CELLULAR ENGINEERING AND CELLULAR THERAPIES

DEVELOPMENTS IN HEMATOLOGY AND IMMUNOLOGY

Volume 38

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Cellular Engineering and Cellular Therapies

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edited by

C.TH. SMIT SIBINGA
Sanquin Consulting Services,
Groningen, The Netherlands

and

L.F.M.H. DE LEIJ
Groningen University Institute for Drug Exploration,
Groningen, The Netherlands

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MODERATORS AND SPEAKERS

Moderators

L. de Leij (chairman) – Department of Pathology and Laboratory Medicine, Medical Biology Section, University Medical Centre Groningen, and GUIDE, University of Groningen
Groningen, The Netherlands

G.D. M. Beun – Sanquin Blood Bank North-East
Groningen, NL

P.L.M. Jansen – Department of Hepatology and Gastroenterology, University Hospital Groningen
Groningen, The Netherlands

H.C. Kluin-Nelemans – Department of Haematology, University Hospital Groningen
Groningen, The Netherlands

P. Law – Director, Cell Process Development
Dendreon Corporation
Seattle, WA, USA

F. Miedema – Sanquin CLB Division Research
Amsterdam, The Netherlands

S.D. Noga – The Johns Hopkins University, and Alvin & Lois Lapidus Cancer Institute, Sinai Hospital of Baltimore
Baltimore, MD, USA

P. Rebulla – Cell Factory ‘Franco Calori’, Milano Cord Blood Bank, Centro Trasfusionale e di Immunologia dei Trapianti
Milan, Italy

Speakers

L.A. Drayer – Sanquin Blood Bank North-East
Groningen, NL

C.G. Figdor – Center for Molecular Life Sciences,
University of Nijmegen
Nijmegen, The Netherlands

G. de Haan – Department of Stem Cell Biology,
University of Groningen
Groningen, The Netherlands

H.J. Haisma – Dept of Therapeutic Gene Modulation,
University Center for Pharmacy,
University of Groningen
Groningen, The Netherlands

P.L.M. Jansen – Department of Hepatology and Gastroenterology,
University Hospital Groningen
Groningen, The Netherlands

P. Law – Director, Cell Process Development
Dendreon Corporation
Seattle, WA, USA

L. de Leij – Department of Pathology and Laboratory
Medicine, Medical Biology section
University Medical Centre Groningen
Groningen, The Netherlands

N.H. Mulder – Dept. of Medical Oncology,
University Hospital Groningen
Groningen, The Netherlands

S.J. Noga – The Johns Hopkins University, and
Alvin & Lois Lapidus Cancer Institute,
Sinai Hospital of Baltimore
Baltimore, MD, USA

F.A. Ossendorp – Department Immunohematology and Blood
Transfusion, Leiden University Medical Center
Leiden, The Netherlands

P. Rebulla – Cell Factory 'Franco Calori',
Milano Cord Blood Bank, Centro Trasfusionale e
di Immunologie dei Trapianti
Milan, Italy

E.K. Read – Department of Transfusion Medicine,
Warren G. Magnusen Clinical Center,
National Institutes of Health
Bethesda, MD, USA

P.H. Roddie – Consultant Haematologist,
Department of Haematology,
Western General Hospital
Edinburgh, UK

J.W. Semple – Toronto Platelet Immunobiology Group Toronto,
Ontario, Canada

I. Slaper-Cortenbach – Department of Hematology,
University Medical Center Utrecht
Utrecht, The Netherlands

C.Th. Smit Sibinga – Academic Institute for International Development of Transfusion Medicine,
University of Groningen, and
Sanquin Consulting Services
Groningen, The Netherlands

S. Solomon – Stem Cell Allotransplantation Section,
Hematology Branch, National Heart, Lung and
Blood Institute, NIH
Bethesda, MD, USA.

FOREWORD

Welcome to the City of Groningen, the center of the North of the Netherlands. Groningen is proud of the long lasting tradition of scientific symposia organised by the Sanquin Blood Bank.

These Sanquin International Symposia on Blood Transfusion have become a true traditional event in Groningen, marking the early academic year and have contributed to the specific reputation of Groningen and its University in the scientific field of Transfusion Medicine. The growing tradition has also contributed to initiatives of both University, Province and the City of Groningen to bring science and industry together – BioMedCity Groningen. Such reputation does not just happen, but is the result of creative and scientific leadership, of vision and an open mind, to explore in a team spirit horizons. Groningen is particularly proud of this reputation thanks to its leadership, the Sanquin Blood Bank North-East.

This year in particular the theme chosen some two years ago is extremely timely as it illustrates the activities and scientific interest of an integrated team which includes our regional Sanquin Blood Bank North-East and fits in the City initiatives within the concept of BioMedCity, Groningen.

Transfusion medicine is the bridging science involved in both the processing and engineering of harvested cells and the response to potential application of cellular therapies – in practice vein to vein. Specifically harvested cells are attractive targets for cellular therapies and gene transfer, because

1. cells can be easily obtained and manipulated, and
2. stem cell transplantation has already been successfully applied for treating a number of genetic disorders.

Thus, the transfusion medicine centres of excellence, such as the Sanquin Blood Bank NorthEast in Groningen, should no longer be regarded as simple production facilities but should be academically integrated and become centres for translational research.

Since cellular therapies including gene transfer entail both cellular processing and genetic modification under strict pharmaceutical conditions securing optimal quality, there is a need for integrated processing and clinical laboratories where these activities can be carried out and properly managed. In Groningen such an integration has started with an joint endeavour of University, Academic Hospital and Sanquin Blood Bank and will undoubtedly lead to

new developments in cellular engineering and cellular therapies. The City of Groningen has great interest in this project and will support where possible.

It is evident that a complex of conditioning requirements need to be fulfilled, including a highly developed infrastructure, cost containment and above all team spirit and leadership. The clinical outcome – the patient care - of a joint effort in an academic setting and an infrastructure climate within the City of Groningen should be based on strictly controlled and well governed production processes for which expertise is available within our city and province.

I wish you all a successful symposium and hope to see you back many more times in Groningen to enjoy the science, the city and the culture of the North of the Netherlands.

Arnoud Guikema, JD
Department of ROEZ
Municipality of Groningen

I. DEFINING THE FIELD

CELLULAR ENGINEERING AND CELLULAR THERAPIES – AN OVERVIEW

L.F.M.H. de Leij, M.J.A. van Luyn, M.C. Harmsen[1]

Introduction

Cellular therapy offers a challenging approach for the treatment of a variety of diseases. Although some types of cellular therapy, like the generation and application of appropriately differentiated embryonic or adult stem cells, still await the successful outcome of a lot of further experiments, others have been established and clinically applied already a long time ago. So, a lot of clinical experience has been obtained with both autologous and allogeneic bone marrow transplantation protocols and various kinds of immunotherapeutical approaches employing ex vivo activated and cultured NK cells, cytotoxic T lymphocytes or dendritic cells. These trials have shown that cellular therapy can be given in a safe and controlled way. The current Good Manufacturing Practice (cGMP) procedures already established for the preparation, culturing and ex vivo manipulation of cells in these types of applications should be taken into account in implementing appropriate policies and procedures leading to cGMP procedures for future types of cellular therapy such as the use of manipulated stem cells.

Recent advances in stem cell biology have raised hopes that also non-haematopoietic stem cells or derivatives hereof can be given to patients directly or after appropriate ex vivo culturing and differentiation inducing protocols [1]. This may open new avenues for therapy, i.e. new forms of regenerative medicine. For a better evaluation of such a prospect it is timely, firstly, to evaluate the present progress in a number of selected models. In these models a large variety of different cell types like neurons, endothelial cells, insulin-producing cells, cardiac muscle cells, hepatocytes, and Schwann cells can be derived from embryonic stem cells (ESC) or cultivated from stem cells derived from or present in adult tissue [adult stem cells (ASC)]. In specifically designed animal models these cells can be used successfully to reconstitute diseased tissues or organs. Secondly, hints for future directions in this line of research can be expected from knowledge about embryogenesis and from in vitro ESC manipulation experiments. This includes the identification and manipulation of specific molecular switches leading to guided differentiation.

1. Department of Pathology and Laboratory Medicine, Medical Biology sectionUniversity Medical Centre Groningen, Groningen, NL.

Diseases in which only one type of cell is afflicted such as genetically defined anaemia and immunodeficiency cases, or, alternatively, diseases of other aetiology like Park nson's disease, ischemic heart disease or diabetes, represent the most obvious first targets for stem cell based therapeutics. In the case of genetically based diseases this should include the use of ex vivo genetically modified or allogeneic cell preparations. The use of adult stem cells versus embryonic stem cells merits further discussion. It is clear that a subset of tissues, such as the haematopoietic, gastrointestinal, liver, pancreas and integumental systems, show a remarkably capacity of regeneration throughout life indicating the presence of abundant, easily inducible and/or potent ASC. This can be taken as an argument that these tissues are amenable to ASC based therapies in the near future. An important factor that will guide proper in situ cell function after ASC therapy is the fact that cells function in vivo in a three-dimensional way, in which cells interact not only with other cells, but also with (preformed) intercellular matrixes. In therapeutic settings preformed and, in time, resolving matrices could provide a scaffold in which these conditions might be mimicked.

Selected Disease Models

A large array of both clinical and animal studies on stem cells has been published during the past few years. These include studies using both ESC and ASC giving rise to neurons, oligodendrocytes, microglia, astrocytes, hepatocytes, epithelial cells, cardiomyocytes, skeletal muscle cells and endothelial cells [2]. Since clinical studies have only limited possibilities to evaluate the course and outcome of therapy, it is important to use appropriate animal models to establish the principles underlying this new type of treatment. In the following only a few models will be reviewed.

Parkinson's disease

For still unknown reasons, dopaminergic neurons in the Substantia Nigra progressively die in Parkinson's disease patients during the course of disease. Lack of locally produced dopamine by the basal ganglia will precipitate eventually into the typical movement disorder symptoms (rigidity, bradykinesia and resting tremor), and, subsequently, brain degeneration and cognitive malfunctions. Restitution of dopamine producing cells in animal models of Parkinson's disease showed great promise for treatment. Also clinical studies, in which in situ injections of cells isolated from the embryonic ventral mesencephalic tissue of multiple donors were given, showed that this kind of cellular therapy could reverse symptoms [3]. Apart from ethical and logistical considerations, the donor tissue employed in these studies is not well defined and limited, however. An alternative source could be derived from stem cells. In vitro, mouse ESC can be induced to differentiate into dopaminergic neurons. Following a protocol in which ESC are cultured and induced to differentiate in media supplemented with both neurotropic and survival promoting factors a significant part of all formed neurons proved to be dopaminergic [4,5]. When ESC are genetically modified to express a transcription factor that guides differentiation into dopamine producing cells, the percentage of dopamine producing cells is even higher

[6]. This type of genetic modification has great potential in guiding the differentiation into other cell lineages, since it ensures higher percentage of 'intended' cells without concomitant contamination with 'undesirable' cells. When such mouse ESC derived neurons were transplanted into an animal model of Parkinson's disease, i.e. into the striatum of immunocompromised (nude) rats made devoid of dopamine producing cells, this transplantation proved to reverse the Parkinson's disease like symptoms [6,7]. Although functional dopaminergic neurons could be demonstrated to be present in the majority of these animals, a major drawback of the procedure was that in about 25% of the treated animals teratomas had developed [7]. These findings indicate that in the case of the use of ESC derived cells purging should be done, i.e. prior to transplantation all remaining undifferentiated ESC should be removed. Also human ESC can be induced to become dopaminergic neurons and to be functional after grafting into neonatal mice [8]. Before clinical trials can be started with such cells, the safety of the procedure, i.e. the annihilation of the risk of teratoma formation, has to be firmly established however. A more attractive approach will be the development of protocols starting from bone marrow derived ASC. Dopaminergic cells can be cultured from stem cells or neural cell progenitors taken from the brain as well as from bone marrow. Since bone marrow is the most obvious source to be used in clinical protocols, it is anticipated that such a procedure might become possible in the near future.

