First International Workshop

CELL THERAPY:
FILLING THE GAP BETWEEN BASIC SCIENCE
AND CLINICAL TRIALS

Istituto Superiore di Sanità
Rome, 15-17 October 2001

ABSTRACT BOOK

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The Istituto Superiore di Sanità, in collaboration with the Istituto Dermatopatico dell’Immacolata (Rome), the Istituto Nazionale Ricerca sul Cancro-Centro Biotecnologie Avanzate (Genoa) and the University G. D’Annunzio (Chieti), has organized this international meeting to provide an overview on the most recent developments in Cell Therapy, the future perspectives of these clinical trials, the regulatory issues involved and a progress report on the clinical protocols which have been approved up to now in Italy. The meeting includes 6 scientific sessions (Immunotherapy, Epithelium, Osteo and cartilage regeneration, Hematopoiesis, Future directions and Regulatory issues) which involve national and international scientists.

**Key Words:** Cell therapy, National health program, Immunotherapy, Epithelium, Osteo and cartilage regeneration, Hematopoiesis, Regulations

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SCIENTIFIC PROGRAMME

Monday, 15 October 2001

8.30  Registration

9.30  Welcome address: E. Garaci

Session 1: Immunotherapy
Chairpersons: F. Belardelli, M. Cianfriglia

9.50  Dendritic cells and immunotherapy
      A. Lanzavecchia

10.30 A new type I IFN-mediated pathway for the rapid differentiation of monocytes into highly active dendritic cells
        S.M. Santini(1) (Young Investigator)

10.50 Coffee break

11.20 Immunotherapy of HIV-1 infection
        G. Pantaleo

12.00 Immunotherapy with dendritic cell hybrids
        R.H. Ringert

12.40 Cancer vaccines
        G. Parmiani

13.20 Lunch break

Session 2: Epithelium
Chairpersons: Y. Barrandon, M. De Luca

14.20 Stem cells and skin morphogenesis
        Y. Barrandon

15.00 Stem cells in the gastrointestinal mucosa
        C. S. Potten

15.40 Ocular surface stem cells: from clones to clinic
        G. Pellegrini

16.20 Coffee Break

(1) Presentation selected from the abstracts submitted
16.50 Towards epidermal stem cell-mediated gene therapy  
M. De Luca

17.30 Isolation and clonal analysis of human epidermal stem cells in long term culture  
S. Papini (Young Investigator)

Tuesday, 16 October 2001

Session 3: Osteo and Cartilage Regeneration  
Chairpersons: A. Facchini, R. Quarto

9.00 Generation of organ tissue and specific cells from peripheral blood derived MSC  
R. Huss

9.40 Repair of large bone defects by autologous bone marrow stromal cells  
R. Quarto

10.20 Tissue engineered osteochondral composites  
I. Martin

11.00 Coffee Break

11.30 Bridging the gap between the lab and the clinic  
S. Bruder

12.10 A novel hyaluronan biomaterial (HYAFF-11) as scaffold for chondrocytes and bone marrow stromal cells  
L. Roseti (Young Investigator)

12.30 Lunch Break

Session 4: Hematopoiesis  
Chairpersons: S. Amadori, G. Leone

14.00 Clinical applications of ex vivo expanded hematopoietic cells  
I. McNiece, J. Hotzefeld

14.40 Ex vivo manipulation of human hematopoiesis: from concept to clinic  
P. Simmons
15.00  Coffee break

16.00  KRN 7000: a novel dendritic cell activator
       A. Shimosaka\(^{(1)}\)

16.40  The role of MSC in hematopoietic engraftment of cord blood derived CD34+ cells in NOD-SCID mice
       W. Fibbe

17.00  Cell Therapy with IL-10 energized T cells in hematopoietic stem cell transplantation
       R. Bacchetta\(^{(1)}\)

17.30  Identification of a new hematopoietic growth factor: hemangiopoietin
       Z.C. Han\(^{(1)}\)

Wednesday, 17 October 2001

Session 5: Future directions
Chairpersons: C. Peschle, M. Orlando

9.00   Cell therapy for muscle
       G. Cossu

9.40   Cell Therapy and transfusion medicine
       M. Narla

10.20  Cord blood: An expandable source of human stem cells
       W. Piacibello\(^{(1)}\)

11.00  Coffee Break

11.30  Cell Therapy for the hematopoietic stem cell and the hemoangioblast
       C. Peschle

12.10  Towards the selective expansion of transplanted normal hepatocytes
       E. Laconi\(^{(1)}\)

13.00  Lunch Break

Session 6: Regulatory issues
Chairpersons: G. D'Agnolo, C. Pini

14.00 - 16.00  Round Table

Closure of the conference
LIST OF SELECTED ABSTRACTS SUBMITTED\(^{(2)}\)

**Immunotherapy**

1. A new type I IFN-mediated pathway for the rapid differentiation of monocytes into highly active dendritic cells

**Epithelium**

2. Isolation and clonal analysis of human epidermal stem cells in long term culture
   S. Papini, D. Campani, R.P. Revoltella

3. Optimization of the microencapsulation of human parathyroid cells *in vitro*
   L. Picariello, S. Benvenuti, R. Recenti, A. Falchetti, A. Morelli, M. Fazi, F. Tonelli, M.L. Brandi

**Osteo and cartilage regeneration**

4. A novel hyaluronan biomaterial (HYAFF-11) as scaffold for chondrocytes and bone marrow stromal cells

5. Interactions and cytocompatibility between 3D polyurethane scaffolds and bone compartment cells for bone regeneration
   E. Piscitelli, S. Benvenuti, R. Recenti, A. Tanini, M.C. Tanzi, M.L. Brandi

**Hematopoiesis**

6. KRN700: A novel dendritic cell activator
   A. Shimosaka, Y. Koezuka, N. Nishi

7. Cell therapy with IL-10 anergized T cells in hematopoietic stem cell transplantation
   R. Bacchetta, E. Zappone, K. Fleischauer, E. Zino, B. Blazar, S. Narula, C. Bordignon, M.G. Roncarolo

\(^{(2)}\) The presenting authors of the abstracts selected for oral communications are indicated in bold
8. **Biological characterization of Hemangiopoietin, a factor capable of stimulating bone marrow hematopoietic stem cells and endothelial cells**
   Y.J. Liu, Y.L. Cai, S.H. Luo, Z.C. Han

9. **Preliminary study for a clinical approach in ucb ex vivo expansion.**
   T. Bonfini, L. Costarelli, R. Giancola, E. Liberatore, F. Di Penta, G. Di Leve,
   T. Rotondo, A. Iacone.

