity. The number of CAFC 28–35 and LTC-IC in femoral bone marrow doubled in PTH-treated mice. On the contrary the CFU-S 9 and CFU-S 13 number decreased about 2-fold while their self-maintenance ability (measured by the number of daughter CFU-S per 13 day-old spleen colony) was 1,5–2 fold higher. Concentration of CFU-GM and BFU-E did not change. These results suggest the ability of PTH to govern the function of “niches”. This suggestion was supported by revealed changes in bone marrow seeding efficiency (f-factor-24 hours) of normal hematopoietic precursors in PTH-treated mice. CFU-S seeding decreased approximately 5-fold from 5% to 0,6% while CAFC-28 f-24 increased 1.2-fold. In spleen f-24 for CFU-S was unchanged in treated and control mice (5–6%) however for CAFC-28 it decreased from 7,6% to 1,5% in PTH-treated recipients. For the best of our knowledge the polar changes in niche function for precursors of different maturity after experimental treatment were revealed for the first time. PTH did not affect the number of mesenchymal stem cells measured by their ability to transfer hematopoietic microenvironment while it doubled the weight of de novo formed bone shell in the ectopic foci. In normal mice circulating hematopoietic precursors with lower self-maintenance ability as compared to bone marrow precursors repopulate the ectopic hematopoietic foci. The self-renewal capacity of CFU-S in the foci formed in PTH-treated mice increased 2–3 fold. It is uncertain whether PTH treatment induces mobilization of more immature precursors from bone marrow or activates the precursors during foci formation. The mechanism of hematopoietic effects of PTH is needed to be scrutinized.

Poster 27:

Comparative Analysis of Telomerase Activity in Hematopoietic Cells from Thymectomized Mice and in Cells from Normal Hematopoietic Systems

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Purpose: The telomeric repeat amplification protocol (TRAP - PCR-ELISA) assay was used to measure telomerase activity (TA) in several populations of hematopoietic cells (bone marrow -BM, day 9 colony-forming units spleen colonies - CFU-S, spleen) from normal (N) and adult thymectomized (A.Tx) mice in term of steady state hematopoiesis and during serial transplantation.

Methods: Four weeks old mice were thymectomized and were used as a donor of BM cells 1–22 months after thymectomy. Normal mice were the same age as A.Tx mice. BM was collected from femurs of the same mice by aspiration throughout 23 months period and injected into irradiation recipients for the analysis of day 9 CFU-S. In the second sets of experiments BM from A.Tx mice was used for reconstitution of the lethally irradiated recipients 12 months later after thymectomy. The secondary recipients were obtained from the 9 months primary chimeras. TA in cells from primary and secondary radiation chimeras was investigated 9 and 12 months after reconstitution of hematopoiesis, accordingly. Cell cycle status was analysed by flow cytometry on FACScan.

Results: Low level of TA (2–5%) was detected in BM of normal mice and in the chimeras, reconstituted with BM from normal mice. Age-dependent correlation of TA was not observed. However, telomerase is expressed much more in mice after thymectomy. TA was 2–3 times higher BM cells from either A.Tx donors (7–19%) and in the primary (8%) but not in the secondary recipients, reconstituted with BM from A.Tx mice. The thymus implantation to the A.Tx mice is abrogated effect of thymectomy. It was not observed correlation of the TA with the number of cycling BM cells. In contrast to BM it revealed no significant differences in the level of TA from the cells of day 9 colonies. The both, normal and A.Tx mice exhibited high level of TA, which was almost equal to the basal level of TA in the BM from A.Tx mice (about 8%). There is no correlation between the increased enzyme activity and the number of cells per colonies.

Conclusion: These data suggests that under steady-state conditions (natural ageing) mouse hematopoietic cells express telomerase over their life and the level of TA is not strong associated with thymus involution. However, the activation of the enzymes level is detected in BM cells after thymectomy and in the A.Tx primary chimeras, or in spleen colonies without lymphocyte lineage of differentiation. The dramatic reduction of TA after second round restoration of donor-derived cells from A.Tx mice suggests a diminished repair mechanism for the long-term replicative capability of BM cells. These new findings suggest of unexpected influence of T-cell depletion on the telomerase expression in mouse haemopoietic cells. The immunological status of animals became one of the factors for regulation of telomerase activity.

Poster 28:

Molecular Signature of Mesenchymal Stem Cells

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Various preparative protocols have been proposed for acquisition and cultivation of mesenchymal stem cells (MSC). Surface antigen markers have failed to precisely define this population. Microarray analysis might provide another dimension for characterisation of MSC.
Here, we have analyzed molecular profiles of human MSC isolated from adipose tissue (AT), from umbilical cord blood (CB) and from bone-marrow (BM) under two growth conditions and have compared them to terminally differentiated human fibroblasts (HS68). Cultured with the appropriate conditions osteogenic and adipogenic differentiation could be confirmed in all MSC preparations but not in fibroblasts. No phenotypic differences were observed by flow cytometry using a panel of 22 surface antigen markers. Global gene expression profiles were then compared using our Human Genome Microarray representing 51.144 different cDNA clones. Whereas MSC derived from different donors but using the same culture procedure yielded a consistent and reproducible gene expression profile, many genes were differentially expressed in MSC from different ontogenetic sources or from different culture conditions. Pair wise comparisons of microarray data from AT, CB and BM showed that 23 genes were overlapping and up-regulated in all MSC preparations as compared to HS68 fibroblasts. These genes included fibronectin, ECM2, glypican-4, ID1, NF1B, HOXA5 and HOXB6. These genes are known to be involved in extracellular matrix, cell division and development, whereas several inhibitors of the Wnt-pathway (DKK1, DKK3, SFRP1) were higher expressed in fibroblasts.
This study reports on differences in the transcriptome of MSC isolated from AT, CB and BM, while similar populations were reproducibly isolated under standardized conditions. Our comparative approach provides the foundation for a reliable quality control using genotypic analysis for clinical applications.

Poster 29:

Long-term hematopoietic stem cell engraftment in non-myeloablated c-KitW/Wv mice lacking all lymphocyte lineages.

