HEMATOPOIESIS

Organizers: David Golde and Jerome Groopman

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Hematopoiesis

Hematopoietic Stem Cells

A 001 ISOLATION AND BIOLOGY OF HEMATOPOIETIC STEM CELLS, Gerald J. Spangrude, NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT 59840.

Hematopoiesis depends upon a continual source of progenitors for a variety of cell lineages. These progenitors are derived from a small pool of hematopoietic stem cells, which are defined as cells possessing multilineage differentiation and high proliferation potentials. Although these cells constitute a tiny fraction (less than 0.1%) of normal hematopoietic tissue, several laboratories have been successful at enriching them to very high levels of purity. With these cell populations, it should be possible to answer questions regarding the cell biology of early hematopoiesis. To investigate the repopulating potential of isolated hematopoietic stem cells, small numbers of genetically marked cells (1 to 25) have been transferred into irradiated recipients in the presence of a rescue dose of normal unmarked bone marrow. Progeny of the transferred stem cells are identified in the blood of the recipient animals by expression of an allele of the CD45 molecule. The results indicate that single stem cells can make significant contributions to the peripheral blood population as early as 5 weeks post-transplant, the earliest time evaluated in these studies. Further, long-term studies showed that such early-acting stem cells can continue to function for the lifespan of the animals, suggesting that the original cells have established a colony of self-renewing hematopoiesis. These results demonstrate that long-term repopulating stem cells are not necessarily limited in their ability to rapidly respond to environmental stimuli by proliferating and differentiating, and that such responses do not preclude the ability of the cells to establish long-term hematopoiesis in radiation chimeras. Interestingly, our single cell studies do not provide evidence for a quiescent state of stem cells following transplantation into irradiated animals, since animals that failed to demonstrate detectable progeny of the transferred cells early after transplant continued to be negative for up to 6 months. This may be a result of a high hematopoietic demand upon a relatively small number of cells, such that every cell that localizes in a hematopoietic site after intravenous transfer is induced to proliferate. Single cell studies suggest that the efficiency of seeding to such hematopoietic microenvironments after intravenous transfer is extremely high, since approximately one cell in ten was able to produce sufficient progeny to be detected in our assays.

Myeloid Growth Factors I

A 002 HEMATOPOIETIC GROWTH FACTORS ON HUMAN CHROMOSOME 5: GENES AND THEIR RECEPTORS, K. Arai[1], E. S. Masuda[2], A. Muto[1], H. Tokumitsu[2], H. J. Lee[1], N. Koyano-Nakagawa[1], M. Muramatsu[1], Y. Naito[2], I. Matsuda[1, 2], N. Sato[1, 2], S. Watanabe[1], Y. Nakagawa[1], H. Kosugi[1], S. Watanabe[1], T. Yokota[1], A. Miyajima[2], and N. Arai[2]
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Genes encoding IL-3/GM-CSF/IL-5 are clustered on human chromosome 5 along with IL-4, IL-9 and IL-13. The expression of these genes in T cells requires two signals (PMA/calcium). In the GM-CSF gene, CLE2/GC and CLE0 interact with PMA-inducible GM-κB (or NF-κB p50/p65) and NF-CLE0 (or AP1/NFAT), respectively. HTLV-I encoded Tax promotes translocation of NF-κB from cytoplasm to nucleus and activates the GM-CSF gene, independent of CLE0 and PMA/calcium signals. The presence of CLE0- or NFAT-like elements in IL-2, IL-3, IL-4, IL-5, GM-CSF promoters suggests that AP1/NFAT-like factors contribute to coordinate the activation of these genes during T cell activation. Interaction of purified AP1/NFAT with CLE0-like elements will be discussed. We also found that activation of IL-5 gene requires a cAMP-dependent signal(s) and CLE0, but is independent of NF-κB. GMR, IL-3R, and IL-5R have an a subunit specific to each and α common β subunit, both of which are members of the cytokine receptor superfamily. The genes for IL-3Rα and GMRα are composed of 11 and 13 exons, respectively. Our results indicate that 1)reconstituted GMRα/β transduces signals in hematopoietic cells and fibroblasts, 2) cytoplasmic domains of both α and β are required to transduce signals, and 3) *c-fos/c-jun* activation and *c-myc* activation/cell proliferation are regulated by distinct pathways. By constructing chimeric receptors between extracellular and cytoplasmic domains of α/β subunits, we show that β cytoplasmic domain alone can transduce signals. These results suggest that the cytoplasmic domain of β forms an oligomer in functional GMR and that the cytoplasmic domain of α subunit triggers oligomerization of the cytoplasmic domain of the β subunit.

A 003 GROWTH FACTORS, TYROSINE KINASES AND HOMEOBOX GENES IN MYELOPOIESIS, Judith C. Gasson, Division of Hematology-Oncology, Departments of Medicine and Biological Chemistry, UCLA School of Medicine, Los Angeles, CA.

The processes of hematopoietic cell proliferation, differentiation and maturation are linked in ways that are not clearly understood. To better understand these events, we examined granulocyte-macrophage colony-stimulating factor (GM-CSF) signal transduction as a model for the mechanism by which myeloid growth factors stimulate this process. We generated mutants in conserved regions of the GM-CSF binding subunit (GM-CSFRα) of the heterodimeric receptor complex and identified the role of conserved amino acids in ligand binding, internalization, tyrosine phosphorylation and transduction of a proliferative signal. Members of the hematopoietic growth factor receptor superfamily do not contain intrinsic tyrosine kinase activity, yet one of the earliest measurable responses is increased phosphorylation of proteins on tyrosine residues. c-Fes is a Src-like tyrosine kinase expressed in myeloid cells, which has been proposed, using biochemical analyses, to associate with the GM-CSF, interleukin 3 (IL-3) and erythropoietin receptors. To determine the function of c-Fes in myelopoiesis, we created a dominant negative mutant and examined the effects of this mutation on tyrosine phosphorylation and colony formation in a factor-dependent cell line stimulated with GM-CSF, IL-3, erythropoietin and interferon. To study early events regulating changes in gene expression induced by myeloid growth factors, we characterized the transcriptional induction of the GM-CSF- and IL-3-responsive primary response gene, EGR-1. We identified an apparently novel DNA recognizition element in the EGR-1 promoter that is essential for transcriptional activation by GM-CSF and IL-3. Finally, in an effort to determine molecular mechanisms directing the lineage determination and maturation of myeloid cells, we used degenerate primers to the conserved region of the homeodomain and employed the polymerase chain reaction to identify homeobox-containing proteins that are induced by myeloid growth factors (GM-CSF, granulocyte colony-stimulating factor (G-CSF) and IL-3) in CD34-positive human hematopoietic progenitor cells. Anti-sense oligonucleotides as well as over-expression strategies are being employed to define a role for these specific homeobox genes in the maturation and lineage commitment of myeloid cells.

A 004 STRUCTURE-FUNCTION STUDIES OF HEMOPOIETIN RECEPTORS, Nicos A. Nicola, Meredith J. Layton, Catherine A. Owczarek, Peter Lock and Donald Metcalf, The Cooperative Research Centre for Cellular Growth Factors and the Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Parkville, VIC 3050, Australia.

The hemopoietin family of cytokine receptors is characterized by at least one copy of a 200 amino acid domain in the extracellular region which forms a double β-barrel structure and has conserved cysteine residues and, the Trp-Ser motif WSXWS. The prototypic model of these structures is embodied in the X-ray crystallographic structure of the growth hormone/receptor complex which shows that two identical hemopoietin domains interact with two different sites on growth hormone. For some hemopoietic receptors the two interacting hemopoietin domains are provided by distinct α- and β- receptor subunits. The α-subunit interaction with the growth factor is formally equivalent to site I, and the β-subunit interaction occurring only after the α-subunit interaction is equivalent to site II of the growth hormone/receptor complex. Moreover, the β-subunit can be shared in common with several different types of ligand-specific α-subunits. A determination of growth factor interaction sites with α- and β-subunits may allow the development of selective agonists and antagonists of growth factor action. We have generated a large series of human/mouse chimaeric molecules of leukemia inhibitory factor (LIF) and taken advantage of the one way species cross reactivity to determine the amino acid residues in LIF that confer the ability to bind to the LIF receptor α-chain. Five amino acid residues in the predicted C-helix, B-C loop and C-D loop of human LIF when present on a mouse LIF backbone contributed at least 80% of the binding energy of authentic human LIF to the LIF receptor α-subunit and molecular modelling of the LIF structure suggested that these residues form a continuous patch of interaction surface on the LIF molecules. Similar studies in the GM-CSF/receptor system have identified interaction surfaces on GM-CSF that are important for interaction with the α- or β- receptor subunits. Mutagenesis studies of the human GM-CSF receptor β-subunit have identified a single residue (Histidine 367) in the loop between the second b' and c' β strands of the hemopoietin domain that is critical for interaction with Glutamate 21 of human GM-CSF in forming the high affinity receptor/ligand complex.

Myeloid Growth Factors II

A 005 THE IL-3 AND GM-CSF RECEPTORS AND SIGNAL TRANSDUCTION, Atsushi Miyajima, Alice Mui, Noriko Sato*, Kazuhiro Sakamaki, Taisei Kinoshita, Toshio Kitamura, Ikuko Miyajiima, Takashi Iwamoto, Toshio Ogorochi, Daniel Gorman, Sumiko Watanabe*, Ken-ichi Arai*, Masatoshi Ichihara, Mineo Takagi, and Takahiko Hara. DNAX Research Institute, Palo Alto, CA 94304 and *Institute of Medical Science The University of Tokyo, Minatoku, Tokyo, Japan.

Pleiotropic cytokines, IL-3, GM-CSF, and IL-5 exhibit similar function on their common target cells, such as eosinophils. High affinity receptors for these cytokines are composed of a cytokine specific α subunit and the common β subunit. The α subunits bind their respective cytokine with low affinity by themselves. In contrast, the common β subunit (β_c) does not bind any cytokines by itself but forms high affinity receptors for all the three cytokines with cytokine specific α subunits. The β subunit is not only required for high affinity binding, but is also essential for signal transduction. Thus, the common β subunit with signalling function provides a basis for common biological activities of IL-3, GM-CSF and IL-5 and the expression of the α subunits is responsible for specificity to cytokines. Although all these cytokines induce rapid protein tyrosine phosphorylation, neither the α nor the β subunit has an intrinsic tyrosine kinase, suggesting that the receptors activate a tyrosine kinase(s) directly or indirectly. Deletion analysis identified two distinct cytoplasmic domains of β_c for signaling. The membrane proximal region is responsible for induction of c-myc and is indispensable for proliferation. The distal region is responsible for activation of Ras, Raf, MAP kinase as well as induction of c-fos and c-jun which can be also activated by serum. Thus, the distal region is dispensable for proliferation of BaF3 cells in the presence of serum. Role of these signaling pathways will be discussed. In addition, we will describe a unique murine mast cell line that lacks normal expression of the IL-3 receptor α subunit (IL-3Rα). Although this mast cell line does not proliferate in the presence of either IL-3 or stem cell factor (SCF) alone, the cells proliferate in the presence of both factors, indicating IL-3 dependent signaling. The impaired expression of IL-3Rα, characterization of the IL-3 *receptor*, and synergism between IL-3 and SCF in this unique mast cell line will be presented.

A 006 TRANSCRIPTIONAL REGULATION OF IL-3 AND GM-CSF EXPRESSION, Stephen D. Nimer[1], Jin Zhang[1], Wei Zhang[1], Julia Chen[1], Maurice Wolin[2], Roy Lau[2], Karen Kwan[2], Richard Frank[2], Masayo Kornuc[2]. [1]Memorial Sloan-Kettering Cancer Center, New York, NY 10021, [2] UCLA School of Medicine, Los Angeles, CA 90024.

Human granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) have overlapping but distinct biological activities and the high affinity receptors for these molecules share a common beta subunit. The genes encoding these hematopoietic growth factors are located 9kb from each other on the long arm of chromosome 5 and both contain an AT-rich mRNA destabilizing 3' flanking region. GM-CSF and IL-3 are expressed by activated T-cells, NK cells, mast cells and megakaryocytes yet the 5' flanking regions of these genes share little sequence homology. We have been studying the regulatory elements that control the expression of GM-CSF and IL-3 in activated T-cells, HTLV infected T-cells and megakaryocytic cell lines and have characterized the DNA-protein interactions in these regulatory regions. We have identified the repeated sequence CATT(A/T), that is critical for GM-CSF promoter activity, and characterized interactions between this element and an adjacent ets binding site.

We have also used DNase I footprinting experiments and transfection studies to identify regulatory sequences in the 5' flanking region of the IL-3 gene including two regions which we call footprint region "A" and "B". We have recently cloned a leucine zipper containing transcription factor that can bind to the "A" element and have characterized and partially purified other factors that bind to these regulatory regions.

The coordinate and gene specific regulation of these two lymphokine genes will be discussed.

A 007 PRECLINICAL AND CLINICAL BIOLOGY OF PIXY321, Douglas E. Williams[1], Linda S. Park[1], Ann Farese[2], Thomas J. MacVittie[2], [1]Immunex Research and Development Corporation, Seattle, WA, [2]Armed Forces Radiobiology Research Institute, Bethesda, MD.

PIXY321 is a GM-CSF/IL-3 fusion protein which is currently being tested clinically for the reversal of multilineage myelosuppression. Pre-clinical studies have shown that the biological activities of the fusion protein are unique, and cannot be reproduced with the simple mixing of GM-CSF and IL-3 which approximate the amount of these cytokines present in PIXY321. PIXY321 has a higher specific activity on leukemic cell lines and normal hematopoietic progenitor cells, binds to the IL-3 receptor with enhanced affinity, and interacts with a unique 100 kDa protein on the surface of KG-1 cells. In preclinical models of myelosuppression, PIXY321 accelerates the recovery of both neutrophils and platelets. Recent data has shown that PIXY321, given to rhesus monkeys after lethal whole body irradiation, can promote hematopoietic recovery whereas GM-CSF + IL-3 cannot. Data from Phase I/II clinical studies indicates that PIXY321 promotes recovery of neutrophils, platelets, and red blood cells following chemotherapy or bone marrow transplantation.

Red Cells and Megakaryocytes

A 008 TYROSINE KINASES IN MEGAKARYOCYTOPOIESIS, H. Avraham[1], S. Avraham[1], D.V. Goeddel[2], B.D. Bennett[2], and J.E. Groopman[1], [1]Division of Hematology/Oncology, New England Deaconess Hospital, Harvard Medical School, Boston, and [2]Department of Molecular Biology, Genentech, Inc., California.

Protein-tyrosine kinases play critical roles in cell signal transduction. An understanding of the regulation of megakaryocyte growth and maturation will be assisted by studying gene products relatively unique to cells of this lineage. We have isolated a cDNA clone encoding a novel human intracytoplasmic tyrosine kinase, termed *matk* (megakaryocyte associated tyrosine kinase). Expression of *matk* mRNA was primarily observed in cells of megakaryocytic lineage and in adult brain tissue. The *matk* cDNA clone encodes a polypeptide of 527 amino acids, and has closest homology to the *csk* tyrosine kinase. Sequence analysis also demonstrates that *matk* contains *src*-homology (SH) region 2 and 3 domains but lacks the N-terminal myristylation signal, the negative regulatory tyrosine (Tyr-527), and the autophosphorylation site (Tyr-416) corresponding to those found in *src*. Antibodies directed to the amino terminus of *matk* immunoprecipitated a 60 KDa protein from the CMK human megakaryocyte cell line. Expression of *matk* mRNA was upregulated in megakaryocytic cells induced to differentiate by the phorbol diester PMA. The 5'-flanking region of the *matk* gene has been cloned. To date, cis-acting motifs that have been identified in the region include GATA-1, SP-1, and NF-E2. Given the relatively restricted expression of *matk* and its upregulation during *in vitro* differentiation, it is likely that *matk* transduces growth and maturation signals during megakaryocytopoiesis.

A 009 THE ERYTHROPOIETIN RECEPTOR (EPO-R) DRIVES ERYTHROID SPECIFIC DIFFERENTIATION IN A HEMATOPOIETIC PROGENITOR CELL LINE, Alan D. D'Andrea, Martin Carroll, Yuan Zhu, and Bernard Mathey-Prevot, Division of Pediatric Oncology and Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston.

Ectopic expression of the erythropoietin receptor (EPO-R) in Ba/F3 cells, an interleukin-3 (IL-3) dependent progenitor cell line, confers EPO-dependent cell growth. To examine whether the transfected EPO-R could affect differentiation, we isolated Ba/F3 EPO-R subclones in IL-3, and assayed for the induction of β-globin mRNA synthesis following exposure to EPO. Detection of β-globin mRNA was observed within three days of EPO treatment, with peak levels accumulating after ten days. Surprisingly, growth in EPO plus IL-3 suppressed the EPO-induced accumulation of β-globin mRNA. When EPO was withdrawn, expression of β-globin mRNA persisted in most clones, suggesting that commitment to erythroid differentiation has occurred. Growth in EPO also resulted in the upregulation of the erythroid transcription factors, GATA-1 and EKLF, suggesting that EPO is driving not only β-globin mRNA accumulation but also other events related to erythroid differentiation. Although EPO-R expression will also support EPO-dependent proliferation of CTLL-2, a mature T-cell line, those cells do not produce β-globin transcripts, presumably because they lack requisite cellular factors involved in red cell differentiation. Since expression of the EPO-R in Ba/F3 cells drives erythroid specific differentiation, we next employed this cell system to identify EPO-induced or IL-3 induced patterns of gene expression. Specifically, Ba/F3-EPO-R cells were starved and restimulated with IL-3 or EPO for eight hours. Total RNA was prepared and examined by differential display (Peng and Pardee, Science 257: 967-971, 1992). Using this approach, we have isolated several cDNAs which are specifically induced by either IL-3 treatment or EPO treatment. We are currently investigating the role that these cDNAs play in IL-3 induced proliferation versus EPO-induced erythroid differentiation.

A 010 INDUCTION OF EMBRYONIC HEMATOPOIESIS IN XENOPUS AND ZEBRAFISH, Claire Kelley[1], Peter Hahn[1], Oscar Piedad[1], H. William Detrich III[2], and Leonard I. Zon[1], [1]Division of Hematology, Children's Hospital, Harvard Medical School, and [2]Northeastern University, Boston, MA. 02115

During embryogenesis, ventral mesoderm is induced to form blood by spatially distributed signals. We have isolated and characterized *Xenopus* and zebrafish cDNA clones for several hematopoietic transcription factors, including the zinc finger transcription factors GATA-1 and GATA-2, and the helix-loop-helix transcription factor SCL (tal-1). mRNAs encoding the Xenopus DNA-binding proteins are initially expressed in the presumptive hematopoietic axis of the embryo before blood is histologically evident. GATA-1, SCL, and globin expression are induced and maintained in cultures of ventral marginal zone explants, and not in explants of other regions. Therefore, hematopoietic induction is likely to be regulated by localized mesodermal inducing signals present in the ventral region of the embryo. The zebrafish and most other teleosts form blood in a dorsal-lateral compartment of the embryo known as the intercellular mass (ICM) of Oellacher (described in 1872), rather than in the yolk sac. Zebrafish GATA-1 expression suggests the remarkable origin of this ICM from apparent extra-embryonic mesoderm which lines the posterior border of the embryo bilaterally. During development, this mesoderm appears to fuse medially and to become incorporated into a lateral position in the embryo proper prior to the onset of hematopoietic differentiation. GATA-2 mRNA is detected in the same extra-embryonic mesoderm as GATA-1 mRNA. GATA-2 is also expressed in the early ectoderm and the central nervous system. We have studied the expression of GATA-1 and GATA-2 in a zebrafish mutant of blood formation called *BLOODLESS*. Early in development (15 hrs), GATA-1 is expressed at a very low level in presumptive *BLOODLESS* homozygous mutants compared to wild-type embryos, and is not expressed in later staged embryos. GATA-2 RNA is expressed in the central nervous system in late stage *BLOODLESS* homozygotes at the same level as wild-type embryos, but not in most of the ICM; instead, expression is retricted to the extreme posterior region of the embryo where undifferentiated hematopoietic stem cells have been proposed to exist. Therefore, the *BLOODLESS* mutation may either affect the differentiation or migration of the embryonic hematopoietic stem cell pool.

Cytokines, Microenvironment and Differentiation

A 011 **TNF RECEPTOR SIGNAL TRANSDUCTION**, David V. Goeddel[1], Mike Rothe[2], Louis A. Tartaglia[2] and Merrill Ayres[2], Dept. of Molecular Biology, [1]Tularik, Inc. South San Francisco, CA 94080, [2]Genentech, Inc., South San Francisco, CA 94080

The biological activities of tumor necrosis factor (TNF), a potent cytokine produced primarily by activated macrophages, are mediated by two specific cell surface receptors, TNF-R1 (55 kD) and TNF-R2 (75 kD). We have shown previously that both TNF receptors are active in signal transduction. TNF-R1 mediates signals for cytotoxicity and many other TNF activities in diverse cell types. In these systems TNF-R2 contributes to the cytotoxic signal that is initiated through TNF-R1 by facilitating the binding of TNF to TNF-R1 in a non-signaling mechanism ("ligand-passing model"). Direct signaling by TNF-R2 has so far been restricted to a small subset of TNF activities in some lymphoid cells such as the stimulation of thymocyte proliferation and proliferation of the murine cytotoxic T-cell line CT6.

To investigate the signaling mechanisms of both TNF receptors functional transfection-based assays were developed. The aggregation of TNF-R1 intracellular domains, which are not associated in the absence of ligand, is an important component of the signal for cellular toxicity. Mutational analysis of the human TNF-R1 intracellular domain has defined a region near the C-terminus that mediates the signal for cytotoxicity. This "death domain" encompasses a region of weak homology that has been noted between the intracellular domains of TNF-R1 and the Fas antigen, a cell surface receptor that mediates a programmed cell death very similar to that signaled through TNF-R1. Surprisingly, when both of these receptors are transfected into murine CT6 cells they elicit opposite biological activities: TNF-R1 induces cell proliferation whereas the Fas antigen causes cell death.

In a search for targets of TNF-R2 signaling we have shown that in CT6 cells TNF-R2 activates the transcription factor NFκB and mediates transcriptional activation of the GM-CSF gene. Interestingly, the same gene is activated through TNF-R1 in fibroblasts. A mutational analysis of the intracellular domain of the human TNF-R2 will also be presented.

A 012 IL-4 AS A MEDIATOR OF LOCALIZED INFLAMMATION AND TUMOR CYTOTOXICITY, Robert K. Hurford, Jr., Shazib Pervaiz, Robert Kleinfield and Robert I. Tepper, Laboratory of Tumor Biology, Massachusetts General Hospital Cancer Center, Charlestown, MA 02129

We have demonstrated that the expression of an activated murine IL-4 gene locally at the subcutaneous transplantation site in mice of a wide variety of murine and human tumors results in a potent antitumor effect that is not observed on tumor cells in vitro. The action of IL-4 is dose-dependent, with complete eradication of transplanted tumor cells requiring high concentrations of the cytokine. Associated with tumor cell death is the extensive infiltration of the tumor within 18 hours by eosinophils, and later by macrophages. In a murine plasmacytoma (J558L) or melanoma (B16) model, both eosinophil infiltration and tumor cytotoxicity can be blocked by pretreating mice with neutralizing antibodies to IL-4 or an antibody (RB6-8C5) which specifically depletes host granulocytes, including eosinophils, but not macrophages or lymphoid cells. These results suggest that the local infiltration of tumors by host eosinophils is an important mechanism of IL-4-mediated tumor killing. IL-4 has been shown to stimulate, by itself, or synergistically in conjunction with IL-1 or TNF-α, the upregulation on human endothelial cells of VCAM-1, an adhesion molecule whose ligand is the leukocyte integrin VLA-4. While VLA-4 is expressed on both T and B lymphocytes and monocytes, it is of interest that eosinophils, but not neutrophils, possess this integrin. It has been recently shown that VCAM-1 activation of human endothelium in vitro promotes adhesion of purified eosinophils, but not neutrophils. Taken together, these findings suggest that the characteristic inflammatory infiltrate induced by IL-4 may relate to the specific induction of adhesion molecules, such as VCAM-1, on the endothelial surface. To test this hypothesis, we have administered in vivo monoclonal antibodies which have the capacity to neutralize the activity of VLA-4 and VCAM-1. Eosinophil accumulation at the site of IL-4-producing tumors can be significantly inhibited by treatment with these antibodies, suggesting that the induction of VCAM-1 is an necessary step for the characteristic inflammatory response induced by IL-4.

To exploit the antitumor activity associated with IL-4-induced eosinophilic inflammation, we have utilized a high-efficiency retroviral vector to achieve in vivo targeting of IL-4 to experimentally-induced tumor metastases established in the liver. The relative selectivity of tumor versus normal tissue is based on the requirement for cell division in order to achieve retroviral integration and expression. Hepatic metastases were established in mice by hematogenous delivery through the portal circulation, followed two to four days later by the delivery of IL-4-transfected retroviral producer cells by the same route. Histological analysis of hepatic tissue from IL-4-treated mice revealed a 70-80% reduction in tumor burden compared with control animals, following a single producer cell injection. These experiments support the notion that in vivo retroviral gene targeting of established metastatic lesions can be obtained and the tumoricidal effect of non cell-autonomously acting genes, such as IL-4, can be significant when delivered in this manner.

Signal Transduction of Differentiation

A 013 JAK KINASES AND HEMATOPOIETIC CELL PHOSPHATASE IN SIGNALLING THROUGH THE CYTOKINE RECEPTOR SUPERFAMILY, James N. Ihle[1], Bruce A. Witthuhn[1], Frederick W. Quelle[1], and Taolin Yi[2], [1]St. Jude Children's Research Hospital, Memphis, TN 38105, [2]The Cleveland Clinic, Cleveland, OH.

Hematopoiesis is regulated through the interaction of growth factors with receptors of the cytokine receptor superfamily. Although lacking intrinsic protein tyrosine kinase activity, ligand binding rapidly induces tyrosine phosphorylation. In the response to a number of cytokines, the protein tyrosine kinase JAK2 is phosphorylated and its kinase activity activated. JAK2 physically associates with a membrane proximal region of the cytoplasmic domain of the EPO receptor, a region that is required for mitogenesis. Utilizing a series of mutant Epo receptors, there is a tight correlation between the ability to activate JAK2 kinase and to induce mitogenesis. One of the substrates of tyrosine phosphorylation in the response to IL-3 is the IL-3 receptor β chain while the Epo receptor is phosphorylated in the response to Epo. Phosphorylation of the Epo receptor occurs in the carboxyl region, a region that negatively influences the response to Epo both in tissue culture and *in vivo*. The phosphorylated receptors are binding sites for hematopoietic cell phosphatase (HCP) and binding occurs through the amino terminal SH2 domain of HCP. Thus following activation, HCP is recruited to the receptor complex and is hypothesized to down regulate the response. Consistent with this hypothesis over expression of HCP in IL-3 dependent cell lines suppresses growth in IL-3. Similarly, genetic deletion of HCP, which occurs in mice with the *motheaten* mutation, results in increased responsiveness of a number of hematopoietic cell lineages. Thus growth of hematopoietic cells is hypothesized to be regulated through a balance of induced tyrosine phosphorylation, through the activation JAK2 kinase, and tyrosine dephosphorylation by HCP.

A 014 STUDIES ON THE MECHANISM OF ACTION OF INDUCERS OF DIFFERENTIATION OF TRANSFORMED CELLS: POLAR/APOLAR AGENTS, Paul A. Marks, Victoria M. Richon, Hiroaki Kiyokawa and Richard A. Rifkind, DeWitt Wallace Research Laboratories, Memorial Sloan-Kettering Cancer Center, N.Y.C.

Some years ago, we discovered a series of hybrid polar/apolar compounds, of which hexamethylene bisacetamide (HMBA) is the prototype, that are potent inducers of differentiation of various transformed cells. The mechanism of action of HMBA has been explored using as a model Friend murine erythroleukemia cells (MELC). HMBA induced differentiation of MELC is a multistep process involving an initial latent period (ca. 10-11 hrs) during which various metabolic changes occur including early translocation of protein kinase C activity from the cytosol to the membrane, cessation of transcription of c-myc and c-myb genes and down regulation of p53 protein (WT and mutant p53), and prolongation of the initial G_1 following transit of the cells through the cell cycle in the presence of the inducer. These changes appear to occur in almost all, if not all, cells in culture with HMBA and are required for subsequent commitment to terminal differentiation, but they are not sufficient since removal of inducer during the latent period blocks subsequent commitment. During the HMBA induced prolongation of G_1 there is accumulation of underphosphorylated pRB, down regulation of cyclin A, cdk4 proteins and cdk2 activity. Cells committed to terminal differentiation, including onset of active transcription of globin genes, are first detected during this prolonged G_1. Using inhibitors of cell cycle progression, we obtained evidence that HMBA causes changes by an action early in G_1 presumably prior to the restriction point. During the initial prolonged G_1, only about 15% of cells become committed to terminal differentiation. We hypothesize that a critical level of a factor(s) regulating transit through G_1 is required for commitment, such as the level of pRB, and/or cyclin dependent kinase activity. It requires an additional 2 to 4 cell cycles in the presence of HMBA for recruitment of approximately 100% of the cells to differentiate. The amount of cdk 4 proteins remain suppressed during these subsequent cycles. We suggest that with each subsequent cycle additional cells achieve the critical level of the factor(s) that causes the cells to arrest in G_1 and differentiate.