Diabetes

Diabetes type I (Insulin-Dependent Diabetes Mellitus, IDDM) is a disease in which the patient's pancreatic islet cells have become destroyed in time as a result of ongoing autoimmune reactions. In Diabetes type 2 (Non-Insulin Dependent Diabetes Mellitus, NIIDM) increased insulin resistance of target tissues is probably causing increased islet cell activation eventually leading to loss of functional islets. In both cases, when the number of ß-cells in their islets falls below a critical level, diabetic patients can't produce enough insulin to control their blood glucose levels. Current therapy using various kinds of insulin administration is efficient in the short run, but, since it does not provide the necessary tight control of blood glucose levels, will run into severe, late-stage complications. Transplantation protocols using the whole pancreas, or more recently and probably more appropriate, isolated islets, have shown some successes, but suffer from the need to install long term immunosuppression, and, more importantly, from the limited supply of donor organs. In diabetes the gradual loss of ß-cells is due to a shift in the balance between enhanced loss of cells and insufficient cell renewal. In adult life ß-cells have a great capacity to proliferate. It has been shown in vitro that it is possible to generate ß-cells also from adult human pancreatic duct cells [9]. Furthermore, using elaborate culture and differentiation protocols, in which a number of 'stages' could be discerned, cells expressing insulin and other pancreatic endocrine hormones were obtained from mouse ES cells [10]. These cells proved to be capable to self-assemble in three-dimensional clusters similar in topology to normal pancreatic islets. Glucose triggered insulin release from these cell clusters in vitro and when injected into diabetic mice, these clusters maintained the islet-like organization, became vas-

cularized and were able to produce insulin. However, also in this case the number of 'undesirable' cells is high; meaning that genetic engineering with a gene encoding a transcription factor guiding cells into ß-cell differentiation would be helpful. The pancreatic and duodenal homeobox-1 (Pdx-1) gene could be such regulator gene. Interestingly, this same gene also appeared to be operative, after appropriately enhancing its function by genetic engineering, in trans-differentiation of hepatic cells to pancreatic cells [11]. Whether differentiated ESC can be used for cellular therapy in diabetes or that autologous ASC offer better possibilities, is still uncertain. ASC could be derived from pancreatic duct cells, from liver precursor cells or, possibly from bone marrow cells. Before such a therapy can be given, at least in the case of diabetes type I, the underlying autoimmunity has to be overcome, either by appropriate immune suppression or by the induction of tolerance.

Ischemic heart disease

One of the clinical endpoints of atherosclerosis is myocardial infarction, i.e. progressive occlusion of the vessels that supply blood to the myocardium. The myocardial tissue lying downstream of such occlusions will start to suffer from lack of nutrients and oxygen and, eventually, will die. In these ischemic areas a remodelling process starts which involves replacement of dead cardiomyocytes by fibroblasts resulting in the formation of rigid, scar-like tissue. To compensate the resulting loss of function the remaining myocardium will becomes hypertrophic and eventually this process will precipitate in a heart attack. When the patient survives major parts of the heart remain fibrotic and non-functional, since adult cardiomyocytes are unable to proliferate. The prognosis is not very good, since the patient is at great risk to attract additional infarctions and heart attacks. For the moment no appropriate therapy, except whole organ transplantation, is available. Alternative treatments are sought such as the use of local VEGF gene therapy to induce angiogenesis and restoring in this way blood supply to infarcted regions. This might be helpful in early, but not in late stages of disease. A more attractive approach is the use of stem cells, which in the end could replace the non-functional cells in the scar tissue by functional cardiomyocytes.

Spontaneously differentiating ESC, obtained in culture by withdrawal of leukaemia inhibitory factor (LIF) in the case of mouse ESC, display contractile properties. Therefore it was thought that these cells could be used to repopulate the infarcted areas. Also human ESC can be induced to cardiomyocyte differentiation [12]. However, only a small proportion of these cells actually display cardiac muscle-specific antigens [12]. Also ASC can be induced to differentiate into cardiomyocytes and such cells were shown to be able to repopulate ischemic areas in a mouse model [13]. A complication is that not only cardiomyocytes have to repopulate the infarcted areas, but that also new blood vessels have to be formed. This can be achieved by inducing angiogenesis from existing blood vessels (e.g. by adding VEGF) or by vasculogenesis from endothelial precursor cells in combination with angiogenesis.

An attractive possibility is the replacement of scar tissue by the implantation of in vitro engineered, in time degradable 3-D matrices. In vitro it has been

shown that cardiomyocyte precursor cells can be seeded in such matrices and cultured for some weeks. During this period of time the precursor cells populated the construct, established proper cell-cell contacts and became functional cardiomyocytes [14].

Bone Marrow As a Source for ASC

The discovery that a small fraction of the cells present in bone marrow can differentiate into endothelial cells has shed a different light on bone marrow which is classically known as a site of hematopoiesis. To date, the bone marrow is considered as a stem cell compartment with the potential to generate and mobilize stem cells and precursor cells to virtually every tissue and organ (see also figure 1). Bone marrow derived stem cells have been shown to differentiate into endothelial cells, neurons, liver cells, muscle cells and in due time many more possibilities to differentiate are anticipated to be found. Bone marrow stem cells can be mobilized into the peripheral blood circulation by the application of specific growth factors. Consequently, bone marrow derived stem cells can be relatively easily purified from peripheral blood. Two main populations of suitable stem cells for differentiation into endothelial cells appear to exist in bone marrow: CD34+ stem cells and multipotent adult precursor cells (MAPC) [15]. The CD34+ stem cells are best known as haematopoietic stem cells, but a fraction of these cells can also differentiate into e.g. endothelial cells. These cells have become known as endothelial precursor cells or EPC. MAPC are a rare fraction in bone marrow preparations, but can also be isolated from other tissue compartments such as brain or muscle [16]. MAPC are isolated as fibronectin adhesive cells that lack CD34, CD45 (present on all leukocytes) and glycophorin-A. In serum-free medium supplemented with growth factors such as

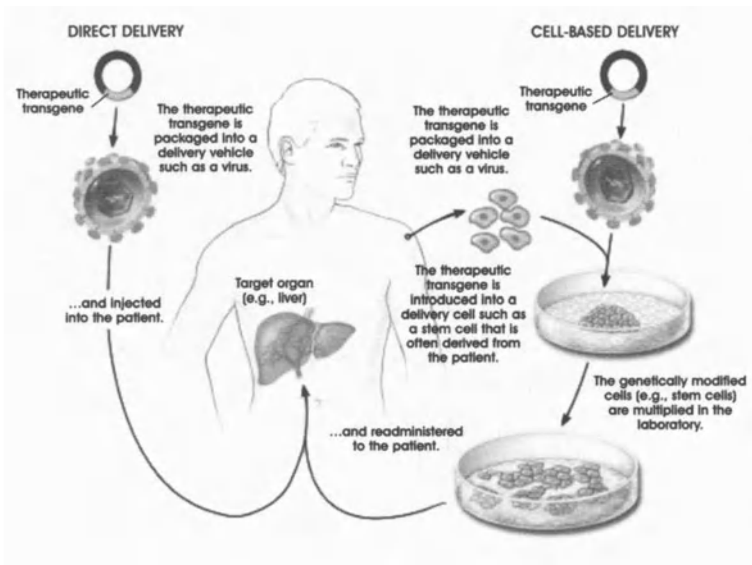

Figure 1: Plasticity among adult stem cells (courtesy of T. Winslow, L. Kibiuk and C. Duckwall [17], printed with permission).

IGF, EGF and PDGF-BB they proliferate virtually in definitively. Cultured MAPC are pluripotent, because upon injection in murine blastocyst they give rise to animals with chimeric organs and tissues (except for cardiomyocytes). In addition, cultured MAPC express telomerase and also a number of other markers typical for primitive stem cells, such as Oct-4 and Rex-1.Patients suffering from major trauma often show higher levels of endothelial precursor cells than healthy controls. Whether other precursor cells are also present, or not, is still unknown.

The use of autologous bone marrow derived stem cells is an advantage when tissue grafts are to be developed. These cells can be modified in vitro. Genetic manipulation is facilitated by several molecular tools such as plasmid transfection and infection with viruses (see also figure 2). These molecular genetic vehicles can be programmed such that they encode marker proteins which are

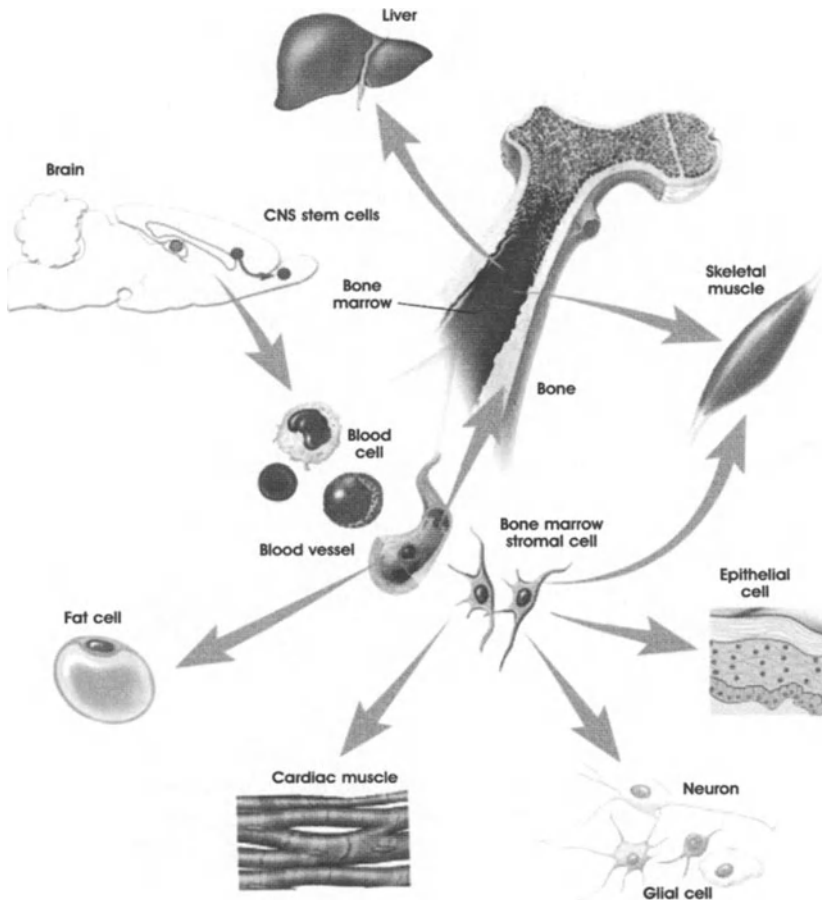

Figure 2: Strategies for delivering therapeutic transgenes into patients (courtesy of T. Winslow [17], printed with permission)

instrumental to following the fate of differentiated stem cells once these are grafted into animals. Moreover, molecules that facilitate binding to the growth substrate or which promote differentiation into specific cell types can be introduced into stem cells using these vehicles.Artificial grafts can be constructed with natural and synthetic polymers as scaffolds. The development of artificial vascular or other grafts critically depends on a suitable growth supporting matrix for the stem cells. After seeding stem cells will differentiate into specialized adult cells which continue to proliferate until the graft is fully occupied. Grafts can be modified by binding growth factors or adhesion factors to enhance binding and differentiation of stem cells.