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Mice deficient for the receptor kinase c-Kit show defects in various cellular compartments including the hematopoietic system. There, c-Kit is mainly expressed on stem cells and on progenitors. Despite its wide usage as stem cell marker the biological function of c-Kit in hematopoietic stem cells (HSC) is largely unknown. Bone marrow (BM) cells of c-Kit null (W/W) mouse mutants show defects in HSC as shown by lack of radioprotective or colony-forming-unit-spleen activity after transfer into lethally irradiated mice. Nevertheless, c-Kit$^{W/W}$ HSC contribute to hematopoiesis after transfer into irradiated mice. Reversely, syngeneic c-Kit$^{+/+}$ bone marrow cells reconstitute all hematopoietic lineages upon transfer into mildly- or non-irradiated c-Kit$^{W/Wv}$ mice, indicating that c-Kit$^{W/Wv}$ HSC are inferior to c-Kit wild type HSC. Due to the MHC-haplotype of c-Kit$^{W/Wv}$ mice (H-2^{b} from the C57Bl/6 mouse strain, and H-2^{i} from the WB mouse strain) these mice can only serve as recipients for syngeneic B6 x WB F1 donor mice.
Mice deficient for recombination activation genes (Rag) and the common cytokine receptor gamma chain (γ_c) lack mature T-, B-, and NK-cells, and are, therefore, widely used as recipients for BM transplantations. Lack of host versus graft reactions on one side and easy monitoring of developing T-, B- and NK cells on the other side make them ideal recipients for BM cells after mild irradiation (4Gy).
We combined Rag- and γ_c- deficiency with a “weak” stem cell compartment in order to generate a ‘universal’ recipient for BM transplantation. Rag-2- γ_c- c-Kit$^{W/Wv}$ mice indeed are easily reconstituted with allogene c BM cells without previous myeloablation. Wild type BM donor cells form colonies in Rag-2- γ_c- c-Kit$^{W/Wv}$ mice 12 days after transplantation (CFU-S$_{d12}$) and replace the endogeneous stem cell compartment of host mice for at least 4 months.
In addition to serving as a highly receptive stem cell host, our combined mutants will be analyzed to gain insight into the mechanism(s) underlying stem cell engraftment without conditioning.

Non-Hematopoietic Stem Cells

Poster 30:

Embryoid bodies in long term culture as resource of hematopoietic progenitor cells

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Human pluripotent embryonic stem cells might be used extensively in studying the establishment, differentiation and some gene functions. The cells derived from primordial germ cells of embryo by nature are thought to be pluripotent too. In our study EG cells derived from gonads region of 4–5 weeks old human embryos were cultured by “hanging drop” technique. Cells were maintained in medium with leukemia inhibitory factor (LIF), otherwise known as differentiation inhibiting activity (DIA). Embryoid bodies (EB) were obtained in drops on 3–5-th day of cultivation and were passed in 96-well culture dishes where cultured over 2 months in vitro in DMEM (Gibco/BRL) supplemented with 15 % fetal bovine serum (Gibco) without LIF. Morphology of EB-derived cells during further differentiation to hematopoietic lineage have been studied. Cloning efficiency of hematopoietic cell from supernatant of embryoid bodies' culture was investigated in semisolid agar and granulomonocytic and erythroid progenitors were found. The results testify that human embryonic germ cells isolated from early embryo give rise to EB which during long term cultivation in vitro provide the generation of hematopoietic precursors. These properties allow using EB technique as model system for investigation of early stages of hematopoiesis during embryonic development and powerful potential stem cell resource for further clinical application .

Poster 31:

Adhesion and Maintenance of Bone Marrow-Derived Stem Cells in Neural and Stromal In Vitro Environments is Non-Selective and Depends on the Expression of Integrin- and Notch-Ligands, Respectively

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Several studies in animal models demonstrated that bone marrow-derived stem cells (BMDSC) can home in neural tissues and ameliorate inherited or acquired defects. Since interaction with the niche is known to play an essential role for the differentiation fate of stem cells we studied the early events of interaction of $CD34^+/CD133^+$ BMDSC in stromal and neural in vitro environments, i.e. the fetal murine stromal liver cell line *AFT024*, human marrow stromal cells (MSC), murine astrocytes and murine neurons.

We found that neural as well as stromal feeder layers induced homing behaviour as soon as 20 min. after starting the coculture. Up to 92.5 ± 10.4% of the BMDSC exhibited podia formation, directed SDF-1 induced migration as well as continuous seeking for homo- and heterotypic cell-cell contacts. Despite this similar behaviour within the various in vitro environments adhesion kinetics revealed significant differences in the affinity of BMDSC for stromal and neural feeder layers. A strong and stable adhesion for at least 12h was found to MSC (31.7 ± 6.1% adherent cells) and *AFT024* (37.6 ± 12.7% adherent cells), but not to neurons or astrocytes. Broad immunophenotyping (CD18, CD29, CD33, CD34, CD38, CD44, CD45, CD54, CD90, HLA-DR, CD117, CD133) and functional characterization of the BMDSC by means of CFC assay, LTC-IC assay and PKH-26 based division analysis as well as serial and cross-over adhesion experiments revealed that the observed differences are not due to an adhesion of different subpopulations. Since immunophenotyping showed a preferential adhesion of CD18 (beta 2-integrins) and CD29 (beta 1-integrins) positive BMDSC we characterized the various feeder layers for the expression of appropriate ligands, i.e. CD54/ICAM-1, CD106/VCAM-1 and fibronectin by means of immunofluorescence microscopy, immunoblotting and immunophenotyping. In comparison to the neural feeder layers we found an exclusive expression of fibronectin and a superior expression of CD106/VCAM-1 with 59.9 ± 2.0% positive cells for *AFT024* and 41.2 ± 1.8% for MSC within the stromal layers. Pretreatment of the BMDSC with integrin-blocking antibodies revealed a significant reduction of integrin-mediated adhesion.

Furthermore we performed time-lapse studies and LTC-IC assays of the adherent cells to follow up the fate of BMDSC within the stromal and neural in vitro environments. We found that about 60% of initial LTC-IC were maintained by *AFT024* and MSC, whereas neural feeder layers did not maintain LTC-IC at all. This correlated with a lacking expression of the Notch-ligands Jagged-1 and Delta by the neural feeder layers.

Taken together these results demonstrate that in vitro homing of BMDSC within neural environments is a non-selective, integrin-dependent process and maintenance of primitve, adaptable cells correlates with expression of Notch-ligands by the feeder layers.