10. **Ex vivo manipulation of cord blood stem cells in view of clinical applications**
    L. Lazzari, T. Montemurro, S. Lucchi, R. Giordano, A. Giorgetti, R. Pacchiani,
    L. Porretti, L. Lecchi, P. Rebulla

11. **Ex vivo expansion of CIK cells from fresh or cryopreserved-PBLs samples**
    P. Olioso, R. Giancola, M. Di Riti, A. Iacone

**Future directions**

12. **Megakaryocyte engraftment and platelet production in Nod/Scid mouse model by injection of fresh and expanded cord blood CD34+ cells and their megacaryocyte-differentiating progeny**
    S. Bruno, M. Gunetti, L. Gammaitoni, G. Cavalloni, F. Sanavio, M. Aglietta,
    W. Piacibello

13. **Bone marrow and mobilized peripheral blood CD34+ cell ex vivo expansion and telomere length**
    M. Gunetti, L. Gammaitoni, S. Bruno, F. Sanavio, F. Fagioli, M. Aglietta,
    W. Piacibello

14. **Towards the selective expansion of transplanted normal hepatocytes**
    S. Laconi, S. Pitzalis, D. A. Shafrtz, P. Pani, E. Laconi

15. **The proliferation/differentiation switch of the progenitor cells:implications for cell therapy**
    L. Maccioni, R. Galanello, G. Migliaccio

**Regulatory issues**

16. **Lesson learnt from designing an ex vivo somatic cell therapy protocol**
    R. Giordano, L. Lazzari, T. Montemurro, S. Lucchi, A. Giorgetti, R. Pacchiana,
    P. Rebulla.

17. **Immunological characterization of anti-F. VIII antibodies by a sandwich-Elisa and the Bia-technology**
    G. Iannone, P.G. Davoli, A. Traldi, C. D’Urso, S. Arrighi, R.P. Rivoltella
Session 1
Immunotherapy
Chairpersons
F. Belardelli, M. Cianfriglia
A NEW TYPE I IFN-MEDIATED PATHWAY FOR THE RAPID DIFFERENTIATION OF MONOCYTES INTO HIGHLY ACTIVE DENDRITIC CELLS

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We recently reported that addition of type I IFN to freshly isolated GM-CSF-treated human monocytes resulted in the generation of partially mature dendritic cells (IFN-DCs) (J. Exp. Med., 191:1777, 2000). We have characterized the phenotypic and functional features of IFN-DCs as compared to the immature DCs obtained after GM-CSF/IL-4 stimulation (IL-4-DCs). IFN-DCs rapidly acquired the morphologic and functional features of DCs within 3 days, expressed the typical markers of mature DCs (CD83, CD25, TRAIL) and behaved as potent APC.

IFN-DCs markedly migrated in response to β-chemokines (especially MIP-1β) and expressed the Th-1 chemokine IP-10. Notably, IFN-DCs showed an up-regulation of CCR7 as well as of its natural ligand MIP-3β characteristics typical of mature DCs. Of interest, IFN-DCs exhibited a marked chemotactic response to MIP-3β in vitro and strong migratory behaviour in SCID mice. In SCID mice reconstituted with human PBL, IFN-DCs induced a potent primary human antibody response and IFN-γ production, indicative of a Th-1 immune response. These results define the highly specialized maturation state of IFN-DCs and point out the existence of a “natural alliance” between type I IFN and monocyte/DC development, instrumental for ensuring a prompt and efficient connection between innate and adaptive immunity.
Session 2
Epithelium

Chairpersons
Y. Barrandon, M. De Luca
ISOLATION AND CLONAL ANALYSIS OF HUMAN EPIDERMAL STEM CELLS IN LONG TERM CULTURE

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We developed a procedure for growing normal human epidermal keratinocyte stem cells in long term culture retaining normal cell differentiation capabilities. Primary skin epithelial cells were maintained onto collagen-coated plates with irradiated human dermal fibroblasts as a feeder in medium supplemented with serum for over 120 days, approximately 115 population doublings, before they reached senescence and ceased to proliferate. Selected colonies were then serially subcultured to determine their limiting proliferation potential. Clonal analysis revealed three predominant cell types: 1. Holoclones, endowed with the highest proliferative potential and extensive capacity for division, revealed the expected properties of stem cells, accounting for the prevailing long term proliferation of the original cultured mass. In 3-dimensional cultures, either on a gelatin matrix or on a collagen layer-containing fibroblast feeder, the cells of these clones formed stratified layers and expressed differentiation associated markers. 2. Another large cell fraction, classified as transient-amplifying keratinocytes, formed colonies with variable proliferative potential (meroclones) with the general tendency to develop terminally differentiated keratinocytes. Additionally we found cells producing mucin in cultures with high proliferative capacity, indicating that both keratinocytes and mucin producing cells derive from a common bipotent progenitor. The number of mucin producing cells increased after confluence, suggesting that committent for their differentiation occurs late in the life of a single clone. 3. Finally we identified other clones (paraclones) including mature keratinocyte progenitors capable of only a limited number of divisions (CNR.PF-MADESS 2°; MURST– Stem cells).
OPTIMIZATION OF THE MICROENCAPSULATION OF HUMAN PARATHYROID CELLS IN VITRO