These in vitro studies have had application to clinical therapy of cancers. In a phase II trial of HMBA in 28 patients with myelodysplastic syndrome and acute myelogenous leukemia, complete remission in 3 and a partial remission in 6 patients (lasting 1 to 16 mos.) was induced by the agent. (Andreeff et al., Blood, 80:2604,1992). There was morphological and cytogenetic evidence that the inducer caused differentiation of transformed precursor cells to mature granulocytes.

A 015 D-TYPE G1 CYCLINS AS INTEGRATORS OF GROWTH FACTOR-INDUCED SIGNALS, Charles J. Sherr,[1,2] Jun-ya Kato,[2] Martine F. Roussel,[2] and Dawn E. Quelle,[1,2] Howard Hughes Medical Institute[1] and Department of Tumor Cell Biology,[2] St. Jude Children's Research Hospital, Memphis TN 38105

Mitogenic growth factors enforce progression through the first gap (G1) phase of the cell cycle by driving cells through a late G1 restriction (R) point, after which cells committed to replicate DNA (S phase) can complete cell division even if growth factors are subsequently withdrawn. During G1, growth factor-induced signals regulate the expression of mammalian D-type cyclins (D1, D2, and D3), which are differentially expressed in various cell lineages. When coexpressed in insect Sf9 cells, *each of the D-type cyclins can act as a regulatory subunit for the cyclin-dependent kinase, cdk4; cyclins D2 and D3 (but not* D1) also functionally interact with cdk2. The formation of cyclin D/cdk complexes in mammalian cells is presumed to lead to the phosphorylation of key substrates, the retinoblastoma protein among them, whose modified activities ensure G1 progression and S phase commitment. In proliferating cells, the formation of cyclin D/cdk4 complexes is nonperiodic, and they persist as long as cells are continuously stimulated with growth factors. However, the bound D-type cyclins turn over rapidly ($t_{1/2}$ < 20 min), whereas cdk4 is much more stable ($t_{1/2}$ = 4 hrs), suggesting that the cyclins continuously move into and out of complexes with their catalytic subunits. Fibroblasts engineered to overexpress D-type cyclins exhibit a shortened G1 interval, smaller size, and a reduced serum dependency for S-phase entry, but they remain contact-inhibited, anchorage-dependent, and undergo growth arrest in serum-free medium. Microinjection of antibodies to cyclin D1 into these cells during G1 prevents their entry into S phase, but injections performed near the G1/S boundary are noninhibitory. Thus, D-type cyclins appear to be both necessary and rate-limiting for G1 progression. In 32D myeloid cells, IL-3 induces the synthesis of cyclins D2 and D3 (but not D1), but when cells are shifted to G-CSF, proliferation stops, and D2, D3, and cdk4 synthesis terminate as cells undergo G1 arrest and differentiate to neutrophils. When either of the three D-type cyclins were ectopically expressed in cells growing in IL-3, G1 was contracted with a compensatory prolongation of S phase. In G-CSF, cells overexpressing cyclins D2 and D3 were unable to differentiate to granulocytes and instead lost viability, whereas D1 was without effect. Therefore, the contraction of G1 (observed with cells overexpressing each D-type cyclin) cannot explain the selective effects of D2 and D3 on differentiation. When cells were shifted to G-CSF, ectopically expressed cyclins moved into complexes with cdk2. The failure of cyclin D1 to functionally interact with cdk2 might explain its inability to prevent differentiation in this system. These data underscore linkage between the actions of *bona fide* cell cycle regulators and the execution of differentiation programs in hematopoietic cells.

Gene Transfer and Expression

A 016 CYTOKINE GENE TRANSFER INTO TUMOR CELLS, MODULATION OF TUMORIGENICITY AND HOST IMMUNITY. B. Gansbacher, L.Minasian, R.Motzer, N.Bander, A.Houghton, D. Golde. Memorial Sloan Kettering Cancer Center, New York/NY 10021.
There are several hypotheses why cytokine gene therapy could show same beneficial effects in cancer patients. First, it has been shown by P. Van der Bruggen et al. (Science, 254, 1643, 1991) that some melanoma cells express tumor associated antigens recognized by CTL. Second, melanoma patients do have circulating CTL precursors in their blood (P.Coulie,et al, Int.J.Cancer, 50, 289, 1992) capable of giving autologous tumor cells. Third, in a mouse tumor model CTL were enriched 2-4 fold by having the tumor cells secrete IL-2 (V.Ley, et al, Eur. J. Immunol., 21, 851 1991). Our working hypothesis was that in some HLA-A2 positive patients tumor cells express CTL defined antigens and CTL precursors circulate. By injecting allogeneic HLA-A2 positive tumor cells that express the same antigen and secrete IL-2, clonal expansion of the tumor specific CTL should be induced. CTL do have access to systemic circulation and should reach residual tumor in this way.
Our initial studies were done in a murine tumor model. We used recombinant retroviral vectors to introduce the IL-2 cDNA into the murine fibrosarcoma CMS-5. The resulting IL-2 secretion by these tumor cells had a potent effect on the host immune system inducing specific CTL's and memory (Gansbacher et al., J.Exp.Med., 172, 1217, 1990). Subsequent studies done in human melanoma and renal carcinoma cell lines showed that it was possible to use retroviral vectors to introduce and stably express cytokine genes in human tumor cells. One advantage of retroviral gene transfer was that because of the high transduction efficiency most the starting tumor cell populations were transduced with the IL-2 gene. In this way one can use a bulk infected population maintaining the tumor cell heterogeneity, a problem if one chooses a clonal population. The amounts of IL-2 secreted by bulk tumor cell population transduced with our retroviral vectors were in the range of 40-100 U/IL2/10^6 cells/24 hrs. In addition it was possible to irradiate the transduced renal carcinoma cells and they continued to secrete cytokine for several weeks (Gastl et al, Cancer Res.,52, 6229-6236, 1992). Irradiated transduced melanoma cells secreted cytokines for up to 35 days and were able to generate specific CTL's in vitro coculture (Gansbacher et al, Blood, 80, 11, 2817-2825, 1992). A phase I study was opened at Memorial Hospital in March 1993. Fourteen patients with melanoma or renal cell carcinoma have been enrolled in the two protocols.

A 017 MOLECULAR GENETIC INTERVENTIONS FOR MALIGNANCY AND AIDS, G.J. Nabel[1], E.G. Nabel[1], G.E. Plautz[1], Z. Yang[1], X. Gao[2], L. Huang[2], D. Gordon[1], B. Fox[1], S. Shu[1], and A. Chang[1], [1]The University of Michigan, Ann Arbor, MI., [2]The University of Pittsburgh, Pittsburgh, PA.

The immune system confers protection against a variety of pathogens and can contribute to the destruction of neoplastic cells. Several cell types participate in the recognition and lysis of tumors, and appropriate immune stimulation provides therapeutic effects in malignancy. Foreign major histocompatibility complex (MHC) proteins also serve as a potent stimulus to the immune system. Foreign MHC genes have been introduced directly into malignant tumors in vivo in an effort to stimulate tumor rejection. In contrast to previous attempts to induce tumor immunity by cell-mediated gene transfer, the recombinant gene was introduced directly into tumors in vivo. Expression induced a cytotoxic T cell response to the foreign MHC protein and, more importantly, to other antigens present on unmodified tumor cells. This immune response attenuated tumor growth and caused complete tumor regression in many cases. Direct gene transfer in vivo can therefore induce cell-mediated immunity against specific gene products which provides effective immunotherapy for malignancy and can be applied to the treatment of cancer and infectious diseases in man. Progress in a phase I human trial using direct gene transfer for patients with melanoma will be summarized. In separate studies, we have recently prepared retroviral vectors containing a Rev gene encoding a dominant negative inhibitor. Expression of this gene product confers significant resistance to HIV infection without alteration of normal T cell function. This gene therapy approach has received approval for a human clinical trial which will assess the potential of this gene product to protect against HIV infection in T cells of AIDS patients.

Clinical

A 018 INTERLEUKIN-3 IN THE CLINIC, Arnold Ganser, Department of Hematology, University of Frankfurt, D-60590 Frankfurt am Main, Germany

Preclinical in vitro and in vivo studies have demonstrated the capacity of IL-3 and sequential therapy of IL-3 with later-acting cytokines to stimulate the proliferation and differentiation of hematopoietic progenitor cells as well as to induce an increase in the number of circulating peripheral blood cells, including neutrophils and platelets. Clinical trials have repeated the preclinical observations with a predominant stimulation of granulocyte and platelet recovery after myelosuppressive therapy, although it is inferior to G-CSF or GM-CSF with regard to acceleration of granulocyte recovery. When given to patients with hematopoietic failure states, i.e. aplastic anemia and myelodysplastic syndromes, and those with prolonged cytopenia due to preceding chemo-/radiotherapy, IL-3 can restore hematopoietic function in patients with secondary hematopoietic failure. In patients with aplastic anemia, treatment with IL-3 is largely ineffective, and only when combined with immunosuppressive therapy it appears to improve bone marrow function. In patients with myelodysplastic syndromes, platelet responses are only seen in a minority of patients, but induction of secondary cytokines, e.g. TNF-α, might be responsible for the treatment failures. Furthermore, stimulation of blast cell proliferation limits the role of IL-3 in these patients, although this effect is being used in acute leukemia treatment to render the blast cells more sensitive to the action of cytostatic drugs. Compared to IL-3 alone, sequential therapy with IL-3 and GM-CSF leads to a rapid rise of circulating progenitor cells and to a pronounced increase in circulating neutrophils in addition to the rise in platelet numbers. The circulating progenitor cells can then be collected for autologous stem cell transfusions, thus allowing intensification of anti-tumor therapy in cancer patients.

A 019 **CLINICAL POTENTIAL OF STEM CELL FACTOR, George Morstyn[1], [1]Amgen Inc., Thousand Oaks, CA and UCLA Medical Center, Los Angeles, CA.**

Stem cell factor (SCF), a ligand for c-kit, has a broad range of activities and can effect cells at or near the level of the multipotential stem cell. It can act on committed cells, as well.[1] SCF was purified in 1989 and the human gene was first cloned at Amgen Inc. Recombinant methionyl human SCF (r-metHuSCF) is produced in *E coli.*

Preclinical studies have been done. In a murine model, recombinant rodent (rr)SCF pretreatment protected against lethal irradiation, [2] while in a canine model, recombinant canine (rc)SCF enhanced survival and accelerated hematologic recovery from total body irradiation.[3] When r-metHuSCF 200 µg/kg/day was given to normal baboons for ≤28 days, it elicited a multilineage response in peripheral counts and increased bone-marrow cellularity.[4,5] Also in the baboon model, 25 to 200 µg/kg/day for ≤28 days increased circulating progenitor cells (PBPCs) up to 300-fold in a dose-dependent manner.

Early clinical results suggest that doses of r-metHuSCF ≤10 µg/kg/day given by the subcutaneous route are well tolerated. At higher doses, adverse events are seen; these events may possibly be related to the effects of r-metHuCSF on mast cells.[6,7]

Recombinant-metHuSCF has major clinical potential because of its synergy with other growth factors, and potentially useful combinations may include r-metHuG-CSF, Epoetin alfa (EPO), Interleukin (IL)-6, and IL-7. Anticipated areas of clinical utility will capitalize on combinations of cytokines with r-metHuSCF to expand bone-marrow cellularity and peripheral blood progenitor cells. These include expansion of progenitor cells before harvesting to accelerate engraftment, mobilization of PBPCs for transplantation after multiple cycles of high-dose chemotherapy, and treatment of bone-marrow failure syndromes such as aplastic anemia.

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7. Demetri GD, Costa J, Hayes D, et al. Proc ASCO. 1993;12:142a

A 020 PARVOVIRUSES IN BONE MARROW FAILURE, Neal S. Young, Kevin Brown, Giorgio Gallinella, Sachiko Kajigaya, Koichi Miyamura, and Mikio Momoeda, Hematology, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892.

Parvoviruses are the smallest DNA viruses to infect human cells. B19 parvovirus is the etiologic agent of fifth disease, a rash illness of childhood and a rheumatoid syndrome in adults. B19 causes transient aplastic crisis, temporary erythropoietic failure in patients with underlying hemolysis; pure red cell aplasia due to persistent infection in the immunosuppressed host; hydrops fetalis after transplacental transmission; and congenital pure red cell aplasia in infants surviving hydrops. In normals, B19's clinical manifestations are due to the immune response and immune complex deposition in skin and joints. Persistent infection results from a defective humoral antibody response. Anemia is secondary to viral destruction of erythroid precursors in the marrow. B19 has extraordinary tropism for human erythroid progenitor cells; the virus infects cells at the CFU-E stage and by expression of its nonstructural protein effects cell lysis. While cytotoxicity is the usual effect of parvovirus infection, we have observed dysplasia in marrow samples from infected humans and from monkeys suffering fatal anemia due to a novel, closely related simian parvovirus. The cellular and molecular biology of the virus has been determined in cultures of human bone marrow. Similar to other parvoviruses, the viral genome of 5 kb of single stranded DNA is replicated through a double stranded intermediate. Transcription differs from the pattern of other *Parvoviridae* in its initiation from a single promoter, complexity, extensive splicing, and failure to coterminate all mRNA at the 5' end. Viral protein products include a single nonstructural protein and two capsid proteins, VP1 (minor) and VP2 (major), which differ by 227 amino acids. The basis of erythroid specificity is now well understood. Only nonstructural protein is expressed early after infection or in abortive infection; in nonpermissive cells, transcription is abortive, resulting in nonstructural protein gene expression (and cell death) without capsid protein synthesis or virus replication. In addition, the cellular receptor for the virus has been identified as erythrocyte P antigen or globoside, a tetrahexose glycolipid also present on erythroid precursors, megakaryocytes, and endothelial cells, as well as on fetal liver and cardiac cells. Virus binds with high affinity to the carbohydrate moiety of globoside. Individuals who genetically lack P antigen (p or Tja- phenotype) uniformly lack serologic evidence of previous B19 infection, and their bone marrow grows normally in vitro in the presence of high concentrations of virus. To circumvent the tissue tropism of the virus, we have produced by genetic engineering in baculovirus recombinant empty capsids that mimic virions antigenically and immunogenically. The antibody response to virus is restricted, and the dominant epitopes cluster within a short region of the minor structural protein, which constitutes less than 5% of the capsid. Capsids enriched for the minor protein elicit strong neutralizing antibody responses in animals and should serve as a suitable vaccine reagent in humans. Because VP1 is not required for capsid formation, the unique region of the protein can by substituted by large amounts of this protein can be substituted by heterologous protein, including the hemagglutinin protein of influenza virus and hen egg white lysozyme.

Hematopoietic Stem Cells; Lymphopoiesis

A 100 INTRATHYMIC LYMPHOID PRECURSOR CELLS DURING FETAL THYMUS DEVELOPMENT,

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Our previous studies have demonstrated the presence, in adult mouse thymus, of a population of early precursor cells able to give rise to T and B lymphocytes but not myeloid or erythroid cells (the "low CD4 precursors"). In order to determine whether a similar low CD4 precursor forms part of the pathway of T cell development during ontogeny, the embryonic and neonatal thymus was examined. A population with the phenotypic characteristics of the adult low CD4 precursor was found from day 15 of embryonic development, although the expression of low levels of CD4 was apparent only from embryonic day 17. Functional tests of these putative precursors showed they had no reconstituting ability for the adult thymus when isolated from thymuses at any time during embryonic life, and very low reconstituting ability even 24 days after birth. These results raise questions about the adult low CD4 precursor as an obligatory stage in the development of T cells in the thymus.

A 101 DEVELOPMENT OF B-LINEAGE COMMITTED PRECURSORS FROM EMBRYONIC STEM CELLS IN VITRO.

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Embryonic stem (ES) cells can efficiently undergo differentiation *in vitro*, generating embryoid bodies (EBs) which consist of cells from many different lineages, including those of the hematopoietic system. Most studies, this far have focussed on the myeloid potential of the cells within the EB. Although several reports suggest that cells expressing surface markers, characteristic of lymphopoietic cells develop within the EBs, conclusive evidence demonstrating their lymphoid nature does not exist.

The aim of this study is to explore the B lymphoid potential of the EBs *in vitro*, using DJ_H or V_HDJ_H rearrangements as an assay for B lineage commitment. EBs differentiated *in vitro* for 28 days were disrupted and cell populations expressing CD44 were isolated by cell sorting. These $CD44^+$ cells were cultured on the stromal cell line S-17, in the presence of interleukin-7 (IL-7), conditions known to support B-lymphopoiesis.

In three independent experiments we have been able to detect DJ_H rearrangement in the $CD44^+$ population following 4-5 weeks of culture on S-17. V_HDJ_H rearrangements were not present. Current experiments are aimed at determining whether or not extended culturing on S-17 will enable the cells to progress to the V_HDJ_H stage of differentiation. To further characterize the B lineage committed precursor, different hemato/lymphopoietic markers have been used to further subdivide the EB-derived $CD44^+$ population. These populations are presently under analysis.

The results presented herein demonstrate that ES cells can in a reproducible fashion generate cells of the B lymphoid lineage in culture.

A 102 A RARE B220$^+$ THYMOCYTE POPULATION EXPRESSES VARIABLE LEVELS OF THY1 AND MAY BE ENRICHED FOR A-MuLV TRANSFORMATION TARGETS,

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Preferential transformation of immature B-lymphocytes by the v-*abl* expressing Abelson Murine Leukemia Virus (A-MuLV) has been instrumental in the study of early B-cell development in bone marrow and fetal liver. In bone marrow, a rare B-progenitor population expressing the B-lineage antigen, B220, and low levels of the T-lineage antigen Thy1 ($Thy1^{lo}$), has been shown to be highly enriched for A-MuLV transformation targets. From the thymus, A-MuLV transformed cell lines have been derived that represent early stages of T-cell development. However, some A-MuLV transformed thymic cell lines resemble $B220^+$, $Thy1^-$ pre-B cells and one A-MuLV transformed $B220^+$, $Thy1^{lo}$ thymic cell line has been reported to be able to rearrange both immunoglobulin (Ig) and T-cell receptor (TCR) genes, which suggests that an A-MuLV transformation target may be a lymphoid progenitor common to the T and B-lineages. Since $B220^+$ cells are exceedingly rare in the normal thymus, the nature and significance of B-cell A-MuLV transformed cell lines from the thymus is not clear. We have found that $B220^+$ cells in primary thymomas induced with v-*abl* or *BCR-ABL* also express low levels of Thy1 and resemble the highly preferred B-progenitor A-MuLV transformation target in bone marrow. To clarify the origin of $B220^+$ A-MuLV transformed cells in the thymus, we examined the lineage and stage of differentiation of $B220^+$, $Thy1^{lo}$ cells from A-MuLV induced thymomas. $B220^+$, $Thy1^{lo}$ thymoma cells were surface IgM^-, $CD43^+$, $CD5^-$, $CD2^{lo/-}$, and had partially rearranged Ig heavy chain genes, but maintained Ig light chain κ, TCR γ and TCR β genes in germline configuration. Thus, these cells express a phenotype similar to intermediate stage B-progenitors found in bone marrow. In some tumors, a minor subpopulation expressed $B220^+$, $Thy1^{hi}$, a phenotype that has not been previously described in normal murine T or B-lineages. From these data, it was predicted that a rare B-progenitor population may exist in normal thymi and provide an enriched source for A-MuLV transformation targets. We found infrequent $B220^+$ thymic lymphocytes that express a continuum of Thy1, from low to high, and variable levels of surface IgM and CD43. These data suggest that several stages of B-cell development may be present in normal thymus. Efforts are underway to learn whether B-cell maturation occurs in the thymus and whether the $B220^+$ thymic population is enriched for A-MuLV transformation targets. The findings show that A-MuLV may be useful for revealing early stages of lymphopoiesis in the thymus.

A 103 IN VITRO GENERATION OF NK CELLS FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS DEPLETED OF LYMPHOCYTES WITH A MATURE NK CELL PHENOTYPE,

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To begin analyzing the pathway of differentiation of Natural Killer (NK) cells, we have investigated the development of these cells in vitro from populations of human peripheral blood mononuclear cells, depleted of the mature $CD3^-/CD16^+/CD56^+$ lymphocytes mediating spontaneous and antibody-dependent (ADCC) cytotoxicity, upon coculture with B lymphoblastoid cell lines previously shown to support preferential proliferation of mature NK cells. Depletion of NK cells from the starting populations was achieved using a combination of cell separation techniques based on expression of specific surface antigens, and was confirmed by flow cytometric analysis using a panel of monoclonal antibodies to lymphoid and myeloid markers, spontaneous cytotoxicity and ADCC assays with IL-2-stimulated effector cells, and reverse transcriptase-polymerase chain reaction (RT-PCR) to detect CD16 mRNA. Mononuclear cell populations depleted of functional and phenotypically mature NK cells on the basis of these criteria were analyzed after 10 to 12-day in vitro coculture with the EBV-transformed RPMI-8866 B cell line with or without added cytokines (IL-2 and/or IL-12). A significant proportion (10-20%) of the cells was detected that expresses CD16 and/or CD56 in the absence of surface CD3 and mediates both spontaneous cytotoxicity and ADCC. These results indicate that immature NK cells are present, in peripheral blood from healthy individuals, the differentiation of which is supported by the in vitro culture conditions used. Similar results were obtained when the mononuclear cell populations used were a) depleted also of CD2-bearing lymphocytes, and/or b) enriched for $CD34^+$ cells and cells lacking other lymphoid or myeloid markers. Although it is not clear which stage of differentiation the repopulating cells may represent, our data suggest that NK cell precursors are contained within either or both of these populations. The nature of these precursors and their growth/differentiation requirements are under investigation.

A 104 α4β1 INTEGRIN EXPRESSION ON SUBSETS OF HAEMATOPOIETIC STEM CELLS,

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Adhesion of cells to the extracellular matrix and to other cells is largely mediated by members of the integrin receptor family. These integrins are heterodimeric transmembrane proteins consisting of an α and β subunit. The integrin α4β1 is involved in adhesion of haematopoietic cells and is likely to play a pivotal role in the regulation of haematopoietic cell proliferation and differentiation,in haematopoietic stem cell homing following transplantation and in maintenance of stem cell quiescence. Interaction of the α4β1 integrin with the CS-1 fragment of fibronectin mediates adhesion to the extracellular matrix while binding of this receptor to vascular cell adhesion molecule-1 mediates adhesion to stromal and endothelial cells. We have shown that murine bone marrow cells express the α4β1 integrin. We have also shown that lineage negative cell populations (that is, cells which do not express lineage-restricted markers such as $B220^-$, Lyt-2^-, L3/$T4^-$ and $7/4^-$ and which are enriched for transplantable primitive haematopoietic stem cells and high proliferative potential-colony forming cells) express this integrin. We have expanded this study to investigate the expression of the α4β1 integrin on various other subsets of haematopoietic cells. These sub-populations include: (1) purified primitive haematopoietic stem cells (lineage-negative, Rhodamine 123-dull, Hoechst 33342-low); (2) committed lineage restricted macrophage progenitors (GR-1^+, $7/4^+$) and (3) peripheral blood primitive haematopoietic stem cells mobilised by cytokines, such as interleukin-1. The results of this investigation and the possible functions of the α4β1 integrin in haematopoiesis will be discussed.

A 105 TNF*α* PROTECTS PURIFIED HUMAN HEMATOPOIETIC PROGENITOR CELLS FROM THE INHIBITORY EFFECTS OF QUINONES,

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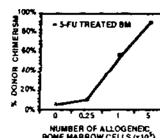
The quinone antitumor agent doxorubicin (DX) and the benzene metabolite hydroquinone (HQ) appear to have common mechanisms of hematotoxicity; most likely through the induction of oxidative stress. In addition, tumor necrosis factor-*α* (TNF*α*) has been shown to protect against agents or conditions which induce oxidative stress. Therefore, we have conducted studies evaluating the inhibitory effects of DX and HQ on human hematopoietic progenitor cells (HPC) and the influences of TNF*α* on DX- or HQ-mediated inhibition. This was done *in vitro* by pretreating human bone marrow mononuclear cells (BMMNC) or immunoaffinity-selected $CD34^+$ cells with either 25-200nM DX, 10-50μM HQ, 25ng/ml TNF*α* or with the combination of TNF*α* and HQ or TNF*α* and DX in liquid cultures for 22hr at 37°C, 5% O_2, 7% CO_2 (low O_2). The pretreated cells were washed, plated in semisolid medium in the presence of GM-CSF, IL-3, and kit ligand, cultured for 14 days at 37°C in low O_2 and colonies of ≥ 50 cells were counted. Following the 22hr pretreatment, viability of either the BMMNC or the $CD34^+$ cells was unaffected. However, DX inhibited colony-formation with an IC_{50} of 50-100nM and the HQ ID_{50} was 10-30μM. HPC in the BMMNC fraction were not protected by TNF*α*, possibly due to the inhibitory effects of TNF*α* alone. In contrast, when purified $CD34^+$ cells were used TNF*α* protected HPC from both DX and HQ inhibition. A TNF*α* dose response analysis showed that the protective effect plateaus at 10ng/ml TNF*α*. Furthermore, colony type was determined by dual staining, *in situ*, with monocyte/macrophage- and granulocyte-specific monoclonal antibodies. Results showed that the relative proportion of CFU-G CFU-M, and CFU-GM was not affected by pretreatment with TNF*α*, HQ or TNF*α* and HQ. These results suggest that TNF*α* protects purified human HPC from the inhibitory effects of oxidative stress-inducing compounds *in vitro*.

A 106 5-FLUOROURACIL TREATMENT ENRICHES FOR A BONE MARROW CELL POPULATION THAT FACILITATES ENGRAFTMENT OF ALLOGENEIC BONE MARROW STEM CELLS

Yolonda L. Colson, Christina L. Kaufman, Suzanne T. Ildstad, Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15261

It has previously been demonstrated that purified bone marrow stem cells rescue lethally irradiated syngeneic, but not allogeneic, recipients from radiation-induced aplasia. We have recently identified and characterized a unique donor bone marrow-derived cell that facilitates the engraftment of purified stem cells in an allogeneic environment. This cell is distinct from the allogeneic stem cell, but must be MHC-matched for facilitation to occur. Invivo treatment of animals with 5-fluorouracil (5-FU) has been shown to deplete the bone marrow compartment of mature, proliferating hematopoietic cells thought to cause GVHD, while sparing the pluripotent stem cell necessary for bone marrow reconstitution. We now present data that 5-FU treatment of allogeneic bone marrow also enriches for the "facilitating cell" population and enhances allogeneic stem cell engraftment. B10 or BALB/c mice were lethally irradiated and reconstituted with 5 x 10^6 syngeneic and 5 x 10^6 allogeneic (B10.BR) bone marrow cells depleted of T cells using rabbit anti-mouse brain (RAMB) sera. This model of reconstitution resulted in a 5.25 ± 10.5% level of allogeneic chimerism, with 75% of recipients exhibiting only syngeneic reconstitution. The addition of 1 - 5 x 10^5 bone marrow cells from 5-FU treated donors results in significant facilitation of allogeneic engraftment to levels of 55.5 ± 38.9% and 89 ± 15.5% donor chimerism respectively (Figure).



Animals reconstituted with this inoculum did not exhibit external features compatible with GVHD. In contrast, the addition of 10 x 10^6 RAMB-treated allogeneic bone marrow cells, which are depleted of the "facilitating" population, results in only 50% donor chimerism despite transplantation of a greater number of total donor bone marrow cells. Moreover, there was a 3-fold increase in the expression of cell surface markers consistent with the facilitating cell phenotype (CD8+ /CD45R+) as detected by flow cytometric analysis (9.79% vs. 3.5% untreated). These results suggest that invivo 5-FU treatment of donor bone marrow can remove mature hematopoietic elements to decrease the risk of GVHD without removing the critical population(s) of "facilitating cells" necessary for engraftment in an allogeneic environment. Studies are in progress to isolate the specific "facilitating cell" population(s) from 5-FU treated bone marrow.