Poster 32:

Implantation of Human Bone Marrow Derived Stromal Cells in a Nude Rat Model of Spinal Cord Injury

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Our aim in this study was to develop a new transplantation regime to stimulate sparing and regeneration of descending neural pathways following moderate acute (1 week) contusive spinal cord injury (SCI). Our novel repair strategy involves using multipotent human bone marrow stromal stem cells (hBMSCs) from SCI patients which have been transduced with a retrovirus encoding the reporter gene Green Fluorescent Protein (GFP). HBMSCsGFP were transplantated into immunologically deficient (Nude) rat hosts subjected to a moderate SCI (10g from 12.5mm) using an NYU impactor device. The therapeutic potential of hBMSCsGFP has been assessed both behaviourally (recovery of function) and anatomically using immunohistochemistry and tracing of lesioned axon pathways using the retrograde tracer fluorogold. Preliminary data show that transplanted hBMSCsGFP survive well in the injured spinal cord, induce axonal growth around the transplants, produce growth promoting molecules and co-exist with host glial cells such as astrocytes and Schwann cells within the lesion site. Neural marker expression profiles show that in acute SCI, by 1wk after transplantation, RT97, GFAP, p75, beta-III tubulin and S100 is often expressed in close proximity to transplanted hBMSCsGFP, that also produce laminin and fibronectin. Functionally, host nude rats transplanted with donor hBMSCs show an improvement in open field BBB scoring (ranging from 13–15) up to 2 months post operative either with or without CsA treatment. Further functional analysis using a computerized catwalk gait analysis system is in progress. Further in vitro immunocytochemical analysis of hBMSCs show they express a number of phenotypic markers such as beta-III tubulin, laminin and fibronectin. The knowledge gained from these ongoing studies may provide the possibility of isolating and transplanting back an SCI patient's own multipotent cells to repair spinal cord injuries and therefore provide a clinical treatment for such injuries in the future.

Poster 33:

Promoter regions diverse methylation profile of pluripotency-related genes in newly derived hESC lines.

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Human embryonic stem cells (hESCs) are a promising model for studying mechanisms of regulation of early development and differentiation. Genes required to maintain pluripotency of hESCs are largely unknown, with the exception of few recently described such as OCT4, NANOG, OCT4-related genes and some others. The less is known about molecular mechanisms involved in their regulation. Apart of genetic regulation of gene expression epigenetic events play an important role in early development. DNA methylation is one of the major epigenetic modifications of DNA in mammalian organism. In the current study we have analyzed the epigenetic status of previously derived hESC lines. All cell lines proliferate without differentiation for more than 2 years, have normal karyotype and express markers characteristics for hESCs. We demonstrated that derived hESC lines give rise to the cells of three germ layers in vitro. Analysis of methylation profiles of promoter or putative regulatory regions of pluripotency-maitaining genes OCT4 and NANOG demonstrated that expression of these genes correlates with their methylation status, thus indicating that likely in mouse ES epigenetics contributes to gene silencing during cell differentiation. However, methylation of putative regulatory regions of DPPA3 and DPPA5 genes varied between cell lines not significantly affecting their expression profile. Our finding indicates that isolated hESC lines have different epigenetic background that may impact on differentiation potential of the hESC lines.

Poster 34:

Genetic cell tracing of hematopoiesis origins

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Hematopoietic lineage development in mammalian embryogenesis proceeds through multiple steps and requires a number of specific cellular environments. The relationship between different nascent blood cell populations is poorly defined and the contribution of primary hematopoietic sources in embryo to mature hematopoietic system is disputed. Cell tagging of early embryonic progenitors using a genetic switch system offers the possibility for efficient cell fate analysis. To this end, Mer-Cre-Mer recombinase fusion gene was placed by targeting under the control of the endogenous Runx1 proximal promoter, which is active specifically at the sites of earliest embryonic hematopoiesis. Mice bearing the Cre-recombinase knock-in construct were crossed with ROSA26R LacZ-reporter mice. Pregnant females were then injected with a single dose of tamoxifen at different stages of gestation to activate Cre-recombinase. The resulting LacZ-positive hematopoietic cells were followed during embryo development to term and beyond. No Cre-recombinase activity was detected in absence of tamoxifen, whereas the tamoxifen-dependent activation was strictly stage-specific. Descendants of early blood island cells were detected in endothelium of dorsal aorta and umbilical arteries/veins. Their liver contribution commenced at around E10.5, and later was observed in multiple definitive hematopoietic lineages. Substantial fraction of E16.5 lymphoid cell population originated in E7.5-8.0 yolk sac blood islands. A limited contribution to hematopoietic system of one-month old mice was also detected. Descendants of AGM region progenitors were labeled by tamoxifen injection at E9.5 and their contribution to adult hematopoiesis is now under investigation.
The data suggest that early angioblasts are capable of movement through blood stream over large distances within the embryo. This further corroborates the concept of a hemogenic endothelium as an adaptation of nascent hematopoietic cells to the luminal microenvironment. The in vivo tracing analysis suggests a common anatomical origin for primitive embryonic hematopoietic lineages and definitive multipotential progenitors.

Poster 35:

Hematopoietic Progenitor Cells and their Niche; Behavioral and Molecular Changes upon Interaction with a Cellular Microenvironment.

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Cell-cell contact between stem cells and cellular determinants of the microenvironment plays an essential role in controlling cell division. Using human hematopoietic progenitor cells (CD34+/CD38-) and a stroma cell line (AFT024) as a model, we have studied the initial behavioral and molecular sequel of this interaction. Time-lapse microscopy showed that CD34+/CD38- cells actively migrated towards and sought contact with stroma cells and 30% of them adhered firmly to AFT024 stroma through the uropod. CD44 and CD34 are co-localized at the site of contact. Gene expression profiles of CD34+/CD38- cells upon cultivation with or without stroma for 16h, 20h, 48h or 72h were analyzed using our Human Genome cDNA Microarray. Chk1, egr1 and cxcl2 were among the first genes up-regulated within 16h. Genes with the highest up-regulation throughout the time course included tubulin genes, ezrin, c1qr1, fos, pcna, mcm6, ung and dnmt1, genes that play an essential role in re-organization of the cytoskeleton system, stabilization of DNA and of methylation patterns. Our results demonstrate directed migration of CD34+/CD38- cells towards AFT024, adhesion through the uropod and that upon interaction with supportive stroma, re-organization of the cytoskeleton system, regulation of cell division and maintenance of genetic stability represent the most essential steps.