L. Picariello, S. Benvenuti, R. Recenti, A. Falchetti, A. Morelli, M. Fazi, F. Tonelli, M.L. Brandi
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Parathyroid transplantation in humans has long been investigated as a possible mean of treating patients affected by hypoparathyroidism caused by thyroid or parathyroid surgery, in which substitute therapy with exogenously administered vitamin D and calcium prevents long-term hypocalcemia. However, the main obstacle is represented by tissue rejection. A reliable method to allow long-term survival of the graft, experimented in rats, is the encapsulation of the transplanted tissue or cells in sodium alginate-poly-L-lysine membranes, permeable to nutrients but impermeable to immunoglobulins when reimplanted in vivo. We describe, for the first time, the microencapsulation of cultured human parathyroid cells, derived from adenoma or hyperplastic glands. Purified epithelial cell cultures were obtained by serial filtrations of dispersed cells using an appropriate growth medium, low in bovine serum supplementation and rich in growth factors, with physiological concentrations of Ca$^{2+}$ (1 mM) and Mg$^{2+}$ (0.5 mM). Spherical sodium alginate gel drops containing parathyroid cells were formed by syringe-pump extrusion and collected in calcium chloride solution and then cultured in the appropriate growth medium, in a humidified 95% air and 5% CO$_2$ atmosphere at 37°C. Morphological investigation by phase-contrast light microscopy revealed the round shape of the capsules with an average diameter of 2 mm. Microencapsulated cells maintained their integrity for over three months in culture and were able to grow (DNA content of the drops significantly (*p< 0.05) varied from 2.075±0.025 to 7.1±1.9 ng DNA/bead after 1 and 12 days, respectively). In addition, cultured parathyroid microencapsulated cells produced stable amounts of PTH for over three months in a range of concentrations between 46.8±1.5 to 84±0.7 ng/µg DNA after 1 and 15 days respectively, to 83.3±2.25 ng/µg DNA after 105 days. On the contrary, PTH release from parathyroid cells in monolayer culture was more variable (240±30.4 pg/µg DNA to 50.89±6.77 ng/µg DNA) and limited up the 20$^{th}$ day in culture. Microencapsulated parathyroid cells maintained sensitivity to increasing concentrations of extracellular Ca$^{2+}$ with significant (*p< 0.05) reduction of PTH release (70% and 80% reduction vs control, after 1 and 6 days respectively) after exposure to 2 mM Ca$^2$. Parathyroid cells in monolayer culture showed a reduction of 50% and 55% vs control after 1 and 6 days, respectively *p<0.05. In conclusion, this method represents a crucial step toward the obtaining of functional bioartifical parathyroid organoids for the treatment of human hypoparathyroidism.
Session 3
Osteo and cartilage regeneration

Chairpersons
A. Facchini, R. Quarto
A NOVEL HYALURONAN BIOMATERIAL (HYAFF-11) AS SCAFFOLD FOR CHONDROCYTES AND BONE MARROW STROMAL CELLS

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The field of tissue engineering is rapidly expanding and has numerous potential application in orthopaedics. This therapeutic strategy combines isolated cells with scaffold/cell carriers in order to promote cartilage or bone formation. The ideal scaffold should prevent cells from floating out of the defect, provide mechanical strength, make uniform cell spreading possible and stimulate the chondrogenic and osteogenic phenotypes of the transplanted cells. Furthermore, the matrices of both biologic and synthetic origin, must be biocompatible, biodegradable and malleable so they can fit the defects. Aim of this study was to evaluate in vitro the potential of Hyaff-11, a recently developed hyaluronic acid-based biodegradable polymer, to maintain chondrocyte phenotype and facilitate mineralization of bone marrow stromal cells (BMSC). Human chondrocytes (10^6cells/cm²) and rat BMSC (2x10^6cells/cm²) were seeded on HYAFF-11 and their proliferation and differentiation were assessed at different time points. Chondrocyte gene expression for collagen I, II and aggrecan was revealed by RT-PCR evaluating the presence of the specific mRNAs while histochemical analysis for these proteins were performed by the use of specific monoclonal antibodies. Osteogenic differentiation of rat BMSC was investigated analysing the expression of calcium, alkaline phosphatase, osteopontin (OP), bone sialoprotein (BSP) and collagen type I.

Our data indicate that HYAFF-11 is capable of: 1) maintaining the characteristics of differentiated chondrocytes expressing the cartilage-specific marker collagen type II; 2) permitting differentiation and mineralization of rat BMSC, thereby favouring development of bone-like tissue positive for calcium, OP and BSP. These results provide an in vitro demonstration of therapeutic potential of HYAFF-11 as a delivery vehicle in tissue-engineered repair of articular cartilage and bone defects.
INTERACTIONS AND CYTOCOMPATIBILITY BETWEEN 3D POLYURETHANE SCAFFOLDS AND BONE COMPARTMENT CELLS FOR BONE REGENERATION

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Bone reconstruction is essential to provide functional integrity to the patient's skeleton in a variety of clinical situations. Regenerative capacity to form bone can be an alternative mechanism to autologous bone graft. Polymer-cell constructs also hold great promise in the field of tissue engineering. They provide a scaffold on which cells can grow, organize and begin to secrete their own extracellular matrix for natural tissue replacement. In this study we investigated the biocompatibility and the interaction of the biostable polymer polyurethane (PU) with different physical properties (density, cells size and percentage of open cells) and osteoblast-like cells (Saos-2), human bone marrow stromal cells (HBM) and chondrocytes. Cells (5x10⁵/ml) were cultured on disks (2mm thick, 15mm ∅) of PU foams and checked after two to eight days to evaluate cell morphology, adhesion by scanning electron microscopy, proliferation by ³H-thymidine uptake, and production of alkaline phosphatase. All cell types were able to adhere and synthesize DNA. After 48 hours of culture, HBM stromal cells showed the highest rate of cell adhesion. Particularly, cells proliferated better onto the PU foams with the largest cell size. Both chondrocytes and Saos-2 seemed to attach preferentially to foams exhibiting the highest percentage of open cells. Saos-2 cultured for 2, 4, and 8 days were able to adhere and grow onto the foams, showing a time-dependent increase in cell proliferation. Scanning electron microscopy analysis revealed both HBM stromal cells and chondrocytes closely adherent to the foam substrates, evidencing long thin cytoplasmatic extrofilament. Moreover, adherent HBM cells exhibited a number of vesicles on their surface, indicating a strong cell activity. Adherent Saos-2 cells exhibited a flattened shape with a wide spread morphology and grow to form a monolayer. Alkaline phosphatase assay revealed a time-dependent increase of activity in Saos-2 cultured on PU foams for 2, 4, and 8 days. All the experiments were carried out in triplicate and were statistically significant. According to our data from in vitro studies PU foams are biocompatible materials for bone transplantation in terms of bone cell proliferation and adhesion.