A 107 IDENTIFICATION OF HOMEOBOX GENES EXPRESSED IN HUMAN HAEMOPOIETIC PROGENITOR CELLS,

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Homeobox genes have been shown to regulate commitment and differentiation in fungal, invertebrate and vertebrate systems. CD34 positive cells are a complex mix of early, stem and progenitor cells at various stages of commitment to the many haemopoietic lineages. We have cloned and sequenced 31 homeodomain sequences, identified using using redundant oligonucleotide primers, in a PCR screen of cDNA derived from CD34 positive human haemopoietic cells. Sixteen of these sequences correspond to previously identified sequences, 13 of these corresponding to genes within the HOX A, B and C clusters. 10 of the clones most likely represent homologues of genes identified previously in other species. 5 of the clones represent novel homeobox sequences. Sequence comparisons indicate that 2 of the 5 novel human sequences represent distinct new classes of homeobox genes. We also recovered three pairs of related homeodomain sequences. One pair of sequences correspond to the human genes for *TCL3* and *Murl0F*, while the other two pairs have not been described in haemopoietic cells previously. The identification of 5 new homeobox genes using a novel 5' primer, designed from the *engrailed* and *Xanf1* sequences, suggests that there probably still remain several uncharacterised homeobox genes in the human genome. Haemopoietic cells purified on the basis of CD34 expression are a rich source of regulatory genes consistent with their ability to differentiate into diverse haemopoietic cell types.

A 108 IL-10 SYNERGIZES WITH IL-7 TO ENHANCE MURINE PRE-B CELL PROLIFERATION AND ACCELERATES RECOVERY OF SPECIFIC ASPECTS OF B-LYMPHOPOIESIS IN CYCLOPHOSPHAMIDE-TREATED MICE, Jay S. Fine, Heather D. Macosko, Michael J. Grace and Satwant K. Narula, Department of Cell Biology, Schering-Plough Research Institute, Kenilworth, NJ 07033.

Previous work in our laboratory has found that IL-10 enhances IL-7-mediated bone marrow CFU-pre-B activity at in vitro concentrations of 0.1 to 10 ng IL-10/ml. To better understand the mechanism by which IL-10 stimulates B lymphocyte development, we further investigated the action of this cytokine in vitro and in vivo. The effect of IL-10 in this system was observed with unfractionated as well as with purified B220+ IgM- bone marrow cells. Single cell analysis of CFU-pre-B revealed that a higher percentage of cells from IL-10 + IL-7-stimulated cultures were CD43+ B220+ IgM- blasts. The effect of IL-10 was not blocked by the addition of anti-stem cell factor antibodies.
The influence of IL-10 in vivo was examined in BALB/c mice given a single sublethal dose of cyclophosphamide (CY) followed by daily treatment with recombinant IL-10. Mice administered 0.1 to 10 µg IL-10 per day demonstrated an accelerated recovery of marrow CFU-pre-B compared to vehicle-treated animals which was observed as early as day 8 post-CY and was maximal by day 16. In some experiments, the number of CFU-pre-B in IL-10-treated animals was greater than in normal, age-matched animals. The number of CFU-pre-B remained increased in IL-10 treated animals through day 22 post-CY. These data suggest that IL-10 may influence specific events in B-lymphopoiesis. We are currently investigating the effect of IL-10 treatment on mature B cell populations in the periphery.

A 109 PROTEOGLYCAN SYNTHESIS IN HUMAN AND MURINE MULTIPOTENT HAEMATOPOIETIC CELL LINES - ISOLATION AND CHARACTERIZATION OF HEPARAN SULFATE PROTEOGLYCANS AS THE MAJOR PROTEOGLYCANS FROM THE HUMAN HAEMATOPOIETIC CELL LINE TF-1
Georg Stöcker, Zofia Drzeniek, Ursula Just*, Wolfram Ostertag*, Helmut Greiling and Hans-Dieter Haubeck, Institute for Clinical Chemistry and Pathobiochemistry, University of Technology, D-52057 Aachen and * Heinrich-Pette-Institute for Experimental Virology and Immunology, 20251 Hamburg, Germany

Whereas the importance of growth factors and colony-stimulating factors in the control of growth and development in the haematopoietic system has been shown by numerous studies, only recently the role of the microenvironment of the haematopoietic tissue, e.g. the bone marrow stromal cells and their extracellular matrix was realized. It has become clear that stromal cells play a critical role in haematopoiesis. Furthermore it has been shown recently, that proteoglycans might be involved in the interaction of haematopoietic stem and stromal cells. Here we report on the isolation and characterization of proteoglycans from two multipotent haematopoietic cell lines, the murine FDCP-Mix A4 and the human TF-1 cell line. Proteoglycans were isolated from metabolically labelled cells and purified by several chromatographic steps, including anion exchange chromatography and size exclusion chromatography. Biochemical characterization was performed by electrophoresis prior and after digestion with glycosaminoglycan-specific enzymes and fluorographic detection. Whereas FDCP-Mix A4 cells synthesize a homogeneous chondroitin-4-sulfate-proteoglycan, isolation and characterization of proteoglycans from the human cell line TF-1 revealed, that TF-1 cells synthesize in addition to a chondroitin sulfate proteoglycan at least two heparan sulfate proteoglycans as major proteoglycans.

A 110 **EX-VIVO EXPANSION OF HUMAN MARROW AND CORD BLOOD CD34+ PROGENITOR CELLS.**
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CellPro, Inc., Bothell, WA.

The ability to grow human progenitor cells ex-vivo has broad potential for therapeutic benefit. This study focuses on defining conditions to make ex-vivo expansion practical in a clinical setting. The CEPRATE™ immunoaffinity system has been used to isolate large numbers of CD34+ progenitor cells from human bone marrow or cord blood, and the effect of various changes in culture conditions on the growth of these cells in suspension culture was evaluated. The cultured cells were analyzed for overall expansion (fold-increase over starting value) of total cells, total CD34+ cells, and in-vitro colony-forming activity (CFU-GM, BFU-E, HPP-CFC and LTC-IC). Our results indicate that CD34+ purification enhances expansion >10 fold as compared with unseparated marrow or cord blood, and dramatically reduces the volume of media required for a full-scale clinical procedure. Supplementing serum-free media with 5% human plasma augments growth an additional >5-fold. Optimal expansion requires combinations of at least three cytokines. Using SCF+IL-1+IL-3+IL-6 results in a 30-50 fold increase in CFU-GM and HPP-CFC progenitor cells by 7-14 days of culture. When no media exchange expansion is best at starting cell densities of $\leq 1x10^4$ cells/ml. Frequent refeeding with fresh media and cytokines allows some expansion at plating cell densities of 1-4 x 10^6 cells/ml. On a per CD34+ cell basis cord blood progenitors expand 2-4 fold more than bone marrow. Finally, a total of 10^7 CD34+ cell in a 1 liter bag were expanded 20-fold which demonstrates that clinical-scale expansion ex-vivo for subsequent transplantation is now feasible.

A 111 ISOLATION OF DEVELOPMENTALLY REGULATED GENES FROM A HAEMOPOIETIC PROGENITOR CELL LINE USING A RETROVIRAL GENE-TRAP-VECTOR, Marc Hotfilder and Fred Sablitzky, Max-Delbrück-Laboratorium in der MPG, Carl-von-Linné-Weg 10, 50289 Köln, Germany.

All mature blood cells are derived from a common haemopoietic stem cell located in the bone marrow. The molecular mechanisms which regulate self-renewal, determination and differentiation into myeloid or lymphoid cell lineages are not well understood. It is likely, however, that developmentally regulated genes are involved in these processes.
In order to identify such genes we constructed a self-inactivating retroviral gene-trap-vector (pGT3). This vector contains a splice acceptor site in frame with a lacZ/NEO-fusion gene. The NEO-gene permits selection of integration sites within active genes. The lacZ-gene facilitates the identification of trapped genes, which are developmentally regulated during the *in vitro* differentiation of infected haemopoietic progenitor cells.
A model system for myeloid differentiation is the cell line FDCP-mix. Under defined culture conditions this cell line can differentiate into neutrophiles, monocytes, macrophages and erythroid cells.
We infected FDCP-mix cells and established G418 resistant cell lines. Approximately 60% of the G418 resistant clones express a functional ß-galactosidase. Premliminary results indicate that a high percentage of trapped genes are developmentally regulated.
We are currently cloning the fusion transcripts using the 5'RACE procedure and will present data on the characterization of the trapped endogenous genes.

A 112 RADIATION SENSITIVITY OF A NOVEL CELL POPULATION THAT FACILITATES ENGRAFTMENT OF ALLOGENEIC STEM CELLS Christina L. Kaufman, Yolonda L. Colson, and Suzanne T. Ildstad, Transplantation Institute, Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15621

We previously reported the characterization of a cellular population from donor bone marrow that facilitates engraftment of purified bone marrow stem cells in MHC-disparate allogeneic recipients. This cellular population is CD8+, CD3+, CD45R+, Class II $^{dim/intermediate}$, but $\alpha\beta$TCR-, and $\gamma\delta$TCR-. This cell population can be purified from the lymphoid gate, (low forward and side scatter) using live sterile multiparameter cell sorting. We have now examined the radiation sensitivity of this facilitating cell population. The administration of a mixture of 5 x 10^6 B10 and 5 x 10^6 B10.BR bone marrow cells, depleted of T cells using rabbit anti mouse brain (RAMB) sera plus complement, into lethally irradiated (950 cGy) B10 mice results in mixed allogeneic host/donor chimerism. 57% of recipients repopulate as totally syngeneic (B10) and the mean allogeneic donor chimerism overall is 13 ± 17%. The addition of 1 x 10^6 untreated allogeneic (B10.BR) bone marrow cells to the mixed bone marrow inoculum, as a source of facilitating cells, results in ≥ 97% B10.BR allogeneic chimerism. To examine the radiation sensitivity of facilitating cells, the augmenting allogeneic bone marrow was exposed to varying doses of irradiation (2000 to 125 cGy), in vitro after harvest. The facilitating effect was eliminated if the augmenting allogeneic (B10.BR) inoculum was irradiated with ≥ 250 cGy (Table: Groups A-D), but not 125 cGy (Group E). A similar dose-titration effect was observed when the donor for the B10.BR augmenting inoculum was irradiated in vivo prior to bone marrow harvest. Surprisingly, bone marrow treated with ≤ 700 cGy successfully rescued 100% of syngeneic recipients from radiation-induced aplasia (Group G). These data suggest that the facilitating cell population is more radiation sensitive than are bone marrow stem cells.

RADIATION SENSITIVITY OF THE FACILITATING EFFECT

(5 x 10^6RAMB B10 + 5 x 10^6 RAMB B10.BR + 5 x 10^6 Irradiated B10.BR → B10)

Group	Donor	Irradiation	N	Recipient	Donor Chimerism(Range)
A	B10.BR	2000 cGy	8	B10	9 % (1-26)
B	B10.BR	1000 cGy	8	B10	18% (3-35)
C	B10.BR	500 cGy	8	B10	14% (11-29)
D	B10.BR	250 cGy	8	B10	27 % (21-54)
E	B10.BR	125 cGy	8	B10	91% (21-100)
F	B10.BR	None	8	B10	97% (96-100)
G	B10.BR	700 cGy	4	B10.BR	100% (Syngeneic)

A 113 HEMATOPOIETIC PROGENITOR CELL GROWTH IS DETERMINED BY THE BALANCE BETWEEN THE SYNERGISTIC EFFECTS OF MULTIPLE STIMULATORS AND THE COOPERATION OF MULTIPLE INHIBITORS. J.R. Keller, F.W. Ruscetti, M. Ortiz, J.M. Gooya and S.E.W. Jacobsen, BCDP-PRI/Dyncorp, LLB-BRMP,NCI-FCRDC, Frederick, MD 21702 and Department of Immunology, Norwegian Radium Hospital, Oslo, Norway.

The proliferation of hematopoietic progenitor cells is thought to be regulated, in part, by the interaction between stimulatory and inhibitory growth factors. This study investigated the balance of positive and negative growth signals in directly regulating hematopoiesis in vitro. IL-3 combined with steel factor (SLF) optimally stimulated the proliferation of Lin$^-$Thy-1$^+$ murine bone marrow progenitors in single cell assays and TGF-β inhibited more than 90% of the colony formation. CSF-1, GM-CSF, IL-1 or IL-6 were each incapable of counteracting the TGF-β1-mediated inhibition of IL-3-plus-SLF-induced growth, while G-CSF slightly enhanced the number of TGF-β-resistant clones. As a fourth factor, only IL-1 could partially counteract TGF-β-mediated inhibition. While the addition of a cocktail of five additional stimulatory growth factors did not enhance the frequency of single Lin$^-$Thy-1$^+$ progenitors proliferating in response to IL-3 plus SLF, the number of responding progenitors in the presence of TGF-β was enhanced 9-fold. In addition, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein 1α (MIP-1α) or interferon gamma (IFN-γ) alone could not overcome the action of multiple stimulatory cytokines. However, TNF-α and IFN-γ but not MIP-1α cooperated with TGF-β to reverse the proliferative effects of multiple stimulatory cytokines. Thus, the direct effects of single inhibitory factors on hematopoietic progenitor cell growth can be reversed by multiple stimulatory growth factors, and inhibitory growth factors can directly cooperate to suppress the proliferation induced by multiple positive acting factors. The dominant biological effect is determined by the relative concentrations of growth stimulating and inhibiting cytokines.

A 114 THE ROLE OF HEPATOCYTE GROWTH FACTOR IN MURINE AND HUMAN HEMATOPOIESIS
T. E. Kmiecik[1], F. W. Ruscetti[1], J. M. Gooya[2], and J. R. Keller[2]. [1]LLB, BRMP, DCT, NCI-FCRDC. [2]PRI/DynCorp, Inc., NCI-FCRDC, Frederick, Md.

Hepatocyte Growth Factor (HGF) was identified through its ability to induce hepatocytes to proliferate. It has more recently been shown to be identical to scatter factor, which causes epithelial cells to dissociate and migrate. The met tyrosine kinase functions as a receptor for HGF. We have reported an ability of human HGF to synergize with IL-3 and GM-CSF to increase colony formation of murine Lin$^-$ bone marrow progenitors. More recently, we have found that murine HGF can inhibit colony formation of murine Lin$^-$ progenitors. Human HGF can inhibit the formation of purified human CD34+ progenitors, while no effect is seen with unfractionated human bone marrow cells. Murine Lin$^-$ progenitors sequentially treated with 100 ng/ml murine HGF and then with 30 ng/ml IL-3 show an HGF-induced decrease in colony formation of 49%. However, if 30 ng/ml IL-3 is added to the cells followed by 100 ng/ml HGF one day later, no effect of HGF is seen. To examine potential mechanisms for the inhibitory effect seen with bone marrow progenitors, we have examined the effect of HGF in NFS-60 leukemia cells, using growth arrest to mimic the cell cycle state of the bone marrow progenitors. Interestingly, addition of HGF for 10 minutes to the growth arrested murine progenitor cell line NFS-60 results in a general decrease in cellular phosphotyrosine levels compared to media controls. No decrease in cellular phosphotyrosine is seen if HGF is added to NFS-60 in log phase. These results indicate that the effect of HGF upon bone marrow progenitors varies with the growth or differentiation state of the progenitor.

A 115 AN IN VIVO ASSAY FOR HUMAN AML STEM CELLS USING SCID MICE Tsvee Lapidot, Christian Sirard, Josef Vormoor, Trang Hoang, Mark Minden, Michael Caligiuri, and John E. Dick, Dept. of Genetics Hosp for Sick Children and Dept of Molecular and Medical Genetics, Univ of Toronto; Clinical Research Inst of Montreal; Ontario Cancer Institute; Roswell Park Cancer Instute

Over the last several years we have developed a system for studying human hematopoietic cells by transplantation into immune-deficient mice. Immature human progenitor/stem cells of the phenotype CD34$^+$THY$^+$ can home to and engraft the murine bone marrow indicating that many components of the murine microenvironment are cross reactive to the immature human cells. Stimulation of the engrafted mice with human cytokines permits these immature cells to repopulate the murine bone marrow with high levels of multilineage pre-B, myeloid, and erythroid cells including progenitors for each of these lineages. This system permits the possibility of creating animal models of human diseases by direct transplantation into mice. We have now created animal models for human myeloid leukemia. Mice have been transplanted with cells obtained directly from patients with AML and CML. The AML cells proliferate in the SCID mice producing the same leukemic morpholgy as the human disease and in many cases the dissemination to peripheral organs appears to reflect the normal course of the disease in patients. For example FAB AML-M4 are much more infiltrating that AML-M1, this feature of the disease is also seen in SCID mice. These mice also have similar numbers of AML progenitors (AML-CFU) as the donor. One of the major problems in AML has been to develop an assay for the leukemic stem cell that has extensive self-renewal and proliferative capacity. Significant progress has been made to develop an in vivo assay for the AML leukemic stem cell by transplanting AML cells into SCID mice at limiting dilution. The engraftment was quantitative resulting in a new assay that defines a SCID-LEUKEMIA Initiating Cell, SL-IC, based on its ability to initiate human AML in SCID mice. Cell purification indicates SL-IC express CD34. An animal model for human leukemia provides an important opportunity to purify SL-IC and to identify molecular mechanisms important in transformation and progression and to evaluate new therapeutic treatments in vivo.

A 116 DIFFERENT CELL KINETIC EFFECTS OF TGF-ß ON CD34+ PROGENITOR CELL GROWTH DEPENDING ON THE APPLIED STIMULUS.

Filip Lardon, Hans-W. Snoeck, Griet Nijs, Marc Lenjou and Dirk R. Van Bockstaele, *Laboratory of Experimental Hematology, University of Antwerp (UIA/UZA), Belgium.*

The immediate cell kinetic response of highly purified human bone marrow progenitor cells (CD34+ sorted fraction) to the inhibitory effects of TGF-ß was studied using the BrdU-Hoechst flow cytometric technique. The progenitor cells were stimulated with either IL-3 alone or with IL-3 in combination with IL-1, SCF or IL-6 and the inhibitory action of TGF-ß was evaluated in each phase of the first three consecutive cell cycles. Semisolid methylcellulose cultures were also performed to compare these initial events to the effects observed after 7, 14 and 21 days of incubation.

Within the CD34+ compartment, we can discriminate the progenitor cells on a functional basis, i.e. in terms of TGF-ß sensitivity. Very primitive progenitors, recruited out of the G_0-phase by IL-3 plus an early acting factor (IL-1, SCF) are, upon addition of TGF-ß, arrested specifically in the G_1-phase of the second cell cycle. In the clonogenic assays, the increased colony formation due to IL-1 or SCF was completely abolished by the counteracting effect of TGF-ß that diminished colony output back to the level of TGF-ß + IL-3 supplemented colony growth.

Addition of TGF-ß to CD34+ progenitors responding to IL-3 alone resulted in an overall retardation, but without an apparent specific accumulation of cells in any of the cell cycles.

Finally, within the CD34+ compartment there exists a subset of IL-3 responsive, but TGF-ß insensitive progenitor cells which were, upon addition of TGF-ß, not arrested at all.

A 117 MOLECULAR CLONING OF A LIGAND FOR THE FLT3/FLK-2 TYROSINE KINASE RECEPTOR THAT IS BIOLOGICALLY ACTIVE ON PRIMITIVE HEMATOPOIETIC CELLS. Stewart D. Lyman, Laura James, Tim VandenBos, Ken Brasel, Peter de Vries, Kathleen S. Picha, Terry Farrah, Tamy Hollingsworth, Brian Gliniak, Hilary J. McKenna, Frederick A. Fletcher, Roxanne Splett, Eugene Maraskovsky, Douglas E. Williams, and M. Patricia Beckmann. Immunex Research and Development Corp., Seattle, WA 98101.

Cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor was undertaken using a soluble form of the receptor to identify a source of ligand. A murine T cell line, P7B-0.3A4., was identified that appeared to express a ligand for this receptor on its cell surface. A cDNA expression library was constructed from mRNA isolated from these cells. Screening of this expression library led to the isolation of a single cDNA clone that was capable of conferring binding of soluble flt3 when transfected into cells. The cDNA encodes a transmembrane protein that is similar in both size and overall structure to Steel factor and colony stimulating factor 1. Cross hybridization of the murine ligand to a human T cell cDNA library allowed us to isolate a human flt3 ligand cDNA, which is 72% identical to the murine protein at the amino acid level. Since the flt3/flk-2 receptor has been reported to be expressed on murine stem cells, we tested a soluble form of the murine flt3 ligand for its biological effects on *c-kit* positive murine bone marrow stem cells as well as AA4.1 positive murine fetal liver cells. The flt3 ligand stimulates the proliferation of each of these cell types, and synergizes with some hematopoeitic growth factors. The murine factor also stimulates the proliferation of human CD34 positive bone marrow cells.

A 118 IN VITRO EXPANSION AND MATURATION OF HUMAN CORD BLOOD-DERIVED PRIMITIVE HEMATOPOIETIC PROGENITORS ($CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ CELLS) EXPRESSING INTERMEDIATE vs LOW/UNDETECTABLE LEVELS OF THY-1. **Hector Mayani & Peter Lansdorp. Terry Fox Laboratory. BC Cancer Research Centre. Vancouver, Canada.**

We have previously demonstrated that the most primitive human hematopoietic cells are included within a cell subpopulation expressing high levels of CD34 and low/undetectable levels of CD45RA and CD71. To further characterize the in vitro biological properties of these cells, we have subdivided them based on their expression of the Thy-1 antigen, and assessed their proliferation and differentiation in response to a mixture of hematopoietic cytokines in a serum- and stroma-free environment. $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells expressing intermediate levels of Thy-1 (25% of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells), were particularly enriched (up to 41% of the clonogenic cells) for high proliferative potential colony-forming cells (HPP-CFC), whereas $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ $Thy\text{-}1^{lo}$ cells were enriched (up to 45% of the clonogenic cells) for multipotential colony-forming cells (CFU-MIX). When both subpopulations were cultured, for up to 40 days, in serum-free liquid cultures supplemented with a cytokine mixture that included steel factor, interleukin-6, GM-CSF/IL-3 fusion protein, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and erythropoietin, $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ $Thy\text{-}1^+$ cells showed a much higher expansion in $CD34^+$ (30,000-fold) and colony-forming cell (4,700-fold) numbers, than the one observed in cultures initiated with $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ $Thy\text{-}1^{lo}$ cells (900-fold increase in $CD34^+$ cell numbers and 241-fold increase in colony-forming cell numbers). Cells co-expressing CD34 and Thy-1 were only transiently expanded (up to 29-fold) and were not detected after day 22 of culture. The results of the present study demonstrate that, among primitive human $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells, those expressing intermediate levels of Thy-1 possess a much higher $CD34^+$ and clonogenic cell expansion potential than those expressing low levels of this antigen. Our results further support the notion that HPP-CFC are more immature than CFU-MIX, and suggest that, in our experimental system, long-term hematopoiesis is sustained by a primitive cell population that includes HPP-CFC. The present study also suggests that $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ $Thy\text{-}1^+$ cells may be the most adequate target cells for in vitro hematopoietic cell expansion prior to transplantation.

A 119 HEMATOPOIETIC LINEAGE COMMITMENT GENES IN THE FETAL YOLK SAC, Terrill McClanahan, Margaret Barkett, Anna Voronova and Frank Lee, DNAX Research Institute, Palo Alto, CA 94304

During early post-implantation development in the mouse, hematopoietic cells first arise in the yolk sac of the day 7.5-8 embryo. Our main interest is to define the earliest events of lineage commitment to the hematopoietic lineage. To this end, we have constructed cDNA libraries from day 8.5 yolk sac and day 8.5 embryo, a point in development when hematopoietic stem cells are present in the yolk sac and absent in the embryo, in order to search for candidate lineage commitment genes expressed only in yolk sac. One candidate class of genes are the basic helix-loop-helix (bHLH) family of proteins which are involved in lineage commitment during myogenesis and neurogenesis. Using a degenerate PCR primer strategy, we have identified transcripts of several known bHLH proteins which are present in day 8.5 yolk sac and day 8.5 embryo. Additionally, we found that the hematopoietic-specific bHLH tal-1 gene is highly expressed in yolk sac but is undetectable in day 8.5 embryo. By day 9.5, tal-1 expression can be detected in the embryo; this pattern of expression mirrors the transition from embryonic to fetal hematopoiesis and thus makes tal-1 a candidate as a hematopoietic lineage commitment gene.

A 120 HEMATOPOIETIC STEM CELLS CAN BE DISTINGUISHED FROM TRANSIENT HEMATOPOIETIC PROGENITORS BY MAC-1 EXPRESSION, Sean J. Morrison and Irving L. Weissman, Departments of Pathology and Developmental Biology, Stanford University, Stanford, CA 94305

Thy-1.1^{lo}Lin$^{-/lo}$Sca-1^{hi} cells, representing 0.05% of C57BL/Ka-Thy1.1 bone marrow, are highly enriched for hematopoietic stem cells; however, the functional reconstituting activity of this fraction is heterogeneous, containing both long term and transient multipotent hematopoietic reconstitution. Transient multipotent reconstitution is typically characterized by declining levels of B and T cell reconstitution, and the disappearance of donor derived myeloid cells between 6 and 8 weeks after reconstitution. In an effort to resolve the transient and long term reconstituting fractions within the Thy-1.1^{lo}Lin$^{-/lo}$Sca-1^{hi} population, the progenitor activities associated with each lineage marker within the Thy-1.1^{lo}Sca-1^{hi} fraction were assayed. The only lineage markers associated with progenitor activity were Mac-1 and CD4. The Thy-1.1^{lo}Lin$^{-/lo}$Sca-1^{hi} population was resolved into three subpopulations: Thy-1.1^{lo}Sca-1^{hi}Mac-1^{-}CD4^{-}Lin^{-}; Thy-1.1^{lo}Sca-1^{hi}Mac-1^{lo}CD4^{-}Lin^{-}; and Thy-1.1^{lo}Sca-1^{hi} Mac-1^{lo}CD4^{lo}. The Thy-1.1^{lo}Sca-1^{hi}Mac-1^{-}CD4^{-}Lin^{-} population is highly enriched for long term reconstituting hematopoietic stem cells. 25 Thy-1.1^{lo}Sca-1^{hi}Mac-1^{-}CD4^{-}Lin^{-} cells long term reconstituted 5 of 7 lethally irradiated mice. Both progenitor populations expressing low levels of Mac-1 were highly enriched for transient reconstitution activity. Nine of 10 lethally irradiated mice exhibited transient reconstitution by 30 Thy-1.1^{lo}Sca-1^{hi}Mac-1^{lo}CD4^{-}Lin^{-} cells. No progenitor activity was observable from cells coexpressing Thy-1.1^{lo}Sca-1^{hi} and either Gr-1, CD5, B220, CD8, or high levels of CD4. The Thy-1.1^{lo}Sca-1^{hi} populations which expressed either Ter119, CD3, or high levels of Mac-1 were each less than 0.01% of whole bone marrow, and were not assayed for progenitor activity. Thus lineage marker expression is correlated with functional activity among Thy-1.1^{lo}Sca-1^{hi} hematopoietic progenitors.

A 121 CD4 DULL POSITIVE MURINE HEMATOPOIETIC PROGENITOR CELLS, Mayumi Onishi and Hiromitsu Nakauchi#, The Third Department of Medicine, National Defense Medical College, Saitama, Japan, #Laboratory of Cell Growth and Differentiation, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Tsukuba, Japan.

CD4 positive cells comprise approximately 3 to 6 % of murine bone marrow cells. The majority are CD4^{dull} positive, but there are two distinct sub populations; CD4 brightly positive Gr-1 negative cells (CD4^{hi}Gr-1^{-}) and CD4 positive Gr-1 positive cells (CD4^{lo}Gr-1^{lo}). CD4^{hi}Gr-1^{-} cells are thought to be mature T cells by cell surface antigen expression and morphology. CD4^{lo}Gr-1^{lo} cells, which comprise approximately 0.6 % of the bone marrow cells, express small amount of B220 and Thy 1 antigens. But *c-kit* is undetectable on the cell surface. CFU-S and CFU-C are not enriched in this population. However, when injected into lethally irradiated mice, CD4^{lo}Gr-1^{lo} cells were shown to differentiate into T cell, B cell, and myelo-monocyte lineages when assayed 26 weeks after the transplantation. Furthermore, donor derived CD4^{lo}Gr-1^{lo} cells were present in the recipients bone marrow at least 4 months after transplantation. These observations suggest that murine CD4^{lo}Gr-1^{lo} cells in bone marrow have self-renewal capability and retain the ability to differentiate into at least three lineages in long-term hematopoiesis. It is possible that broad commitment of cells to the immature lineage results in "pre-activation" of many of those chromosomal loci that might potentially be expressed, or that these cell surface antigens serve functions in early cells that differ profoundly from their ascribed functions in more mature cells.