Conclusion

Cellular therapy using differentiated cells without further culturing or manipulation is an 'established' technique. New types of cellular therapy employing ESC- or ASC-derived cells need further establishment of appropriate cell selection, culturing and/or genetic manipulation procedures. Animal models and in vitro manipulation are needed to establish the principles underlying this kind of manipulation. In the end, when human derived ESC or, more likely, bone marrow derived ASC will be used for clinical purposes, appropriate cGMP/cGTP procedures have to be established. Although a lot of preclinical and pilot clinical studies are done already, it is clear that this kind of therapy needs a lot of further research and should not proceed too quickly into the clinic.

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GENE THERAPY 2002: A NEW START

N.H. Mulder[1]

Introduction

Following the turbulent end of the twentieth century when one protocol violation led to a most regrettable toxic death threatening clinical gene therapy in its very existence, it was hoped that easier navigation would lie ahead. Complications arising in the initial successful study of X-linked combined immune deficiency negate this hope. In this review we will produce some evidence for a promise of chances of success on a clinical level in the foreseeable future.

Gene therapy can be defined as the intentionally caused selective changes in DNA brought about by the use of genetic material. An important problem for the development of clinical gene therapy is the immense and overwhelming amount of bureaucracy and red tape that is rapidly suffocating this form of clinical research even faster than it does for other forms.

On the various elements of gene therapy, the gene of interest, its promoter, the vector, targeting principles, the proteins and their immunological consequences, new data have been gathered in recent years and we will discuss some of them.

Vectors

Initially the field of viral vectors was limited to the retroviral vectors, clinical experience with this system is still the largest available, but as adenovirus, adeno-associated virus and lentivirus also find clinical use today, these groups will be discussed.

Especially in this field rapid changes occur: although safety of the retroviral vectors was almost taken for granted until now, their very low transfection efficiency seems to render them unfit for clinical practise in vivo. They may however still have a place in ex vivo gene transfection. In this area the use of foetal cells that have a corrected gene defect will provide an important future for the combination of gene- and stem cell therapy.

Most of the more then 500 active gene transfer protocols now apply the adenovirus. The early generations had deletion in the E1 region, but later generations have deletions in the E1, E2 and E4 genes, rendering them less immuno-

1. Dept. of Medical Oncology, University Hospital Groningen, Groningen, NL.

genic and permitting longer gene expression. The patient who died in September 1999 received a high viral load by the hepatic artery to a failing liver; the adenovirus used was of the first generation, eliciting a rapid immune response, with subsequent coagulopathy and multi-organ failure. It is expected that risks of later generations of adenovirus are considerably reduced. New in this field is the use of replication competent cytolytic virus either alone or in combination with a suicide gene. The earliest successes of this approach concern the P53 binding Onyx virus, however further experience does not suggest that this approach even if it is interesting, is a major breakthrough [1].

Local application of a lytic virus with two suicide genes did however lead to interesting responses in relapsed prostate cancer [2].

Newer developments in adenoviral technology concern the elimination of all protein production related genes "gutless adenovirus", these vectors require a helper system, but toxicity should be marginal and the room for inserted genes is increased [3].

Although adenovirus has selectivity for some organs by its homing characteristics, especially to the liver, selectivity can be greatly enhanced by manipulating the targeting of the virus that naturally occurs through the common adenoviral receptor system. Tissues that lack this system can be transfected when for instance bispecific monoclonal antibodies are used [4].

The small adeno-associated virus, in contrast to adenovirus can transfect non-dividing cells and lead to stable integration. In humans its track record so far is good as far as toxicity is concerned, however in mice liver tumours have been recorded after exposition to this virus [5].

The lentivirus has as its main attraction also the possibility of stable integration in non-dividing cells; its potency to transfect haematopoetic and probably stem cells is interesting [6]. The safety of this HIV related virus has been brought about by eliminating all but two genes (Gag and Pol) from the genome, however ultimately safety has to be determined in clinical trials, especially as animal models of HIV are scarce. The complexity of the human lentivirus, with 9 genes offers greater opportunities for modifications that reduce the emergence of replication competent recombinants (RCR) than the animal lentivirus variants.

Safety issues have become extremely complex in recent years, for individual patients safety may be increased with transient expression and eliminating antibodies. High transfection and recombination rates will be a risk for the environment. Risk of germ cell insertion of lentivirus compared to AAV is difficult to estimate. The main attraction of non-viral technology is the evasion of real or assumed toxicity risks. As all important side effects of gene therapy are so far caused by viral vectors this field of research deserves much wider support.

In Table 1 some of the competitive or additive aspects of various vector systems and their relative merits or demerits are mentioned.

Whatever vector is used, the transfection efficiency into the target cells will remain a limiting factor. Devices such as the gene gun have been developed primarily for transfection in vitro, but transfection of skin cells and subcutaneous tumours has been demonstrated to occur in vivo. Interesting recent develop-

Table 1. Vectors in comparison

Vectors	Targets	Clinic	Advantage	Problems	Logistics
Lenti-virus	Neurons Pancreas Haemo-poiesis	Monogenetic Reconstruction	Stable transfection resting cells	RCR reverse transcriptase stip	helper system required
Adeno-virus	Liver Muscle	Rapid, single immunisation Suicide genes	Efficient targettable	immunity	easy-unless gutless
A.A.V.	Liver Muscle Brain	Secreted proteins Monogenitic Reconstruction	Stable no immunity no human disease cause	slow expression small size liver tumours in mice?	helper system required

ments include the use of ultra sound [7] and in vivo electroporesis to facilitate gene delivery for instance into muscles [8]. Cationic polymers and various other chemicals have also been used to evade viral vectors. Although usually easier to produce, these systems can not always be considered to be a priori safe [9].

In general the ultimate goal of the therapy dictates the vector to be used: monogenetic diseases require stable transfection with an AAV virus or a Lentivirus and when used ex vivo with a retrovirus. Immune modulation can be achieved through transient methods, with adeno virus or various naked DNA methods; the same systems can be used for suicide genes in oncology. (Table 1). Transfection of muscle tissue with AAV was shown in the early phases of development of this vector to lead to prolonged expression and secretion of proteins [10].

Clinical Goals and Experience

Despite the increase in hurdles raised for clinical gene therapy trials in recent years, their number has increased above expectations and probably also above the number essential to answer present day questions. (Table 2).

Table 2. Numbers of gene therapy trials

Year	1999	2000	2001
Cancer	194	234	331
Other	83	116	201
Total	277	350	532

Table 3. Monogenic Targets

Phase 3	Phase 2	Phase 1	Pre-clinical
Cardiac ischaemia VEGF	Haemophilia	Muscular dystrophy	Diabetes
Peripheral ischaemia VEGF	Scid	Fanconi	Thalassemia
Glioblastoma TK	Gaucher		Amauroris
	OTC def.		Hypertension

Gene therapy started its clinical career with an attempt at transfer of the adenosine deaminase gene ex vivo, a goal, although widely publicised that is still unfulfilled today.

Over the years and unabated today, cancer is the most common target for studies with minimal results, however the relatively few studies in other areas (Table 3) have led to the still rare positive results.

Clinical gene therapy has specific requirements as whole organs and systems need to be transfected this involves large cell numbers, sometimes specific cells in the organs are the target requiring specificity and protein production must be regulated and controlled.

Cell numbers

In all situations sufficient cells will have to be transfected with the gene and this common problem is only partly dealt with by the viral vectors of today.

Some cell systems such as stem cells may be expanded in vitro, if permitted by stem cell technology. Even expansion in vivo has been considered though not achieved for instance in the attempts to transfect bone marrow with a resistance gene for chemotherapy. In this area of research stem cell technology and gene therapy can be greatly synergistic.

Specificity

Presently specificity is usually achieved by the crude method of injecting into the organ of interest or into the supplying vessel. Adenovirus naturally homes to the liver, herpes virus to the brain.

Non viral vectors can relatively easily be targeted to tissue receptors by means of antibodies. In viral vector technology this has been especially successfully attempted with the adenovirus.

More sophisticated is the physiological specificity on tissue or organ level. This can be brought about at the promoter level, as can be achieved for tissue specificity, for instance in the case of the prostate specific antigen promoter [11] but also at a disease level in the case of anoxia dependent expression [12]. Another form of specificity occurs when a farmaco-sensitive promoter is used for instance an element responding to tetracycline, or to ultrasound or irradiation.

Usually such models have a basic "leaky" expression, limiting their specificity.

Control of expression

An important goal of gene therapy is the production of a protein that is secreted into the circulation. Examples are growth factors, insulin and clotting factors.

For reasons still largely unknown the skeletal muscle is exquisitely suited for the production of proteins, this has been found with viral vectors as well as with plasmide technology.

For some proteins regulation by their natural half-life is sufficient: for example with clotting factors, but for insulin strict regulation is of paramount importance.

This specific problem, gene therapy for diabetes has extensively been studied. The gene for insulin production can be transfected and the protein has been

produced in a number of tissues: liver, muscle, glands and others. Crucial is the ability of the production to respond to blood glucose levels. Various elements of insulin synthesis have been scrutinised for optimal control: the promoter region, the transcription and the conversion of pro-insulin to insulin, exocytosis of the hormone and (counter) regulation by glucagon. Most studies use regulating elements in the promoter region to correct insulin production, however this invariably leads to slow adaptation of insulin production to blood glucose levels, due to the slow time course of transcriptional control. An interesting alternative was the transfection of K cells [13]. These cells in the upper digestive tract have naturally glucose responsive elements in order to produce the glucose dependent insulinotropic polypeptide (GIP) that closely follows insulin secretion in vivo. If these cells are transfected with the insulin gene under control of the GIP regulatory gene and injected in mice, a working model of glucose regulation resulted.

Cancer

Any therapy of cancer requires elimination of all or nearly all cancer cells to be of value. This seems to be incompatible with the main problem of gene therapy today, the lack of transfection efficiency. Two strategies circumvent this problem, the suicide gene therapy concept and the immune gene intervention.

The suicide gene modality increases its quantitative effect with the "bystander effect": neighbouring cells transmit toxic products without having been transfected. Table 4 gives most of the genes that have been studied in this respect.

Table 4. Suicide gene therapy in cancer

Gene	Prodrug
E Coli cylosine Deaminase	5 Fluorocytosine
Herpes Thymidine kinase	ganciclovir
E coli Deo D	6 MP-desonyribonucleoside
E coli g.p.t.	6 Thioguanine
Py50	cyclophosphamide
XGPRT	6 Thioxanthine
β lactumase	Vinca-cephalosporine
β glucosidase	amygdaline (CN)
5 DFUR	Thymidine (5FU)
nitroreductase	EB 1954 (alkylator)
carbotypeptidase	CMDA (alkylator)

Although the suicide concept should usually lead to low systemic toxicity most of the products used have inherent side effects and most of the end products have limited cancercell toxicity. It is not always evident that the concept surpasses the effect of local injection of the end compound. The toxic elements of the bystander effect may however not be the only mechanisms involved as in general immune competent animals respond better to this strategy.