The possibility to employ these materials in patients should be supported by in vivo studies.
Session 4
Hematopoiesis
Chairpersons
S. Amadori, G. Leone
ABSTRACT

KRN7000: A NOVEL DENDRITIC CELL ACTIVATOR

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Introduction

KRN7000 is a glycosyl-ceramide compound which activates immune system via dendritic cells (DC), NKT cells, T-cells and macrophage. Its mother compound, an agelasphin family compound, was isolated from ocean sponge, Agelas mauritianus found in southern part of Japan. The mother compound, Agelasphin, was screened using in vivo B16 melanoma model animal. It was not active in in vitro screening system because it has no cytotoxicity as well as KRN7000. The modification study proved that KRN 7000 is the best candidate molecule for further development. KRN7000 can be totally synthesized. It is only active in normal mice with normal immune system and not active in nude mice.

These information suggested that the molecule is active through animal immune system. Further studies to learn its mechanism of action revealed that the molecule is active through dendritic cells (DC) and NKT cells in stimulating NK cells, T-cells and monocytes (1,2).

Biological activities

KRN7000 is active in animal model against B16, colon 26, EL-4 and other solid tumors as well as metastatic models. Cytotoxic T-Cells are activated with KRN7000 administration. Cell-lytic activity is dose dependently increased associated with increased endogenous IL-2 and Interferon γ production. It stimulates autologus mixed lymphocyte reaction (MLR) almost equal to allogeneic MLR. It alone can not cure the animal when the tumor is already bulky. Together with cytotoxic agent, it is possible to cure the animal with extensive tumor mass which can not be eradicated only by cytotoxic agent or KRN7000. It is possible for KRN7000 to eradicate small tumor mass like other methods based on immune system activation. Tumor recognition by DC when stimulated by KRN7000 is specific to the immunized tumor. The animal treated with KRN7000 after colon 26 inoculation survived tumor free over 100 days although control animal died within 45 days after tumor challenge. Then same colon 26 was challenged to the survived animal, tumor was not accepted by the animal. But when Meth A was challenged, tumor grew and killed the animal. (3) The study on the mechanism of action revealed that KRN7000 stimulate NKT cells, T-cells and macrophages through DC activation. Ex vivo stimulation of DC with KRN7000 is also effective against metastatic tumors in animal as well as in vivo direct administration. Ex vivo vaccination of DC with tumor lysate can be enhanced with KRN7000.

Mechanism of action

The mechanism of action of KRN7000 is unique. We studied the target cells which is responsible for further immune stimulation. It stimulates specific cohort of DC only, specially antigen presenting cells (APC). The activated DC binds to Vα14 NKT cells in
mice (Human case is Vα24 NKT). This binding needs CD40 and CD40L, B7-1/B7-2 and CD28/CTLA4 receptor/ligand binding. Any antibodies to those receptor/ligand inhibits KRN7000 activity. It is not active in animal with Vαl4 NKT gene knocked out (4).

Safety/ Toxicology

KRN7000 has no cytotoxicity and studied 1. µg/kg~4mg/kg in animals without any symptoms except increase in WBC. KRN7000 was safe up to 4800 µg/m2 in normal healthy volunteers and patients with cancer. Increase in NKT cells were observed.

Further study

The role of NKT cells has become known to be important in antigen specific immune response control. In patients with auto-immune disease, their immune response is not controlled. Some patients with auto-immune disease have decreased NKT cells. (5) KRN7000 increases the number of NKT cells in vivo and in vitro. Clinical applications of KRN7000 are expected for the antigen specific activation of DC either in vivo or ex vivo, enhance autologus MLR to expect GVL effect and the treatment of auto-immune disease. We would like to explore the benefit of KRN7000 for the activation of DC ex vivo with specific antigen in comparison with DC/antigen alone activation

References
CELL THERAPY WITH IL-10 ANERGIZED T CELLS IN HEMATOPOIETIC STEM CELL TRANSPLANTATION


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Allogeneic bone marrow transplant is the only cure for many patients affected by acute leukemias or aggressive lymphomas. A minor proportion of these patients have an HLA compatible donor in the family and can not afford to wait the time necessary for a successful search of an HLA matched donor in the international bone marrow donor registry. Most patients, instead, have an haploidentical donor in the family, but so far transplant of haploidentical bone marrow cells has been hindered by the high incidence of acute graft versus host disease (GVHD), graft rejection, and post transplant immunodeficiency. Although the use of purified haploidentical stem cells in high number results in complete engraftment, without GVHD, the patients experience a long period of profound and life threatening immunodeficiency. On the other hand, adoptive transfer of unmanipulated donor lymphocytes causes acute GVHD, even at very low doses. In vitro induction of tolerance against host alloantigens (alloAg) could lead to the use of donor lymphocytes in haploidentical transplants to ameliorate the immunodeficiency status without the risk of fatal GVHD. Interleukin-10 (IL-10) is an anti-inflammatory and suppressive cytokine. In primary MLR performed with HLA mismatched donors, IL-10 induces alloAg specific unresponsiveness in human CD4+ T cells, and promotes the generation of T regulatory type 1 cells (Tr1), which inhibit Ag specific responses of naive T cells. Furthermore, adoptive transfer of IL-10+TGF-β anergized T cells in mice transplanted with MHC mismatched BM, results in a significant GVHD reduction. These data provided us with a strong rationale for the development of a clinical protocol for adoptive transfer of ex vivo IL-10-anergized T cells of donor origin in patients undergoing allogeneic BMT. Anergy can be induced by IL-10 in total donor PBMC and it is consistent among different donors. The inhibition of Ag-specific proliferation ranged from 63 to 95%, compared to that of control cells (mean value 81 sd 7%, p<0.005) and it corresponded to a similar decrease in frequencies of CTLp specific for the alloAgs. Most importantly, anergized T cells preserve the ability to respond to nominal Ags, such as Tetanus Toxoid and Candida Albicans, and to viral Ags, such as EBV, indicating the Ag-specificity of IL-10 induced anergy. These findings were confirmed also in haploidentical pairs, although the levels of anergy showed variability among donors (mean value 77sd 22%). Similarly to mismatched donors, anergized cells from haploidentical donors had a decreased frequency in alloAg specific CTLp and responded normally to other Ags. The procedure was scaled up and validated for GMP production, to render it suitable for clinical application. A protocol for a pilot clinical trial enrolling patients transplanted with haploidentical hematopoietic stem cells has been submitted for approval.
BIOLOGICAL CHARACTERIZATION OF HEMANGIOPOIETIN, A FACTOR CAPABLE OF STIMULATING BONE MARROW HEMATOPOIETIC STEM CELLS AND ENDOTHELIAL CELLS