A 122 EXPANDED CULTURES OF UNDIFFERENTIATED MURINE YOLK SAC CELLS EXHIBIT HEMATOPOIETIC POTENTIAL IN VIVO S.R. Opalenik and J.A. Thompson. Department of Surgery, The University of Alabama at Birmingham. Birmingham, AL 35294.

Hematopoiesis is a migratory phenomena of embryonic development initiated in the extraembryonic yolk sac (YS) through blood island formation. A limited number of transplantation experiments with primary YS tissue have advanced the hypothesis that the YS is the precursor of both myeloid and lymphoid stem cell populations. To further evaluate the potential to develop a stable YS-derived hematopoietic system in vivo, YS cells were isolated from C57/B6 (H-2K^{b}) embryos and expanded in culture. YS cells (10^6) were introduced intra-peritoneally into sub-lethally irradiated adult SCID recipients (C.B-17*scid*, H-2K^{d}). Peripheral blood sampling and routine FACS analysis for the donor specific MHC class I molecule (H-2K^{b}) were performed at defined time points. YS cells maintained in vitro fail to express H-2K^{b}. In vivo the percentage of H-2K^{b} positive cells varied with time between experimental animals (0%-10%), however a stable YS-derived population within any one animal was not identified. Long-term (18-23wk) animals developed ascites with extensive tumor formation within the peritoneal cavity frequently associated with hematopoietic organs such as liver and spleen. FACS analyses of tumor and organ tissues identified high percentages (up to 25%) of H-2K^{b} positive cells. Subsequent analysis of H-2K^{b} positive ascites cells determined that 17.8% were also positive for CD3. In addition, double positive populations of H-2K^{b} in combination with either CD4 or CD8 were detected. Histological analyses of solid tumor and organ masses revealed the presence of a primitive gelatinous extracellular matrix containing undifferentiated stem cells, lymphocyte precursors, and monocytes. In addition, histology demonstrated significant angiogenesis characterized by endothelium lined vessels reminiscent of blood island formation. These preliminary results are consistent with the hypothesis that under defined culture conditions, YS cells retain the potential to repopulate the hematopoietic system following in vivo transplantation. Future studies will focus on providing transplanted YS cells a more controlled environment for differentiation.

A 123 ANTISENSE ACETYLCHOLINESTERASE OLIGONUCLEOTIDE COUNTERACTS HEMATOPOIETIC APOPTOSIS, Deborah Patinkin, Hermona Soreq, Efrat Lev-Lehman, Dalia Ginzberg and Fritz Eckstein, Department of Biological Chemistry, Life Sciences, Hebrew University, Jerusalem, Israel 91904 and Max Planck Institute of Experimental Medicine, Gottingen, Germany

Hemopoietic stem cell differentiation is induced by a complex interaction of cytokines, stromal elements and apoptosis and is disturbed in malignant and toxic conditions, such as exposure to certain insecticides, acetylcholinesterase (ACHE) and butyrylcholinesterase (BCHE) inhibitors. To analyze the link between CHE gene expression and hematopoiesis we introduced antisense oligodeoxynucleotides (AS-oligos) targeted to CHEmRNAs into murine bone marrow (BM) serum-free cultures. Addition of AS-ACHE to CFU-GEMM cultures caused a 5-fold increase in colony numbers and a 2-fold increase in cell numbers at a peak of 12 µM, suggesting either increased stimulation of stem cells or their increased survival. AS-ACHE introduced into CFU-MK cultures, reduced colony numbers by 2-fold but increased cell number by 2-fold, implying a higher proliferation potential of fewer stem cells. AS-BCHE had far smaller effects on CFU-GEMM colony and cell counts than AS-ACHE. Differential analysis of AS-ACHE-treated CFU-GEMM revealed a 7-fold increase in macrophages and corresponding decreases in megakaryocvytes and erythroid cells. In CFU-MK, AS-ACHE induced a rise of up to 50% in primitive blast cells. To determine whether AS-ACHE increased cell survival by reducing cell death, DNA was extracted from AS-ACHE-treated CFU-GEMM and CFU-MK cultures and proved to be 5-fold higher in yield than non-treated or sense-oligo DNA. Upon electrophoresis and hybridization of these DNAs with a ^{32}P genomic DNA probe, DNA from AS-ACHE-treated cells exhibited very few DNA fragments while control and sense-oligo-treated cells showed the typical ladders of apoptotic DNA fragments, demonstrating improved cell survival and circumvention of apoptosis with AS-ACHE.

A 124 HEMATOPOIETIC, LYMPHOPOIETIC AND IMMUNOTHERAPEUTIC PROPERTIES OF RECOMBINANT HUMAN INTERLEUKIN-7 POST AGGRESSIVE SPLIT DOSE POLYCHEMOTHERAPY.

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We examined the activity of recombinant human interleukin-7 (r Hu IL-7) on hematopoietic and lymphopoietic reconstitution following the administration of a maximum tolerated split dose polychemotherapy protocol of cyclophos-phamide (60 mg/kg; qdx3), cisplatinum (2.05 mg/kg; qdx3), and BCNU (20 mg/kg; 1x on the third day) by phenotypic and functional analysis on subpopulations in the blood, thymus, bone marrow (BM) and spleen. The chemotherapy protocol was modeled in mice after the Duke protocol for breast cancer. The injection of r Hu IL-7 (1 ug/A/day bid for 20 days) following split dose polychemotherapy introduced a significant margination of stem cells (CFU-GM and HPP) to the spleen and also resulted in a reduction in BM stem cell activity. The administration of r Hu IL-7 also resulted in a significant increase in the T cell mitogenic response (Con-A) of cells in the bone marrow and thymus, but not the spleen. FACS analysis revealed an accelerated reconstitution of CD-4$^+$ and CD-8$^+$ cells in the thymus and a significant increase in B220 cells in the BM and spleen. R Hu IL-7 also had significant therapeutic activity for the treatment of moderate to large metastatic mammary tumor burdens when used in combination with the polychemotherapy protocol. In these studies tumor bearing mice received a bone marrow transplant (1 x 10^6 syngeneic cells) and r Hu IL-7 therapy was initiated 24 hrs following completion of polychemotherapy. The greatest effect was observed in animals with a moderate tumor burden where r Hu IL-7 therapy was initiated on day nine following the iv injection of 100,000 tumor cells from the clone 66 mammary tumor cell line. Significant, but lesser therapeutic activity was observed in animals treated with r Hu IL-7 beginning on day 13 following the iv injection of a similar number of clone 66 tumor cells. Saline treated mice had a median survival time of 26 to 30 days following iv injection of clone 66 tumor cells. Preliminary studies suggest that mice with a heavy tumor burdens needed a higher dose of r Hu IL-7 (optimal dose of 10 ug/A/day compared to 1 ug/A/day). We conclude that r Hu IL-7 has significant hematopoietic mobil-ization properties, immunorestorative and therapeutic activity following aggressive chemotherapy. Research supported in part by the Nebraska Cancer & Smoking Disease Research Program.

A 127 INTERFERON-γ IS A SPECIFIC INHIBITOR OF VERY PRIMITIVE CD34+CD38- HUMAN HEMATOPOIETIC PROGENITOR CELLS AND NOT OF MORE MATURE CD34+CD38+ PROGENITOR CELLS.

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We compared the effects of interferon-γ (IFN-γ) on the proliferation and differentiation of human CD34+CD38- and CD34+CD38+ cells. CD34+CD38- cells did not form colonies in methylcellulose, but proliferated and generated large numbers of secondary colony-forming cells (CFC), including secondary high proliferative potential-CFC, after 14 days liquid culture in the presence of IL-3, SCF and either IL-1, IL-6 or G-CSF or combinations of these cytokines. No proliferation or secondary CFC were observed when the cells were cultured in the presence of single cytokines or of combinations of two cytokines. IFN-γ profoundly inhibited the proliferation as well as the generation of secondary CFC from CD34+CD38- cells (> 90 % at 5.10^3 U/ml) in a dose dependent way, and this was a direct effect since it was also seen when the primary liquid cultures were performed at 1 cell/well using an Automatic Cell Deposition Unit (ACDU). CD34+CD38+ cells on the other hand proliferated vigourously but did not give rise to secondary CFC when cultured for 14 days in liquid cultures supported by IL-3, SCF, and either IL-1, IL-6 or G-CSF. IFN-γ however did not inhibit the number of primary colonies generated from CD34+CD38+ cells in liquid single cell colony assays in the presence of the above mentioned cytokine combinations and stimulated the number of colonies supported by IL-3 alone. IFN-γ furthermore increased the number of macrophage colonies and of erythroid bursts in single cell methylcellulose cultures supported by GM-CSF, IL-3, IL-1, IL-6, SCF and erythropoietin. These results indicate that IFN-γ has no effect or a stimulatory effect on most progenitor cells, depending on which cytokines are present to induce proliferation of these cells, but that it acts as selective and powerful inhibitor only of very primitive CD34+CD38- progenitor cells, whatever cytokine combinations are present in the cultures.

A 125 EFFECT OF ANTI-APO1 (aAPO1) ON SPONTANEOUS APOPTOSIS AND BCL2 EXPRESSION IN B CHRONIC LYMPHOCYTIC LEUKAEMIA (BCLL) CELLS,

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The cell surface protein APO1 is expressed on various cell types including malignant lymphoid cells. Triggering of APO1 protein with antibody (ab; aAPO1) induces apoptosis in APO1-expressing cells. Bcl2 is important in enhancing cell survival by inhibiting apoptosis. Interleukin 4 (IL4) also inhibits apoptosis. In this study we investigated the expression of APO1 and the effect of aAPO1 on spontaneous apoptosis (SA) and bcl2 expression in BCLL. We also examined the anti-apoptotic activity of IL4. APO1 expression was investigated by flow cytometric analysis of cells stained with a polyclonal ab to human APO1 protein, followed by addition of FITC-conjugated ab. Purified BCLL cells were cultured in medium with or without aAPO1 (10 U/ml) and/or IL4 (100 U/ml). Apoptosis was measured by incorporation of propidium iodide into nuclei using flow cytometry. Expression of bcl2 was determined by flow cytometric analysis of fluorescence of cells stained with ab to bcl2 and FITC-conjugate ab. Our results show that APO1 is not expressed on fresh BCLL nor is its expression induced in culture or with steroids. However flow cytometry revealed a significant increase in the percentage (%) of apoptotic nuclei in cultures with aAPO1 when compared with those in the absence of aAPO1 (SA). The mean % (range) increase of SA with aAPO1 in 6/7 BCLL was 94% (17-196) on day2 and 55% (17-96) (5/6) on day3. No increase was seen in 24h cultures. IL4 had a protective effect against aAPO1-induced apoptosis; the % reduction was 41% (23-71) on day2 (5/6) and 32% (18-56) on day3 (4/6). Bcl2 expression in untreated and in aAPO1-treated cells was similar to that of controls (i.e fresh cells); 105% (61-170) and 107% (60-143) respectively.In conclusion, although BCLL cells do not express APO1, they are sensitive to aAPO1-induced apoptosis by a pathway that is independent of bcl2 expession. IL4 partially protects against such effect.

A 126 ACETYL-SER-ASP-LYS-PRO HAS HEMATOPROTECTIVE ACTIVITY FOR SUBLETHAL AND LETHAL IRRADIATION: MECHANISTIC STUDIES.

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We report that the tetrapeptide N-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) has radioprotective (sublethal irradiation) activity for early hematopoietic precursors and can prolong the survival of mice receiving *lethal* irradiation. When AcSDKP is administered by continuous infusion it has radioprotection properties for mice receiving lethal irradiation in a dose, timing, and duration dependent manner. AcSDKP has optimal activity when administered at one ng per day by continuous infusion (Alzet pumps) and significantly prolongs the survival of mice (C57BL/6) following lethal irradiation (950 rads). Higher and lower doses of AcSDKP had significantly less biological activity suggesting a bell shaped dose response curve. In addition prophylactic treatment with AcSDKP provides myeloprotective effects against sublethal irradiation. The optimal dose of AcSDKP (one ng per animal per day) when administered by continuous infusion results in a significant acceleration in leukocytic reconstitution of the peripheral blood. The increase in peripheral blood cellularity is a general phenomenon with an increase in the absolute number of not only neutrophils but also lymphocytes and monocytes. In addition there is an accelerated reconstitution of spleen cellularity but not bone marrow cellularity. As much as a 60% increase in spleen cellularity was seen as compared to the cellularity of irradiated control mice. There is also an increase in the absolute number of hematopoietic precursors with an significant increase in the total number of high proliferation potential precursors (HPP) in the femur and spleen (>2 fold increase) but only a slight (and not significant) increase in colony forming unit-granulocyte-monocyte (CFU-GMs). These results confirm the in vitro observation that AcSDKP has hematoregulatory properties with an apparent specificity for early progenitors i.e., colony forming unit-spleen (CFU-S). This observation has been extended to the demonstration of biological activity in vivo and suggests a clinical potential as a myeloid protective agent for AcSDKP. This research was supported by a contract with Henri Beaufour, Inc.

A 128 Rapid and sustained hematopoietic recovery in lethally irradiated mice transplanted with purified Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} hematopoietic stem cells

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Hematopoietic stem cells (HSCs) are believed to play a critical role in sustained repopulation of all blood cells following bone marrow transplantation (BMT). However, understanding the role of HSCs versus other hematopoietic cells in the quantitative reconstitution of various blood cell types has awaited methods to isolate HSCs. A candidate population of mouse HSCs, Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} cells, was isolated several years ago; recently, these Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+}have been shown to be the only HSCs in C57BL/Ka-Thy-1.1 BM. As few as 100 of these cells can radioprotect 95-100 % of irradiated mice with long-term multi-lineage reconstitution. We examined the kinetics of reconstitution of irradiated mice transplanted with purified Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} bone marrow cells. Donor derived peripheral blood (PB) WBC were detected as early as day 14 when 100, 500 or 1000 Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} cells were used, with minor dose dependent differences. The reappearance of PB platelets by day 14 and thereafter was also seen at all 3 HSC doses, with a slight dose dependence. All 3 HSC doses also allowed PB RBC levels to recover, although at the 100 cell dose a delay in hematocrit recovery was observed at day 14. When irradiated mice were transplanted with 500 Thy-1.1^{lo} Lin$^{-/lo}$Sca-1^{+} cells compared to 1 x 10^{6} bone marrow cells (the equivalent amount of cells that contain 500 Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} cells as well as progenitor and mature cells), very little difference in the kinetics of recovery of PB WBC, platelets and hematocrit was observed. Surprisingly even when 200 Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} cells were mixed with 4 x 10^{5} Sca-1^{-} BM cells in a competitive repopulation, most of the early (14d) PB myeloid cells were derived from the HSC genotype, indicating the superiority of the Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} cells over Sca-1^{-} cells even in the early phases of reconstitution. These findings indicate that HSCs can account for the early phase of hematopoietic recovery, as well as sustained hematopoiesis, and raise questions about the role of non-HSC bone marrow populations in the setting of bone marrow transplantation.

A 129 ER-MP12, A NEW MONOCLONAL ANTIBODY REVEALING HETEROGENEITY IN THE MURINE HEMATOPOIETIC STEM CELL COMPARTMENT.

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Two-color immunofluorescence analysis of murine bone marrow cells (BMC) with rat monoclonal antibodies ER-MP12 and ER-MP20 revealed 6 distinct subpopulations which were isolated using magnetic and fluorescence activated cell sorting. The populations were tested *in vitro* and *in vivo* for cobblestone area forming cells (CAFC), CFU-C, day-12 CFU-S, prothymocytes and for cells with long-term repopulating ability (LTRA) using an α-thalassemic chimeric mouse model.

Hemopoietic stem cells and uncommitted progenitor cells appeared to be exclusively present in the 2 subpopulations which expressed the ER-MP12 surface marker at intermediate and high levels but lacked the expression of the ER-MP20 antigen. The ER-MP12^{+}20^{-} population (~30% of BMC) contained approximately 90% of the LTRA-cells and primitive day-28 CAFC, about half of the CFU-S-12 and low numbers of day-7 and day-14 CFU-C. From the ER-MP12^{++}20^{-} cells (1-2% of BMC), we recovered 80-90% of the less primitive CAFC, 80% of the CFU-C (SCF, IL-3, IL-11, IL-12 and EpO stimulated), the majority of prothymocytes (21 days after i.t. or i.v. injection), half of the CFU-S-12 and only 10% of the LTRA cells. Phenotypic analysis of this population showed it was enriched for cells expressing high levels of c-kit, Sca-1 and Thy-1, as well as for cells with a high retention of rhodamine-123 and high affinity for wheat germ agglutinin. Our data indicate that the expression of ER-MP12 on stem cells increases upon maturation and is lost on most mature blood cells except for mature B and T lymphocytes.

Although the antigen is still unknown, ER-MP12 may prove useful for further subfractionation of the different hematopoietic activities in murine bone marrow.

A 130 INHIBITION OF PURIFIED STEM CELLS *IN VIVO* AND *IN VITRO* BY THE HEMOREGULATORY PEPTIDE SB108636,

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The hemoregulatory pentapeptide pEEDCK (HP5) was purified from mature granulocytes by Paukovits and Lærum and was shown to inhibit CFU-GM colony formation. By replacing the -SH group of cystein with an isosteric methylene group a stabilised form of HP5 has been obtained named here as SB108636.

We have evaluated the effects of SB108636 on sorted Lin^{-}Sca1^{+} cells. When seeding 400 Lin^{-}Sca1^{+} cells in HPP-CFC assays in the prescence of IL-1, IL-3 and SCF a 50% reduction in the colony numbers is seen in cultures given SB108636. However, when stimulating these cells with IL-1, IL-3 and mCSF no inhibition HPP-CFC colony formation by SB108636 can be observed in the dose range tested. Thus, it seems that the SB108636 responding cell is dependent upon SCF. When these cells were injected into lethally irradiated mice, 50% of the animals were rescued with 100 Lin^{-}Sca1^{+} cells. Lethally irradiated mice were given 2000 Lin^{-}Sca1^{+} cells on day 0 and treated with variuos doses of SB108636 for 11 days. The development of CFU-GM, CFU-S, HPP-CFC and preCFU-S was measured on day 12. We found that SB108636 treated animals had fewer CFU-GM, HPP-CFC and preCFU-S in their femurs than control animals. These results show that the hemoregulatory peptide SB108636 is able to inhibit proliferation of primitive hematopoieitc stem cells in vivo as well as in vitro, and that the target cell seems to be SCF responsive.

Myeloid Growth Factors I & II

A 200 MEGAKARYOBLAST/ENDOTHELIAL RECEPTOR TYROSINE KINASES IN HEMATOPOIESIS AND ANGIOGENESIS

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In order to get insight into the growth regulation of hematopoietic and leukemia cells and to characterize growth factor receptors of megakaryoblasts, we have cloned novel tyrosine kinase cDNAs from human leukemia cells with a bipotential erythroid/megakaryoblastoid differentiation potential. This resulted in the identification of several novel receptor tyrosine kinases including a potential anti-oncogene and members of the fibroblast growth factor (FGF) and FLT1/KDR/FLK-1 receptor families. One of the novel receptor tyrosine kinases (Tie) is abundantly expressed in all fetal endothelial cells. After the fetal period its expression is downregulated in several organs, but again increased during neovascularization associated with e.g. the ovulatory cycle and wound healing. Several of the cloned genes are expressed in leukemia cells and at least some normal hematopoietic cells. - Ligands binding to these receptors are being characterized. These receptors may be involved in hematopoiesis and in angiogenesis. They may provide a potential to regulate the growth of hematopoietic and endothelial cells by strategies that will be discussed.

Proc. Natl. Acad. Sci. 87: 8913, 1990; ***EMBO J.*** 10: 1347, 1991; 11: 2919, 1992; in press; ***Oncogene*** 6: 2013, 1991; 8: 2009, 1993; in press; ***Blood*** 80: 2548, 1992; ***Nucl. Acids Res.*** 19: 5096,1991; ***Mol. Cell Biol.*** 12: 1698, 1992; ***Cancer Res.*** 52: 746, 5738; 1992; ***Progr. Growth Factor Res.*** 4: 69, 1992.

A 201 INDUCTION OF GM-CSF EXPRESSION BY THE *V-SRC* ONCOGENE IN FACTOR DEPENDENT MYELOID CELLS

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Introduction of *v-src* or *c-src*^{527F}, a transforming mutant of the *c-src* proto-oncogene, into the growth factor-dependent cell line FDCP-1 resulted in growth factor-independence. Studies with FDCP-1 cells carrying the *ts*LA29 mutant of *v-src* demonstrated that growth factor independence was oncogene dependent; that is, the cells were growth factor independent only at the permissive temperature. Introduction of the *c-src* proto-oncogene did not result in growth factor independence. The *c-src*^{2A,527F} mutant, which encodes an activated tyrosine kinase but does not transform fibroblasts due to a mutation in the membrane localization sequence, also induced growth factor independence suggesting that the presence of an activated tyrosine kinase was necessary, however membrane localization was not. The following data suggests that *v-src* and activated mutants of *c-src* induce an autocrine growth cycle involving GM-CSF: 1) conditioned media from *v-src*-infected FDCP-1 cells stimulated proliferation of parental FDCP-1 cells; 2) anti-GM-CSF antibody inhibited this growth stimulatory activity; 3) quantitative ELISA detected GM-CSF in conditioned media; 4) RT-PCR detected the presence of GM-CSF mRNA in FDCP-1 cells infected with active *src* mutants; 6) a reporter plasmid containing sequences upstream of the GM-CSF gene showed elevated expression in *v-src*-infected FDCP-1 cells relative to controls; and 7) a novel protein complex that bound to the region of DNA that regulates the transcription of GM-CSF in T lymphocytes was detected by a gel shift assay in *v-src*-infected FDCP-1 cells. These results suggests that activated tyrosine kinases may contribute to leukemogenesis by inducing autocrine expression of cytokines.

A 202 CLEO, the GM-CSF Promoter Cis-acting Element, Mediates Induction Signals in T Cells and is recognized by Factors Related to AP1 and NF-AT

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Expression of the GM-CSF gene in T cells is activated by the combination of phorbol ester (PMA) and calcium ionophore (A23187) which mimic antigen stimulation through the T cell receptor. We have previously shown that a fragment containing -95 to +27 of the mouse GM-CSF promoter can confer inducibility to reported genes in human Jurkat T cell line[1]. We concluded that a cis-acting element, CLEO (positions between -54 and -40) is a target for two distinct signals[2] because of following observations. i). Induction was observed with templates containing intact CLEO but not with templates with mutated CLEO. ii). Two distinct signals were required for the stimulation through CLEO since only extracts from cells treated with both PMA and A23187 supported optimal stimulation in *in vitro* transcription assay. iii). Stimulation was mediated by CLEO-binding proteins because depletion of these proteins specifically reduced GM-CSF transcription. We recently obtained evidence that NF-CLEOγ, one of the CLEO-binding factors, is similar to the nuclear factor of activated T cells, NF-AT[3]. Affinity-purified NF-AT from nuclear extracts of human Jurkat T cells stimulated with both PMA and A23187 bound strongly to the CLE 0 element to form a NF-CLEOγ complex. This DNA-protein complex was competitively inhibited by oligonucleotides containing NF-AT and AP-1 binding sites, suggesting that CLEOγ complex identical to NF-AT and contains AP-1 proteins. The purified 120 kDa component of NF-AT through Mono Q column reconstitutes NF-AT binding with recombinant cFos/cJun heterodimer. Furthermore, binding of this 120 kDa protein to both the NF-AT and CLEO sequences can be reconstituted with the addition of affinity-purified Jurkat AP-1 proteins. The presence of CLEO-like elements in the promoters of IL-3, IL-4, IL-5, GM-CSF, and NF-AT sites in the IL-2 promoter, suggests that the coordinate induction of these genes during T cell activation[4].

1). Miyatake et al. MCB 11:5894,1991 2). Masuda et al. submitted 3). Tokumitsu et al. submitted 4). Arai et al. Pharmac. Ther. 55:303, 1992

A 203 INTERACTION OF THE FIRST AND FOURTH HELICES OF INTERLEUKIN-3 WITH THE α- AND β- CHAINS OF ITS RECEPTOR

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Interleukin-3 (IL-3) is a member of the family of haemopoietic growth factors which stimulates the proliferation and differentiation of haemopoietic progenitor cells and the function of mature cells such as basophils, eosinophils and monocytes. Our recent findings of endothelial cell activation and leukocyte transmigration in response to IL-3 indicate that this factor is likely to play an important role in chronic inflammation and allergic disease.

The structure of IL-3 has not yet been reported but it is thought to exist as a four helix bundle of topology similar to that of GM-CSF and growth hormone and to function via a cell-surface heterodimeric receptor consisting of an α- and a β-chain.

We have modelled IL-3 and performed site-directed mutagenesis of IL-3 and its receptor to determine the residues involved in binding and function. Within the first presumptive helix of IL-3, mutations of $Asp^{21}{\rightarrow}Arg$ and $Glu^{22}{\rightarrow}Arg$ led to molecules with severely decreased biological activity. In the case of the $Asp^{21}{\rightarrow}Arg$ mutation, this was associated with a loss of binding to the α component of the receptor whereas the $Glu^{22}{\rightarrow}Arg$ mutation was defective in binding to the β component of the receptor. Partial restoration of binding of the $Asp^{21}{\rightarrow}Arg$ mutant was observed when a mutation ($Arg^{234}{\rightarrow}Glu$) was introduced into the α-chain of the receptor. A mutant in the receptor β-chain ($Glu^{366}{\rightarrow}Ala$, $His^{367}{\rightarrow}Ala$) is unable to bind IL-3 with high affinity and molecular modelling suggests that residue His^{367} may interact with residue Glu^{22} in IL-3.

Mutations in the region of the fourth presumptive helix of IL-3 have identified residues important for function. Mutants $Arg^{109}{\rightarrow}Glu$, $Lys^{110}{\rightarrow}Glu$, $Lys^{116}{\rightarrow}Glu$ and $Glu^{119}{\rightarrow}Arg$ showed significant decreases in biological activity. The $Glu^{119}{\rightarrow}Arg$ mutant was found to exhibit reduced binding to receptor α-chains and to the α/β receptor complex. A model of IL-3 interacting with the α- and β-chains of its receptor will be presented.

A 204 EXPRESSIUN AND FUNCTIONALITY OF THE trkA PROTOONCOGENE PRODUCT IN UNDIFFERENCIATED HEMOPOIETIC CELLS.

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Expression of the low affinity NGF receptor (P75) and trkA protooncogene product was analyzed in human hematopoietic cell lines at protein and RNA levels. We could not detect any form of NGF receptor in cell lines displaying a myelomonocytic phenotype, in contrast cells displaying a more immature erytroleukemic phenotype (TF1, K562, KG1) expressed TRKA in the absence of detectable p75. Scatchard analysis revealed a single high affinity site for NGF (10^{-10}M),with a copy number ranging from 300 to 3,000 depending on the studied cell line. In addition, NGF could induce autophosphorylation of TRKA and substitute to GM-CSF to trigger the proliferation of the TF1 cell line with a half-maximal effect observed at 50pM, indicating that that the second subunit of NGF receptor, p75 was not required for DNA synthesis. The physiological relevance of NGF in early hematopoiesis was ascertained by showing that 12-15% of the progenitor cells from mice treated with 5-fluorouracil expressed TRKA and that these cells could be induced to proliferate and differenciate in response to NGF in association with CSFs. Similar observations were achieved in human after the characterization a 5-10% CD34+ subset expressing TRKA. Interestingly we recenly found that long term supporting hematopoiesis stromal cell lines were able to produce NGF.