The clinical data with suicide gene therapy have as a common characteristic that localised tumours have been chosen, brain tumours, prostate, cavities, head and neck and liver. In most of these patients a retroviral vector has been used and only the herpes simplex thymidine kinase has, in sufficient numbers of patients, been tested. Although generally non-toxic, the effect on tumour growth has been limited. By far the largest study ever done in gene therapy, a phase III study in 248 patients, found no effect of HSV-TK in the adjuvant setting in patients with glioblastoma multiforme [14]. In some of the prostate cancer studies a prolonged decline of circulating PSA levels has been noticed [15].

The limits of transfection efficiency of present day vectors is also less of a problem in immunological gene therapy studies. Numerous studies using cytokine genes alone or in combination with a tumour antigen have been published. Occasional responses and induction of immunity has been noticed.

The problems of transfection efficiency are prominent in the field of suppressor gene transfection mainly with P53. The sound concept is to supply wild type P53 to mutated tumour cells. This should help these cells to go into apoptosis following DNA damage by conventional anti-cancer treatment. As no bystander effect is expected to occur, as many cells as possible should be transfected. Most studies therefore have been done with an adenovirus as vector. Again, as with the suicide gene approach, mainly localised tumours have been studied [16].

One of the stimulating observations of in vitro studies with P53 in in vitro models was, that transient expression without further intervention sometimes led to tumour cell death provided that enough wild type protein was present. A strong promoter such as the CMV promoter is therefore commonly used.

Most data published to till now concern toxicity evaluation and combinations with chemo- and or radiotherapy. It is evident that gene transfection is possible, also in the presence of adenovirus antibodies, that toxicity is minimal, and not increased in the case of concurrent chemo- or radiotherapy. At present it is not possible to speculate on a positive outcome of phase III studies: although some impressive remissions have been presented they do not seem to be common.

Circulation

Various interesting interventions are conceivable at gene level in ischaemic diseases. At the basis of such diseases hypertension is often encountered. The genetic background and possible interventions of this syndrome, for instance at the level of transfer of the kallikrein gene, the atrial natriuretic peptide gene and others are the subject of intense studies [17]. In this field also gene therapy of diabetes plays a role, as many of these complications occur in diabetic patients.

Part of the problem of vessel obstruction is proliferation. In line with the suicide gene attempts in cancer this has been attacked with the Thymidine kinase and ganciclovir approach, and also animal studies have been done aimed at interfering with the cell cycle and induction of apoptosis.

Most clinical studies however concern the angiogenic growth factor VEGF. Three homodimers of the gene were isolated, resulting in four isoform proteins. Two are in a diffusable form: 121 and 165. Two other subtypes 189 and 206 are cell bound. Two types of receptors have been found on macrophages and endothelial cells. Initial clinical studies with VEGF in ischaemic disease date from

1994, pioneered by Isner. The first study used a catheter system but later studies applied plasmide DNA or adeno viral vector encapsulated DNA into muscle. Also other growth factors have been used, such as fibroblast growth factor. In cardiac ischaemia various approaches have been used: catheters, direct injection, concomitant with bypass surgery. Toxicity and feasibility can be determined from the work of the pioneering group [18]. In general toxicity seems to be limited, although innate mortality in these elderly and often ill patients can make recognition of side effects difficult. The most common side effect was peripheral oedema occurring in approximately 30% of the patients.

The response rate as seen in table 5 is extremely high, both in peripheral and heart ischaemic disease. These results should however be considered critically, as responses are mainly clinical. Improvement of pain is known to be frequent with placebo treatment, and even wound healing and angiographic improvement can not be judged outside a randomised study. Indeed the first randomised study in peripheral disease was negative [19].

In this study specifically increased angiogenesis was seen with digital subtraction angiography in the VEGF treated patients, but clinical improvement occurred frequently also in the placebo treated group leading to no significant differences between both groups.

Table 5. Clinical effects of VEGF gene therapy

Ischaemic disease	**Agent**	**n**	**Response %**
Critical peripheral	Plasmide	50	80
Cardiac	Plasmide	30	90
Cardiac	Adeno	21	≈ 100

Monogenic Diseases

Cystic fibrosis

On first glance, cystic fibrosis is a suitable target for gene therapy. It is a monogenetic disease, the gene was identified in 1989, its activity is located relatively superficially in the airways, it is a severe disease, and no satisfactory treatment is available. Moreover, a limited activity of the gene is probably sufficient for a clinical effect.

Relatively early (1994) clinical studies on gene therapy in this disease have started, until now however with marginal success.

For this disease an otherwise rare way of application of the gene has been chosen extra cellular application of the vector in the upper airways. Non viral vectors, as well as adeno and adeno-associated virus have been used.

In the clinical studies with CF patients, the drawbacks and limitations of the adenoviral vector have become especially obvious. Clearly in this chronic disease repeated applications of the gene have to be given. With the adenovirus this has led to antibody production and also to symptomatic immunological side effects. More unexpected this has also been seen with non-viral vectors.

The AAV vector has led to gene transfer in approximately 10% of the cells of the paranasal sinus, at this level clinical effects might be possible. Probably these data are useful starting points for the next generation of trials. The groups working in this field generally stick to local delivery of the gene, be it with more sophisticated devices. [20] Given the more persistent nature of AAV vector transfer attempts by way of the bronchial circulation might also be considered.

Haemophilia

Haemophilia, in the manifestation of both factor VIII and factor IX deficiencies have been intensely studied for the applicability of gene therapy. Important stimuli for this research have been the unique nature of the protein in that no strict regulation of its production seems necessary, and the fact that a small increase in the circulating levels of approximately 2% would have important beneficial clinical effects. A draw back for this research is the size of the factor VIII gene that requires trimming to fit in some vectors.

It is clear that haemophilia gene therapy would benefit from stable transfection therefore adeno viral vector transfection is unlikely to become of importance. The retroviral, adeno-associated and lentiviral approach are the systems of choice, but non-viral systems could be interesting. Drawbacks, even with stable transfection, are the silencing of the gene by promoter inactivation and the production of antibodies. Factor VIII activity requires expression close to or in the circulation, by a large vector even if the gene is depleted of its B domain.

Factor IX has been studied after intramuscular injection in the AAV vector in 6 patients in a dose escalation study. So far three patients have shown probable clinical benefit, also evidence of gene expression and plasma level increases have been documented. This study is going to be continued and will also involve intra hepatic delivery [21].

Factor VIII deficient patients have been treated with ex vivo cell transfection with electroporetion and laparoscopic reinjection of positive clones. So far 6 patients have been treated, 4 show some production and 2 some benefit [22].
A retroviral vector is used for intravenous infusion of the factor VIII gene so far in 13 patients in a dose escalation setting. Six patients show some increase in factor VIII.

Although some 25 patients have now been enrolled in these studies it is too early to predict if gene therapy can be successful in this disease. The in vitro approach is complicated, but long-term safety issues will haunt the viral approach for many years in these life long applications.

X linked immune deficiency

The in vitro transfection by retrovirus of cells of children with this rare disease was highly successful in restoring immunity. Very recently however a T cell lymphoma has developed in one of these patients. This is the first such occurrence in over 1500 patients treated with a retroviral vector, but it will effectively stop this program.

Ethical Considerations

Throughout its short life gene therapy has led to heated discussion regarding its ethical merits or demerits. In comparison to other treatments that have the genome as a goal, oncology, radiotherapy this discussion seems to be excessive. Probably it is the eventual perspective of germ line gene therapy that fuels the ethical debate, as individual chosen somatic alterations of DNA, be it therapeutically or as toxic effects of alcohol and drugs and tobacco seldom do.

Germ line gene therapy is many decades away from our technical abilities and in the light of the rapid development in genetic selection techniques in combination with in vitro fertilisation this discussion is often directed at the wrong forum.

The ethical problem considered to be inherent to gene therapy is in reality a toxicity problem that has to be discussed and handled in the same context as in oncological and virological research.

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STEM CELLS: POTENTIAL, SELECTION AND PLASTICITY

G. de Haan, A. Wiersema[1]

Recently, we have witnessed a surge of reports describing the unprecedented and unexpected biological properties of a class of cells that collectively is referred to as "stem cells". This flurry of reports and the potential clinical utility of stem cells for a variety of human diseases merits a thorough analysis of biological concepts that are believed to rule stem cell functioning. To discuss some of the recent findings properly, it may be appropriate to reconsider carefully how stem cells are defined. Historically, a stem cell is considered to be a functionally immature cell capable of generating large numbers of functionally mature, committed progeny, while simultaneously producing cells that are indistinguishable from the stem cell itself [1]. This latter process, generally referred to as self-renewal, ensures that the stem cell population itself is never exhausted. Stem cells are typically divided in two main classes, embryonic stem cells and adult, or tissue-specific stem cells.

Embryonic stem cells are isolated from the inner cell mass of in vitro cultured blastocysts. They have the potential (and are actually functionally defined) to contribute to any tissue if transplanted into a developing embryo. For obvious ethical reasons, surrogate assays have been developed to test the multipotentiality of human ES cells [2].

Adult, tissue-specific stem cells are capable of producing a variety of distinct cell types that collectively function to form a specific tissue. A well known, arguably the best, example are hematopoietic stem cells, which are capable of producing massive numbers of lymphocytes, granulocytes, erythrocytes, monocytes, platelets, and additional derivatives hereof [3]. Also neural stem cells would qualify for this category, as they have been shown to be able to produce neurons, astroglia, and oligodendrocytes [4]. In fact, most tissues contain a population of such tissue-specific stem cells. The emergence of tissue-specific stem cells during embryonic development is carefully controlled, both in time and in space. For example, the first definitive hematopoietic stem cells in the mouse have been shown to arise early in development in a very specific region, called the Aorta-Gonad-Mesonephros (AGM), 10 days after conception [5]. During ontogeny, and continuing throughout adult life, tissue-specific stem cells generate large numbers of committed stem cells, that are capable of producing

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1. Department of Stem Cell Biology, University of Groningen, Groningen, NL.

large numbers of fully differentiated, post-mitotic cells which carry out specific biological functions.

Such a hierarchical stem cell model implies that fate decisions, either made instructively or stochastically, are of a top-to-bottom nature, and that developmental properties are gradually lost during differentiation. Furthermore, this concept favors the idea that properties once lost cannot be regained. Although this dogma had been challenged earlier, only recently the definitive experimental proof has been obtained by a large number of exciting findings that possibly imply that adult tissue-derived stem cells in fact may not have lost all developmental properties once possessed by their ancestors. Thus, adult somatic stem cells may prove to be far more "plastic" than previously imagined. Although "plasticity" has also been observed for neural stem cells, hepatic cells, epidermis stem cells, gut stem cells, and probably cells from other sources, for the sake of this review we will focus on the developmental potential of bone marrow-derived stem cells.