Yong Jun Liu*, Ying Lin Cai*, Su Hong Luo*, Zhong Chao Han°
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°Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China

The hemangiopoietin (HAPO), a polypeptide acting on hematopoietic and endothelial cell lineages, was cloned from human fetal liver mRNA. Sequence analyses indicated that the first exon of HAPO coded signal peptide, and the third exon coded a somatomedin B homology domain. To initiate structure-function studies by protein engineering, the part of HAPO cDNA (786bp) coding N-terminal region (262 amino acids) was amplified by PCR, cloned into the pET32c(+) expression vector downstream to the his-tag site. After transformation into Escherichia coli (BL21), the HAPO protein was expressed as soluble protein, with the yield of 5mg per liter of Escherichia coli cells. Purification of HAPO protein involves a combination of DEAE sepharose fast flow, his-tag affinity and size-exclusion columns. After enzymatic digestion, the recombinant HAPO was further isolated and tested in vitro and in vivo for its biological activities. It was found that HAPO support significantly the proliferation and survival of murine bone marrow hematopoietic cells and endothelial cells in long-term bone marrow culture. In the presence of HAPO, the number of CD34+ and Flk-1+ cells was significantly increased. HAPO was also able to promote the growth of KG1a cells, a CD34+ leukemia cell line, and ECV304 cells, an endothelial cell line. In vivo, HAPO provided stimulation of hematopoiesis when injected for 7 days after total body irradiation of 4.5 Gy. Mice given HAPO had a significant increased bone marrow CD34+ cells, megakaryocytes and mononuclear cells compared with control mice. Although the full understand of biological function of HAPO requires further study, our data demonstrate for the first time the presence of a previously not described cytokine acting on both hematopoietic and endothelial cell lineages.
ABSTRACT

PRELIMINARY STUDY FOR A CLINICAL APPROACH IN UCB EX VIVO EXPANSION

Tiziana Bonfini, Laura Costarelli, Raffaella Giancola, Elisabetta Liberatore, Fabiola Di Penta, Giulia Di Leve, Tiziana Rotondo, Antonio Iacone

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Umbilical cord blood (UCB) is an alternative stem cell source to bone marrow or growth factor mobilized peripheral blood for allogeneic transplantation. However, major disadvantage of UCB is the relatively low number of cells available causing delayed engraftment kinetics and limiting the use of UCB to children and small size adult patients. Ex vivo stem cells expansion seems to be a promising strategy to ensure an adequate cell dose and to improve post transplant outcome. Efforts on expansion programs have concerned mainly the amplification of progenitor cells; however, differentiated cells could play a potential role providing a microenvironment helpful for engraftment as well as providing effector functions in the critical post-transplantation aplasia period; this issue should be taken into account. The clinical approach requires the effective capacity to expand, in a serum-free, stroma-free short term culture system, primitive and committed progenitors as well as precursor cells, simultaneously. Aim of our study was to evaluate a short term culture system suitable for clinical practice.

UCB was collected from full term deliveries after maternal informed consent and mononuclear cell fraction was obtained by density centrifugation; CD34+ cells were separated by MiniMACS isolation kit (Miltenyi, Biotec) according to manufacturers’ instructions, resulting in a final purity ranging between 83 to 92%. CD34+ cells (1x10^5 cells/ml cell concentration) were cultured up to 14 days with SCF, TPO, FLT-3, G-CSF (100 ng/ml each) and IL-6 (50 ng/ml) in pre-selected commercial serum-free medium (X-VIVO 20, BioWhittaker). Fresh medium and cytokines were fed every 2 days; demi-population was performed on day +7.

The expanded cell population was assessed by cell count, immunophenotyping and clonogenic test; viability was evaluated by trypan blue exclusion and morphology by cytospin preparation. Nucleated cells resulted amplified by 655 fold (140-890), CD34+ cells by 16 fold (4-21) and CFC by 69 fold (21-135); cell viability was 86% (73-91). Median percentages of CD34+ and CD34+38- cells were 1.8% (0.3-3.3) and 0.16% (0.01-0.3), respectively.

Immunophenotyping analysis of lineage commitment showed mostly myeloid lineage cell markers, 80-95% being CD33+ cells.