A 205 SIGNALLING THROUGH THE ALPHA SUBUNIT OF THE HUMAN GM-CSF RECEPTOR, Dawn X Ding, Coralia I. Rivas, Mark L. Heany, Maribeth A. Raines, Juan Carlos Vera, and David W. Golde, Program of Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor that stimulates myelopoiesis and enhances the function of mature granulocytes and mononuclear phagocytes. The receptor for GM-CSF is composed of an alpha and beta subunit, which together form the high-affinity receptor. The alpha subunit by itself binds ligand at low affinity whereas the isolated beta subunit does not bind GM-CSF. It is generally believed that the high-affinity receptor is responsible for the multiple functions of GM-CSF and that the isolated alpha subunit does not transduce a signal. In order to address the question of alpha subunit function, we expressed the alpha subunit of the human GM-CSF receptor in Xenopus oocytes and assessed the effects of ligand. Oocytes injected with full-length RNA encoding human alpha subunit expressed up to 10^{10} low-affinity sites for GM-CSF (Kd 6nM). GM-CSF binding to the alpha subunit expressed in Xenopus oocytes caused activation of 2-deoxyglucose transport through endogenous glucose transporters. 2-Deoxyglucose transport was stimulated by similar low concentrations of GM-CSF in HL-60 cells as well as normal human neutrophils and Xenopus oocytes expressing the alpha subunit. Engagement of the isolated alpha subunit in oocytes did not lead to tyrosine phosphorylation of MAP kinase although MAP kinase was activated by progesterone treatment. Genistein, a specific inhibitor of protein tyrosine phosphorylation, inhibited GM-CSF induced phosphorylation of proteins in human neutrophils and HL-60 cells without affecting the capability of GM-CSF to stimulate increased uptake of 2-deoxyglucose. These results indicate that the isolated alpha subunit signals for hexose transport and can do so without engagement of the MAP kinase cascade. Our data also suggest that signalling for hexose uptake may occur in a tyrosine-phosphorylation independent manner in cells expressing the high affinity GM-CSF receptor.

A 206 ROLE OF THE BETA SUBUNIT OF THE HUMAN GM-CSF RECEPTOR IN RECEPTOR INTERNALIZATION AND SUBUNIT ASSOCIATION. Parul Doshi, Jyothi Venepalli, Aaron Rapoport and John DiPersio, Hematology Unit Univ. of Rochester, Rochester, NY 14642.

The β subunit (β_c) of the human GM-CSF receptor (GM-R) does not bind GM-CSF but reconstitutes high affinity receptors when coexpressed with the ligand binding α subunit of hGM-R.We have examined the regions of β_c involved in the generation of high affinity receptors, receptor internalization, and ligand-induced signaling using a number of cytoplasmic deletions in β_c. Deletion of the cytoplasmic domains of both GM-Rα and β_c does not affect high affinity GM-CSF binding. COS cells transiently expressing GM-Rα alone do not internalize ^{125}I-GM-CSF efficiently. Deletion of the entire cytoplasmic region of GM-Rα has no effect on internalization, however the cytoplasmic region of β_c, appears to be important in receptor internalization and signaling. Deletion of the entire cytoplasmic domain or only the C-terminal 55 amino acids (AA) of β_c reduce but do not completely inhibit receptor internalization. Elimination of 366 AA proximal to these C-terminal 55 AA of β_c consistently increases internalization suggesting that Y^{750}, the assumed ligand-induced tyrosine phosphorylation site of β_c, is not essential for internalization and that the region between AA# 518-842 may contain sequences which negatively regulate internalization. The same effects on internalization of ^{125}I-IL-3 were seen when β_c mutants were coexpressed with wild-type and cytoplasmic deletion mutants of IL-3Rα. Sodium azide inhibits only 30-40% of receptor internalization in both COS transfectants and in TF-1 cells suggesting that a significant portion of ligand-induced internalization is energy-independent. We have also examined the role of kinase inhibitors on internalization in TF-1cells. Ligand-induced internalization is dramatically inhibited by staurosporine suggesting that GM-CSF internalization in TF-1 cells may be dependent on serine/threonine phosphorylation. Tyrosine kinase inhibitors have no effect on receptor internalization but dramatically inhibited ligand-induced tyrosine phosphorylation of β_c. These studies suggest that internalization of GM-CSF can occur without cytoplasmic domains of GM-Rα and β_c. Specific regions of β_c have both positive and negative effects on ligand-induced internalization. We are currently examinig the role of the transmembrane domain of both GM-Rα and β_c in subunit association ligand induced internalization, and signaling by generating chimeric receptors which are anchored on the cell surface through a glycophospholipid linkage. The effect of these mutants on generation of high affinity receptors and receptor internalization will be presented.

A 207 POTENTIAL ROLE FOR THE IL-2 RECEPTOR γ CHAIN IN RECEPTOR COMPLEXES OF OTHER MEMBERS OF THE CYTOKINE RECEPTOR SUPERFAMILY. N.L. Farner[1], S.D. Voss[1], T.P. Leary[1], J. Gan[1], W.L. Farrar[2], G.A. Evans[2] and P.M. Sondel[1], [1]Dept Human Oncology, Univ Wisconsin, Madison, WI 53792 and [2]National Cancer Inst, Frederick, MD 21702-1201.

Evidence exists that the IL-2 receptor (IL-2R) γ chain is involved in the formation of both high- and intermediate-affinity IL-2R and plays a central role in cellular responses to IL-2. T, B, and NK lymphocytes both respond to IL-2 and express IL-2R . However, several cell types which do not appear to functionally utilize IL-2 have been found to express IL-2R γ, and we have hypothesized that this receptor component may be shared among other members of the Cytokine Receptor Superfamily. In order to test this hypothesis, we obtained the human myelomonocytic cell line, Tf-1 which functionally responds to IL-3, IL-5, GM-CSF, and Epo, but not IL-2. Based on PCR and Northern blot analyses, this cell line was found to express mRNA encoding IL-2R γ, despite no expression of IL-2R β and very low levels of IL-2R α. Following infection of Tf-1 cells with a retrovirus containing the cDNA encoding IL-2R β, a cell line was derived--Tf-1β--which expresses relatively high levels of IL-2R β on the cell surface. Scatchard analysis of ^{125}I-IL-2 binding to Tf-1β revealed predominantly intermediate-affinity IL-2 binding with approximately 5000-7000 binding sites/cell. Functional responses to IL-2 by Tf-1β (done with J.Hakimi) occur predominantly through these intermediate-affinity IL-2R and are completely inhibited by mAb directed against IL-2R β. Anti-IL-2R α mAb, in contrast, had little effect, nor was there an effect of these same mAb on the ability of Tf-1β to respond to GM-CSF. Preliminary cold competition and radiolabeled ligand/receptor crosslinking studies are consistent with the possibility that IL-2R γ may also have a role in the GM-CSF receptor. Tf-1 and the derivative, Tf-1β thus provide a unique model with which to evaluate the role that IL-2R γ may play in the structure and function of the receptors for IL-3, Epo, and GM-CSF.

A 208 INVOLVEMENT OF GP130/IL-6 TRANSDUCING PROTEIN IN IL-11 RECEPTOR.

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Recent studies have defined a new emerging family of cytokines sharing biological properties, and which is composed by Interleukin 6 (IL-6), Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM) and Ciliary Neurotrophic Factor (CNTF). Some redundancies of their biological effects is explained by the presence in their multimeric receptor complexes of a common gp130 transducing protein. We studied the possibility for the Interleukin 11 , and which displays many properties of IL-6, to belong to this family.
We observed that IL-11 triggered the TF1 erythroleukemic cell line proliferation in a dose dependent manner. The proliferative response to IL-11 was specifically blocked by monoclonal antibodies raised against the gp130 signal transducer. In addition, immunoblotting studies with antiphosphotyrosine antibodies showed that both IL-6 and IL-11 induced tyrosine phosphorylation of gp130. Antibodies against IL-6 receptor/gp80 failed to block the biological activities of IL-11, indicating that the cytokine may use a different receptor binding protein(s) to elicit its effects. Similarly we didn't observe any displacement between IL-11 and IL-6, in competition experiments using radiolabelled IL-6. These results indicate that in addition to gp130 an other binding component is required to mediate the Il-11 activities. These results showed that IL-11, along with IL-6, LIF, OSM, CNTF, belongs to this newly emerging family of cytokines.

A 209 THE DIRECT SYNERGISTIC EFFECTS OF LEUKEMIA INHIBITORY FACTOR ON HEMATOPOEITIC PROGENITOR CELL GROWTH.

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Leukemia inhibitory factor (LIF) is a multifunctional cytokine that is produced by a variety of cells including activated T cells, monocytes, fibroblasts and transformed cells. While LIF receptors have been detected on murine monocytes and megakaryocytes, and LIF has been shown to enhance retroviral-mediated gene transfer into bone marrow cells, no effect on normal murine bone marrow cell (BMC) proliferation has been observed. The studies presented here were designed to examine the effects of LIF in combination with other hematopoietic growth factors (HGFs) on unseparated and purified normal BMCs and determine whether these effects were direct or indirect. While LIF had no effect on GM-CSF-induced (previously shown) or IL-3-induced colony formation of normal BMCs, it enhanced CSF-1- and SLF-mediated colony formation. In comparison, LIF enhanced the growth of purified lineage negative (LIN^-) cells in response to GM-CSF, IL-3, CSF-1 and SLF in colony assays. While LIF affected the proliferation of hematopoietic progenitors, it did not affect their differentiation. In addition, these effects were direct since LIF increased the frequency of isolated LIN^- cells that proliferate in response to GM-CSF, IL-3 and CSF-1. We also examined the effects of other members of the LIF receptor family on LIN- cell growth and found that while IL-6 and IL-11 are potent synergistic factors, CNTF-ciliary neurotrophic factor and OSM-oncostatin M have no effects. LIF can directly increase the frequency and size of the more primitive $Thy\text{-}1^+LIN^-$ or $c\text{-}KIT^+LIN^-$ cells that proliferate in response to SLF plus IL-3. Finally, LIF directly increased the frequency of purified $CD\text{-}34^+$ human progenitors that proliferate in response to GM-CSF or IL-3 plus SLF. Thus, LIF directly promotes the growth of hematopoietic stem/progenitor cells in the presence of other growth factors.

A 210 THE LIGAND FOR FLT3/FLK2.

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Flt3 (also called Flk2) is a receptor tyrosine kinase most homologous to c-kit and c-fms, the receptors for stem cell factor and M-CSF, respectively. Flt3 messenger RNA is found in populations of cells highly enriched for hematopoietic stem cells as well as in progenitor cells. It is also found in fetal liver and brain, and adult brain, thymus, and placenta. The ligand for this receptor is unknown, but it may share similarities with SCF and M-CSF. We will report the purification, sequencing, and partial characterization of the Flt3 ligand. The source of the molecule is the mouse thymic stromal cell line TA4. The purified ligand was digested with endoproteinases, and the resulting peptides were purified by narrow-bore HPLC and individually sequenced. The partial protein sequence defines a novel molecule with the expected biochemical properties of the Flt3 ligand. Biological characterization of the ligand will be presented.

A 211 Molecular evolutionary principles of hematopoietic growth factors

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Previously we demonstrated two molecular evolutionary principles: Development-Related Evolution of CSFs and Concerted Evolution between cytokines and their receptors(Fuchu He & Chutse Wu, Exp Hematol 21:521-524, 1993). Here we analysed molecular evolution of 21 hemopoietic growth factors(HGFs). The result indicated that: (1) at amino acid sequence level, IL-3(multi-CSF) evolved at the highest rates, followed successively by IL-6, IL-4, GM-CSF, IL-9, IL-1α, IL-13, IL-12(p35), IL-2, IL-12(p40), IL-7, M-CSF, IL-5(Eo-CSF), G-CSF, IL-1ra, SCI(MIP-1α), LIF, IL-1β, EPO, IL-11 and SCF(MGF); (2) IL-3, -6 and -4, which have the highest evolutionary rates, evolved at much lower rates in gene sequence, flanking sequence of gene and 5',3' NTRs(nontranslated regions) of cDNA; (3) HGFs evolved at slowing-down rates in species phylogeny, i.e. they evolved faster in lower mammals, much slower in primates and slowest in human. The results showed that the principle of development-related evolution was taken for almost by all the differentiating HGFs(not by proliferating HGFs), and that the regulators of stem cell proliferation might be more important in evolution than differentiating HGFs; implied that the regulating sequences of the genes of those HGFs might play a more important role than their encoding regions; and indicated that HGFs did not follow the "molecular evolution clock".

A 212 EFFECTS OF ACETYL-SER-ASP-LYS-PRO (AcSDKP) ON BONE MARROW HEMATOPOIETIC PROGENITOR CELLS FOLLOWING SHORT-TERM AND LONG-TERM *IN VITRO* TREATMENT. John D. Jackson, Yun Yan, Cynthia Ewel, Linda Kelsey, and James E. Talmadge, Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198 and Henri Beaufour Institute, Inc., Washington, DC 20037
The tetrapeptide AcSDKP is a potent inhibitor of hematopoietic stem cell proliferation. In these studies, the effects of AcSDKP was examined in long-term bone marrow (LTBM) cultures and in short-term liquid (STL) cultures in the absence or presence of cytokines. In STL cultures, mouse bone marrow was incubated with AcSDKP in the presence or absence of IL-3 or GM-CSF for various time periods. The percent of CFU-GM in cell cycle was inhibited following 48 hours in culture; however, cultures containing exogenously added cytokines (IL-3 or GM-CSF) required 72 hours in culture to inhibit the number of progenitors in cycle. Dose response studies showed effects at 10^{-12} M and 10^{-14} M AcSDKP with no effects at higher or lower concentrations. In the LTBM culture studies, we investigated the effects of AcSDKP on the production of granulocyte/macrophage colony forming cells (CFU-GM) and high proliferative potential colony forming cells (HPP). AcSDKP was added daily to LTBM cultures at various concentrations (10^{-8} M to 10^{-16} M) for up to five weeks. Cultures were assayed at week one, three and five. AcSDKP was active at 10^{-12} M with little activity seen at higher or lower concentrations. The number of non-adherent CFU-GM per LTBM culture was not changed at one week of treatment with 10^{-12} M AcSDKP but decreased at weeks three and five. HPP progenitors were decreased throughout the treatment period. The number of CFU-GM and HPP in cell cycle was significantly decreased from week one. AcSDKP had no effect on the number of adherent CFU-GM, HPP or adherent cellularity per culture or percent in cell cycle. These studies indicate that the concentration of AcSDKP and the timing of exposure to the tetrapeptide is critical in eliciting an effect on hematopoietic progenitors. The presence of cytokine or stromal cells also affect the response of progenitor cells to AcSDKP.

A 213 IL-7 ENHANCES CSF-INDUCED PROLIFERATION OF MURINE BONE MARROW MACROPHAGES AND MAC-1$^+$ MYELOID PROGENITORS IN VITRO. Frede W. Jacobsen[1], Ole P. Veiby[2], and Sten E.W. Jacobsen[1]. [1]Department of Immunology, The Norwegian Radium Hospital, 0310 Oslo, Norway. [2]Nycomed Bioreg, 0371 Oslo, Norway.
The role of interleukin 7 (IL-7) as an important stimulator of the growth of B and T cell precursors, as well as mature T cells, is well established. In contrast, no myeloproliferative effects of IL-7 have previously been reported, and IL-7 has therefore been regarded as a lymphoid lineage restricted cytokine. However, recently we have found that IL-7 can enhance CSF-induced proliferation and myeloid differentiation of primitive murine (Lin$^-$Sca-1$^+$) hematopoietic progenitors in vitro. In the present study IL-7 enhanced the CSF-1-induced colony formation from individually plated bone marrow macrophages 90%, while IL-7 alone had no effect. Furthermore, IL-7 increased CSF-induced proliferation of mononuclear cells expressing the Mac-1 antigen (Mac-1$^+$MNC), while IL-7 did not significantly affect proliferation of Mac-1$^-$MNC. IL-7 increased Mac-1$^+$MNC CFU-Cs induced by CSF-1, GM-CSF, and IL-3, 3-fold, 2-fold, and 5-fold, respectively, while G-CSF-induced proliferation was not affected by IL-7. The CSF-induced myeloid differentiation of the Mac-1$^+$MNC cells was however, not influenced by IL-7, suggesting that IL-7 can provide proliferative signals to subpopulations of committed myeloid progenitors without altering their differentiation.

A 214 INTERLEUKIN-12 DIRECTLY ENHANCES THE PROLIFERATION, MYELOID DIFFERENTIAION AND VIABILITY OF PRIMITIVE MURINE AND HUMAN BONE MARROW PROGENITOR CELLS. Jacobsen SEW, Veiby OP, Lien E, Smeland EB. Department of Immunology, The Norwegian Radium Hospital, 0310 Oslo, Norway.
Interleukin-12 (IL-12) has been demonstrated to be a growth factor for mature lymphoid cells. We now demonstrate that IL-12 also is a potent synergistic factor for colony stimulating factor (CSF) and stem cell factor (SCF)-stimulated proliferation of Lin$^-$Sca-1$^+$ murine bone marrow progenitors, increasing the cloning frequency up to 9-fold. The stimulatory effects of IL-12 were dose-dependent with an ED_{50} of 2-20 ng/ml, and directly mediated since the effects could also be observed at the single cell level. The enhanced proliferation observed in response to IL-12 was due to formation of mature granulocytes and macrophages. However, IL-12 did not affect the proportion of granulocytes and macrophages formed in response to CSFs or SCF. Furthermore, IL-12 alone directly enhanced the viability of single Lin$^-$Sca-1$^+$ cells many-fold without inducing proliferation. The ability of IL-12 to maintain the viability of Lin$^-$Sca-1$^+$ progenitors was comparable to IL-6, while SCF was significantly more potent than IL-12 as a viability factor. Finally, IL-12 also directly enhanced the proliferation of human CD34+ bone marrow progenitor cells in combination with myeloid growth factors. Thus, in addition to its ability to stimulate lymphopoiesis, IL-12 is a modulator of the viability and growth of murine as well as human hematopoietic progenitor cells.

A 215 DIFFERENTIAL REGULATION OF M-CSF GENE EXPRESSION IN STIMULATED CORD VS. ADULT MONONUCLEAR CELLS. Sun min Lee, Lori Ishizawa, Carmella van de Ven, and Mitchell S. Cairo, Children's Hospital of Orange County, Orange, CA.
The increased susceptibility of neonates to infection may be due to multiple deficiencies. We have recently reported reduced expression and production of GM-CSF, G-CSF, and IL-3 from stimulated cord (C) vs. adult (A) mononuclear cells (MNC) (Cairo, et al, *Pediatr Res* 30:362, 1991, and 31:574, 1992). M-CSF regulates the proliferation, differentiation, and functional activation of monocytes. In the present study, the regulation of M-CSF gene expression was compared from stimulated C vs. A MNC. MNC were isolated by Ficoll-hypaque density centrifugation and stimulated with rhGM-CSF (20 ng/ml, 18 hrs). Total RNA was purified and M-CSF mRNA level was detected by Northern blot hybridization using M-CSF cDNA probe p3ACSFRI (Steven Clark, Genetics Institute). Adhesion of cells to flasks for 18 hrs caused constitutive expression of M-CSF mRNA in both C and A MNC. Adult MNC expressed four times higher level of M-CSF mRNA than C MNC without any stimulation. After 18 hrs of stimulation with rhGM-CSF, M-CSF expression was up-regulated in both C and A MNC. Stimulated A MNC expressed approximately 1.5 times higher level of M-CSF mRNA than stimulated C MNC. In order to determine whether the reduced expression of M-CSF from cord MNC was mediated at the level of gene transcription, the relative rates of transcription of M-CSF gene were determined in C and A MNC by nuclear run-on assays using short fragment cDNA (880 bp EcoRI/BamHI fragment from p3ACSFRI) as target. The basal level signal of M-CSF gene was similar between C and A MNC. The transcriptional rate upon stimulation of cells appeared to increase to a similar extent in both C and A MNC (C: 130 ± 10% vs. A: 150 ± 15%, n=3, Mean ± SD). These findings indicate that C MNC transcribe M-CSF genes at the same level as A MNC. In order to determine if transcript stability is involved in regulation of this gene, M-CSF mRNA half life was compared in C and A MNC by Actinomycin D decay studies. The half life of M-CSF mRNA from stimulated A MNC was 70 ± 7.0 min. (n=4) compared with 47 ± 2.8 min. (n=3) from stimulated C MNC (Mean ± SD, $p<0.05$). The involvement of labile protein factors in post- transcriptional regulation was next assessed by the treatment of stimulated C and A MNC with cycloheximide (CHX) for 3 hrs. There was a significant induction of M-CSF mRNA by CHX treatment in both C and A MNC. The fold increase of M-CSF mRNA induction by CHX was approximately 2 times higher in C MNC compared to that in A MNC. These results indicate that there are one or more labile proteins that regulate transcript stability. Taken together, these findings suggest that the reduced M-CSF mRNA expression from stimulated C vs. A MNC is not transcriptionally regulated. Instead, post-transcriptional regulation involving the synthesis of labile proteins that affect the stability of M-CSF transcript could account, in part, for these differences between C and A MNC.

A 216 CHARACTERIZATION OF C-KIT POSITIVE INTRATHYMIC STEM CELLS THAT ARE RESTRICTED TO LYMPHOID DIFFERENTIATION,

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We have previously reported that c-kit+,Thy-1^{lo}, lineage marker negative cells (c-kit+ Thy-1^{lo} Lin$^-$) in mouse bone marrow contains primitive stem cells displayed a capacity to reconstitute all hematopoietic lineages in long term. We found that these cells are present not only in bone marrow but also in thymus ("BM c-kit" and "Thymus c-kit" cells). Although the two cell types are phenotypically similar, only BM c-kit cells showed the potential to form colonies in vitro as well as in vivo. However, both of them revealed extensive growth and differentiation potential to T cells after direct transfer into an irradiated adult thymus, or a deoxyguanosine treated fetal thymus. Time course analysis showed that Thymus c-kit cells differentiated into CD4CD8 double positive cells approximately 4 days earlier than BM c-kit cells did. In addition, anti-c-kit antibody blocked T cell generation of BM c-kit cells but not of Thymus c-kit cells. Intravenous injection of Thymus c-kit resulted in the generation of not only T cells, but B as well as NK1.1+ cells. These data provide evidence that Thymus c-kit cells represent common lymphoid progenitors with the differentiation potential to T, B and possibly NK cells. The c-kit mediated signaling appears to be essential in the transition from BM c-kit to Thymus c-kit cells.

A 217 GENES FOR ALPHA SUBUNITS OF HUMAN IL-3 AND GM-CSF RECEPTORS: EXON-INTRON ORGANIZATION CONSERVED AMONG THE CYTOKINE RECEPTOR SUPERFAMILY

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High-affinity receptor for IL-3 or GM-CSF is composed of a common β subunit and a unique α subunit (hIL3Rα and hGMRα). These three subunits have structurally conserved domains shared by the members of cytokine receptor superfamily, each of which is predicted to have seven β strands and is related to the fibronectin type III domain. The common β subunit gene is mapped on chromosome 22 and the genes encoding hIL3Rα and hGMRα are located on X-Y pseudoautosomal region.
We have isolated genomic clones that cover the entire coding sequences of the genes encoding hIL3Rα and hGMRα and have characterized their exon-intron organization and 5'-upstream regions. The genes for hIL3Rα and hGMRα consist of 11 and 13 exons respectively, and both span at least 40 kilobases. Like most members of this gene family, each conserved domain of hIL3Rα and hGMRα genes is encoded by two exons, with one exception in hGMRα gene. Based on these data we will discuss possible mechanisms how this gene family has evolved.

A 218 EFFECTS OF DELAYED G-CSF ADMINISTRATION ON THE ACCELERATION OF HEMOPOIETIC RECOVERY IN RADIATION-INDUCED APLASTIC MICE.

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Granulocyte colony-stimulating factor (G-CSF) has been shown to significantly enhance hemopoietic regeneration following myelosuppressive radiation exposure when administration is initiated within 24 hours after irradiation. The purpose of this study was to evaluate the effects of delayed G-CSF administration on hemopoietic recovery following radiation injury. Female B6D2F1 mice were exposed to an aplasia-inducing high-sublethal dose of cobalt-60 radiation (7.75 Gy @ 0.4 Gy/min). Daily on days 1-17, days 4-17, days 7-17, or days 10-17 postirradiation, mice were subcutaneously administered rhG-CSF (100 μg/kg/day; Amgen); irradiated control mice were subcutaneously administered saline on days 1-17. Bone marrow (BM) and splenic (SPL) CFU-s and GM-CFC recoveries as well as peripheral blood white cell (WBC), red cell (RBC), and platelet (PLT) recoveries were evaluated on days 10, 14, 17, and 22 postirradiation. As expected, when G-CSF therapy was initiated on day 1 postirradiation, significantly accelerated recovery of all hemopoietic parameters was observed, with effects detected as early as day 10 postirradiation. G-CSF therapies initiated on either day 4 or day 7 postirradiation were also quite effective at accelerating hemopoietic regeneration; effects, however, were less dramatic than those observed with the day 1-17 G-CSF protocol. Delaying G-CSF therapy until day 10 postirradiation had only marginal effects on hemopoietic recovery compared to the effects induced when therapy was initiated earlier. For example, on day 17 postirradiation, mice treated with G-CSF on days 1-17, 4-17, 7-17, and 10-17, respectively, exhibited BM GM-CFC recoveries that were 262%, 211%, 174%, and 102% of the recovery observed in saline-treated mice and BM CFU-s recoveries that were 357%, 216%, 201%, and 129% of the recovery observed in saline-treated mice. These results illustrate, that following severe radiation-induced hemopoietic aplasia, G-CSF therapy can be used to significantly enhance CFU-s, GM-CFC, WBC, RBC, and PLT recoveries even when initiation of therapy is delayed until day 7 postirradiation. Best results, however, are obtained with therapy initiated within 24 hours of irradiation.

A 219 TNF-α POTENTLY INHIBITS PRIMITIVE HUMAN HEMATOPOIETIC PROGENITOR CELLS: INVOLVEMENT OF p55 AND p75 TNF RECEPTORS,

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Tumor necrosis factor-α (TNF-α) has previously been reported to have both inhibitory and stimulatory effects on hematopoietic progenitor cells. Specifically, TNF-α has been proposed to stimulate early hematopoiesis in humans. In the present study TNF-α was found to preferentially and directly stimulate day 7 colonies induced by IL-3 and GM-CSF, while day 14 colonies were less prone to stimulation by TNF-α. Furthermore, we demonstrate for the first time that TNF-α is a potent inhibitor of high proliferative potential colony-forming cells regardless of the hematopoietic growth factors stimulating their growth. Using agonistic antibodies to the p55 and p75 TNF receptors, we show that both receptors can mediate this inhibition. In contrast, the stimulatory effects of TNF-α on GM-CSF and IL-3-induced colony formation, as well as the inhibition of G-CSF-induced colony growth, are mediated exclusively through the p55 TNF receptor. Taken together, our results suggest that the inhibitory effect of TNF-α on immature bone marrow progenitor cells are mediated through both p55 and p75 TNF receptors, while the p55 receptor exclusively mediates the bidirectional effects on more mature, single-factor responsive bone marrow progenitor cells.

A 220 POTENT INTERLEUKIN-3 SYNTHOKINES WITH ENHANCED AFFINITY FOR ALPHA SUBUNIT OF RECEPTOR, John W. Thomas, Michael B. Steketee, William F. Hood, Nicholas R. Staten, Lyle E. Pegg, Joseph B. Monahan, and John P. McKearn, Inflammatory Diseases Research, G.D.Searle /Monsanto Co, St. Louis, MO 63198.

The systematic combination of structure-activity information lead to the construction of a series of synthetic interleukin–3 receptor agonists (synthokines) with increased biological potency and improved physiological properties. Members of this series (*e.g.,* SC-55494) exhibit 20- to 40-fold greater biological activity in AML cell proliferation assays, but these same synthokines have shown only slightly enhanced binding to the high-affinity IL–3 receptor as expressed on AML cells (the heterodimeric alpha-beta complex). We now report that these same molecules exhibit a marked increase in their binding to the alpha subunit of the IL-3 receptor.

Native IL-3 inhibited binding of iodinated IL–3 to the alpha subunit with an IC_{50} in the 100 nM range as expected. In contrast, the synthokine, SC-55494, inhibited binding with an IC_{50} of 5 to 10 nM (10- to 20-fold lower). Consistent with this result Scatchard analysis of data obtained using a related radiolabelled synthokine gave a Kd value of 6 to 9 nM. For this series of synthokines, cell proliferative activity correlates better with alpha subunit binding than with high-affinity receptor binding. The results suggests a mechanism of action in which enhanced initial binding to the alpha subunit leads to increased signal transduction. The subsequent steps are currently under investigation. In addition, these higher affinity synthokine ligands improve the alpha receptor assay and are aiding in the definition of the alpha receptor contact residues.