Most cells in the bone marrow belong to the hematopoietic lineage. Among these are the rare hematopoietic stem cells, for which frequency estimates range from one in every 10^4 to 10^6 bone marrow cells. Notwithstanding their scarcity, hematopoietic stem cells are very well characterized, both in terms of function as well as in their antigenic repertoire. This latter feature has allowed to separate stem cells from non-stem cells by staining with a panel of fluorescently labelled monoclonal antibodies and subject stained cells subsequently to flow cytometry. Table 1 provides a summary of markers that are used most commonly in the field of hematopoietic stem cell purification.

Also in bone marrow there are cells that have non-haematopoietic characteristics, that is, they are not considered to contribute to blood cell production. These non-haematopoietic cells are far less well characterized than their haema topoietic counterparts. Such relatively poorly defined cells are referred to as mesenchymal cells or stromal cells, and include endothelial cells, adipocytes,

Table 1. Markers used to purify hematopoietic stem cells

Marker	**Used for human stem cells**	**Used for mouse stem cells**	**Use**
CD34	Yes	Yes	Used widely in clinical transplants. Use for murine cells questionable
CD38	Yes	No	Most primitive cells are CD38$^-$
HLA-DR	Yes	No	Most primitive cells are HLA-DR$^-$
CD133	Yes	No	Increasingly used as substitute for CD34
c-kit	Yes	Yes	Identifies stem cell factor receptor
Sca-1	No	Yes	Used in conjunction with c-kit
Lineage-markers	Yes	Yes	Stem cells do not express a variety of lineage-specific markers
Hoechst 33342	Yes	Yes	Stem cells can actively "pump" this dye out of the cell.

chondrocytes, and fibroblasts [6,7]. There are some markers that can be used to detect at least some mesenchymal subsets, but their best identification results from the fact that these cells do not express the pan-haematopoietic cell marker CD45, and the fact that mesenchymal cells in vitro are strongly adherent, whereas most haematopoietic cells, and particularly primitive haematopoietic cells, are non-adherent in tissue culture. Ofcourse, it is not at all obvious whether the differential in vitro adherence properties of haematopoietic stem cells and mesenschymal cells has any relevance for their in vivo behavior. In general though, it has been proposed for the longest time that mesenchymal cells provide the microenvironment, or niche, in which they "feed" and "nurse" haematopoietic stem cells. Most researchers would agree that in vivo there can be no haematopoietic stem cells without stromal cells.

As mentioned earlier, haematopoietic stem cells normally contribute to blood cell production only, whereas mesenchymal cells do not. However, a large body of recent studies has shown that this paradigm is not of universal validity. To interpret these data and to ask the question which cells in bone marrow contribute to the production of non-haematopoietic cells, it is important to carefully consider the exact technical details of the experiments. Essentially, three types of experimental approaches have been employed in papers claiming stem cell plasticity:

- in vivo transplantation studies of purified haematopoietic stem cells;
- in vivo transplantation studies of unfractionated bone marrow cells;
- in vitro generation of non-haematopoietic cells from mesenchymal cells.

Table 2 provides a summary of these reports. So far, there have been no reports on the in vitro generation of non-haematopoietic cells from purified haematopoietic stem cells. It is beyond the scope of this paper to extensively review all recently published studies that test the potential of bone marrow-derived cells to contribute to non-haematopoietic tissue. Instead, we will highlight some of the most notable studies, particularly those that have tested the potential of cells enriched for haematopoietic stem cell activity to contribute to non-haematopoietic tissues.

One of the earliest reports which tested purified haematopoietic stem cells described the potency of male "side-population" cells transplanted in female *Mdx* mice [16]. Due to a genetic mutation myofibres of *Mdx* mice are unable to express wild-type *dystrophin*, providing an animal model for Duchenne's muscular dystrophy. The side population of cells is identified by flow cytometry after staining cells with the fluorescent dye Hoechst 33342 [17]. Upon cellular uptake, hematopoietic stem cells can actively transport ("pump") this dye out of the cell, which renders hematopoietic stem cells Hoechst 33342 dull. Transplantation of 2,000 –5,000 bone marrow-derived side population cells resulted 12 weeks after transplant in the presence of ~4% male, dystrophin-expressing fibres in the tibia anterior of female recipients.

The use of genetically deficient recipient mice to test the potential of purified haematopoietic stem cells was also applied in a study where low numbers (10-1000) lineage-depleted, Thy^{low}, c-kit^{+} and Sca-1^{+} (KTLS-cells) bone marrow cells? A potential answer may be that although there is phenotypic overlap be

Table 2. Overview in vivo transplants of BM-derived cells

BM cells used	**Marker to detect donor cells**	**Animal model**	**Cell types / markers /organ**	**Reference**
Unfractionated BM suspensions containing 10^7 cells/animal from wild type male mice	Y-chromosome	Knock out strain lacks macrophages neutrophils b,t-cells ; cells given intraperitoneally	Neurons / Neun, NSE / brain	8
MAPCs mesenchymal cells	β-galactosidase	Postnatal animals, cells given by tail vein	Hepatocytes / β-gal+, albumin in liver	9
Mononuclear GFP+ cells	GFP+	Lethally irradiated mice cells given by tail vein	Neurons, by Neun, Nf-H, β-tubulin III in brain	10
KTLS-cells	β-galactosidase	Lethally irradiated FAH-mice, with liver defect	Hepatocytes / FAH+ & morphology / liver	11
BM, myogenic progenitors	β-galactosidase	Lethally irradiated scid mouse, MLC3F-nlacZ bm transplanted back	Fiber regeneration, β-gal+, muscle	12
Sorted BM cells	Y-chromosome	Lethally irradiated mice	Type II pneumocytes / surfactant B / lung	
Unfractionated BM cells	Y-chromosome	Lethally irradiated mice cells given by tail vein ISH	tubular epithelium / staining for cytochrome p450 / kidney	13
Unfractionated BM cells	Y-chromosome	Lethally irradiated, ISH	Endothelium / factor VII / liver	14
Sorted BM (lin- c-kit+)	Y-chromosome and GFP+	Sham-mice, myocardial infarction left ventricle,	Myocardium / expressing cardiac proteins / heart	15
Side population BM cells	Y-chromosome	Dystrophin deficient Mdx mice	Muscle, dystrophin expression	16

stem cells were transplanted in mice deficient for *fumarylacetoacetate hydrolase* (FAH), a mouse model of fatal hereditary tyrosinemia type 1 [11]. Most animals transplanted with 10 to 50 stem cells died prior to analysis, but animals transplanted with 100 to 1000 cells remained alive and showed extensive contribution of transplanted stem cells to the liver, after in vivo pharmacological selection of wild type cells. The authors demonstrated that hepatopoietic potential of transplanted cells was closely correlated with haematopoietic engraftment, and conclude that hepatopoietic potential resides in the haematopoietic stem cell compartment. However, transplantation of 10^6 unfractionated bone marrow cells proved to be more efficient than purified cells. As the authors report that KTLS cells constitute ~0,015% of total bone marrow, it can be calculated that 1×10^6 bone marrow cells contain only ~150 KTLS cells, which immediately raises the question why purified stem cells perform worse compared to unfractionated tween haematopoietic stem cells and bone marrow-derived cells with hepatopoietic potential, in fact these are two distinct cell populations. Only transplantations with single cells can resolve this issue, and it is interesting to note that the same authors have recently documented that transplantation of a single, highly purified haematopoietic stem cell results only in very marginal liver contribution (Wagner). Now the authors question the potential of haematopoietic stem cells to contribute to non-haematopoietic tissue, but clearly this conclusion is entirely dependent on the results obtained with cells purified according to the KTLS phenotype. In other words, it could very well be that cells with non-haematopoietic potential have only partial overlap with the KTLS phenotype.

Enriched cells were also used to test the ability of haematopoietic stem cells to restore cardiac function after an experimental myocardial infarction in mice [15]. To this end large numbers of Lin^- $c\text{-}kit^+$ bone marrow cells were injected shortly after coronary ligation in the contracting wall bordering the infarct. It was noted that newly formed myocardium was largely bone marrow derived shortly after transplant, again confirming the potential of bone marrow-derived cells to contribute to non-haematopoietic tissue.

The ability of single bone marrow derived stem cells to contribute to other tissues was probably most rigorously addressed by Krause et al [18]. These authors documented that descendants of very low numbers, possibly of a single, male bone marrow-derived stem cell are present in significant numbers and in multiple tissues after transplant in irradiated females. Donor-derived contribution was variable, and ranged from less than 1% in esophagus, stomach, intestines, and liver, to ~2% in skin and ~15% in lung. It should be noted that these authors used a particular stem cell purification technique that is only employed by this group. It entailed a physical separation by elutriation, followed by a lineage depletion, and labeling of cells with the membrane dye PKH26. Very large number of cells were subsequently transplanted in lethally irradiated primary recipient animals. Two days later PKH26 labeled cells were retrieved from the bone marrow of recipients by flowcytometry and very low numbers of cells were transplanted in a limiting dilution fashion, such that individual secondary recipients were likely to be transplanted with a single stem cell. A simultaneously transplanted graft of female cells ensured that the recipient animals did not die. Of the 30 animals that were transplanted using this method, only 5 survived

long enough to assess the contribution of bone marrow-derived cells to non-haematopoietic tissue. It is of relevance to note that the exact phenotype of the single cells that were transplanted and that showed evidence of plasticity, is not known. The cells were retrieved from the bone marrow of primary recipients on the basis of PKH26 labeling, but were transplanted without detailed further analysis.

These studies, together with an ever increasing number of additional reports, clearly illustrate that in the bone marrow cells exist that have the potential to differentiate to non-haematopoietic cells. However, the exact mechanism by which this occurs, nor the origin of such multipotent cells, has remained completely obscure so far. Four distinct models can be proposed that would explain these phenomena [19] (Figure 1). If tissue A and tissue B both function normally (left panels), tissue-specific stem cells A and B contribute to their respec-

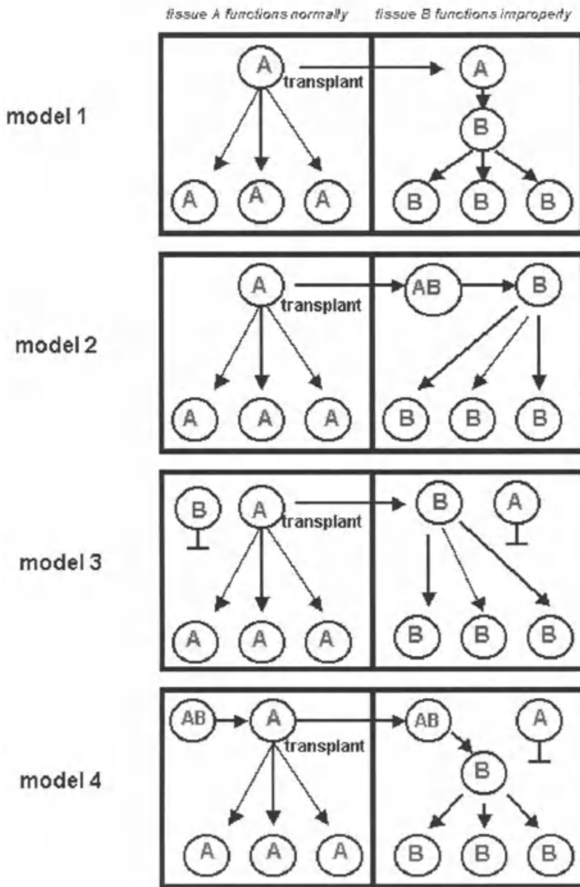

Figure 1.

tive lineages exclusively. However, if tissue B functions improperly and cells from tissue A are transplanted into tissue B, B-type cells may be produced by various mechanisms (right panels).Model 1 considers the possibility that an A-type stem cell only behaves A-like because it is located in tissue A. Upon transplantation to tissue B, it receives novel cues from the B-type environment which directly reprograms the transcriptional activity from an A-type to a B-type, and converts the stem cell identity from A to B.