Poster 36:

Molecular Composition of Intercellular Contacts in Human Mesenchymal Stem Cells

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The long-term fate of stem cells depends on their interaction with the niche. Mesenchymal stem cells (MSC) from human bone marrow have been demonstrated to differentiate into various tissues, such as bone, cartilage, muscle and fat. As the interaction with the microenvironment plays a major role in differentiation, we have characterized the cell-cell contact among MSC. We have demonstrated the occurrence of a novel kind of adhering junction, consisting of slender, villiform-to-vermiform cell projections (processus adhaerentes). Continuing this line of research, we have systematically analyzed the molecular composition of these junctions.

A panel of antibodies specific for various components of tight junctions, gap junctions, adherens junctions and desmosomes was used. The expression of these antigens was verified by light and electron microscopy and by biochemical analysis, including immunoprecipitation and RT-PCR.

We demonstrate that MSC were interconnected by occasional gap junctions and frequent adhering junctions. Additionally, we found a unique molecular composition of these adhering junctions, as they comprise the transmembrane glycoproteins cadherin 11 and N-cadherin, together with the cytoplasmic plaque proteins alpha- and beta-catenin and p120ctn.

Our data indicate that MSC communicate with each other through junctions and junctional complexes. We hypothesize that MSC can embark on alternative differentiation pathways with specific junctional and cytoskeletal patterns. Characterization of and understanding the role of such intercellular contacts and their correlation to specific differentiation programs are being conducted.

Hematopoietic Stem Cell Transplantation

Poster 37:

A Panel of Polymorphic Gene Alleles: Their Application on Haematopoietic Stem Cell Transplantation

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Many human genes are represented as common allelic variants, or polymorphisms. Such genetic variants affect either transcription rates, or activity of specific protein products, thus predisposing for some clinical disorders. E.g., significant associations were previously noted between certain polymorphisms of some cytokine and protease genes and GvHD occurrence. Hence, such bi-allelic human gene variants may provide a \"soft\" clinical model of experiments with "knockout genes". Objectives. The aim of present study was to extend clinical applicability of typing of physiologically important genes in allogeneic hematopoietic stem cell transplantation (HSCT), using a panel of six unique polymorphic genes. Patients and methods. The study included a total of 213 patients with various oncohaematological disorders treated at the BMT departments of Hamburg and St.Petersburg (80 familial and 83 unrelated transplants, nonmyeloablative conditioning regimens been applied in 58 cases. Allelotyping was performed with genomic DNA of patients and donors by means of allele-specific PCR for MMP-1 (1G/2G-1607) and PAI-1 (4G/5G-675). In a subgroup of 66 patients from St. Petersburg, MMP-3 (5A/6A-600), MDR-1 (C3435T), MTHFR (C677T), and GSTM1 (+/null alleles) have been tested. Globin gene-specific PCR was used as a control. Assays of genetic chimerism were performed in qualitative and semi-quantitative manner.
Results. 1. Serial allelotyping of the six mentioned genes allowed us to find informative mismatches between donor and recipient in over 80% of the HSCT cases (i.e., the allele was present in the patient and absent in donor). GSTM1 (plus-to-null allele ratio of approx. 1:1) may be especially useful, in addition to widely used chimerism detection with sex chromosome-linked genes. 2. In extended group of patients, we have confirmed a dictinct association between the presence of aGvHD and homozygous state of MMP-1 (2G/2G) in the patients. Moreover, a significant association was found between the variant allele of MDR-1 gene (T/T) and more severe aGvHD (grades II to IV), thus presuming altered pharmacokinetics as a probable reason for this complication. 3. The post-transplant reactivation of herpes simplex virus (HSV) did also correlate with MMP-1 genotype, i.e. 78% of HSV-positive patients were homozygous for 2G/2G, compared to 22% in HSV-negative group ($p=0.02$, $n=63$). Less pronounced correlation trends ($p=0.07$-0.09) were found between CMV infection and MDR-1 (C allele) or PAI-1 (4G allele). When analyzing other complications in transplanted patients, we have revealed higher incidence of the less active MMP-1 1G/1G genotype in the patients with gross infections following HSCT ($p=0.04$, $n=56$), thus confirming the role of matrix metalloproteinase system in natural immune defence system.
Conclusions. The data obtained show that certain gene alleles may predict higher risk of severe complications post-HSCT. These findings should be tested in other ethnic groups and clinical conditions. In addition, a panel of these alleles could be effectively used for the quantification of genetic chimerism in post-transplant patients.

Poster 38:

Current results and perspectives of high dose chemotherapy with autologous stem cell transplantation in multiple sclerosis patients

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Objectives: High dose chemotherapy with autologous stem cell transplantation (HDCT+ASCT) is a new and promising therapy for multiple sclerosis (MS) patients. At present, the information about its efficacy in patients at different disease stages is lacking. In current clinical trials HDCT+ASCT is usually performed for the advanced MS. We suggest that transplantation in early disease stage may lead to the recovery. The aim of the study was to search for the optimal time for HDCT+ ASCT in MS and to evaluate treatment outcomes in patients with different strategies of HDCT+ASCT. Methods: 17 patients with MS were included in the study (mean age - 32.3, SD - 6.6; male/female - 4/13). Median EDSS at base-line was 6.0 (range 2.0 - 7.5). The median follow-up duration was 18 months (range 3–60 months). All of the patients had previously undergone conventional treatment. Neurological and quality of life (QoL) evaluation was provided at baseline, at discharge, 3, 6, 9, 12 months, and then every 6 months after HDCT+ASCT. MRI was conducted at baseline, at 6, 12 months, and at the end of follow-up. Clinical improvement was defined as a decrease in the EDSS score by at least 0.5 points on two consecutive visits 6 months apart as compared with baseline; disease stabilization – as no change in EDSS score during follow-up; disease progression was defined as an increase by at least 0.5 points after 6 months and/or appearance of new lesions on MRI. QoL was assessed by FACT-BMT and FAMS. QoL response was evaluated by the method of integral profiles using Integral QoL index. Results: Based on the experience of the Russian Cooperative Group for Cellular Therapy we proposed three strategies of HDCT+ASCT in MS depending on the disease stage and degree of disability. Early ASCT. Indications: confirmed MS; no or minimal signs of disability; EDSS – less than 3.5. Goals: to prevent irreversible lesions in the central nervous system (CNS), to prevent disability, to preserve QoL. Conventional ASCT. Indications: confirmed MS; evident signs of disability; EDSS - 3.5-6.5. Goals: to stop the disease progression, to prevent exacerbation of disability, to stop the decline in QoL or to improve it. Salvage ASCT. Indications: confirmed MS; high disease activity; multiple irreversible lesions in the CNS; pronounced disability; EDSS - 7.0-8.5. Goals: to stop the disease progression, to save a patient from a complete disability, to improve a severe decline in patient's QoL. One out of 17 patients underwent early transplantation (male, 21 years old, secondary progressive type of MS, EDSS at base-line - 2.0) and showed both clinical (EDSS decreased by 0.5 points) and QoL improvement. One patient underwent salvage transplantation (female, 49 years old; primary progressive type of MS; base-line EDSS 7.5; follow-up 48 months). As a result, significant clinical improvement (EDSS decreased from 7.5 to 6.0) and excellent QoL response were achieved at the end of follow-up. Other patients underwent conventional transplantation: 9 out of 13 patients (2 patients were not included in the analysis because of 3 months follow-up) experienced a clinical stabilization or improvement. All of the patients with clinical stabilization and improvement exhibited negative MRI scans. One patient continuously worsened and died 3 years after the transplantation. Three other patients worsened by 0.5 points on their EDSS. Analysis of QoL response showed that at one year after HDCT+ASCT and during follow-up the majority of patients with clinical stabilization and improvement exhibited good or excellent QoL response. Conclusion: HDCT+ASCT in MS patients resulted in clinical stabilization and improvement in 73.3 % of patients under observation. In addition, dramatic improvement of QoL took place. Thus, our results have demonstrated a feasibility of early, conventional, and salvage HDCT+ASCT in MS patients; further studies are needed to confirm the aforementioned concept.