Particularly, CD15, CD14, CD61 and Glycophorin A markers were 91%, 20%, 4% and 2% respectively. Lymphoid differentiation markers (CD3, CD19 and CD16/56) were expressed on less than 5% of cells. Morphological evaluation showed a heterogeneous population of early and myelomonocyte precursors. In a practical approach this expansion protocol may be suitable for clinical purposes.
EX VIVO MANIPULATION OF CORD BLOOD STEM CELLS IN VIEW OF CLINICAL APPLICATIONS

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Cord blood (CB) is an attractive source of stem cells for hematopoietic and non-hematopoietic cell replacement. With regard to the hematological applications, we investigated the ability of a cocktail containing TPO, FL, IL-6, IL-11 and a serum-free medium, to sustain long term expansion of the more uncommitted progenitor cells. Moreover, as CB is usually banked in the cryopreserved state, we compared the expansion obtained using in parallel fresh and cryopreserved aliquots of CB units. An additional objective of our in vitro studies was to evaluate if cryopreservation and thawing damage megakaryocyte (Mk) progenitors and impair their clonogenic capacity. To this aim, we investigated if our ex vivo expansion protocol induces Mk maturation. After 2 weeks we observed a three- to- five log fold expansion of Mk precursors both in fresh and cryopreserved CB samples. The evidence so far collected supports the possibility to expand cryopreserved CB cells with the same efficacy as fresh cells. These results, if supported by in vivo evaluations, may be useful to design clinical protocols. In this regard, we collected evidence of the multilineage reconstitution capacity of the ex vivo expanded cells in NOD/SCID mice in collaboration with the group of Dr. A. Thrasher, London. Moreover, experiments of transplantation of the expanded cells in fetal sheep are in progress together with Dr. B. Péault in Paris.

Finally, the ultimate clinical use of ex vivo manipulated CB stem cells requires: (1) adequate scale-up experiments; (2) laboratories compliant with relevant standards, norms and regulations and (3) prospective trials with sufficient statistical power.
EX VIVO EXPANSION OF CIK CELLS FROM FRESH OR CRYOPRESERVED-PBLS SAMPLES

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Background
Immunotherapy represents a promising treatment strategy for many types of cancer. Recently Schmidt-Wolf and Negrin developed a protocol for the generation of very large numbers of highly efficient cytotoxic cells, termed CIK cells, since their phenotype express both the T-cell marker CD3 and the NK-marker CD56. CIK cells kill a variety of tumor cell lines, multidrug-resistant and autologous tumor cells. Some of these results concern the fresh-expanded cells, while data regarding the frozen-thawed ones are lacking.

Aims
We compared ex vivo expansion, immunophenotype and cytotoxic activity of CIK cells generated from fresh versus cryopreserved-PBLS samples.

Methods
Fresh-PBLs samples were obtained from 2 patients and 3 healthy blood donors. Cryopreserved-PBLs samples were obtained from 2 patients undergoing apheresis, in preparation for autologous PBSCT, and 3 healthy DLI donors. CIK cells were generated by growing PBLs in the presence of IFN-γ followed by IL-1β, OKT3 and IL-2. Expansion was assessed between day 21 and 28. A CytoTox 96 non-radioactive cytotoxicity assay was performed, on days 21 and 28. K562 and A-20 cell lines were used as targets.

Results
At day 0 fresh and cryopreserved derived CIK cell products contained: 76.2% CD3+(range: 49 to 86.3%) and 4.58% CD3+CD56+(2.6-9.5%) vs 76.5%CD3+(69-90.5%) and 5.33%CD3+CD56+ cells (3.5-6.5%), respectively. 28 day median culture was 99% viable (98-99.6%) vs 95% viable (80–99.5%), p=0.01, 98.7% CD3+(98.2-99.7%), 58.6% CD3+CD56+(42-82.7%), vs 98.9%CD3+(98-99.8%), 36.4% CD3+CD56+(26.9-42%), respectively. The absolute number of CD3+CD56+ cells expanded 1668 fold(421-3599) and the CD3+ 169 fold(47.1-144.8) vs 28 day 98.7%CD3+(98-99.8%), 36.4% CD3+CD56+(26.9-42%), respectively. The expression of HLA-Dr and LFA-1/CD 11a was 84.8%(77.7-96.7) and 55.87%(31-99.6%) vs 51.63(33.1-73.8) and 67.2(60-80.3), respectively.

At a E:T ratio of 20:1 and 40:1 we found a mean value of 44.3%(9-75.2%) and 57.9%(37.7-86.3%) cell killing vs a mean value of 42.6%(25-75.5%) and 58.3%(43.6-83.7%), respectively.
Conclusions

If confirmed in larger scale experiments, these preliminary results may have clinical relevance in the immunotherapy with CIK cells. In fact, expansion of CIK cells from cryopreserved PBLs is feasible and no statistical significant differences are detected in immunophenotyping and cytotoxic activity. There is a significant difference in the fold of expansion. Therefore, because the lesser capability of expansion, frozen-thawed cells require a larger starting cell population to obtain a sufficient number.
Session 5
Future directions

Chairpersons
C. Peschle, M. Orlando
MEGAKARYOCYTE ENGRAFTMENT AND PLATELET PRODUCTION IN NOD/SCID MOUSE MODEL BY INJECTION OF FRESH AND EXPANDED CORD BLOOD CD34+ CELLS AND THEIR MEGAKARYOCYTE-DIFFERENTIATING PROGENY

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Cord blood (CB) is an alternative source of hematopoietic stem cells for marrow reconstituting in young patients treated with high-dose chemotherapy; unfortunately CB transplantation is characterized by a slow platelet recovery. In our work, we have compared the NOD/SCID mouse short- and long-term megakaryocyte (Mk) reconstitution and platelet production by unmanipulated (U) and 4-week-expanded (in the presence of FLT-3 ligand, Thrombopoietin, and Stem Cell Factor) and re-isolated (4w) CB CD34+ cells. In vivo studies showed that there was no difference in long-term (6-8 weeks after transplantation) Mk repopulation ability between unmanipulated and expanded CD34+ cells; in fact in both cases in the Bone Marrow (BM) of injected mice Mk progenitors and Mk mature cells were found. Short-term (1, 2 and 3 weeks after transplantation) engraftment showed that UCD34+ cells did not reconstitute rapidly the Mk lineage (only at week 2); by contrast expanded cells were able to completely reconstitute the Mk lineage in 7 days.