A 221 INTERLEUKIN 10 SUPPORTS THE GROWTH OF GRANULOCYTE/MACROPHAGE COLONY FORMING CELLS (CFU-GM) IN MOUSE BONE MARROW CULTURES BY BLOCKING ENDOGENOUS TGF-β1 SYNTHESIS. Van Vlasselaer, P., Activated Cell Therapy, Inc 219 North Bernardo Avenue, Mountain View, California 94043

Cultures of bone marrow derived from 5-fluorouracil (5-FU) treated mice support osteogenesis in the presence of β-glycerophosphate and vitamin C. Under these conditions the ability to generate CFU-GM is lost after two weeks of culture. In contrast, IL-10 supported the growth of CFU-GM in these cultures for more than six weeks. Orcein staining showed that the majority of the colonies contained cells with a macrophage morphology. Only a minority of the colonies contained granulocytes or were mixed. FACS analysis supported this observation and showed increased numbers of $Mac\text{-}1^+$, $Mac\text{-}2^+$and $Mac\text{-}3^+$ macrophages in cultures performed in the presence of IL-10. Furthermore, cells from IL-10 treated cultures supported CFU-GM colony formation for five weeks when seeded on irradiated bone marrow stroma whereas control cultures supported CFU-GM formation for only one week. This suggests the presence of pluripotent hematopoietic cells in the IL-10 treated but not the control cultures. The increased number of c-kit$^+$ cells observed in the IL-10 treated cultures may be in support of this idea. A direct proliferative effect is unlikely to explain the effect of IL-10 on CFU-GM since neither cells from untreated nor cells from IL-10 treated bone marrow cultures showed increased [^{3}H]- thymidine incorporation or CFU-GM numbers when exposed to IL-10 alone. Consequently, it appears that IL-10 acts via an indirect pathway by inhibiting the synthesis of TGF-β1, which is known to suppress hematopoiesis. Exogenous TGF-β1 suppressed the IL-10 induced increase of CFU-GM numbers in cultures treated with neutralizing anti-TGF-β blocking antibodies (1D11.16). Taken together these results show that IL-10 is able to support hematopoiesis in osteogenic cultures by inhibiting endogenous TGF-β1 production

A 222 SYNERGISTIC STIMULATION OF MYELOID PROGENITOR CELL PROLIFERATION BY A NOVEL β-GLUCAN AND GM-CSF, Eric Wakshull, Heidi Correira, and Tracy Sidloski, Alpha-Beta Technology, Inc., Worcester, MA 01605.

β-Glucans have pleiotropic immunomodulatory properties, including anti-infective and hematopoietic activity. Previous reports have indicated enhanced protection and recovery from myelosuppressive treatments following glucan administration to mice and primates. The present studies have examined the effect of a novel β-glucan (PGG-glucan; BETAFECTIN™) on a) *in vitro* stimulation of CFU-GM production in combination with GM-CSF and b) *in vivo* response to cyclophosphamide (CYP)-induced myelosuppression in mice. The results show that PGG-glucan stimulates a hematopoietic response both *in vitro* and *in vivo*.

A) Bone marrow from naive mice were plated in methylcellulose-containing RPMI medium with a sub-optimal GM-CSF level (0.5ng/ml) with or without PGG-glucan. In the absence of GM-CSF, PGG-glucan did not support colony formation. The number of CFU-GM increased 2-fold in the presence of PGG-glucan and GM-CSF, with an $E.C._{50}$ ranging from 10-50ng/ml. Increased CFU-GM production (1.7-fold) was also seen when PGG-glucan was added to optimal GM-CSF (2-5ng/ml). B) Mice were injected with PGG-glucan (0.5mg/kg, s.c.) at various times before or after CYP (200mg/kg, i.p.) administration. Following CYP administration, WBC counts, bone marrow (bm) cellularity, and bm-derived CFU-GM production in the presence of optimal GM-CSF (2ng/ml) were measured. By Day 5 after CYP, a 2-fold increase in bone marrow cellularity, >3-fold increase in bm-derived CFU-GM, and a 4-fold increase in WBCs (primarily PMNs), relative to CYP alone, were observed. Similar results were seen whether PGG-glucan was administered 24hr before or after CYP. Thus, *in vitro* in the presence of GM-CSF, or *in vivo* under conditions of myelosuppression, where an increased expression of local CSFs would be anticipated, PGG-glucan has potent hematopoietic activity for cells of the myeloid lineage. These data support the use of PGG-glucan in reversing the myelosuppressive effects of chemotherapy.

A 223 SIGNAL TRANSDUCTION OF HUMAN GM-CSF RECEPTOR CHARACTERIZED BY EMPLOYING POLYOMA REPLICON, Sumiko Watanabe[1], Atsushi Miyajima[2], Yoshiaki Ito[3], Ken-ichi Arai[1], [1]Institute of Medical Science, University of Tokyo, Tokyo, Japan, [2]DNAX Research Institute, Palo Alto, CA, [3]Virus Research Institute, Kyoto University, Kyoto, Japan

hGMR consists of α/β chains and induces activation of early response genes, protein tyrosine phosphorylation and proliferation in hemopoietic cells. The hGMR α/β chains reconstituted in NIH3T3 fibroblasts is functional indicating that molecules unique to hemopoietic cells is not essential to transduce signals[1]. Studies with deletion mutants and inhibitors of tyrosine kinase indicated that distinct regions of β chain are involved for proliferation/c-*myc* activation and for c-*fos*/c-*jun* activation[2]. To analyze more detailed mechanism leading to DNA replication, we employed polyoma (Py) replicon in IL-3 dependent mouse hamopoietic cells. Large T antigen (LT) is the only molecule encoded by Py genome required for replication. Plasmids containing Py origin/enhancer and Py LT linked to RSV-LTR were transiently co-transfected into mIL-3 dependent BA/F3 cell expressing hGMR. Py replicates in response to hGM-CSF or mIL-3 stimulation in LT-dependent manner as monitored by DpnI assay. Using this system, *cis*-acting regions of Py as well as the region(s) of hGMR β chain required for Py replication were characterized. Our results indicated that the β chain region required for cell proliferation[2] is also required for Py replication. In contrast, hGM-CSF was unable to stimulate Py replication even though it stimulated cell proliferation in NIH3T3 cells.

[1]Watanabe S. et al. (1993) Mol. Cell. Biol. 13, 1440-48
[2]Watanabe S. et al. Mol. Biol. Cell in press

A 224 *IN VIVO* CYTOKINE PRODUCTION DURING THERAPY FOR CHILDHOOD LEUKAEMIA

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In vitro studies have consistently demonstrated that a spectrum of cytokines are involved in the development of specific haematopoietic cell lineages. This study was designed to investigate the *in vivo* temporal expression of cytokines during human marrow regeneration. Bone marrow from paediatric patients undergoing treatment for acute lymphoblastic leukaemia was studied at different phases during the chemotherapy cycle. Samples were obtained at weeks 1, 5, 9, 12, 14, 20 and 100 depending on the treatment protocol used. Cytokine mRNA expression for c-kit, IL-1β, IL-6 and GM-CSF was studied by Northern blot analysis. Individual cell production of IL-1β mRNA was investigated using non-radioactive in situ hybridization.

Northern blot analysis revealed that c-kit message was strongly expressed in all samples examined. IL-1β had a variable level of expression. A strong signal was seen in post-induction samples when morphologically the marrow demonstrated very active haematopoiesis. IL-6 and GM-CSF were detected in a few samples, but did not appear to correlate with timing of therapy or any particular morphological picture in the marrow.

In a small number of samples, in situ hybridization detected 3-10% cells positive for IL-1β mRNA. These were also predominantly post-induction.

These results demonstrate changes in cytokine gene expression during human bone marrow regeneration *in vivo.*

Red Cells and Megakaryocytes; Cytokines and Microenvironment

A 300 EXPRESSION OF c-kit AND STEM CELL FACTOR (SCF) BY HUMAN FLG 29.1 OSTEOCLASTS AND THEIR FUNCTIONAL ROLE IN INTERACTIONS WITH OSTEOBLASTS.

Aldinucci D., Gattei V., Cozzi M., Pistello M.*, Perin V., De Iuliis A., Gloghini A., Carbone A., Pinto A., The Leukemia Unit, Divisions of Medical Oncology and Pathology, C.RO. Aviano, I-33081, *Department of Biomedicine, University of Pisa, Italy.

Although it has been established that human osteoclasts derive from hemopoietic precursors, little is known about the cytokine network regulating their functions within the bone microenvironment. By utilizing a human continuous cell line (FLG 29.1) of osteoclast precursors, able to differentiate in vitro into mature osteoclasts (Gattei et al. J Cell Biol 116, 437, 1992), and the human osteoblast cell line Saos-2, we have investigated the cytokine/receptor circuitries regulating proliferation of osteoclasts and their functional interactions with osteoblasts. A striking finding of our investigation was that FLG 29.1 osteoclasts and Saos-2 osteoblasts expressed high levels of surface c-kit receptors and c-kit specific mRNA. In addition, Saos-2 osteoblasts expressed high amounts of membrane bound (mb)-SCF as detected by the 4B10 MAb, whilst lower levels were evidenced in FLG 29.1 cells. Accordingly, RT-PCR experiments demonstrated the presence of mRNAs related to both the soluble and membrane forms of SCF in FLG 29.1 cells, whereas a single SCF mRNA transcript, related to the mb form of the molecule, was amplified from Saos-2 cells. Co-culture experiments showed that adhesion of FLG 29.1 osteoclasts to Saos-2 osteoblasts, is mediated at least in part by the interaction of mb-SCF and the c-kit protein since soluble SCF or anti-c-kit MAbs specifically inhibited cell-cell binding. Finally, Saos-2 osteoblasts produced GM-CSF when co-cultured with FLG 29.1 osteoclasts and both SCF and GM-CSF stimulated proliferation of this latter cell type, which in turn expressed functional receptors for GM-CSF. Our results indicate for the first time a specific functional role of c-kit and SCF in regulating cellular interactions among specialized bone cell populations and suggest that signals mediated by SCF and GM-CSF are implicated in the regulation of osteoclast generation and proliferation.

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A 301 **EXPRESSION OF Tie RECEPTOR TYROSINE KINASE IN ENDOTHELIAL AND HEMATOPOIETIC CELLS**

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The Tie receptor tyrosine kinase was originally identified as an amplified product in reverse transcription-polymerase chain reaction analysis of human K562 leukemia cell RNA. *In situ* hybridization analysis revealed that the corresponding mouse gene is expressed predominantly in endothelial cells in the early postimplantation mouse tissues. No *tie* mRNA was detected in 7.5 day mouse embryos. In 8.5 day embryos, *tie* expression was observed in differentiating angioblasts of the head mesenchyme, in splanchnopleure, in dorsal aorta as well as in migrating endothelial cells of the developing heart. A *tie* signal was also obtained from hemangioblasts in the blood islands of the yolk sac. In Northern blot analysis the 4.4 kb *tie* mRNA was expressed at high levels in five of five human megakaryoblastic leukemia cell lines studied and in two IL-3 dependent mouse myeloid leukemia cell lines, but not in 42 other human and murine leukemia cell lines representing various hematopoietic lineages. Increased expression of *tie* mRNA and protein was observed upon treatment of the megakaryoblastic leukemia cells with the tumor promoter TPA, known to enhance megakaryoblastic markers. - Analysis of Tie expression in different hematopoietic compartments and lineages will be presented. Our findings thus suggest that among hematopoietic lineages Tie is predominantly expressed in cells with megakaryoblastic properties and in more primitive myeloid cells. Analysis of the ligand of Tie and Tie expression in hematopoietic stem cells is in progress.

Mol. Cell Biol. 12: 1698,1992; Blood 80: 2548,1992; Leukemia 7, in press; Oncogene, in press

A 302 DIETARY CALCIUM AND LEAD EXPOSURE DURING PREGNANCY INTERACT TO INFLUENCE ERYTHROPOIESIS

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Both toxic exposures and dietary factors may influence hematopoiesis; their impact may be especially critical during pregnancy. We studied the effects of dietary calcium and lead exposure on erythropoiesis during pregnancy. Pregnant Sprague-Dawley rats (n=42) were randomly assigned to one of 6 treatment groups of 7 rats each. Half were fed diets of low (0.1%), normal (0.5%), or high (2.5%) calcium and exposed to 250 mg/L of lead in their drinking water for the duration of pregnancy (21 ± 2 days). Three control groups were fed the same diets without lead exposure. Pup body weights and lengths were determined at 1 day and 1 week of age. Blood from the dams, day old pups, and week old pups was analyzed for lead (BPb), the hematocrit (Hct), free erythrocyte protoporphyrin (FEP) and hemoglobin (Hgb). Two way analysis of variance showed that there were statistically significant ($p < 0.01$) effects of lead on BPb, Hct, Hgb, FEP, and pup body weights and lengths. There were also significant ($p < 0.01$) effects of dietary calcium on these parameters, as well as significant lead-calcium interactions. Maternal and fetal blood samples from the groups fed the low calcium diet had the highest BPb, while the lowest concentrations were found in the groups fed the high calcium diet. Lead exposure significantly increased dam and fetal FEP concentrations for animals in the low and normal, but not the high, dietary calcium groups. The reduction in FEP concentrations associated with the high calcium diet was about 240% greater for the lead-exposed pups than for the dams, probably due to the enhanced erythropoiesis associated with rapid fetal and neonatal growth. Dam and pup Hgb and Hct were reduced by lead exposure and by the high calcium diet. The data support the hypothesis that the calcium content of the diet can influence erythropoiesis and can modify the effects of lead on erythrocyte production during pregnancy. (Aided by Reproductive Hazards in the Workplace, Home, Community, and Environment Research Grant No. 15-FY93-0659 from the March of Dimes Birth Defects Foundation.)

A 303 **DISRUPTION OF THE SCI / MIP 1α GENE BY HOMOLOGOUS RECOMBINATION**

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The proliferative status of hematopoietic stem cells and early progenitors is influenced by both positive and negative regulators. Macrophage inflammatory protein (MIP-1α), also known as stem cell inhibitor (SCI), is one such negative regulator that reversibly inhibits the proliferation of CFU-S and other primitive progenitors *in vitro*. To directly investigate its role *in vivo*, we have used homologous recombination to disrupt the gene coding for MIP-α in embryonic stem cells. Correctly targetted E14 TG2A ES cells were injected into C57BL/6 blastocysts which, upon transfer to pseudopregnant recipient animals, gave rise to 10 chimeric mice. Five of these chimeras have transmitted the ES cell genome to their progeny. Mice homozygous for the disrupted locus should provide a good system to study the role of MIP-1α *in vivo*, and may also provide a means of obtaining increased numbers of hematopoietic stem cells both *in vivo* and *in vitro*.

A 304 *TUMOR-PROMOTING PHORBOL ESTER SUPPRESSES* ERYTHROID AND STIMULATES MEGAKARYOCYTIC GENE EXPRESSION IN CULTURES OF HEMATOPOIETIC PROGENITORS FROM HUMAN PERIPHERAL BLOOD, Nadya L. Lumelsky, Department of Medicine, University of Wisconsin, Madison, WI 53706

The effect of a potent activator of the protein kinase C (PKC) signal transduction pathway, tumor-promoting phorbol ester (PMA), on erythropoiesis and megakaryocytopoiesis has been investigated in the semi-solid cultures of human hematopoietic progenitors derived from normal peripheral blood. Exposure of the cells to PMA in the presence of erythropoietin (EPO) resulted in the complete inhibition of both the erythroid colony formation and the expression of the erythroid-specific markers. This inhibitory effect was observed only when PMA was added within several days after the cultures were established. This indicates that only early erythroid progenitors are sensitive to negative regulation by PMA. Later addition of PMA to the EPO-containing cultures resulted in activation of the expression of a number of megakaryocytic-specific markers. This effect was not observed, however, when PMA was added early during the life of the culture. The PMA effect on expression of erythroid and megakaryocytic genes was similar in cultures of unfractionated mononuclear cells and in cultures of partially purified late erythroid progenitors. These results may suggest that erythroid progenitors can be diverted from erythroid to megakaryocytic pathway of differentiation relatively late in the course of the erythroid progenitor maturation. The same model system has been used for investigation of factors involved in the regulation of erythro- and megakaryocytopoiesis to elucidate the mechanisms of selectivity between these hematopoietic pathways. To this end, a recently developed differential display PCR methodology was applied in search of genes uniquely expressed under the conditions promoting erythroid *vs.* megakaryocytic gene expression. The results of these studies will be discussed.

A 305 **STRUCTURE AND REGULATION OF THE HUMAN C-MPL GENE.**

Vincent Mignotte, Erik Boucher de Crèvecoeur, Isabelle Vigon and Stany Chrétien[1], INSERM U.91, Hôpital Henri Mondor, 94010 CRETEIL, FRANCE and [1]INSERM U.76/INTS, Rue Alexandre Cabanel, 75015 PARIS, FRANCE.

The human c-mpl proto-oncogene encodes a member of the cytokine receptor superfamily, expressed mainly in CD 34-positive hematopoietic progenitors and in the megakaryocytic lineage. To investigate the elements required for this tissue-specific expression, we cloned the human c-mpl gene. This gene contains 12 exons distributed over 18 kb of DNA. Each of the two "cytokine receptor domains" of Mpl is encoded by a set of four exons, the trans-membrane domain by a single exon and the cytoplasmic domain by two exons. We describe how three types of mRNA, encoding different proteins, are generated. The major species contains all twelve exons, mRNAs encoding a protein with a smaller cytoplasmic domain are produced by termination of the transcript within intron 10, and mRNAs encoding a putative soluble form of the c-Mpl protein lack exons 9 and 10. The promoter was also characterized. It is GC-rich and contains putative binding sites for proteins of the Ets and GATA families. A 200 bp fragment of the human c-mpl promoter displays partial tissue specificity: it is active in the erythroid/megakaryocytic HEL cell line and in the K562 erythroid cell line, but inactive in the non-hematopoietic HeLa cell line and the Jurkat T lymphoid cell line. In HeLa cells, the 200 bp promoter can be efficiently trans-activated by ectopic expression of the GATA 1, Ets 1 and Fli 1 transcription factors. This strengthens the importance of these proteins for megakaryocyte specific gene expression.

A 306 EXPRESSION OF TYPE II ACTIVIN RECEPTOR GENES DURING DIFFERENTIATION OF HUMAN K562 CELLS, Olli Ritvos, Kristiina Hildén and Timo Tuuri, Department of Bacteriology and Immunology, University of Helsinki, 00290 Helsinki, Finland

Recent studies have indicated that activin A/erythroid differentiation factor is a physiologic hematopoietic growth and differentiation factor mainly for cells of the erythroid lineage. We studied the expression of the two type II activin receptor mRNAs during the differentiation of K562 erythroleukemic cells which are known to be induced toward the erythroid lineage in response to activin or toward the megakaryoblastic lineage by phorbol myristate acetate (PMA). The cDNA of the human activin receptor type IIB (hActR-IIB) was cloned and sequenced by reverse transcription-polymerase chain reaction (RT-PCR) from two RNA sources, the K562 cells and the human fetal brain. The cDNA encodes a predicted 512 amino acid protein containing an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular serine/threonine kinase domain. The amino acid sequence is 99.2% and 98.4% homologous in the coding region to the previously described mouse and rat ActR-IIBs, respectively, and 69% identical to the other human activin serine/threonine kinase receptor, hActR-II. Northern analysis revealed that the 10 and 2.5 kb transcripts of hAct-RIIB are more abundantly expressed than the 6.0 and 3.0 kb transcripts of hActRII in K562 cells. No changes in the steady-state levels of hActR-II and IIB mRNAs were detected upon differentiation of K562 cells by activin A or by PMA. Similarly, the receptor mRNA levels remained constant in HL-60 cells induced to either monocyte/macrophage or granulocyte-like cells by PMA or dimethyl sulfoxide, respectively. Thus the mRNA expression levels of both receptors do apparently not correlate with the differentiation status of these cells.

A 307 CYTOKINE PRODUCTION BY NORMAL AND APLASTIC ANAEMIA BONE MARROW STROMAL CELLS, John Scopes, Steven Daly, Edward E.C. Gordon-Smith and Frances M. Gibson, Division of Haematology, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, London, UK

Cross-over LTBMC have demonstrated abnormal stromal function in some aplastic anaemia (AA) patients. Abnormal colony-stimulating activity (CSA) and burst-promoting activity (BPA) production by AA stromal cells has been demonstrated using these cells in semi-solid clonogenic culture in the absence of exogenous factors and in this study specific cytokine production defects have been identified using neutralising antibodies.

Normal (N) marrow (n=31) and from patients with AA (n=15) was grown in LTBMC until confluence. The stroma was then irradiated, harvested and co-cultured ($2.5x10^4$ stromal cells) with 10^5 BMMC or $3x10^3$ $CD34^+$ cells from allogeneic N marrows in the presence of 2U/ml EPO alone. On day 14, CFU-GM (CSA) and BFU-E and CFU-GEM (BPA) were counted. Identical cultures were set up containing antibody to individual cytokines, G-CSF, GM-CSF, IL-3, IL-6 and MGF, and neutralisation of CSA and BPA was evaluated. The table below shows the numbers of N and AA marrows producing each cytokine.

	G-CSF	GM-CSF	IL-3	IL-6	MGF
N	31/31 (100%)	31/31 (100%)	29/31 (94%)	23/31 (74%)	16/24 (67%)
AA	15/15 (100%)	15/15 (100%)	11/15 (73%)	7/15 (47%)	5/7 (71%)

Although the production of G-CSF, GM-CSF and MGF from AA and N stromal cells is similar, a dysfunction in IL-3 and IL-6 production is demonstrated in some AA bone marrows. Further study will be needed to resolve whether this dysfunction can explain the pathogenesis of the disease in these cases or reflect part of a repair mechanism or a secondary effect of an immunopathogenic mechanism.

A 308 ADMINISTRATION OF ERYTHROPOIETIN (EPO) FOLLOWING AUTOLOGOUS BLOOD STEM CELL (ABSC) OR BONE MARROW TRANSPLANTATION (ABMT) ACCELERATES TRILINEAGE HEMATOPOIETIC RECOVERY, J.G. Sharp, D.A. Crouse, C. Weekes, S. Clausen, D. Smith*, Univ. of Nebr. Medical Center, Omaha, NE, and Univ. of Okla. Medical Center, Oklahoma City, OK.

Previous studies in swine and in patients have shown that administration of EPO prior to and during ABSC collection by apheresis resulted in a product which on transplantation to myeloablated recipients resulted in an accelerated trilineage (neutrophils, red cell and platelet) recovery compared to transplantation of a non-mobilized product. EPO doses of 100 to 500 U/kg had equivalent effects on neutrophil recovery in swine, similar to 200 U/kg in man but in swine there was a dose response for platelet recovery with 500 U/kg providing the most rapid recovery. In the present studies, we evaluated the effects on hematopoietic recovery of administration of rHu EPO daily following ABMT in mice (1,000 U/kg for 7 days) or swine (500 U/kg daily for 14 days or until neutrophil recovery) and also in swine following ABSCT (100 U/kg/day for 14 days or until neutrophil recovery). In mice EPO post-ABMT resulted in accelerated peripheral neutrophil, but not platelet recovery and concomitant elevated GM-CFC appearance in spleen and bone marrow. In swine, EPO post-BMT produced a modest acceleration of engraftment of 2-3 days. Following transplantation of a suboptimal (2 aphereses) harvest, 100 U/kg EPO produced a significant acceleration of engraftment reducing the period of cytopenia from over 32 days to 16 days (neutrophils) and 24 days (platelets). Therefore EPO post-transplant accelerates hematopoietic recovery in more than the erythroid compartment and this effect may be more pronounced following ABSCT than ABMT. (Supported in part by a UNMC Seed grant, EPO kindly donated by Ortho Biotech.)

A 309 UNIQUE CIS-ELEMENTS OF THE MURINE β^{MAJ} GLOBIN PROMOTER MEDIATE EPO-INDUCED TRANSCRIPTION IN RESPONSIVE J2E CELLS: A CENTRAL ROLE FOR GATA-1, D. J. Taxman and D. M. Wojchowski, Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802

Using J2E cells as a model, we have performed the first direct transcriptional analyses of induced late erythroid gene activation in an EPO-responsive cell line. Transferrinfection using a novel transferrin/poly-L-lysine conjugate (Taxman et al., in press) of wild-type, point-mutated, and synthetic murine β^{maj} promoter-reporter constructs revealed unique mechanisms for EPO-induced transcription *vs.* DMSO-induction in the prevalent F-MEL cell system. For example, repression at the -165 BB1 element is not observed for the -340 promoter in J2E cells, with a requirement instead for adjacent activating sequences. Furthermore, a promoter comprised of WGATAR-CACCC-TATA elements which is highly inducible in the F-MEL/DMSO system is poorly active in EPO-induced J2E cells, while $[WGATAR]_4$ TATA promoter which is inactive in F-MEL cells is efficiently activated by EPO. This latter finding indicates a direct role for GATA-1 (or an associated co-factor) in mediating EPO-activated transcription at the β^{maj} promoter, and mutations at a -60 GATA site demonstrate a requirement for this proximal element for induced transcription in both the J2E/EPO, and F-MEL/DMSO systems. To directly define mechanisms of GATA-1 action, we also have developed an efficient cell-free transcription system using recombinant GATA-1 expressed in insect SF9 cells. GATA-1-induced transcription from a $[WGATAR]_4$ TATA promoter, as well as more complex erythroid promoters (e.g., $G^{-}106\beta^{maj}$) is demonstrated with activation levels as high as 10 to 12-fold. Thus, this system for the direct and efficient *in vitro* analysis of GATA-1 function and co-factors should serve well to advance an understanding of its action at proximal promoters.

A 310 CHEMILUMINESCENCE ASSAYS FOR HUMAN CYTOKINES,

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Colony Stimulating Factors (CSFs) and other cytokines are now recognized as critical regulators of hematopoiesis, and important intermediaries in the pathogenesis of conditions associated with immune activation and inflammation. However, a major limitation to clinical and epidemiological studies has been the inability to measure these important biomarkers in serum, urine, or other body fluids from normal, healthy subjects. Without this capacity, interpretation of apparently elevated levels is problematic, and evaluation of decreased levels is impossible.

We have applied chemiluminescence (CL) techniques to enzyme-linked immunosorbent assays (ELISA) for CSFs and other cytokines, resulting in a 100-fold increase in sensitivity. For example, the CL-ELISA for human GM-CSF has a limit of sensitivity of less than 1 pg/ml and has been successfully applied to serum and plasma samples. Assays are performed in a microtiter plate format (Microlite luminometer, Dynatech), allowing testing of large numbers of samples for application to clinical and epidemiological studies.

CL-ELISA assays have been developed for a broad range of cytokines, to allow simultaneous measurement of a panel of cytokines on a single small aliquot of serum. This approach will facilitate investigations of interrelationships within the cytokine network in studies of normal homeostasis as well as disease states. Assays are available for both murine and human cytokines, permitting effective cross-fertilization between research in animal and human systems.

Initial laboratory efforts are focused on developing normative data and the influence of relevant host factors. Preliminary data for GM-CSF levels in serum samples (diluted 1:4) from 22 healthy subjects: median 18.5 pg/ml, range 2-233 pg/ml.

A 311 *IN VITRO* COMMITMENT OF ES CELLS UNDER CHEMICALLY DEFINED CONDITIONS TO MESODERM AND HEMATOPOIETIC CELLS, Michael V. Wiles, Basel Institute for Immunology, CH4005 Basel, Switzerland.

Embryonic stem (ES) cells efficiently undergo differentiation *in vitro* to mesoderm and then to hematopoietic precursors (≤ 4 days). Differentiation is rapid, efficient, highly reproducible and appears to recapitulate some aspects of days 6.0 to 8 of mouse development (Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M. V. (1993). MCB.13, 473-86). But for the analysis of growth factors in early commitment this system is flawed as Fetal Calf Serum is present. To overcome this we have developed a *completely* chemically defined medium in which ES cells will differentiate. Contrary to previous studies on ES cell differentiation performed with FCS, added factors now do have an effect. Activin β_A causes a considerably enhancement of mesoderm development over a very narrow concentration range (markers *Brachyury*, EVX1, *Nodal etc*), *in agreement* which similar mesoderm induction experiments on *Xenopus*. In the presence of the basic defined medium hematopoiesis is extremely poor, if however BMP4 (also TGFβ family) is present there is a striking enhancement of hematopoietic development. These data are the first to indicate that the TBFβ family members behave in a similar manner in mesoderm formation as previously reported in non mammals. The development of such an ES based system combined analysis of embryos at the hematopoietic stages is allowing us to dissect the events involved in early commitment, especially in relation to the factors involved in mesoderm formation and hematopoiesis.