Poster 39:

Aldehyde Dehydrogenase Activity for Quality Assessment of Hematopoietic Stem Cell Transplants

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Aldehyde dehydrogenase (ALDH) enzyme activity has been shown to be a characteristic metabolic marker for primitive hematopoietic progenitor, so-called SSCloALDHbr cells. Using a flow cytometry approach based on ALDEFLUOR® we investigated utility of this new marker for the quality assessment of hematopoietic stem cell transplants. First we compared expression kinetics of the standard CD34 marker (as determined by the ISHAGE protocol) vs. ALDH in bone marrow and peripheral blood stem cells (PBSC) samples during a 7-day storage period in relation to their viability and colony-forming ability. This resulted in two important findings: (i) Under stress conditions only ALDH activity but not CD34 expression strongly reflects progenitor (CFU-GM) potential, (ii) If stored at room temperature PBSC transplants lose most of their progenitor cells (CFU-GM) within just 48 hours, a reasonable time period for transportation of PBSC in an MUD setting. In contrast, no such loss was observed if the PBSC were stored in the fridge. Next we retrospectively analyzed ALDH and CD34 expression in stem cell preparations after a freezing/thawing cycle, another type of stress applying in the context of HSCT. Obtained results were related to the engraftment data for the given patients transplanted with frozen/thawed PBSC. Six of the twenty one transplants included so far showed strongly decreased numbers of SSCloALDHbr as compared to CD34+ cells. Notably, in the five affected patients with available data this was associated with delayed establishment or even permanent absence of full hematopoietic donor cell chimerism, whereas all other patients (n=9) showed early complete chimerism.
In conclusion, we suggest (i) to introduce ALDH activity as a convenient surrogate marker for hematopoietic stem cell activity, and (ii) to transport and store PBSC under controlled cooling conditions.

Poster 40:

TCR and Ig gene rearrangements as early markers of relapse in lymphoproliferative diseases.

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) in lymphoproliferative disorders (LPD) is the most radical method of their therapy. Meanwhile, early diagnostics of the developing relapses is of great value during post-transplant period. lonal rearrangements of Ig and T-cell receptors (TCR) genes are the known pathogenetic markers of the LPD. Therefore, the purpose of our study was to assess the significance of clonal rearrangements in the Ig and TCR gamma genes for early diagnostics of molecular relapses in LPD patients following allogeneic HSCT.

Methods: Nine patients participated in this study (five with -ALL, two with T-ALL, one with -NHL, and one with T-NHL), who have undergone allo-HSCT. The period of observation was up to 1.5 years. Genomic DNA was extracted from peripheral blood or bone marrow samples (QIAamp DNA Blood Mini Kit). We analyzed the amplified V-J-gamma junctions (TcR-gamma gene) in the patients with T-cell LPD, and the junction region of Ig heavy chain gene in the patients with B-LPD, by means of heteroduplex analysis with electrophoretic separation in the non-denaturating poly-acrylamide gel. Bone marrow and blood samples were collected at the time prior to conditioning, at the day of hematopoietic reconstitution and at different follow-up time points depends on clinic situation. As a verifying technique, the post-HSCT chimerism was evaluated, using allele-specific PCR for IL-6(C-174G), IL-10(A-552G), DR-1(C3435T) and HFR(C677T) gene polymorphisms.

Results: Clinical features of relapse were observed in six patients (66.7%) during the post-HSCT period. These cases were characterized by detection of monoclonal Ig or, resp., TCR genes prior to conditioning (4 patients, 66.7%). The relapse of disease was diagnosed in all these patients, along with verified mixed chimerism. In 2 patients, full donor cell engraftment was changed to the mixed chimeric state 1 week before the clinical features of relapse did occur. In 2 cases (1 with -ALL and 1 with -NHL) the monoclonal TCR gene was found. Three patients (2 with B-ALL and 1 with B-NHL) exhibited a monoclonal gene of Ig heavy chain, before the clinical signs of relapse appeared. The mean period between detection of clonal genes Ig and TCR and confirmation of relapse was 1 week. A group of relapse-free patients after HSCT (3 patients, 33.3%) was characterized by the presence of TCR gene monoclonality prior to conditioning in one patient with -ALL, full donor chimerism in three patients, and polyclonal Ig and TCR gene sets in 3 patients observed.

Conclusions: Detection of B-, as well as T-monoclonal states before conditioning regime correlated with the high risk of relapse after HSCT. During the post-transplant period, monoclonality was an early marker of developing relapse. Monoclonality was detected a week before the clinical signs of relapse appeared. A reversal from donor chimerism to mixed chimeric state is an indication for studying the lymphocyte clonality.