In the second model an A-type stem cell, upon transplantation from tissue A to tissue B actually dedifferentiates, and thereby gains B-type developmental potential. It converts to a bipotential AB-type stem cell before it is forced by the tissue B environment to exclusively produce B-type cells.

The third model considers the option that tissue A normally contains type-B stem cells, that remain quiescent as they do not receive the appropriate signals from the tissue A environment. Upon transplantation to tissue B, however, these dormant B-type stem cells become active whereas type A stem cells stop dividing.

The fourth model takes into account the possibility that bipotent AB-type stem cells are present in tissue A. These multipotent (embryonic?) stem cells normally may function to produce committed A-type stem cells. However, after transplant these primitive stem cells produce B-type stem cells in their new B environment, and stop producing A-type stem cells.

Whereas model 1 and 2 would be compatible with the notion of actual stem cell plasticity, model 3 and 4 are not. The data that are currently available do not allow a definitive conclusion whether plasticity is really a developmental option for (haematopoietic) bone marrow stem cells. However, if in the near future definitive evidence for stem cell plasticity is obtained, one may consider stem cell plasticity from a molecular perspective by speculating that “stemness” or “primitiveness” is associated with the spectrum of transcriptional activity of a stem cell. Thus, stem cells would be those cells that have the potential to express the widest variety of genes. A broad transcriptional repertoire of stem cells would thus ensure that the cells keep many options open. In contrast, differentiation, which is the progressive loss of developmental options, can be interpreted to reflect the fact that cells lose the ability to express specific genes. Transcriptional silencing can result from a variety of epigenetic modulations, most notably DNA methylation and chromatin condensation. Plasticity, or stem cell reprogramming, would thus reflect the reactivation of silenced genes.

Finally, from a potential therapeutic perspective there are two main challenges in the field of stem cell plasticity. First, if stem cells can be reprogrammed, how can this be enhanced (i.e. made more efficiently)? Second, how can stem cells be propagated ex vivo? It should be noted that only a very limited number of stem cell types can be amplified in vitro. These include embryonic stem cells, neural stem cells, and mesenchymal stem cells. Haematopoietic stem cells, although they are the most widely used cells clinically, have so far proven to be refractory to in vitro amplification. Efficient in vitro amplification protocols of haematopoietic stem cells are urgently needed to fully exploit the developmental potential of haematopoietic stem cells.

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THE POTENTIAL OF STEM CELL TRANSPLANTATION TO RESCUE THE FAILING LIVER

P.L.M. Jansen[1]

The growing shortage of donor organs calls for new approaches in organ transplantation. Hepatocyte transplantation in animal models has revealed the possibility to repopulate a damaged liver with normal liver cells. After injection into the portal vein, hepatocytes become correctly integrated into liver cell plates and are able to divide and perform a number of functions. Interestingly, transplanted hepatocytes become polarized and are able to secrete solutes and metabolites into bile. For instance, mdr 2 -/- knock out mice lack an essential canalicular phospholipid transporter and develop severe liver disease in particular upon feeding a bile salt supplemented diet. Under these conditions they produce a bile acid-rich phospholipid-poor bile that is toxic to hepatocytes and cholangiocytes. Syngeneic mouse hepatocytes containing either wild type mouse mdr2 or transgenic human MDR3 were transplanted into the livers of these mdr 2 -/- mice and were shown to partly repopulate the liver. After transplantation the knock-out mice produced phospholipid-containing bile and appeared protected from ongoing liver damage [1]. This experiment proves several points: healthy hepatocytes transplanted into a damaged liver divide and partly repopulate the liver, the transplanted cells do not form clumps of hepatocytes not connected to the biliary tree but they integrate into the liver cell plates with a correct anatomical orientation so that they can perform the critical function of bile secretion.

Various animal models now have provided evidence for the feasibility of liver repopulation. Examples are bilirubin-conjugating hepatocytes in UDPglucuronosyltransferase-deficient Gunn rats [2], albumin-producing hepatocytes in analbuminemic mice [3,4] tyrosine-metabolizing hepatocytes in fumarylacetoacetate hydrolase-deficient mice (a model for hereditary tyrosinemia [5], healthy hepatocytes into rats with Wilson's disease [6]. Recently Gilgenkrantz et al showed high repopulation rates upon transplantation of apoptose-resistant Bcl-x(L) transgenic hepatocytes into the highly apoptotic model of Fas-agonist antibody treated mice [7]. Thus, resistant hepatocytes not only survive but under conditions of a significant survival benefit they divide, repopulate and perform a variety of liver functions. Growth factors, stimulated by the damaged liver, probably play a major role in inducing the functionally capable cells while they fail to stimulate the growth of damaged cells.

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1. Department of Gastroenterology and Hepatology,University Hospital Groningen, NL.

Hepatocyte transplantation has been carried out in humans [8,9]. Normal UPPglucuronosyltransferase-containing hepatocytes were transplanted in a patient with Crigler-Najjar syndrome typel [10]. The jaundice of this child ameliorated and bilirubin conjugates appeared in the bile. The patient needed to be transplanted after about a year. Also patients with hereditary familial hypercholesterolemia [11] and patients with liver cirrhosis have been treated with liver cell transplantation [9]. Table 1 shows potential indications of hepatocyte transplantation in humans. However, for the human situation one has to realize that procurement of hepatocytes still requires the availability of livers. With the current severe shortage of donor organs it would be much better to use fetal liver or non-hepatic cells. Fetal liver cells, umbilical vein cells, cells from the placenta, pancreas cells and bone marrow cells may be able to do the job. Fetal liver cells may be closest to human application. These cells have been shown to have a considerable potential to repopulate damaged livers and are available [12,13]. Bone marrow cells have the advantage that they can be procured from the same patient and therefore immunosuppressive therapy is not necessary. However, it is clear that only a small fraction of bone marrow cells have the potential to differentiate into hepatocytes [14]. There is debate whether this occurs spontaneously to some degree [15,16]. Apart from this, the potential of bone marrow cells to repopulate a failing liver needs to be proven. Also the mechanism is unclear [17]. It is likely that bone marrow have to be educated to become liver cells. This can be done be administration of certain genes that enables them to survive in the liver.

Table 1. Candidate diseases for hepatocyte transplantation

Alpha-1 antitrypsin deficiency
Disorders of amino acid metabolism
Urea cycle disorders
Crigler Najjar syndrome
Progressive familial intrahepatic cholestasis types 1, 2 and 3
Glycogen storage disease
Familial hypercholesterolemia
Wilson's disease
Acqiored liver disease
Acute liver failure
Chronic viral hepatitis
Cirrhoses

Our laboratory is specialised in ABC transporter research. These are transmembrane transporters that, in addition to the drug metabolising enzymes of the cytochrome P450 system, protect cells from accumulation of toxic metabolites and drugs [18]. Recently, we observed a remarkable strong (50-100 fold) upregulation of ABC transporters (MDR1, MRP1, MRP3) in severe liver disease (i.e submassive necrosis) (Ros et al. J Pathol 2003, in press). Proliferating hepatocytes, cholangiocytes and hepatic progenitor cells (HPCs) show a clear over-

expression of these transporters. This suggests a survival advantage of cells over-expressing certain ABC transporters.

A transgenically reinforced expression of ABC transporters may give donor cells the advantage to survive and repopulate a severely damaged liver. Thus, the transgenic expression of an appropriate set of membrane transporters, and perhaps a set of drug metabolising enzymes, may give bone marrow cells the required advantage to survive a toxic environment and repopulate a failing liver.

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DISCUSSION

Lou de Leij, Frank Miedema

F. Miedema (Amsterdam, NL): Being trained in the haematological environment gradually people are realising that the term 'stem cells' does not always mean the haematopoietic stem cell. In your presentation here and there you use the term also in a sort of synonymous fashion. Is it now clear to you that in bone marrow we also have to think all the time that next to a more differentiated haematopoietic stem cell, there is always a real stem cell present?

L. de Leij (Groningen, NL): Yes, I guess you could say that it is fair to assume that there should be a general precursor cell. Maybe this still elusive cell is a rare mesenchymal kind of stem cell that can give rise to all kind of mesenchymal derived cells including blood cells.

F. Miedema: So this will also come back in the presentation of de Haan and could contaminate many previous experiments. So we have to go back and think about those conclusions?

L. de Leij: I think that this presumed 'mesenchymal stem cell' must be a much more rare cell then the committed stem cells that form the haematopoietic lineages. So I do not think that a lot of research has to be repeated. Not only this is presumably an extremely rare cell, but also very difficult to culture. But I am sure that we come back to that later.

F. Miedema: The other thing that came to my mind: you say that also in adults there are real true stem cells? So you have them in the embryo and the foetus, but also in the adult. This cell is probably not so easy to propagate and to use for a transplantation, for cellular therapy; is that correct?

L. de Leij: Yes, I think that is completely correct. That is also one of the reasons why I say that if you want to think about using stem cell therapy and if you want to use the adult stem cell than you will need cells that are ready available, i.e. stem cell lines that can be cultured in a number of laboratories. This means that you must go to an allogeneic setting. It is probably difficult to imagine that from one patient you can isolate adult stem cells, propagate them into large amounts and than give them back to the same patient. So I guess for that kind of purposes we should rely on the real embryonic stem cell lines.

F. Miedema: It is always wise to learn from the past. In fact people started with bone marrow transplantation and now a lot more is done with stem cells, that are found in the blood. So could one think of a lot of research directed to evade all those bio-ethic problems like working with fetal matter or real true embryo cells by trying to concentrate on stem cells from the blood and that a lot of research research needs to be started to characterize the cell from the blood. In fact like we now do it from peripheral blood, not needing any real bone marrow any more.

L. de Leij: Do you mean mobilisation of adult stem cells and just take the blood. That is a possibility.

I. Slaper-Cortenbach (Utrecht, NL): Dr. Catherine Verfaillie described a multi-potent adult progenitor cell, which she calls MAPC[1]. That is a cell which is able to generate all kind of different cells. But that is a cell we have to culture for over three months or so. So in order to be able to use that therapeutically it will take a long time before you have enough cells to do it anything with those cells. But those are the circulating cells.

F. Miedema: But that it is at this point of time. Of course we know that ten years later there will be new growth factors and there will be a lot of new technology to grow them in a week. That is what we want to do, that is why we want to improve on this and do more research.

I. Slaper-Cortenbach: I do not think that we will be able to speed things up, because it is a very immature cell. It has to go through certain dividing stages before it is able to differentiate. You cannot speed things up by adding in just more cytokines.

L. de Leij: That is exactly in line with what I was saying before. If you want to use such cells, you can not use them in an autologous setting, because in that case it takes too much time before you have the amount of cells you need. So therefore you go to the allogeneic setting.