U and 4w CBCD34+ cells were grown in the presence of various growth factor combinations composed of some megakaryocyte-active cytokines: Interleukin-3 (IL-3), SCF, Interleukin-6 (IL-6) and TPO. Mk progenitor and mature cells output were analyzed during the culture by flow-cytometry. In vitro data showed that the cytokine combination of TPO and SCF was sufficient to expand Mk cells (240-fold) from UCD34+; by contrast the four growth factors (IL-3+SCF+IL-6+TPO) were required to obtain the greatest Mk production by 4wCD34+ (40-fold). Short-term Mk reconstitution and platelet production by Mk-differentiated UCD34+ and 4wCD34+ cells was evaluated. Platelet production and Mk-reconstitution by Mk-differentiating population was transient. Human platelets in the Peripheral Blood (PB) were detect after 3 days but decreased and disappeared after 7 and 14 days posttransplant of UCD34+-differentiating cells. By contrast in the PB of mice injected with 4wCD34+-differentiating cells platelet production was slower but long lasting (after 2 weeks high platelet levels were still found).

Co-transfusion of ex vivo cultured megakaryocyte cells, could be a new potential therapy to shorten the period of thrombocytopenia.
Bone Marrow (BM) and Mobilized Peripheral Blood (MPB) stem cells could be *ex vivo* manipulated and expanded in a stroma-free liquid culture system, in the presence of Flt3 ligand (FL), Thrombopoietin (TPO), Stem Cell Factor (SCF) ± Interleukin-6 (IL-6) or ± Interleukin-3 (IL-3). The presence of IL-6 resulted critical in the BM and MPB CD34+ cell expansion; in this culture condition, adult hematopoietic stem cell could be expanded for up to 12 weeks (up to 3,000-fold) during which they proliferated and produced large numbers of committed progenitors. Limiting dilution assay demonstrated that primitive NOD/SCID mouse repopulating stem cells (SRC) can be expanded 6-fold after 3 weeks and retained the ability to repopulate secondary NOD/SCID mice.

A central issue remains cell senescence during the expansion. Telomere length measurements can be used as a marker of stem cell turnover and aging *in vitro* and *in vivo*; in addition hematopoietic cells from different stages in ontogeny differ in proliferation potential and these functional differences correlate with difference in telomere length. In the present study we investigated the telomere length in human CD34+ cells collected from unmanipulated BM and MPB samples and after several weeks of *ex vivo* expansion.

Telomere length has been evaluated by Flow-FISH methods: 2x10^5 BM, MPB CD34+ cells have been analyzed after a staining with a PNA probe conjugated with FITC at different time points of *ex vivo* expansion. The same number of unmanipulated Cord Blood (CB) and mononuclear cells (MNC) from peripheral blood (PB) has been utilized as examples respectively of long and short telomeres.

The telomere length transiently increased after 1-2 weeks of culture in BM and MPB CD34+ cell expanded with FL+TPO+SCF±IL-6. After that, telomere started to gradually shorten if culture period was prolonged: by contrast, the addition of IL-3 resulted in a rapid decrease of CD34+ cell number, associated with a sharply shortening of telomeres immediately during the culture period.

Further experiments are on progress in order to give a functional explanation to the differences in telomere loss in the presence or not of IL-3, a cytokine probably associated with differentiation input of stem cells.
TOWARDS THE SELECTIVE EXPANSION OF TRANSPLANTED NORMAL HEPATOCITES

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Cell and gene therapy are increasingly being considered as potential strategies to treat chronic disease, including liver disease. In fact, many of the disorders treated by liver transplantation are diseases caused by hepatocyte dysfunction, and it therefore is unnecessary to replace the entire organ. This is particularly the case for genetic deficiencies of hepatic proteins in which selective replacement of hepatocytes would clearly be therapeutic. Clinical trials in humans have been performed, by results have been limited by the quantitative problem of insufficient tissue replacement.

Within this context, we have recently developed a general strategy for near-complete liver repopulation by transplanted isolated hepatocytes, which could be applied to achieve rapid proliferation of normal or any genetically modified hepatocytes. This model is based on two principles: (i) persistent block of resident hepatocyte cell cycle and (ii) subsequent transplantation of normal hepatocytes, which can selectively expand upon appropriate stimulation, since they were not exposed to the blocking treatment. Growth inhibition of resident liver was induced via exposure to retrorsine, a naturally occurring pyrrolizidine alkaloid. Using this approach, we were able to observe >90% repopulation of the resident liver by transplanted hepatocytes within a few months post-transplantation. We have been able to observe near total liver replacement by transplanted cells for up to 2 years in retrorsine-treated animals; transplanted hepatocytes integrated into the recipient parenchyma giving rise to a normal liver structure and function.

The successful application of the above two principles establishes a new concept in the field of cell transplantation: i.e. selective proliferation of donor-derived cells can be achieved via exogenous manipulation of the host, e.g. through drug exposure, which limits the growth potential of resident cells. Normal cells, delivered after drug treatment, can then proliferate selectively when exposed to appropriate stimuli, slowly replacing unresponsive endogenous hepatocytes. These findings are highly intriguing and have far reaching implications, both in basic biology of liver and liver cell repopulation and for their potential application in the field of cell and gene therapy.
ABSTRACT 15

THE PROLIFERATION/DIFFERENTIATION SWITCH OF THE PROGENITOR CELLS: IMPLICATIONS FOR CELL THERAPY


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The massive in vitro production of blood components represents one of the major goals of transfusion medicine.

The specific aim of this study was to investigate the feasibility of in vitro red cell production for autologous blood transfusion of thalassemic patients. Mononuclear cells were purified from peripheral blood of 11 healthy donors and 15 thalassemic patients (10 β-major, 3 β-minor and 2 heterozygous) and grown (1x10^6 cells/mL) under conditions which sustained optimal proliferation of human erythroid cells [stem cell factor, 50 ng/mL; interleukin-3, 1 ng/mL; erythropoietin (EPO) 1U/mL; dexamethasone 10^-6M; estradiol 10^-6M].

Terminal differentiation was induced by transferring the cells into medium supplemented with EPO (1U/mL) and insulin (1 µg/mL). The maturation of the erythroid cell mass was monitored at four days intervals with morphologic, biochemical and cytofluorimetric assays. Under proliferative conditions, 90% of the cells displayed a blast cell phenotype (CD45 low, glycophorin neg, CD71 low) at day 7 of culture and 50-60% of the cells expressed markers of early erythroid precursors (CD45+, glycophorin+, CD71+) by day 9-10. These erythroid cells had a medium hemoglobin (Hb) content of 2.13 pg/cell, unbalanced levels of globin chains (α/non-α synthetic ratio=2.5 by HPLC) and immunofluorescence positivity for foetal (F) Hb (F+ cells: 15-20%).