Poster 41:

Treatment Response in Multiple Sclerosis (Ms) Patients after High Dose Chemotherapy (HDCT) + Autologous Stem Cell Transplantation (ASCT)

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Objectives: The aim of this research was to study clinical and quality of life (QoL) responses in MS patients after HDCT +ASCT.

Background: HDCT+ASCT is a new and promising treatment strategy for MS patients. According to the patients' reported outcome concept, improvement of patients' QoL is an important clinical outcome. We have developed the following grades of QoL response as compared to base-line value of Integral QoL Index: poor (less than 25% improvement compared to the baseline value), average (25–50% improvement), good (50–75% improvement), and excellent (more than 75% improvement).

Methods: Thirteen patients with MS were included in the study. All the patients previously underwent conventional treatment. Clinical and QoL evaluation was provided at baseline, at discharge, at 3, 6, 9, 12 months, and then every 6 months after HDCT+ ASCT. MRI was conducted at baseline, at 6, 12 months, and at the end of follow-up. QoL was assessed by FACT-BMT and FAMS. Integral QoL index was assessed by the method of integral profiles. Median EDSS at base-line was 6.0 (range 1.5–7.5). The median follow-up duration was 36,1 months (range 8–66 months).

Results: Clinical examination and MRI revealed disease stabilization (DS) in eleven patients and disease progression (DP) in two patients. EDSS decreased in eight patients; increased from 6.5 to 7.0 and from 6.0 to 6.5 in patients with DP and remained stable in three patients. Distinct QoL improvement was observed in all patients with DS at the end of follow-up. At one year after HDCT+ASCT all the patients exhibited either average or excellent QoL response.

Conclusion: Clinical response was observed in eleven out of thirteen MS patients after HDCT+ASCT. All patients with clinical response exhibited average or excellent QoL response. These findings confirm that HDCT+ASCT might be considered as an effective treatment for MS patients which results both in clinical and QoL response. Further studies of treatment response in this patient population are worthwhile.

Poster 42:

The detection and quantification of MRD in patients with multiple myeloma after allogeneic HSCT.

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Allogeneic transplantation of hematopoietic stem cells (HSCT) is generally recognized as the most effective therapy for a many malignancies, including multiple myeloma. Allogeneic HSCT induces more molecular remission (MR) of multiple myeloma than autologous SCT. Achieving molecular remission is associated with a long-term freedom of disease. But even low numbers of remaining myeloma cells ("minimal residual disease", MRD) may result in disease relapse. Standard conventional methods will not detect less than 1% myeloma cells. Therefore highly sensitive and specific PCR methods are needed. We have suggested the universal algorithm for allele-specific oligonucleotides (ASO) identification, based on the clonal rearrangement of immunoglobulin heavy chain genes in a given malignant clone of each patient. The algorithm was used for the incidence of MR investigation in the patient group with complete remission (CR). High rates of CR was achieved after an auto-allo tandem approach using fludarabine (150 mg/m^2), melphalan (140 mg/m^2) and anti-thymocyte globulin (ATG: 3 x 10–20mg/kg) three months after a cytoreductive autograft (melphalan 200 mg/m^2). So, specific ASO-primers were generated for twelve patients. As the result, minimal residual disease (MRD) for a median of more than two years after SCT was followed-up. Molecular remission was defined as a negative test of polymerase chain reaction (PCR). For the MRD monitoring in the patients who have not achieved MR we used the simple relative real-time PCR based on the well known consensus probes technique.

Poster 43:

bcl-2 gene expression as a important marker for the early prognosis and monitoring of acute graft-versus-host-disease (aGvHD) in allo-HSCT.

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Our general purpose was to investigate *bcl-2* gene expression in patients treated by unrelated allogeneic haematopoietic stem cells transplantation (allo-HSCT) for the evaluation of aGvHD. Total RNA from the leukocytes of twenty two oncohaematological patients was collected before starting the conditioning regimen (D-T), at the day of hematological recovery (D-R) and at the thirtieth day after allo-HSCT (D+30). An original RT-PCR protocol was developed, by quantifying the signals from the target genes, as compared to beta-actin as a reference gene. We have shown that the levels of *bcl-2* expression in aGVHD patients were higher, as compared to aGVHD-free patients at D-T (p=0,004), like as D-R (p=0,003) time-points. The patients at the D-R and D+30 were divided into two groups, either with or without clinical signs of aGvHD at these observation terms (resp., groups I and II). At D-R point, *bcl-2* expression in the 1st group was two-fold higher (but not significant), than in the patients of second group (p=0,06). By the contrary, *bcl-2* expression by the D+30 in group I was two-fold lower, as compared to the patients of second group (p=0,014). Thus, we suggest the monitoring of *bcl-2* expression to be of certain clinical significance. This criterion could be used as additional approach to prediction of aGVHD development and adjustment of immunosuppressive therapy. We also suppose that the role of the *bcl-2* in the pathogenesis of aGvHD should be analyzed in more details.

Immunology, Immunobiology, Immune Therapy

Poster 44:

Targeting IL-2 to the Endoplasmic Reticulum Confines Autocrine Growth Stimulation to NK-92 Cells

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Objective: Anti-tumor effects mediated by adoptively transferred natural killer (NK) cells are dependent on the presence of interleukin-2 (IL-2). IL-2 is considered to be a survival factor for NK cells and an enhancer of their cytotoxic potential. However, systemic administration of IL-2 is frequently impeded by undesirable side effects, such as high toxicity and non-localized administration. Genetic modification of NK cells expressing IL-2 in a localized and controlled manner could be a powerful tool for overcoming these obstacles.

Methods: Consequently, we have cloned the IL-2 gene using PCR and designed constructs that target IL-2 to specific subcellular compartments. The IL-2-dependent NK-92 cell line was used to verify the functionality of the subcellularly targeted IL-2 constructs.

Results: IL-2 targeted specifically to the ER was sufficient to support growth of NK-92 cells. In such cell lines, IL-2 was verified to be localized to the endoplasmic reticulum. IL-2 was not detected in the supernatant and growth of non-IL-2-modified NK-92 cells was not supported during co-culturing experiments. IL-2-transduced NK-92 cell lines showed comparable functional activity and cytotoxicity to parental NK-92 cells.

Conclusion: We demonstrate the ability of ER-retained IL-2 to provide autocrine growth stimulation to NK-92 cells, without secretion of the cytokine to the extracellular compartment. Therapy with IL-2 gene-modified autoactivating NK cells may avoid side effects imposed by exogenously administered IL-2.