G. de Haan (Groningen, NL): There is unpublished data that I am aware of, that people use agents like tri-costatin A, that opens up chromatin. You can possibly think that if re-programming really occurs, that you have other additional means to basically setting the clock at zero and erase imprinting.

I. Slaper-Cortenbach: Dr. de Haan, you showed plasticity from bone marrow and neuronal cells in mice. Did you also do the same experiments for human bone marrow?

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1. Jiang Y, Jahagirdar B, ReyesM et al. Pluripotent nature of adult marrow derived mesenchymal stem cells. Nature 2002;418:41-49.

G. de Haan: Yes. But we have not as well characterised those cells, which we grow out as well. So I am a little bit hesitant to be fully positive. We see cells growing, that is easy by morphology, but this is just very preliminary. We have to do all kind of functional tests. We are clearly not as far with the human cells as with the mouse cells. But I think theoretically these cells have the potential, they look the same.

M.K. Elias (Groningen, NL): Dr. de Haan, you said that the definitions and nomenclature vary from day to day. But as for today can we consider the mesenchymal stem cells as adult equivalent of the embryonic stem cells? They have the same proliferative potential, they differentiate into the same three tissue types? They proliferate without senescence, and the behaviour seems much alike.

G. de Haan: If you are into the mouse knock out field where many of the mouse embryonic stem cells will come from, you only want to work with an embryonic stem cell line that can go germ line so that you have transmission to the next generation. We are waiting for data from I guess the Verfaillie group to see that if she breeds the animals they are so chimeric and that the cells will they contribute to the germ line. That would be major thing. But there has been another report[1] at least one or to years ago, where people did take neurospheres, and put them also in blastocysts and showed massive contribution to developing embryos. Apparently this is again very difficult to reproduce. Also these cells do not go to the germ line. I am not sure whether that is of any clinical relevance. For that particular purpose they may be fine, but I think it is clear that if that would be a definition of embryonic stem cell that these Verfaillie cells have not yet met.

J.W. Semple (Toronto, C): One of the interesting things about liver transplantation at least in animal models is that you can do it across the MHC barriers quite readily. With hepatocytes it is probably due to the content of dendritic cells within the livers that induce tolerance.

P.L.M. Jansen (Groningen, NL): I can tell you that hepatocyte transplantation to be successful in animal models, and also in humans, you need immunosuppressive drugs. If you stop cyclosporine after hepatocyte transplantation they are quickly rejected. So, rejection is still a problem.

C.Th. Smit Sibinga (Groningen, NL): Prof. Jansen and Dr. de Haan, what should we imagine of hepatocytes that you would inject in the portal vein and then come to a homing and engrafting phenomenon. Is it so that these cells keep a certain potential at a level of either the semantic pluri potent or even one level back the multi potent cells and if so where do they store that capacity? There must be something flexible in the cells that makes them regenerate, to gear

1. Clarke DL, Johansson CB, Wilbertz J, et al. Generalized potential of adult neural stem cells. Science. 2000;288:1660-63.

backwards actually in their development and then start dividing as a kind of self renewal and then mature again.

P.L.M. Jansen: In fact that is already what the early experiments showed – the normal hepatocyte only divides once a year or so, but they have the capacity to go back to a programme where they can divide more easily. In fact if you really stress them like in the experiment I showed with the plasminogen transgene, they are able to divide. In fact they are able to do more than that. They can go back and grow out in the direction of bile duct epithelial cells and hepatocytes.

G. de Haan: I always like to think of a stem cell as a cell that in terms of the number of genes that it can express has many options open. If cells differentiate then based upon the level of gene expression certain genes are just silenced. So it is a potential. If you transplant a lot of cells, there will be some cells that have not necessarily their normal fate, but they have the potential to use certain genes that they would normally not express or express certain genes that would not easily be expressed in another context. So if you transplant these cells, embryonic cells for instance, I guess they have just many options open on the transcriptional level. I think, there are now several papers that are actually looking into the expression potentials on the number of genes and the level of genes[1]. But that is not an initial thing comparing various stem cells from embryonic, neural and hepatic stem cells. It is going to be interesting to see whether there are some common genes or whether it is just the width, if you will, of the special profile that these cells can have.

C.Th. Smit Sibinga: What specific environmental conditions than are needed to attract cells and bring them to a specific stage of maturation into either endo- are ecto- or mesoderm? Is that the ligand system in kind of a stromal basis of environment or is it something else or do we not know anything about this?

G. de Haan: I think at least it is context dependent. So they talk to the environment. It is probably a receptor type of phenomenon. Not easy to conceive any other way, they must talk to their environment. So you put them somewhere else, and they have the capacity although it may be not their normal fate. They have the capacity to express genes that they would normally not do. So I think it is an environmental phenomenon. Clearly I also agree with Prof. Jansen, you have to induce damage, this has been known for a long time, you have to condition your recipients. If you do not condition your recipients, I you give a bone marrow transplant to an unconditioned recipients nothing happens There is no competitive advantage to the incoming cells. So this goes for the liver as well.

P.L.M. Jansen: Another point in your question is, how often does it occur in a normal situation. Why do we have liver disease anyway? Since you have a normal bone marrow why do stem cells not migrate to the diseased liver and re-

1. Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR. A stem cell molecular signature. Science. 2002;298:601-04.

populate the liver with healthy hepatocytes? I think the answer to that is that the damaged liver should produce factors that attract stem or progenitor cells. A severely damaged liver may not be able to do this to a sufficient extent.

G. de Haan: It is interesting to also make the remark of stem cell mobilisation. Why do stem cells leave the bone marrow in the first place? Nobody really knows. It is not really a good question to ask why, but nevertheless you would think that there would be some physiological role why stem cells are mobilised. Very low levels of stem cells normally circulate in the bloodstream, but they can be detected, and anything you inject into a mouse can do it. Some agents do it more efficiently then others, but anything from LPS to sophisticated chemokines will result haematopietic stem cells, leaving the bone marrow and circulate.

C.Th. Smit Sibinga: But we are talking about a very small number. As this is the 95 % of 0.14% or 0.5 % we are talking about a very small number indeed.

I Slaper-Cortenbach: There was recently a publication that stem cells can easily fuse to other cells[1]. So only showing the Y-chromosome in a cell does not mean anything. Is there a chromosome dependence?

G. de Haan: Apparently this is an old idea. It is interesting to read in that paper that there was someone in the laboratory that made the suggestion to look at that. I think it is very artificial that both papers were using co-culturing experiments. So you co-culture cells, embryonic stem cells, with some adult cell and then apparently this happens. I find it very difficult to believe that occurs in vivo, that that is such a common event.

I. Slaper-Cortenbach: If there is a damaged environment and stem cells are going there and than you show that there is a Y-chromosome, it does not prove anything.

G. de Haan: I am sure that everybody who claims plasticity will now do karyotyping.

L. de Leij: Dr. de Haan, if the FGF-receptor gene can be fused to some kind of oncogene, then this stimulus is also applicable in a human situation. You cultured human bone marrow-derived adults cells with fibroblast growth factor alone, but don't you think you need other growth factors like lymphokines.

G. de Haan: It could very well be. I am sure that during our culturing period there are many cytokines being produced by cells because we start with a crude system with unfractioned bone marrow cells that even if you would not add FGF, growth factors would be produced and would accumulate all the time. So we cannot exclude actually that what we are seeing is also LIF-dependent. We

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1. Terada N, Hamazaki T, Oka M,et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002;416:542-45.

would have to do it in a LIF-deficient animals. We have never tested any other growth factor but I am sure that FGF is a potent stimulator of all kinds of cytokines. So it could very well be also the case in our system. We like to do that on purified cells, starting with a single cell. Since we have not been able to do that, I have not mentioned that, but it only works in the context of whole bone marrow cells, so either we did not purified the right cell, which is what I like to believe, or it is dependent upon other growth factors that are being produced all the time, so stromal cells from the bone marrow at present.

L. de Leij: Prof. Mulder, don't you think that treatment with normal radiotherapy, or immunotherapy, will be very successful? Could you imagine that for instance in the leukaemic cell where the retrovirus was inserted just in front of an oncogene, that this had occurred in a random fashion not only in that specific patient, but also for that specific cell type? So maybe 99% of the cells are just normal T cells, and only one percent has become leukaemic. So why not expecting just normal treatment results as in all leukaemia patients?

N.H. Mulder (Groningen, NL): You are right. Probably this was one cell, this is an accident in the population of transferred cells. We cannot be sure of course, there might be more of such accidents. We really know that we have counted wrong. We thought this would not happen, but it does. I hope you will be right. In principle it might be possible if there is a normal population left, but I do not expect this patient will survive.

L. de Leij: There have also been ideas to have TK-gene inserted as a rescue principle. Do you think this approach will get a revival?

N.H. Mulder: First question is whether this program should go on. I do not know. If you put it in perspective, if this is one accident in 100 patients and you compare it to other genetic transplantation situations or intensive chemotherapy it will be acceptable. So the first thing is what happens to the other patients. This is one of the longest survivors. Also, if I say that 1500 patients have gone right, none of them have survived for three years. Those have all been patients who died for instance of their glioblastomas. The problem may be small. We do not know. I think it would be wise to put a hold on this program for a while.

F. Miedema: It nay be of interest for the audience to bring up the presentation of Prof. Jaak Vossen at his recent farewell symposium as a professor of paediatrics in Leiden[1]. At his farewell symposium he showed the impressive improvements over twenty year in the *success rate* of bone marrow transplantation. If you see how little they achieved in the beginning, that was really disappointing. We would now regard that as unacceptable. To see what you do to the patient and what the fate of the patient was 20, 25 years ago, of course it has improved immensely.

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1. J.J.M. Vossen, Tussen hoop en vrees. Dertig jaar beenmergtransplantatie bij kinderen'. Universiteit Leiden, Leiden, NL. 2002.

N.H. Mulder: This accident is a problem of biology. The experience in transplantation concerned logistics. Most problems in allogeneic transplantation where problems of doctors that could be solved. Searching of genes as for instance neogenes, oncogenes or suppressor genes is a biological problem. You can expect that any treatment with activity has a risk of mortality and this will slowly become less toxic. Bone marrow transplantation had a toxicity of 40 % mortality and you can expect that to come down. If one in a thousand patients gets this kind of integration than that will remain. So we cannot do anything about it.

I. Slaper-Cortenbach: I like to add something, because the one patient is one out of 14 patients from Dr. Fisher[1] The patient is from Utrecht so the patient is now treated in the UMC in Utrecht. We are going to do matches, we do a match to unrelated donor transplantation. I do not understand what your arguments are why that does not work, because when there is a matched unrelated donor we usually do a bone marrow transplantation for a SCID patient, because the outcome has improved over the years. Why do you think that the inserted clone cannot be reduce by chemotherapy?

N.H. Mulder: You probably can. I just do not expect that patients in this situation do survive an intensive chemotherapy regimen anymore. But maybe you are right.

I. Slaper-Cortenbach: I would like to have the opinion on this accident from our colleagues from the US and what the FDA is saying about that. Maybe I can invite one of the other speakers to give the US view.

E.J. Read (Bethesda, MD, USA): The only thing I know is that the FDA was going to have a review of this whole situation. They were going to have presentations of the case from the gene therapists and they were going to have some public comments. Then they were going to have a closed session. So presumably they will put out some statement, but I am not sure when.

F. Miedema: The interesting question is, why was this patient enrolled in this gene therapy programme and not just put on the allo-transplantation programme?