A 312 EXPRESSION OF STEM CELL FACTOR BY HUMAN BONE MARROW AND PERIPHERAL BLOOD CELLS, **Alexandra Wodnar-Filipowicz, Michaela Slanicka, Chantal Manz and Catherine Nissen, Department of Research, University Hospital, Basel, Switzerland.**

Stem cell factor (SCF) is a hematopoietic cytokine. Its expression found in bone marrow (BM) stromal cells and its capacity to stimulate early hematopoietic stem cells suggests an important function in maintenance of an optimum hematopoietic microenvironment. SCF exists in two biologically active forms: soluble and membrane-bound encoded by differentially spliced mRNA species. We have measured soluble SCF in plasma of BM aspirates from 20 normal donors. Levels estimated as 1.65 +/- 0.45 ng/ml were not any higher than those reported previously for peripheral blood (PB) serum. This suggests a possibility that PB cells themselves serve as an additional source of SCF, independent of bone marrow stroma. Northern blot analysis revealed expression of SCF mRNA by mononuclear PB cells stimulated with autologous serum. Short-term cultures of T lymphocytes, expanded from PB cells with IL-2, were also able to express this mRNA following cell activation. SCF mRNA species encoding soluble and membrane-bound SCF forms were present in an approximately 1:1 ratio, as shown by PCR analysis. This contrasts sharply with the pattern of SCF mRNAs observed in bone marrow - derived stromal and fibroblast cells expressing predominantly the species encoding a soluble form of the factor. Currently, we are analysing expression of both SCF mRNA forms in response to different modes of cell activation. Our results suggest differences in regulation of SCF expression in the bone marrow and on the periphery, implicating different functions played by SCF in these two compartments.

A 313 CIRCADIAN ORGANIZATION AND RESPONSE OF THE ERYTHRON, **P. Wood, D. Peace, and W. Hrushesky, Stratton V. A. Medical Center and Albany Medical College, Albany, NY 12208.**

Maximizing erythropoietin (EPO) efficacy, minimizing costs and optimizing individual patient response remains a challenge. The number of myeloid precursors in the marrow and their susceptibility to cytotoxic drug injury each vary with time of day. The circadian organization of the erythron is less well defined. We have explored 1) the circadian organization of erythropoiesis in normal CD2F1 female mice and 2) the differential response to circadian timed EPO. Endogenous erythroid activity measured by blood reticulocyte (R) and erythroid precursor cell numbers varied markedly with circadian sampling time. R varied 3 fold throughout the day (p=0.056) with two peaks 12 h apart. Marrow BFU-E (p=0.038) and CFU-E (p<0.001) erythroid colony numbers/femur varied up to 5 fold dependent upon circadian time. Both erythroid colony types showed the identical 12 h rhythm with peaks in early sleep and again in early activity (p<0.05). A similar 12 h rhythm was seen for CFU-E colonies from the spleen. A single dose of subcutaneous EPO (300 U/kg) was administered to animals at one of six times throughout a 24 h span. The R response was highly dependent upon when in the day EPO had been administered (p<0.05). A 350% increase in R occurred when EPO was given in late sleep while no significant rise in R was seen when EPO was given in late activity. The EPO-induced increase in BFU-E and CFU-E colonies in the marrow and spleen, 24 h after EPO, varied up to 12 fold depending upon the time of day of EPO administration (p<0.001). The EPO-induced peak increase in erythroid precursors occurred at the same times of day as the endogenous peaks. The endogenous and EPO-stimulated total body erythroid precursor numbers were likewise higher in early sleep and early activity. This fact strongly supports the idea that actual rhythmic changes in overall erythropoietic activity occur throughout the day and that these patterns are not merely the result of redistribution of erythroid precursor cells. The efficiency of erythroid precursor cell differentiation from early to late committed cell precursors (CFU-E/BFU-E ratio) increased with EPO treatment in the spleen when EPO was given during early sleep (p<0.001) while other times of day showed no effect. Therefore both endogenous and EPO-stimulated erythropoiesis and the efficiency of production of late precursors by EPO are coordinated in circadian time.

A 314 A MEGAKARYOBLASTIC CELL LINE FROM A PATIENT WITH DOWN SYNDROME-ACUTE MEGAKARYOBLASTIC LEUKEMIA (DS-AMKL): IN VITRO DIFFERENTIATION OF MEGAKARYOBLASTS.
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A cell line was established from a patient with DS-AMKL. The primary leukemic cells were classified by electron microscopy as megakaryoblasts by the presence of immunogold-labelled GpIIIa, demarcation membranes and alpha granules. The cell line is growth factor dependent in liquid culture in the presence of Stem Cell Factor (SCF), and proliferates as a pure population of undifferentiated blast cells. The cells have multiple chromosomal abnormalities including monosomy 7, a finding initially present in the megakaryoblasts at diagnosis in vivo. Interphase labelling (FISH) shows that all cells contained only one #7 chromosome. These cells do not express the T-cell antigens CD3 and CD4, the B-cell antigens CD10 and CD19, or monocyte antigen CD14. However they are strongly positive for the stem cell antigen CD34, the monomyeloid antigen CD33 and they also express the megakaryocyte lineage antigens CD41 (GpIIb/IIIa), CD61 (GpIIIa) and CD7 (Pro-T, T-cells and megakaryoblasts). P-selectin (CD62) is not expressed but a few cells express CD42b (GpIba) and all cells express the 8A3 antigen (found on activated platelets). Only a small minority of the cells express glycophorin and SCF. These cells also express genes for the following cytokine receptors: interleukins 1a,2-4,6,7,9,11,G-CSF,TNF-a,SCF. Culture of the cell line with erythropoeitin resulted in the production of normoblasts whose leukemic cell origin was established by the presence of monosomy 7 by FISH. Growth of the primary leukemic cells in SCF and IL3 resulted in the production of basophils/mast cells. These findings prove that the leukemic cells of DS-AMKL originate in pluripotent progenitor cells with the potential of differentiating into erythroid, megakaryocyte and mast cell/basophil lineages.

Signal Transduction and Differentiation

A 400 CONFORMATIONAL STUDIES ON ERYTHROPOIETIN (EPO), THE EXTRACELLULAR ERYTHROPOIETIN-RECEPTOR [EPO-R(t)] AND THE EPO:EPO-R(t) COMPLEX,
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The mechanism of signal transduction of the ligand:receptor interaction is the object of intense research. It has been suggested that a conformational change may be one of the ways to trigger the transduction process. A conformational study of the EPO:EPO-R(t) complex system is reported herein.

EPO-R(t) has been isolated and renatured by using an insoluble EPO-matrix column [R. Betzalel, A. D'Andrea and G.D. Fasman (1993), Protein Society **2**, (Suppl. 1) 62]. The interaction of EPO and EPO-R(t) has been studied by circular dichroism spectroscopy (CD), utilizing both the far UV region (200-250nm, Region A) and the aromatic region (250-330nm, Region B). A change in secondary structure was observed upon forming a 1:1 complex. In Region A, the secondary structure of the complex varied from the average of the two species upon CD deconvolution: Δ α-helix, 1%; Δ β-turns, 5%; Δ random coil, 10%; Δ β-sheet, 7%; and Δ aromatic and disulfide component, 11%. The aromatic region (B) showed significant differences. Differences between the average and observed values were as follows: $\Delta [\theta]_{286}$ = 26 degrees·cm^2/dmol; $\Delta [\theta]_{293}$ = 31 degrees·cm^2/dmol.

Thus it can be concluded that a significant conformational change occurs upon the binding of EPO to EPO-R(t). The conformation of the aromatic region is altered upon complex formation. Transduction of the signal may be brought about by the conformational change discussed in this report.

This research was supported by a grant from Amgen.

A 401 TYROSINE PHOSPHORYLATED SHC ASSOCIATES WITH THE ACTIVATED ERYTHROPOIETIN RECEPTOR IN SOME HEMOPOIETIC CELL LINES BUT NOT IN OTHERS.
Jacqueline E. Damen, Robert L. Cutler, Ling Liu and Gerald Krystal, Terry Fox Laboratory, BC Cancer Agency and University of British Columbia, Vancouver, Canada.
Although the erythropoietin receptor (EpR), like other members of the hemopoietin receptor superfamily, lacks a tyrosine kinase consensus sequence within its proline-rich intracellular domain, addition of its ligand to Ep-responsive cells stimulates the rapid and transient tyrosine phosphorylation of a number of cellular proteins. The characterization of these proteins, which include 5 heavily tyrosine phosphorylated proteins with molecular masses of 145-, 130-, 97-, 72- and 56 Kd is an essential step in understanding the signal transduction pathways utilized by Ep. Recently, we showed that the 72- and 56-Kd proteins are the EpR and recently identified SH2 containing protein, $p52^{shc}$, respectively . In the present study we have investigated whether Shc and the EpR associate following stimulation with Ep. For these studies 1 human (MO7E) and 2 murine (Ba/F3 and DA-3) hemopoietic cell lines were retrovirally infected with the murine EpR and high EpR expressing clones isolated. Ep stimulated the tyrosine phosphorylation of Shc in all 3 cell lines, designated MO7-ER, BA-ER and DA-ER. Moreover, anti-Shc immunoprecipitations revealed that, in its tyrosine phosphorylated state, Shc bound to the major 145 Kd protein that was also tyrosine phosphorylated in these 3 cell lines in response to Ep. Interestingly, the tyrosine phosphorylated EpR was also found to be physically associated with the tyrosine phosphorylated form of Shc, but this apparently took place only in MO7-ER and BA-ER cells. We are currently testing the hypothesis that the activated EpR in DA-ER cells contains a different pattern of tyrosine phosphorylation from that present in the other 2 cell lines, perhaps reflecting a different EpR associated tyrosine kinase, and that this difference is responsible for the lack of interaction between Shc and the EpR in these cells.

A 402 A B-CELL SPECIFIC IMMEDIATE EARLY HUMAN GENE IS LOCATED ON CHROMOSOME BAND 1q31 AND ENCODES AN α HELICAL BASIC PHOSPHOPROTEIN

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To identify potentially novel genes involved in haemopoietic differentiation we previously isolated a panel of cDNA clones representing early response genes (ERGs) from phorbol ester-stimulated human B cells. One such cDNA, designated *1R20* was found to be expressed almost exclusively in cells of the B cell lineage. In addition to phorbol ester, several other agents known to activate B cells such as alpha interferon, anti-immunoglobulin and interleukin-4 also induce the transient expression of *1R20*. Whilst expression of *1R20* in normal B cells and in chronic lymphocytic leukaemia B cells is relatively low, in some other types of malignant B cell such as non-Hodgkin's lymphoma (NHL) and hairy cell leukaemia (HCL) expression of *1R20* is constitutively high and poorly inducible through activators of the protein kinase C pathway. These observations suggest that, as a candidate signalling molecule, 1R20-encoded protein may be involved in the uncoupling of cell growth and differentiation associated with these types of malignant B cell. The cDNA sequence predicts a 196 amino acid open reading frame containing numerous highly basic residues together with several potential alpha helical domains and a canonical DNA binding domain. In addition to a single potential N-glycosylation site there are eight candidate phosphorylation sites suggesting that 1R20 may exist as a phosphoprotein. We have mapped the *1R20* gene locus to the long arm of chromosome 1 at band q31, a region that has been reported to be involved in various haemopoietic malignancies. In addition we have screened a number of transcriptional regulatory elements whose activities are affected by overexpression of 1R20 in DNA transfection assays. The results of these experiments will be presented.

A 403 IL-4 ACTIVATES Raf-1 AND MITOGEN-ACTIVATED PROTEIN KINASE IN A FACTOR-DEPENDENT MAST CELL LINE,

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A number of studies have shown that the Raf-1 and Mitogen-activated protein kinase (MAP kinase) genes may be required for the proliferation and differentiation of hematopoietic cells. In particular, *in vitro* immune complex kinase assays using synthetic peptide and MBP as substrate respectively, have shown that a variety of hematopoietic growth factors, including IL-2, IL-3, GM-CSF, and SCF activate Raf-1 and MAP kinase activities. Interestingly, it has been suggested that IL-4 uses a different signaling pathway. We analyzed the signaling pathways for IL-3 and IL-4 in a growth factor-dependent mast cell line. First, immunoblotting analysis with total lysates revealed that IL-4 like IL-3 induced a shift in mobility of p74 Raf-1 protein suggesting the induction of phosphorylation forms of p74 Raf-1; similarly IL-3 induced a shift in mobility of p42 MAP-kinase, although the shift induced by IL-4 was barely detectable. We are therefore analyzing the enzymatic activities of these two serine/threonine kinases after IL-4 and IL-3 stimulation using *in vitro* assays with Mitogen Kinase Kinase (MKK), the natural substrate for Raf-1 kinase and MBP as substrate for MAP kinase activity.

A 404 BCR-ABL-INDUCED ONCOGENESIS IS MEDIATED BY DIRECT INTERACTION WITH THE SH2 DOMAIN OF THE GRB-2 ADAPTOR PROTEIN.

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BCR-ABL is a chimeric oncoprotein that exhibits deregulated tyrosine kinase activity and is implicated in the pathogenesis of Philadelphia chromosome (Ph1)-positive human leukemias. Sequences within the first exon of BCR are required to activate the transforming potential of BCR-ABL. The SH2/SH3 domain-containing GRB-2 protein links tyrosine kinases to Ras signaling. We demonstrate that BCR-ABL exists in a complex with GRB-2 in vivo. Binding of GRB-2 to BCR-ABL is mediated by the direct interaction of the GRB-2 SH2 domain with a phosphorylated tyrosine, Y177, within the BCR first exon. The BCR-ABL-GRB-2 interaction is required for activation of the Ras signaling pathway. Mutation of Y177 to phenylalanine (Y177F) abolishes GRB-2 binding and abrogates BCR-ABL-induced Ras activation. The BCR-ABL (Y177F) mutant is unable to transform primary bone marrow cultures and is impaired in its ability to transform Rat1 fibroblasts. These findings implicate activation of Ras function as an important and demonstrate that GRB-2 not only functions in normal development and mitogenesis but also plays a role in oncogenesis.

A 405 RESISTANCE TO PHORBOL ESTER-INDUCED DIFFERENTIATION OF A U-937 MYELOID LEUKEMIA CELL VARIANT WITH A SIGNALING DEFECT UPSTREAM TO RAF-1 KINASE

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Previous studies have demonstrated that treatment of human U-937 myeloid leukemia cells with 12-0-tetradecanoylphorbol-13-acetate (TPA) is associated with growth arrest and induction of monocytic differentiation. The present work describes the isolation of a U-937 cell variant, designated TUR, which is unresponsive to the growth inhibitory effects of this agent. The results demonstrate that, in contrast to U-937 cells, the TUR line fails to respond to TPA with induction of the c-jun, jun-B, c-fos and EGR-1 early response genes. The finding that these cells also fail to exhibit adherence or induction of the tumor necrosis factor and c-fms genes, further supports their resistance to TPA-induced differentiation. In contrast, TUR cells responded to 1,25-dihydroxyvitamin D3, another inducer of monocytic differentiation, with growth arrest and induction of early response gene and c-fms transcripts. TUR cells also responded to okadaic acid, an inhibitor of type 1 and 2A protein phosphatases, with similar changes in gene expression. Further characterization of TUR cells has demonstrated decreased expression of protein kinase Cα and β as compared to wild type U-937 cells. We also demonstrate that while treatment of U-937 cells with TPA is associated with activation of the Raf-1 serine/threonine kinase, there was no detectable decrease in electrophoretic mobility of this protein in TPA-treated TUR cells. Taken together, these findings indicate that the TUR variant is defective in TPA-induced signaling events upstream to activation of Raf-1 kinase.

A 406 MUTAGENESIS STUDIES OF AN INTERLEUKIN-2 β HOMOLOGY DOMAIN OF THE ERYTHROPOIETIN RECEPTOR. Craig J. Hilton and Michael V. Berridge, Malaghan Institute of Medical Research, Wellington School of Medicine, Wellington, New Zealand.

The erythropoietin (Epo) receptor has no tyrosine kinase activity or other signalling motif. Despite cloning of the receptor, its mechanism of action remains unclear. We have constructed seven deletion mutant cDNAs from the cloned murine Epo receptor cDNA. These constructs, cloned into the mammalian-expression vector pXM, were co-transfected, with pSV2-*neo*, into the interleukin-3 (IL3)-dependent cell line Ba/F3. G418 resistant transfectants were then tested for Epo dependence.

A cytoplasmic domain of the Epo receptor (amino acids 316-342) shares 50% homology with the interleukin-2 (IL2) β receptor cytoplasmic domain which is essential for IL2 signal transduction (Hatakeyama Cell **59**; 837-845, 1989). Three of the Epo-receptor mutants include all or part of this homology domain while the other mutants flank this region. Deletion of amino acids (316-326) corresponding to the N-terminal part of the IL2β receptor homology domain showed no difference in growth response to Epo from that of cells expressing the wild-type receptor. In contrast, deletion of amino acids 326-336 resulted in decreased proliferative response to Epo. Deletion of the entire IL2β receptor homology domain yielded cells that failed to grow in response to Epo. When amino acids 341-371 or amino acids 358-388, just distal to the IL2β homology domain, were deleted, no change in the Epo growth response was detected. However when amino acids 288-318, just proximal to the IL2β receptor homology domain were deleted, cells showed no growth response to Epo.

Together these results suggest that the C-terminal end (amino acids 336-342) of the IL2β homology domain is essential for Epo signal transduction and that a region N-terminal to the homology domain (amino acids 288-318) is also important for Epo signal transduction.

A 407 SPY75 WITH REPETITIVE SEQUENCES AND AN SH3 DOMAIN BECOMES TYROSINE-PHOSPHORYLATED IN FcεRI-ACTIVATED MAST CELLS, Toshiaki Kawakami, Hiromi Fukamachi, Yuko Kawakami, Libo Yao, and Toru Miura, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

Cross-linking of the high-affinity receptor for IgE (FcεRI) initiates a variety of biochemical and morphological changes leading to degranulation, and synthesis and release of cytokines and lipid mediators. Tyrosine phosphorylation of cellular proteins, including FcεRI β and γ subunits, phospholipase C-γ1, a protooncoprotein $p95^{vav}$, and an SH2- and SH3-containing protein Nck, was previously reported as the earliest signaling event for the FcεRI signal transduction pathway. By amino acid sequence determination and cDNA cloning analysis, a 75 kDa protein, termed SPY75, was identified as a major tyrosine-phosphorylated protein in activated mouse mast cells. SPY75, barely tyrosine phosphorylated in resting cells, was rapidly and transiently tyrosine phosphorylated upon FcεRI cross-linking in an antigen concentration-dependent manner. Similar SPY75 tyrosine phosphorylation was observed when antigen receptors on B and T lymphocytes were cross-linked by appropriate antibodies. However, SPY75 was not tyrosine-phosphorylated by stimulation with growth factors such as IL-3, GM-CSF, or SCF. SPY75 was not physically associated with the receptor or other known signaling molecules. This protein, the mouse homologue of the human *HS1* gene product, has putative repetitive helix-turn-helix motifs found in many DNA-binding proteins and a putative nuclear transport signal. It also has a Src homology 3 (SH3) domain which is found in many signaling molecules and cytoskeletal proteins. These structural features and the rapid tyrosine phosphorylation upon FcεRI cross-linking suggest that the signal generated by FcεRI cross-linking is transmitted through tyrosine phosphorylation of SPY75.

A 408 IL-3 STIMULATES THE TYROSINE PHOSPHORYLATION OF SHC AND ITS ASSOCIATION WITH GRB2 AND A 145 kDa TYROSINE PHOSPHORYLATED PROTEIN. L. Liu, R. Cutler, J. Damen, and G. Krystal. Terry Fox Laboratory, BC Cancer Agency and University of British Columbia, Vancouver, Canada.

The signaling transduction pathway from growth factor receptor to activation of Ras has recently been elucidated for the EGFR in fibroblasts. It has been shown that two newly identified SH2 containing proteins, Shc and Grb2, play important roles in this process. To examine whether hemopoietic cytokines trigger the same signaling pathway in the proliferation of hemopoietic cells we have stimulated various hemopoietic cell lines with IL-3, SF or Ep and found that they all induced tyrosine phosphorylation of Shc and its association, in its tyrosine phosphorylation state, with the 23-kDa protein, Grb2. In the current study we have further investigated the proteins that become associated with the tyrosine phosphorylated form of Shc in the human and mouse IL-3 dependent cell lines, TF1 and $B6SUtA_1$, respectively. Upon IL-3 stimulation, we found, using anti-Shc antibody immunoprecipitation, that Shc becomes associated with a 145 kDa tyrosine phosphorylated protein in both cell lines. Western blot analysis with anti-IL-3Rβ antibodies showed that this 145 kDa protein was not the IL-3Rβ chain. Futhermore, preclearing the cell lysates with anti-IL-3Rβ antibodies prior to anti-Shc immunoprecipitation did not diminish the amount of p145 co-precipitating with Shc. These results suggest that p145 is not the IL-3Rβ. Moreover, unlike the association of tyrosine phosphorylated Shc with the EGFR in fibroblast cells, we could not a detect a physical association between phosphorylated Shc and the IL-3R in our 2 cell lines. We are currently characterizing the Shc associated 145 kDa protein and investigating the possible association of Grb2 with Sos in hemopoietic cells.

A 409 INVOLVEMENT OF SHPTP-1 IN T CELL SIGNALING

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The protein tyrosine phosphatase SHPTP-1 is mainly expressed in hematopoeitic cells. Indirect immunofluorescence studies of endogenous SHPTP-1 in murine and human hematopoeitic cells reveal that this protein is cytoplasmic. Immunoprecipitations identify SHPTP-1 as a 65 kd phosphoprotein. Phosphoamino acid analyses show SHPTP-1 being basally phosphorylated on serine in unstimulated hematopoeitic cells. However in the lymphoma cell line LSTRA, which overexpresses Lck, SHPTP-1 is constitutively phosphorylated on tyrosine. Stimulation of murine T cells and thymocytes by crosslinking of CD4 or CD8 results in increased serine phosphorylation as well as additional tyrosine phosphorylation of SHPTP-1. This crosslinking signals through activation of Lck. *In vitro* kinase assays show that SHPTP-1 is an *in vitro* substrate for Lck. We therefore mapped the tyrosine phosphorylation sites of SHPTP-1 in these three systems; LSTRA cells, stimulated T cells, and *in vitro* phosphorylated by Lck. Tryptic peptide maps show that the same two sites are phosphorylated *in vitro* as well as *in vivo* in LSTRA cells. One of the two sites is targeted in stimulated T cells. This suggests that SHPTP-1 is a direct substrate for Lck in T cells. Deletion and mutation studies localize the phospho-tyrosines to the C-terminus. The effect of tyrosine phosphorylation of SHPTP1 on its activity as well as on its ability to associate with other proteins will be discussed.

It was recently shown (Shultz et al., Cell 73, 1993, Tsui et al., Nature Genetics 4, 1993) that motheaten mice lack SHPTP-1 expression. These mice have defects in a variety of hematopoeitic cell lineages, one of which is impaired lymphocyte development and function. Studies are in progress to analyze signaling in thymocytes and mature T cells isolated from motheaten mice. Comparison of signaling in mutant and normal cells together with *in vitro* studies using different cell lines will help in understanding the role of SHPTP-1 in T cell signaling.

A 410 ACTIVATION OF THE AXL RECEPTOR TYROSINE KINASE INDUCES MITOGENESIS AND TRANSFORMATION IN 32d CELLS.

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axl is a receptor tyrosine kinase isolated by a transfection/tumorigenicity assay using DNA taken from patients with chronic myelogenous leukemia. Association of *axl* expression with myelogenous leukemias and its expression in primitive hamaetopoietic cells suggests a role for *axl* in myeloid biology. To study the cellular function of *axl* we constructed a chimeric receptor tyrosine kinase composed of the extracellular and transmembrane domains of the EGF receptor and the cytoplasmic domain of *axl*. This chimeric receptor tyrosine kinase was expressed in the mouse myeloid progenitor cell line 32D which is absolutely dependent on interleukin-3 for its proliferation and survival. Activation of the chimeric receptor by EGF stimulated the tyrosine kinase activity of *axl* 5X and enabled proliferation through EGF rather than IL-3. Morever, exposure of cells expressing the chimeric receptor to EGF for 14-21 days converted the cells to total factor independent growth. The phosphorylation level of the *axl* in the factor independent cells is 40X greater than the factor dependent cells. This suggests that there are threshold phosphorylation levels of the *axl* kinase for both mitogenesis and transformation. Thus, *axl* can transduce a proliferative signal in myeloid cells and also participate in conversion of these cells to factor independent growth. The ability of *axl* to induce factor independent growth of 32D cells suggests that *axl* can play a role in conversion of myeloid cells to leukemic growth.

Cell Line and Growth Characteristics	Relative Phosphorylation Level of Axl	Factor Independent Growth
32D-EAK + IL-3	1X	No
32D-EAK +EGF	5X	No
32D-EAK + EGF (14 days)	40X	Yes

A 411 C-RAF ANTISENSE OLIGONUCLEOTIDE INHIBITION OF MURINE AND HUMAN HEMATOPOIETIC CELL GROWTH.

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Using antisense oligonucleotides, we have established that Raf-1 is required for the colony stimulating factor (CSF)-induced proliferation of normal murine bone marrow cells and progenitor cell lines stimulated with IL-2, IL-3, CSF-1, G-CSF, GM-CSF, EPO and SCF. We have also demonstrated that Raf-1 is required for IL-4-induced proliferation of FDCP-1 cells. However, previous results indicated that growth factor stimulation of the IL-4 receptor did not activate Raf-1 kinase activity. To resolve this discrepancy, Raf-1 phosphorylation in response to IL-4 was evaluated in an IL-4-dependent FDCP-1 cell line. Results of a mobility shift assay indicated that, consistent with the antisense results, Raf-1 was phosphorylated in response to IL-4. However, in comparison to the IL-3 response, which peaks between 10 and 15 minutes, Raf-1 phosphorylation in response to IL-4 was barely detectable 15 minutes after stimulation and was maximal at 20-30 minutes. The antisense studies have been extended to include another hematopoietic receptor system which includes the receptors for IL-6, LIF and oncostatin M. C-raf antisense oligonucleotide treatment completely inhibits factor-dependent proliferation of DA-1A cells in response to IL-6, LIF and oncostatin M, demonstrating that Raf-1 is also a component of signal transduction pathways activated by growth factor stimulation of receptors associated with gp 130. Finally, c-raf antisense treatment of human bone marrow cells inhibits IL-3-and GM-CSF-induced colony formation of both unseparated and CD34+ purified bone marrow cells. Thus, c-raf expression is required for the proliferation of murine hematopoietic cells in response to growth factor stimulation of multiple classes of hematopoietic receptors and is also required for CSF-induced colony formation by human bone marrow cells.

A 412 **REGULATED EXPRESSSION OF THE Id3 HLH TRANSCRIPTION FACTOR GENE IN HEMATOPOIESIS,**

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Transcription factors characterised by the presence of the helix-loop-helix (HLH) domain play a central role in the regulation of cell growth/differentiation and tumourigenesis. A functionally distinct subset of HLH proteins exemplified by the prototype, Id1, appears to act as "dominant negative" regulators of cell differentiation by forming non-functional hetero-dimers with other, basic HLH transcription factors. We previously isolated a cDNA clone encoding human Id3 (1R21/462/HEIR1) as an immediate early response gene from phorbol ester activated B cells. The Id3 protein shares similar functional properties to Id1 in it's ability to inhibit binding of E2-A HLH proteins to a concensus "E" box recognition sequence *in vitro*. Characterisation of the Id3 gene structure reveals an overall organisation very similar to that of Id1, suggestive of an origin through duplication from a single ancestral Id HLH gene. Although Id3 is expressed in cell types of diverse lineages, either constitutively or following phorbol ester stimulation, within haemopoietic cells its expression appears to be largely restricted to the lymphopoietic compartment. In this respect, Id3 expression appears mutually exclusive to that of its close relative, Id1 and since Id1 is known to function as a negative regulator of myelopoiesis, at least *in vitro*, these observations would be consistent with a role for Id3 as a negative regulator of lymphopoiesis.

A 413 **A Novel Signal Transduction Pathway Utilized by GM-CSF**

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Recent discoveries have elucidated the mechanism through which interferon signal transduction operates. GM-CSF appears to use a similar mechanism. We have demonstrated that GM-CSF treatment of cells rapidly induces formation of a complex that binds to a defined DNA element. This complex contains proteins phosphorylated at tyrosine residues and DNA binding is inhibited by dephosphorylation. Despite these mechanistic similarities, none of the proteins in the GM-CSF complex are identical to those activated by the interferons. Furthermore, the complex induced by GM-CSF can be distinguished from the interferon-induced complex on the basis of mobility in polyacrylamide gels, sequence preferences, and sensitivity to NaCl. Further characterization of this GM-CSF signaling process will be presented.