Poster 45:

A transgenic model reveals the mechanisms and magnitude of CD8+ T cell tolerance to the broadly expressed tumour-associated protein MDM2

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Most human tumour antigens are also expressed at low levels in normal tissues. To assess the level and mechanisms of CD8+ T cell tolerance to a 'real' tumour target antigen we have made T cell receptor (TCR) transgenic mice specific for MDM2, a ubiquitously expressed protein that is over-expressed in human and murine tumours.

H2d mice were used to isolate a high avidity cytotoxic T lymphocyte (CTL) clone specific for an MDM2 peptide presented by allogeneic H2b Class I molecules. The TCR genes were cloned and used to produce transgenic H2d mice, unable to present the MDM2-derived peptide (referred to as auto-Ag -ve mice), and H2dxb mice that did present the MDM2 peptide (referred to as auto-Ag +ve mice). This allowed us to study T cell development in the presence and absence of the TCR-recognised auto-antigen.

Phenotypic analysis revealed that there was substantial clonal deletion of MDM2-specific thymocytes during thymic development in the auto-Ag +ve mice. Despite this, the peripheral T cell pool of auto-Ag +ve mice was dominated by MDM2-specific CTL, consistent with the notion of the preferential peripheral expansion of MDM2-specific CTL that escape central deletion. In spite of this, functional analysis revealed that the MDM2-specific CTL from auto-Ag +ve mice had a phenotype of 'split tolerance', affecting expansion and IFN-gamma production but not the development of cytotoxic effector function. Surprisingly, exogenous IL-2 corrected the defects in expansion and IFN-gamma production but the MDM2-specific CTL from the auto-Ag +ve mice were still of lower functional avidity than MDM2-specific CTL from the auto-Ag -ve mice.

These data suggest that whilst it may be possible to generate MDM2-specific CTL from the autologous repertoire, such CTL have functional defects and display low functional avidity and may therefore be limited in their ability to control tumour growth.

Poster 46:

TCR gene transfer to analyse the role of CD8 in tumour-reactive cytotoxic T-cells

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The tumour-associated antigen MDM-2 is an attractive target for immunotherapy as it is expressed at elevated levels in a large number of tumours. In the past, we have generated two murine allo-restricted CTL clones, one recognising MDM-2 with high avidity and one recognising MDM-2 with a lower avidity. Surprisingly, we found that the function of the high avidity clone was readily blocked by anti-CD8 antibodies, whilst the medium avidity clone was largely CD8-independent. To investigate whether the observed CD8-dependence is a feature of the TCR or whether this feature is determined by the CTL clone, the TCR genes were transferred into polyclonally activated murine splenocytes. The results showed that transduced T-cells expressing the high or medium avidity TCRs were able to kill targets presenting the MDM-2-derived peptide in a highly specific manner. Peptide-specific killing by T-cells expressing the medium avidity TCR was similar in the presence or absence of anti-CD8 antibody. In contrast, killing by T-cells expressing the high avidity TCR was completely abolished by anti-CD8 antibody. Upon introduction of the TCRs into T-cells of CD8 beta-deficient mice, the medium avidity TCR was functionally active and able to trigger antigen-specific cytotoxicity, whilst the function of the high avidity TCR was substantially reduced. The transfer of the medium avidity TCR into CD4+ T-cells revealed that this TCR did not require CD8 expression and was able to mediate direct MHC class I-restricted antigen specificity of T-helper cells. Together, these experiments demonstrate that CD8-dependence is a feature dictated by the TCR itself. The data also indicate that CD8 alpha/alpha homodimers cannot rescue the functional activity of a CD8-dependent TCR. This study suggests that transfer of CD8-independent TCRs may be exploited to produce tumour-reactive CD4+ T cells, which are likely to provide ‘help’ for CTL expressing the same TCR, thus enhancing the efficacy of adoptive T-cell immunotherapy of cancer.

Poster 47:

Crosspresentation of minor histocompatibility antigen HA-1 polypeptides by professional antigen presenting cells – implications for HA-1 peptide vaccinationfor the treatment of hematological malignancies

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Minor histocompatibility antigen (mHag) HA-1 specific CTLs emerging after allogeneic stem cell transplantation (SCT) are highly effective to induce complete remissions of relapsed hematological malignancies. Therefore, HA-1 peptide vaccination in order to boost HA-1 CTLs is a promising concept to enhance the graft-versus-leukemia (GvL) effect. A potential danger of vaccination using nonameric peptides reflecting the mHag HA-1 epitope is the induction of tolerance which can lead to failure of the treatment. This might happen because short peptides can bind to MHC class I molecules of every cell type and are thereby also presented by tolerogeneic non-professional antigen presenting cells (APCs). Long peptides (polypeptides) containing the immunogeneic HA-1 epitope might help to circumvent this problem, because they require “cross-presentation” (i.e. uptake, processing and presentation via the MHC class I pathway) to induce a T cell response. We identified two 30 meric polypeptides that are crosspresented by professional APCs. In contrast to HA-1 nonamer peptides, presentation of polypeptides was dependent on cell viability, time, and temperature which underlines that the presentation of HA-1 polypeptides is an active cellular process. Presentation of HA-1 polypeptides was restricted to dendritic cells, T2 cells and EBV LCLs, but absent in non-professional APCs (T cells). This is the first demonstration of cross-presentation of mHag peptides. In combination with upcoming findings of a current vaccination trial using HA-1 nonamer peptides in leukemia patients after allogeneic SCT these data are of high relevance for the optimal design of follow up mHag HA-1 peptide vaccination trials.

Poster 48:

Halofuginone inhibits NFkB, and p38MAPK in activated T cells

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Introduction: Halofuginone, a low molecular weight plant alkaloid, inhibits collagen α1 (I) gene expression in several animal models and in patients with fibrotic disease, including scleroderma, and graft versus host disease. In addition halofuginone has been shown to inhibit angiogenesis and tumor progression. It was recently demonstrated that halofuginone inhibits transforming growth factor β, an important immunomodulator. Our aim at the present study was to investigate the putative effects of halofuginone on activated T cells.

Methods: Peripheral blood T cells were activated by anti-CD3 monoclonal antibodies (McAbs), in the presence and absence of halofuginone and assessed for: NFκB activity, cytokine production including tumor necrosis factor α, and interferon γ, T cell apoptosis, chemotaxis, and phosphorylation of P38 Mitogen Activated Protein Kinase (MAPK).