In cultures from thalassemic patients, the mean cellular Hb content in the proliferative phase was 1.34 pg/cell with an average α/non-α synthetic ratio of 2.78. Both in cultures from healthy donors and thalassemic patients, the number of cells reached the highest value at day 15 of proliferation (15x10^6 cells from every 10^6 of cells originally seeded) and progressively decreased afterwards, unless mononuclear cells were enriched again by density cut and transferred into fresh culture medium. Once transferred into differentiative conditions, the erythroblasts terminally differentiated (without further proliferation) to mature red cells, 95% of which were CD45neg, glycophorin- and CD71high, in flow cytometry, and diaminobenzidine+, in histochemistry. Once differentiated, the mean Hb content of normal cells increased to 3.3 pg/cell with a balanced α/non-α synthetic ratio (1.29±0.56) while the percentage of F- cells decreased to 8%. Unlike cells from healthy subjects, those from thalassemic patients contained only 1.39 pg of Hb/cell also in the differentiative phase and an α/non-α synthetic ratio of 1.41±0.79.
We are currently investigating the possibility to obtain red cells with a high Hb content from peripheral blood of thalassemic patients by adding to the culture known inducers of γ-chain synthesis. This study could lead to the identification of HbF inducers specifically targeted for the in vivo treatment of each patient, or, in the case of high cytotoxicity, to be used in vitro for massive red cell production for autologous transfusion therapy.
Session 6
Regulatory issues
Chairpersons
G. D'Agnolo, C. Pini
LESSONS LEARNT FROM DESIGNING AN EX VIVO SOMATIC CELL THERAPY PROTOCOL

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The term “cell therapy” means the administration of living, non-germ-line somatic cells to humans for diagnostic or therapeutic purposes. Somatic cell therapy products are generated by ex vivo processes resulting in changes in their proliferation capacity, genotypic and/or phenotypic characteristics. The peculiarity of these products has raised issues at the regulatory, ethical and quality control levels, which have been addressed in official documents published by Regulatory Bodies in various Countries. From the viewpoint of a medical team in charge of designing a phase I/II somatic cell therapy clinical trial, the starting step is to operate in a dedicated facility to avoid microbial contamination of the product and to protect the operator and the environment. Thus, the Italian Guidelines for Somatic Cell Therapy prescribe that all the operations are performed in BL3 laboratories, with controlled access to the laboratories through a series of two self-closing doors and HEPA filtered air. Specific training of the personnel and standard operating procedures must be provided. The second step in designing a cell therapy protocol is the selection of the reagents. In particular, the inclusion of animal-derived substances should be avoided, in favor of non-animal-derived reagents of defined composition, submitted to stringent microbiological control. A close interaction with the producers of the reagents may help to obtain appropriately characterized materials. Moreover, to fulfill the requirements of the current pharmaceutical European legislation the final product should be characterized, to assess the maintenance of the desired phenotype and to exclude undesired effects (e.g. neoplastic transformation, infectious contamination). Unfortunately, most cell products are prepared on an individual patient’s basis, administered to the patient immediately after the manipulation and limited to the clinically necessary dose, with a small aliquot available for quality control. In this regard, the Italian Guidelines prescribe that the entire manipulation process should be validated for sterility, identity, viability, activity and proliferation, using the same process as for the clinical use. Immediately prior to the clinical use, a set of significant rapid tests must be performed. Finally, in order to supply the laboratory of crucial information to improve the product, any adverse effect on the patient must be recorded, analyzed and whenever possible correlated to the composition of the product.
IMMUNOLOGICAL CHARACTERIZATION OF ANTI-F.VIII ANTIBODIES BY A SANDWICH-ELISA AND THE BIA-TECHNOLOGY

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The immunological characterization of both non-neutralizing and inhibitory anti-FVIII antibodies (Abs) which develop in Haemophilia A patients infused with F.VIII, has received increasing attention. Since F.VIII non-neutralizing Abs cannot be directly detected by conventional biological tests, we have developed a sandwich-ELISA and a surface plasmon resonance (SPR)-based biosensor system (BIA technology) for anti-F.VIII total Ab detection and epitope mapping. In the ELISA, an anti-F.VIII MAb was coupled to the plate in order to capture F.VIII subsequently incubated. A rabbit hyperimmune antiserum containing polyclonal anti-F.VIII Abs was then added and the Abs were allowed to bind to the entrapped F.VIII. Enzyme-labelled monospecific Abs were used to determine the presence of bound primary anti-F.VIII Abs, their Ig class and light chain type. Multisite binding assays were performed incubating each time a different MAb raised against a distinct epitope of F.VIII, thus presenting F.VIII with different orientations. Using the biosensor, a rabbit antimouse IgG1-Fc MAb was coupled to an extended hydrogel matrix attached to a gold surface in order to capture an anti-F.VIII MAb injected at saturation. F.VIII was then allowed to bind to this MAb. Multisite binding assays were performed by flowing sequentially other MAbs recognizing distinct epitopes on the F.VIII, and the Ab capacity to interact with the entrapped F.VIII forming multimolecular complexes was monitored in real time with SPR. A set of several different MAbs (all IgG1K) against the vWF, the L- or H-chain of F.VIII was analyzed. By presenting F.VIII with different orientations, we clearly demonstrated that the rabbit polyclonal Abs either failed to bind F.VIII or alternatively interacted with this protein with high or low affinity, depending on the first MAb used for its immobilization. Distinct structural epitopes on F.VIII were recognized by non-neutralising and inhibitory Abs. Comparable results were obtained using the sandwich-ELISA, and the BIA technology. Both methods appear to be accurate and sensitive. Preliminary studies demonstrated that both methods can be successfully applied for the immunological detection and characterization of F.VIII inhibitors present in human sera, revealing as low as 0.3 Bethesda units/ml (CNR.PF-MADESS 2°; MURST– Stem cells).
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