A 414 MOLECULAR AND BIOCHEMICAL STUDIES OF OLEOSINS FROM SEEDS AND POLLEN OF HIGHER PLANTS, Joanne H.E. Ross, Ceri Batchelder, James S. Keddie, Michael R. Roberts, Rod J. Scott and Denis J. Murphy, Department of Brassica & Oilseeds Research, John Innes Centre, Norwich, NR4 7UH, United Kingdom

Oleosins are oil-body membrane proteins previously believed to be located only in the lipid storing tissues of seeds. It has recently been found that a new class of oleosins occurs in the megagametophytic generation of crucifers, where they are found associated with storage oil bodies of mature pollen grains. Sequence analysis suggests that these pollen-specific oleosins comprise a discrete group from, but share a similar origin to the seed-specific oleosin proteins.

The occurrence of oleosins in both sporophytic and gametophytic generations raises questions about the evolution of seed and pollen in higher plants. We are investigating the presence or absence of oleosin-like proteins in the spores of primitive plants, which have similar roles to both pollen and seeds. The functional similarity of seeds and pollen is reflected in their biochemical and structural similarity in crucifers. Both are organs sequestering storage lipids for mobilisation by the rapidly developing seedling/pollen tube. Also, both are capable of withstanding desiccation. This function has recently been correlated with the presence of oleosin in studies with fruits of olives and avocado. Although these fruits store large amounts of lipid, they do not contain detectable oleosin and neither are they able to withstand desiccation.

The regulation of seed oleosin gene expression has been studied using transformation of tobacco plants with a full length *B.napus* oleosin gene driven by 1kbp of its own promoter. The *Brassica* oleosin was correctly targeted to oil bodies in transgenic tobacco plants, as revealed by immunogold labelling. The gene also exhibited a temporal and tissue-specific expression similar to that observed in normal rapeseed development. In contrast, tobacco plants expressing an antisense oleosin construct exhibited gross abnormalities in oil body morphology but no other phenotypic changes.

A 415 GENE TARGETING OF THE MOUSE bHLH PROTEIN LYL-1 AND ITS EXPRESSION DURING MOUSE DEVELOPMENT, Elisabeth Rüpping, Matthias Serwe und Fred Sablitzky, Max-Delbrück-Laboratorium in der MPG, Carl von Linné-Weg 10, 50829 Köln, Germany

Lyl-1 is a member of the basic helix-loop-helix (bHLH) transcription factor protein family, which is expressed in haematopoietic cells of the myeloid and B cell lineage, but not in T cells (1,2,3). However, in a particular type of T cell acute lymphoblastic leukemia (T-ALL), a chromosomal translocation [t(7;19)] leads to a deregulated expression of the lyl-1 gene resulting in the formation of tumor cells.

In analogy to other cell type specific bHLH proteins, like MyoD, myf 5 and myogenin or achaete scute which are crucial for the development of muscle or neuronal cells, respectively, lyl-1 could be involved in the regulation of cell differentiation in the haematopoietic system.

Using the gene targeting approach, we replaced the 3' end of the lyl-1 gene (including its HLH domain) by a lacZ/Neo reporter gene which was introduced in-frame into the fourth exon of the lyl-1 locus. Lacking the HLH domain, the resulting lyl-1/lacZ fusion protein will not be able to form homo- or heterodimers, most likely resulting in loss of function of the lyl-1 protein. In addition, the lyl-1/lacZ fusion protein allowed us to establish the expression pattern of the lyl-1 gene during mouse development (day 8.5 to 16.5 p. c.) by X-Gal staining of whole mount embryos and/or sections of them.

- Heterozygous animals obtained from three independent mutant ES cell lines do not show any obvious phenotype.
- Expression of the lyl-1/lacZ fusion gene was first detected at day 9.5 p.c. in endothelial cells of blood vessels (aortic arch arteries and the arteries in the umbilical cord).
- From day 10.5 p.c. on, cells expressing the lyl-1/lacZ fusion gene develop within the fetal liver which morphologically look like pro-myelocytes, granulocytes and megacaryocytes.
- A third, lyl-1/lacZ expressing location could be detected within the epidermis of the embryos.
- X-Gal staining was not detected in cells of the fetal blood
- Preliminary results obtained from adult heterozygous mice indicate that granulocytes and/or megacaryocytes in the bone marrow express lyl-1/lacZ fusion gene whereas thymocytes, spleen cells and peripheral blood cells don' t express it.
- Homozygous offspring was obtained and is currently analysed.

A 416 CLONING OF *HTK,* A NOVEL HUMAN TRANSMEMBRANE TYROSINE KINASE WIDELY EXPRESSED IN HEMATOPOIETIC AND OTHER TISSUES. Scadden DT[1], Bennett BD[2], Wang Z[1], Wang A[1], Groopman JE[1], Goeddel DV[2]. [1]New England Deaconess Hospital, Harvard Medical School, Boston, MA, [2]Genentech, Inc., South San Francisco, CA

A polymerase chain reaction (PCR)-based strategy was used to identify a novel, human transmembrane tyrosine kinase cloned from a hepatocellular carcinoma cell line which shares amino acid similarity with *ELK, ECK, EPH* and *HEK*. This kinase, hepatoma transmembrane kinase or *HTK*, is located on human chromosome 7. It is expressed in the primary adult tissues kidney, pancreas, liver, lung and skeletal muscle and abundantly in placenta. Of note, *HTK* is expressed in the fetal human brain, but not in the adult. A number of malignant solid tissue cell lines expressed the *HTK* transcript. Among hematopoietic lineages, permanent cell lines of monocytic, megakaryocytic and granulocytic origin expressed *HTK*, while T- and B-lymphoid cells did not. Immunoselected CD34+ bone marrow cells had detectable *HTK* message by reverse transcription polymerase chain reaction (RT PCR). The immunoprecipitated translated product of the 3967bp *HTK* cDNA is approximately 120kD, primarily localized in the cell membrane and autophosphylated on tyrosine. The novel transmembrane tyrosine kinase, *HTK*, expressed on a range of epithelial and hematopoietic cells, appears to be differentially expressed in the developing brain and in hematopoietic elements, and may function as a receptor kinase in the transduction of cell regulatory signals.

A 417 TYROSINE PHOSPHORYLATION AND ACTIVATION OF TEC KINASE COUPLE TO STEM CELL FACTOR-INDUCED SIGNALING THROUGH C-KIT, Bo Tang and James N. Ihle, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38101

The effects of Tec kinase on the signal transduction pathway elicited by interaction of SCF and c-kit in myeloid cell line were studied. By using a growth factor-dependent human myeloid cell lines, MO7E, we demonstrated that Tec kinase became rapidly tyrosine phosphorylated following SCF stimulation, and SCF stimulation induced significant kinase activity of Tec *in vitro* and *in vivo* in MO7E cells. Furthermore, our results indicated that Tec kinase associated constantly with c-kit as measured by co-immunoprecipitation assay using specific antibody to c-kit, indicating that Tec kinase can couple directly to SCF-induced signaling through c-kit. Tec kinase does not seems to participate various signal transduction mediated by a variety of hemopoietic growth factor receptors including receptors for Epo, IL-2, IL-3, IL-4, G-CSF, GM-CSF, as well as IgE receptor cross-linking by its ligand. The signaling processes in which Tec is involved is clearly different from that of CSF-1 receptor which is a member of type III receptor tyrosine kinase family with closely structural relationship with c-kit, suggesting that Tec participates uniquely in signaling events elicited by interaction of c-kit and SCF, which regulates development and differentiation of stem cells and early progenitor cells in hemopoiesis.

A 418 MOLECULAR CLONING OF MGF AND ITS IDENTIFICATION AS PROLACTIN RESPONSE ELEMENT BINDING PROTEIN

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The expression of the milk protein ß-casein gene is under the control of lactogenic hormones, such as insulin, glucocorticoids and prolactin. Our previous results show that these hormones are necessary for the synthesis of ß-casein mRNA in vitro. A DNA fragment retaining the competence to respond to these hormones is isolated from the rat ß-casein promoter. This isolated fragment contains a highly conserved sequence amongst casein gene family. To this sequence binds a trans-acting factor.

The activity of this factor is regulated environmentally and physiologically during the gestation, lactation and post-lactation. This factor is shown to be indispensable for the responsiveness of the ß-casein gene promoter to the lactogenic hormones, and seems to be a mammary gland specific factor(MGF).

In order to investigate multi-hormonal signalling pathway(s) through which these hormones control the ß-casein gene expression via MGF, we have purified this factor from lactating sheep mammary glands by a combination of classical columns and a sequence specific DNA affinity column chromatography. There were two major bands of 84 and 92 kd on SDS gel after a sequence specific DNA affinity column chromatography.

The purified proteins were subjected separately to amino acid micro sequencing analysis. The peptide digestion chart from the two proteins was very close to each other, suggesting that these proteins are highly related. A generated peptide common to both proteins was used to screen a cDNA library of lactating sheep mammary gland.

The deduced amino acid sequence from the cDNA revealed that this factor(MGF) does not belong to any other known protein families except one. Although MGF shares certain homology with this protein, the DNA binding motif of this known protein is absent in MGF. In fact, we do not know yet the DNA binding motif of MGF.

To confirm this cDNA as an authentic MGF clone, we have used it to transfect into COS 7 cells. It has been shown that indeed the transfected COS 7 cells have potential to cause a sequence specific DNA binding upon the ligand binding. Moreover, we have obtained evidences that this factor transactivated a reporter gene in prolactin dependent manner, suggesting that it is a prolactin responsive element binding protein.

A 419 ASSOCIATION OF p85/PI3-K WITH THE EPO RECEPTOR WITHIN THE EXTENDED BOX-2 DOMAIN, AND POSSIBLE ROLES FOR JAK-2 IN PI3-K ACTIVATION, D. M. Wojchowski and T.-C. He, Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802

Using an active, HAI epitope-tagged form of the murine erythropoietin receptor (H-ER) and via direct co-immunoprecipitation, the p85 regulatory subunit of phosphatidyl inositol-3 kinase (p85/PI3-K) is shown to associate with the EPO receptor in transfected FDC-P1 cells (i.e., FDC-HER cells). Co-immunoprecipitation of p85 with the EPO receptor was observed initially in ^{32}P-labeled FDC-HER cells, and association of these factors was confirmed by Western analyses of immune precipitates using p85 antiserum. Interestingly, this association was constitutive, and levels of EPO receptor-associated p85 (and levels of p85 phosphorylation) were not affected following exposure of FDC-HER cells to EPO. However, EPO did stimulate the rapid formation of phosphatidyl inositol ^{32}P-phosphate in FDC-HER, and FDC-ER cell lines. Receptor domains for p85 association were mapped through baculovirus-mediated expression of p85 and EPO receptor forms in SF9 cells. Using HAI-tagged receptor forms with deletions and truncations in cytosolic domains, a putative subdomain for p85 association was delineated to an essential extended box-2 region (including a putative SH2 binding motif, Y^{343}LVL). The observed association of p85(PI3K) with the EPO receptor begins to align this receptor mechanistically with PTK encoding growth factors receptors, and extends interest in the identity of possibly common activation mechanisms. Results of *in vitro* assays of possible modulation of PI3-K by the EPO receptor-associated PTK, JAK2, also will be presented.

Gene Transfer and Expression; Clinical

A 500 ADENO-ASSOCIATED VIRUS: A VECTOR FOR TRANSDUCTION OF PRIMARY CELLS.

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Adeno-Associated Virus (AAV) is a single stranded DNA parvovirus that is endogenous to humans yet has never been associated with any disease. Recombinant adeno associated virus (rAAV) genomes can be engineered to carry approximately 4300 bases of DNA when the internal replication and packaging genes are removed. These recombinant genomes can be packaged into viable transducing viral vectors that can infect a variety of cell types. These rAAV vectors will be used to transduce primary tumor cells for tumor vaccine studies and used to deliver therapeutic genes to T cells and hematopoietic stem cells. We have constructed a number of rAAV vectors which carry one or two inserted genes under the control of different promoters, with and without introns. In particular, we have examined human IL-2 and CAT gene expression under the control of the CMV promoter with and without a hybrid intron. The ability of these rAAV viral vectors to infect primary human CD8$^+$ and primary human CD34$^+$ cells as well as human cell lines and mouse hematopoietic stem cells will be presented. The duration of the transduced gene expression will also be examined. Data using the same rAAV genomes with lipofection of primary tumors will also be presented.

A 501 TRISOMY 8 AND BCR GENE REARRANGEMENT IN MALIGNANT EOSINOPHILIA, L.C. Chan[1], Y.L. Kwong[2], A.K.W. Lie[2], L.M. Ching[1], Haematology Section, Department of Pathology[1], Department of Medicine[2], University of Hong Kong, HONG KONG

Malignant proliferation of eosinophils is recognised as a separate disease entity within the group of disorders classified under idiopathic hypereosinophilic syndrome, HES 'Eosinophilic leukaemia' and 'eosinophilic form of myeloproliferative or myelodysplastic syndrome' have been described and both Philadephilia (Ph) positive and Ph negative cases have been reported.

Case report: A 32 year old male presented with symptoms of anaemia and low grade fever in August 1991. A slight eosinophilia (6% of a total white cell count of 10.0×10^9/l) was noted. The patient was followed up with no specific treatment. In August 92, he was readmitted with fever lower limb edema, hepatomegaly of 10 cm and a splenomegaly of 8 cm. The full blood count showed Hb 9.6g/dl, white cell count of $70\% \times 10^9$/l (52% eosinophils with many vacuolated forms, basophils 5%, blast 2%) and a platelet count of 45×10^9/l. The bone marrow was hypercellular with plentiful eosinophilic myelocytes and eosinophils. There was no relevant drug history and screen for parasites was negative. Cytogenetic analysis on bone marrow revealed the following karyotype:46,XY[2]/48,XY,+der(1)dic(1;12)(p11;p12?p13),+8,-12,+17[8]. Hybridisation of a Southern blot of bone marrow to a probe of the major breakpoint cluster region of the BCR gene showed rearrangement in Bam HI and Bgl II digests. Although a diagnosis of eosinophilic leukaemia can be made, definitive proof that the abnormal karyotype was present in the eosinophils was shown by fluorescent in situ hybridisation studies. Using a biotin labelled chromosome 8 specific α satellite probe, trisomy 8 was detected in the eosinophils.

Discussion: Although trisomy 8 have been reported in malignant eosinophils, the mechanism in our case and other cases of eosinophilic leukaemia is unknown. Cytokines which are known to affect proliferation of eosinophil progenitors and its differentiation include IL3, IL5 and GM-CSF. The ability of p210 BCR-ABL to abrogate factor dependence of IL3 in a haemotopoietic cell lines suggests that this may be a critical event in leukaemic transformation in cases of eosinophilic leukaemia where the Ph chromosome is present.

A 502 COMPARISON OF THE IMMUNE FUNCTION AND PHENOTYPE OF PERIPHERAL BLOOD AND BONE MARROW STEM CELL PRODUCTS AND THE PERIPHERAL BLOOD RECONSTITUTION, Cathy Gordy[1], Greg Perry[2], Michelle Thomas[1], Elizabeth Reed[3], Mark Arneson[3] and James E. Talmadge[1]. [1]Departments of Pathology/Microbiology, [2]Cell Biology/Anatomy, and [3]Internal Medicine, University of Nebraska Medical Center Omaha, NE 68198

We examined the immune function and phenotype of peripheral blood stem cell (PBSC) and bone marrow transplant (BMT) products and the blood of patients with NHL and breast cancer prior to and following transplant. In these studies we observed that the mitogen response of peripheral blood leukocytes (PBL) from patients prior to mobilization was depressed approximately 50% compared to normal samples. Further, the mitogenic response of cells from GM-CSF mobilized PBSC products, BM cells and PBLs of patients post transplant was significantly depressed to levels ~80% below that of normal samples. A comparison of frozen to fresh PBSC demonstrated that freezing was not the source of the loss of immune function. Additional studies revealed that the mitogenic response of PBSC compared to PBL obtained by venipuncture at the time of aphoresis was significantly reduced suggesting that aphoresis was in part responsible for the loss of the mitogen response. PBL at 15, 30 and 100 days post transplant also had a depressed mitogenic function. In contrast natural killer cell activity returned to normal levels by 30 days post transplantation. Prior to mobilization with GM-CSF and aphoresis, the PBL demonstrated little natural suppressor (NS) activity. However, there was an increase in NS activity in PBL's during mobilization, as well as, in frozen and unfrozen PBSC and BMT products. In addition high levels of NS activity was observed in the PBL at least 100 days post transplant. These data suggest the GM-CSF mobilization and aphoresis may up regulate NS activity and thus have a role in the immune dysfunction that occurs post transplantation. In addition to functional abnormalities in the stem cell products and PBL's post transplantation there are substantial phenotypic abnormalities. Both the stem cell products and the PBL's following transplant have a CD4:CD8 inversion. In agreement with the increase in NS activity we observed an increase in TCRα/β+CD4-CD8- cells which are the morphologic homologue of NS activity. In summary, these data suggest that NS cells selected for and or increased by GM-CSF mobilization and aphoresis may have a role in the immune dysfunction observed following induction chemotherapy and transplantation. Supported in part by the Nebraska Cancer and Smoking Disease Research Program.

A 503 THE USE OF IN VITRO CULTURE ASSAYS TO PREDICT IN VIVO RESPONSE TO VARIOUS THERAPEUTIC MODALITIES IN A PATIENT WITH DIAMOND-BLACKFAN ANEMIA. Megason G.C., Moulder S.L, and Pullen D.J. University of Mississippi Medical Center, Jackson, MS 39216

Diamond-Blackfan Anemia is a constitutional pure red blood cell anemia and probably a heterogeneous disorder. Numerous therapies have been proposed including suppression of cell mediated immunity, suppression of humoral immunity, and treatment with growth factors including erythropoietin (EPO) and IL-3. In our newly diagnosed patient who had two HLA-identical siblings, we attempted to use in vitro BFU-E assays to try and determine whether other therapy should be pursued prior to bone marrow transplantation (BMT). Peripheral blood was collected from the patient and two controls. Peripheral mononuclear cells from the patient and controls were placed in media with methylcellulose and erythropoietin at a concentration of approximately 10 cells per ml or dish. The assays were set up in triplicate groups consisting of controls, the addition of cyclosporine to suppress cellular immunity, patient's serum to demonstrate humoral immunity, EPO, GM-CSF and IL-3. After adequate incubation the BFU-E colonies were counted and compared. The cultures treated with the patient's serum demonstrated a significant increase in numbers of BFU-E colonies, possibly due to the patient's high erythropoietin level. This probably eliminates both humoral immunity and a defective EPO molecule as causal factors. Cyclosporine inhibited all cultures but may have been toxic to the assay in a concentration of 200 ng/ml. Inhibition of cellular immunity did not increase BFU-E formation in the patient. Clinically, the patient was also a non-responder to prednisone. Exogenous growth factors, EPO, GM-CSF and IL-3, resulted in only modest increases in patient BFU-E colonies. Failure to enhance erythropoietic potential in vitro with several known treatment modalities, leads us to conclude that BMT may be the best option for this patient. In conclusion, in vitro culture assays may be used to assist in chosing an appropriate course of treatment in Diamond-Blackfan anemia.

A 504 SUPPRESSION OF CFU- L OF CML PATIENTS AND MYELOID LEUKEMIC CELL LINE BY RECOMBINANT HUMAN LIF, Tu Qiang, Sun Jinglu, Zho Guoping, Xiang Peide, Lin Wanming, Center of Molecular Biology, Xidiaoyutai General Hospital, Beijing 100036, China

LIF (Leukemia inhibitory factor), when added to the agar culture up to 2000U/ml, had little observable effect in reducing total HL-60 cluster numbers. But the HL- 60 colony (a cluster with more than 50 cells) number reduced from the control 115± 11 to 94± 10 at dosage 1000 U/ml and from 115± 17 to 3± 3 at 2000U/ml. When 2000U/ml of LIF was added alone to the culture, the mean percentage of dispersed colonies and mature cells increased from 1.7± 0.3 to 33.3± 7.1 and from 1.1± 0.4 to 8.8± 1.5 respectively. LIF incombined with 80U/ml of IL-6 or 20ng/mL of SCF induced a significant decrease in HL-60 colony number in compared with the three factors acting alone. A synergistic reduction of colony number was oberved in HL- 60 culture stimulated incombination of increasing concentrations of SCF and increasing concentrations of LIF up to 20ng/ml and 1000U/ml respectively. When LIF was added to culture of bone marrow mononuclear cells from patients with chronic myeloid leukemia (CML) resulted in a significant suppression of CFU-L. This effect were not affected by addition of IL-6 or SCF. The results suggest that LIF, acting alone or incombination, may be able to suppress human leukemia cells of appropriate type and be of value in the clinical management of myeloid leukemia.

A 505 HEMATOPOIETIC CELL PHOSPHATASE (HCP) ASSOCIATES WITH THE IL-3 RECEPTOR β CHAIN AND DOWN-REGULATES IL-3 INDUCED TYROSINE PHOSPHORYLATION AND CELL PROLIFERATION, Yi, T., L. Alice, G. Krystal and J. N. Ihle. Department of Cancer Biology, The Cleveland Clinic Foundation, Cleveland, OH44195, The Terry Fox Laboratory, British Columbia Cancer Agency, British Columbia, Canada V5Z1L3 and Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38105

Hematopoietic Cell Phosphatase (HCP) is a tyrosine phosphatase with two src-homology-2 (SH2) domains that is predominately expressed in hematopoietic cells, including cells whose growth is regulated by interleukin-3 (IL-3). The potential effects of HCP on IL-3 induced protein tyrosine phosphorylation and growth regulation were examined to assess the role of HCP in hematopoiesis. Our studies demonstrate that, following ligand binding, HCP specifically associates with the β chain of the IL-3 receptor through the amino terminal SH2 domain of HCP, both *in vivo and in vitro*, and can dephosphorylate the receptor chain *in vitro*. The effects of increasing or decreasing HCP levels in IL-3 dependent cells were assessed with dexamethasone inducible constructs containing an HCP cDNA in sense and anti-sense orientations. Increased HCP levels were found to reduce the IL-3 induced tyrosine phosphorylation of the receptor and to suppress cell growth. Conversely, decreasing the levels of HCP increase IL-3 induced tyrosine phosphorylation of the receptor and increased the cell growth rate. These results support a role for HCP in the regulation of hematopoietic cell growth and begin to provide a mechanistic explanation for the dramatic effects that the genetic loss of HCP, which occurs in *motheaten (me)* and *viable motheaten* (me^v) mice, has on hematopoiesis.

Late Abstracts

ANALYSIS OF INTERLEUKIN-6-DEFICIENT MICE.

Stacie A. Dalrymple, Linda A. Lucian, Stacy Fuchino, Dee Aud, Tom McNeil, Frank Lee, and Richard Murray. Department of Molecular Biology, DNAX Research Institute, 901 California Avenue, Palo Alto, California 94304.

Interleukin (IL)-6 is a cytokine with multiple biological activities. Originally described as a B cell growth factor, IL-6 has subsequently been found to play a potential role in a wide variety of immune and inflammatory responses. These processes include the maturation of cytotoxic T cells, stimulation of immunoglobulin synthesis, stimulation of bone marrow colony formation, and acute phase protein induction. High levels of IL-6 are detectable after most traumatic injuries, inflammatory reactions and immunological assaults. To elucidate the role IL-6 may have in these and other responses, we have produced animals deficient in IL-6 production. The IL-6 gene was inactivated in the E14.1 ES cell line via homologous recombination, and these cells were capable of giving rise to germline chimeras. Mice homozygous for the mutation are fertile and appear healthy. The distribution and numbers of various hematopoietic cell populations are normal based on cell surface marker phenotypes. However, several *in vitro* assays reveal differences from normal animals for both lymphoid and myeloid cell function. The generation *in vitro* of cytotoxic T cells from IL-6-deficient splenocytes was slightly impaired. Also, polyclonal stimulation of IL-6-deficient splenocytes with LPS + IL-4 yielded lower levels of IgG1 isotype in comparison to normal littermate controls. Additionally, colony forming unit in culture (CFU-C) assays on IL-6-deficient whole bone marrow using a number of factors showed that, although total colony numbers were similar or slightly decreased, the number of GEMM ("myeloid stem cell") colonies was increased twofold.

EXAMINATION OF INTRA-EMBRYONIC HEMATOPOIESIS OF THE DEVELOPING MOUSE

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Lab. of Physiology and Hematopoiesis, National Research Center for Hematology, Moscow, Russia and Lab. of Gene Structure and Expression, National Institute for Medical Research, London, England.

The classical description of murine developmental hematopoiesis depicts/portrays three different locations which contain hematopoietic stem cells: the yolk sac during embryonic life; the fetal liver acquires HSC activity at day 11 of gestation and around birth the bone marrow.

Since yolk sac hematopoiesis in the avian system has been demonstrated to be transitory, and definitive hematopoiesis originates from cells within the avian embryo body in the region around the dorsal aorta, we examined the homologous region in the developing mouse embryo for hematopoietic activity.

In a first set of experiments we performed CFU-S assays on cells derived from the aorta, gonad, mesonephros (AGM) region of the mouse. We have found both CFU-S_8 and CFU-S_{11} activity in the AGM region. In addition we could show that the precursor frequency in the AGM region exceeds that in the yolk CFU-S_8. Activity first appeared in the yolk sac and the embryo body beginning at the 26-27 somite pair stage (day 9.5 p.c.). Continuing through day 10 p.c. (38-40 s.p.), the CFU-S activity in the AGM region increases more rapidly and is more abundant than in the yolk sac. CFU-S_{11} activity appears in the AGM region at the 31-35 s.p. stage (day 10 p.c.) and at a frequency higher than that found in yolk sac.

The activity of the AGM region to form longterm repopulating cells will be discussed.

These studies demonstrate that in addition to the yolk sac, a pre-liver intra-embryonic source of hematopoiesis exists in the developing mouse embryo.

THERAPEUTIC EFFICACY OF RECOMBINANT HUMAN (rh) LEUKEMIA INHIBITORY FACTOR (LIF) RELATIVE TO THE INTERLEUKINS (IL) 6 AND 3 IN A NONHUMAN-PRIMATE MODEL OF RADIATION-INDUCED MARROW APLASIA, Ann M. Farese[†], Laurie A. Myers[‡], Thomas J. MacVittie[†]. [†]EXH, Armed Forces Radiobiology Research Institute, Bethesda, MD and [‡]Cytokine Development Unit (CDU), Sandoz Pharm. Corp., East Hanover, NJ.

Thrombocytopenia and neutropenia remain as dose limiting consequences following high dose irradiation or cytotoxic drug exposure. IL-6 and IL-3 have been shown to increase circulating levels of platelets (PLT) in preclinical and clinical models of myelosuppression. LIF has recently been shown to enhance circulating PLT levels in the normal mouse and nonhuman primate. The purpose of this study was to investigate the therapeutic efficacy of rhLIF relative to rhIL-6 or rhIL-3 in a non-human primate model of radiation-induced marrow aplasia.

Animals received 450 cGy of total body mixed neutron:gamma radiation and for 23 days (d) thereafter, received daily, single, subcutaneous injections of LIF, IL-6 or human serum albumin (HSA) administered at 15 µg/kg/bw. IL-3 was administered twice daily at a total dose of 15 µg/kg/bw. Cytokines were provided by the CDU, Sandoz Pharm. Corp. Complete blood counts and white blood cell differentials were monitored for 60d post-irradiation and the durations of neutropenia [absolute neutrophil count (ANC) < 1000/uL] and thrombocytopenia (PLT < 30,000/uL) were assessed.

All the cytokines, IL-6, IL-3 and LIF significantly decreased ($p < .05$) the duration of thrombocytopenia (5d, 6.6d and 9.3d, respectively) versus the HSA-treated control animals (12.2d). None of the cytokines significantly altered the duration of neutropenia that was observed in the control animals (17.2d). Cytokine administration did not worsen the radiation-induced anemia observed in the HSA-treated controls. In this model of radiation-induced marrow aplasia, administration of IL-6 or LIF promoted red cell recovery to baseline levels several weeks earlier (d34 and d48, respectively) than the HSA-treated control animals (d70).

These data demonstrate that in a nonhuman-primate model of sublethal irradiation 1.) IL-6, IL-3 and LIF significantly enhanced the regeneration of PLTs without affecting ANC recovery, and 2.) IL-6, IL-3 or LIF did not exacerbate radiation-induced anemia.