Results: Pre-incubation of peripheral blood activated T cells with halofuginone (10–40 ng\\dl) resulted in a significantly decrease level of NFκB activity, in a dose dependent manner (NFκB activity index was reduced by 72% after incubation with 40ng/ml; p=0.002). Additionally, 40 ng/ml halofuginone inhibited the secretion of the proinflammatory cytokines tumor necrosis factor α and interferon γ (by 56% for both; p<0.005). Similarly, 20ng/ml halofuginone was able to inhibit apoptosis in the activated T cells (63% reduction, p=0.005). Moreover, halofuginone inhibited significantly the phosphorylation of P38 Mitogen Activated Protein Kinase (MAPK) in activated T cells (phosphorylation was reduced by 63%, p=0.0001). In contrast, T cell chemotaxis was not affected by halofuginone.

Conclusion: Halofuginone inhibits activated peripheral blood T cell functions, and proinflammatory cytokine production through the inhibition of NFκB activation, and P38 MAPK phosphorylation, making it an attractive immunomodulator and anti-inflammatory agent.

Poster 49:

hOuaR – a novel human drug resistance gene for rapid and efficient selection of gene modified cells

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We have developed a new modified human drug resistance gene that allows for rapid and highly efficient in vitro selection of gene modified cells. The selection regimen is based on ouabain, a selective Na+, K+ ATPase (NKA) inhibitor inducing cell death of human cells at concentrations >10^{-7} M. We aimed to modify the human NKA to gain increased ouabain resistance of the protein. In order to locate possible amino acid substitutions generating resistance to ouabain we made use of the rat alpha 1 subunit, four orders of magnitude more resistant to ouabain than the human, yet 97% homologous. Hence, we prepared a set of human-rat chimeric NKA genes to locate rat sequence regions that could confer increased ouabain resistance in the chimeric setting. Ouabain resistance was analysed by transient transfection of constructs to HeLa followed by 24 hours exposure to 10^{-5} M ouabain. The N-terminal region, in which amino acids differed at ten positions, rendered transfected cells resistant to ouabain. Subsequently, we made combinations of amino acid human-to-rat substitutions by insertional mutagenesis in the human gene at four out of the ten identified positions. Substitutions at two positions were sufficient and required for resistance up to 10^{-4} M ouabain. Transfer of this gene construct (hOuaR) by retrovirus transduction to HeLa and Jurkat allowed the selective elimination of unmodified cells by 24–36 hours of incubation in 10^{-5} M ouabain. Today, there is no optimal selectable marker gene for ex vivo cell selection of gene-modified cells for clinical use. The difference in ouabain sensitivity between human wild type NKA and the hOuaR could represent a suitable selection system to rapidly obtain pure populations of gene-modified cells for this purpose.

Poster 50:

Lymphocyte subset reconstitution in patients, receiving unrelated haematopoietic cell transplantation after different conditioning regimens.

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The retrospective analysis of 122 unrelated haematopoietic stem cell transplantations (HSCT) was provided to study lymphocyte subsets reconstitution in 120 adult patients (median age – 43,5 years, range 18–66 years) with different haematological malignancies (33,3% – CML; 20,8 – AML; 16,7% – MM; 12,5% – ALL; 5,8% – NHL; 4,2% – MDS; 4,2% – OMF; 1,7% – HD; 0,8% – CMML), receiving different chemotherapeutic preparative regimens prior to transplantation. In 65 cases (53,3%) patients received myeloablative and in 57 cases (46,7%) reduced-intensity conditioning regimens. Patients in this two groups differs significantly in age (mean 38,5 years for myeloablative vs. 47 years for nonmyeloablative, $P<0,05$) and not significantly in disease duration (mean 606 days vs. 888 days, respectively, $P=0,09$) Quantitative number of main lymphoid subsets (T-, B- and NK-cells) was evaluated approximately 1 month (median – 34 days, range 20-59 days) and 3 months (median – 100 days, range 80–116 days) after HSCT.

The results of the study show that there was no significant difference in T-, B- or NK-cell levels early after HSCT in groups with different conditioning regimen intensity. Higher numbers of total lymphocytes, CD4 and CD8-positive T-lymphocytes after myeloablative conditioning were not statistically significant one month and were completely equalized up to day +100 post transplant. Despite this there was an evidence of superior 4-years survival for patients with myeloablative conditioning, comparing with nonmyeloablative (62% vs. 34% for EFS ($P=0,03$) and 67% vs. 51% for OS ($P=0,4$), respectively).

In multivariate analysis the intensity of conditioning regimen had no significant influence on different lymphoid subsets reconstitution.

This data show that despite of potentially low toxicity, reduced-intensity conditioning provides the same immunosuppression as conventional regimens with high risk of severe infectious complications, relapse and mortality. This facts acquire extreme importance while providing transplantation for elder, heavily pretreated or otherwise immunocompromised patients.

Poster 51:

Promoter switch during embryonic development for the gene encoding the hematopoietic specific minor histocompatibility antigen HA-1.

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Minor histocompatibility antigens (mHag) are polymorphic peptides presented in the context of HLA molecules, and involved in complications associated with HLA matched transplantations. The mHAg HA-1 is enclosed in a protein encoded by a hematopoietic specific gene. Under physiological conditions the HA-1 gene is not expressed in non-hematopoietic cells. However, transformed epithelial cells were shown to express the HA-1 gene. Since onco-genes are often expressed during embryonic development the expression profile of the HA-1 gene was studied in mouse embryos using in situ hybridization. The mouse HA-1 homologue is expressed early during hematopoietic stem cell development. HA-1 mRNA can be detected in HSCs before they display CD45 positivity. The homology between mouse and human HA-1 is high with the exception of the first 2 exons which are absent in all mouse HA-1 mRNA sequences deposited in the database. Interestingly, exon 1 and 2 are present on the genomic level. In silico analysis predicted the presence of two promoters; one upstream of exon 1 and a second alternative promoter upstream of exon 3. Reporter assays showed promoter activity for both putative promoters. Northern blot analysis and Q-PCR indicated that the alternative promoter is active early in embryonic development and that the promoter upstream of exon 1 takes over the HA-1 transcription in later stages of development. We propose a promoter switch for the expression of the HA-1 gene during embryonic development resulting in two alternative transcripts varying in length and perhaps also in function.