I. Slaper-Cortenbach: Because at the time there was no matched unrelated donor in the registry. But now they try again, because there are more donors in the registry.

F. Miedema: It was as simple as that?

1.Erika Check Regulators split on gene therapy as patient shows signs of cancer. Nature 2002;419:545-46.

I. Slaper-Cortenbach: I think so yes. I do not know all the details, because it happened before I went to Utrecht.

S.J. Noga (Baltimore, MD, USA): Just a comment on this patient. I think your idea on the theory about loosing a matched related might be correct. The problem is the actual biology of the disease. That is why I think, you know, you do not think this persons is going to make it through, especially since they are already immunosuppressed.

I. Slaper-Cortenbach: Yes I know that, but we do not have any data on the gene modified cells.

G. de Haan: It will be interesting to see where the insertion site is. I mean formally, it could very well be that this patient developed leukaemia obviously in cells that survive in the patients. So these will be transduced cells. But that the transgene has been inserted has nothing to do with the leukaemia.

N.H. Mulder: The only thing we now know from this patient is that it has not yet been tested. But the French have said there is in this leukaemic population, evidence of the retroviral genome.

G. de Haan: Yes, but that is really not unexpected as the patient was reconstituted, so basically all T-cells will carry the transgene, because those are the only T-cells which are present in the patient. So that seems to be obvious. The thing is did the transgene cause the leukaemia? Because the whole transplant procedure culture and procedure enzyme, something that went wrong and obviously these cells where also transfected.

F. Miedema: It is already known that there is a virus inserted in front of an oncogene, and that this is having a clonal outgrowth of the leukaemia. Than it is pretty hard to say that there is no causal relation.

II. PROCESSING ASPECTS

THE NEW REGULATORY ENVIRONMENT FOR CELLULAR THERAPY PRODUCTS: CHALLENGES FOR ACADEMIC-BASED MANUFACTURING FACILITIES

E.J. Read[1]

Introduction

Scientific advances in transplantation biology, immunology, and molecular genetics have suggested exciting possibilities for novel cell-based treatments for a variety of malignant and non-malignant diseases. Many of these cellular therapies are already moving from the research bench to the clinic. The development, clinical evaluation, and dissemination of these therapies is largely dependent on the availability of reliable, clinically feasible methods for the procurement, ex vivo manipulation, storage, and administration of human cells and tissues. In academic centers, laboratories engaged in the preparation of cellular therapy products have typically evolved from bone marrow transplant service laboratories, research laboratories, or hospital-based blood transfusion service laboratories. Since 1989, the US Food and Drug Administration (FDA) has published a series of guidance documents and proposed regulations relevant to cellular therapy products. These new expectations have created substantial challenges for manufacturers, especially for academic-based laboratories that support early phase clinical trials not intended to result in a commercial product.

Cellular Therapies at the National Institutes of Health

The Clinical Center Department of Transfusion Medicine (DTM) at the National Institutes of Health (NIH) began its involvement in cellular therapies in 1984, with support of a single protocol for adoptive immunotherapy with autologous lymphokine-activated killer cells [1]. Since then, there has been a rapid increase in demand for complex laboratory services to support clinical cellular therapies. In the past 18 years, over 80 clinical protocols in haematopoietic transplantation, cellular gene therapy, immunotherapy, and pancreatic islet transplantation have been supported by our department. In June 1997, DTM opened a new cell processing facility to support development and implementation of clinical trials in cellular therapies. This facility was designed to meet the rapidly evolving laboratory service needs in an environment that would facilitate compliance with Current Good Manufacturing Practice (cGMP) requirements, as well as evolving quality standards and regulations. Although our initial focus was to address

1. Department of Transfusion Medicine, Warren G. Magnusen Clinical Center, National Institutes of Health, Bethesda, Maryland, USA

Table 1. Summary of Key FDA Documents Relevant to Cellular Therapies

Date	Title	Guidelines or Requirements Covered
1989	Points to consider in the collection, processing, and testing of ex vivo-activated mononuclear leukocytes for administration to humans	Collection and separation of cells Key manufacturing issues Monitoring of recipients
1991	Points to consider in human somatic cell therapy and gene therapy	Definitions of somatic cell and gene therapy Collection & manufacturing issues Clinical trial considerations
1993	Application of current statutory authority to human somatic cell therapy products and gene therapy products	Presented legal authority to regulate these products Presented background for development of new framework
1997	Reinventing the Regulation of Human Tissue: National Performance Review and Proposed Approach to Regulation of Cellular and Tissue-Based Products	Rationale for formulating comprehensive framework for the regulation of HCT/Ps Tiered approach to regulation of cells and tissues based on Public health risk and need for FDA review Presented intent to propose and implement new regulations
1998	Guidance for Human Somatic Cell Therapy and Gene Therapy	Replaced 1991 Points to consider document Current information on production, quality control testing, gene therapy vectors, preclinical testing of cellular products and vectors
1998	Establishment Registration and Listing for Manufacturers of HCT/Ps; Proposed Rule	Proposed a system for registration of establishments and listing of products by manufacturers of HCT/Ps
1999	Suitability Determination for Donors of HCT/Ps; Proposed Rule	Proposed requirements for screening and testing of donors of HCT/Ps
2001	Current Good Tissue Practice for Manufacturers of HCT/Ps; Inspection and Enforcement; Proposed Rule	Proposed requirements for CGTP for manufacturers of HCT/Ps
2001	HCT/Ps; Establishment Registration and Listing; Final Rule	Finalized requirements for establishment registration and product listing, effective April 2001 for tissue and eye establishments, January 2003 for hematopoietic stem cell establishments, ART facilities, sperm banks

physical plant requirements, it quickly became apparent that the physical plant was only one piece of the entire quality infrastructure needed to support cellular therapies at NIH.

Summary of FDA Guidelines and Regulations

Table 1 lists the major FDA regulatory documents relevant to cellular therapies. The first of these, a "Points to consider" document, was issued in 1989 after the FDA had reviewed a series of protocols using activated mononuclear cells for immunotherapy of cancer [2]. It provided general guidelines for the manufacturing process, from cell collection through release testing, as well as for recipient safety monitoring. In 1991, the FDA published "Points to consider for human somatic cell therapy and gene therapy," which expanded the scope of cellular products covered and included more detailed guidance on manufacturing controls, lot release testing, and preclinical and clinical evaluation [3]. This document was updated and published as a guidance in 1998 [4].

In 1993, the FDA published a statement establishing statutory authority to regulate the biologic products associated with somatic cell and gene therapies [5,6]. This statement presented a broad approach to regulation based on existing regulatory requirements for biologics, which included establishment and product licensure, the use of the investigational new drug (IND) mechanism for clinical trials, and conformity with the cGMP regulations [7,8]. Noteworthy was the FDA's exclusion of "minimally manipulated" products – which included the majority of hematopoietic stem cell (HSC) products – from that regulatory scheme. This suggested that development of a more comprehensive system would eventually follow.

In 1997, FDA presented a comprehensive approach to the regulation of all human cells, tissues, and cellular and tissue-based products (HCT/Ps) [9,10]. The proposal was for a tiered system of regulation of HCT/Ps, including tissue for transplantation, cellular therapies, and HSC products. Citing concerns about communicable disease transmission, the effects of tissue processing and handling, and clinical safety and efficacy of these new products, the level of regulation was to be based on risk. Specific risk factors included the degree of ex vivo manipulation, whether the product was autologous or allogeneic, whether it was stored or processed in a facility that handles multiple donors as opposed to a single surgical manipulation, and whether the use included non-homologous function, structural function, or use in combination with other non-tissue components.

Following the 1997 proposed approach documents, three proposed rules were published, specifying how the new system would be implemented [11-13]. These three rules, only the first of which has been finalised [14], comprise the current Good Tissue Practice (cGTP) regulations. The cGTP regulations will in effect amend the cGMP regulations that apply to HCT/Ps currently regulated as drugs, medical devices, or biological products, but would also establish basic requirements for "minimally manipulated" products that had not previously been regulated.

Table 2.

	"361" HCT/Ps (minimally manipulated)	Other HCT/Ps (more than minimally manipulated)
Definition	An HCT/P which is – Minimally manipulated – Intended for homologous use only – Is not combined with a drug or device – and either Does not have a systemic effect, and is not dependent on the metabolic activity of living cells for its primary function or Has a systemic effect or is dependent on the metabolic activity of living cells for its primary function, and is for autologous, related allogeneic, or reproductive use	An HCT/P that does not meet the criteria for a "361" HCT/P
Examples	Autologous or family-related allogeneic cord blood or PBSCs Cadaveric musculoskeletal tissue, cornea, heart valves, dura mater Reproductive tissue (donor eggs, sperm) (all of the above qualify only if minimally manipulated)	Allogeneic unrelated cord blood and PBSCs (even if minimally manipulated) Autologous HSCs delivered for cardiac repair Mononuclear cells activated in culture Gene-transduced HSCs Autologous marrow stroma combined with synthetic scaffolding
Regulatory Requirements	cGTP establishment registration & product listing cGTP donor suitability criteria cGTP manufacturing requirements	cGTP establishment registration & product listing cGTP donor suitability criteria cGTP manufacturing requirements cGMP regs (drugs, biologics) or QS regs (devices) IND mechanism for clinical studies Premarket approval by NDA (drugs), BLA (biologics), or PMA (devices)

HCT/P

Definitions, categories, and requirements

FDA's definition of HCT/Ps includes all products derived from human cells and tissues, but with several exceptions. Blood, blood components, and blood derivatives are excluded because they are already subject to an established set of

FDA regulations [15,16]. Vascularised whole organs for transplantation (e.g., kidney, pancreas, lung) are excluded because they are regulated by the Health Resource Services Administration (HRSA) under separate legal authority [17]. Other exceptions are secreted or extracted human products (such as milk or collagen), ancillary products used to propagate human cells & tissue, and cells & tissue from animals. The FDA also chose to exclude minimally manipulated bone marrow, whether autologous or related allogeneic, from the regulatory scheme. Unrelated allogeneic bone marrow is governed in the US by standards of the National Marrow Donor Program (NMDP), administered by HRSA.

The two major classes of HCT/Ps in the new regulatory framework are (a) minimally manipulated or "361" products and (b) more than minimally manipulated products. Minimal manipulation has been defined as processing that does not alter the relevant characteristics of cells or tissues; this definition has led to considerable confusion and controversy. However, the majority of products can be placed without difficulty into one of the two categories. Table 2 presents the definitions, examples, and basic requirements for these two classes of products. The name "361 HCT/Ps" comes from the fact that these previously unregulated products are now regulated under the new approach using the authority of section 361 of the Public Health Service Act [18]. To qualify for this designation, the products must be minimally manipulated, intended for homologous use only, and cannot be combined with a drug or device. In addition, these products cannot have a systemic effect or be dependent on the metabolic activity of living cells for their primary function, unless they are for autologous, related allogeneic, or reproductive use. Manufacturers of 361 HCT/Ps will only need to comply with the three cGTP regulations, i.e., establishment registration and product listing, donor suitability, and the actual cGTPs for the manufacturing process.

The other category – HCT/Ps that do not meet criteria for a 361 HCT/P – include all HCT/Ps that are more than minimally manipulated (e.g., cultured, activated, gene-transduced) or combined with a drug or device. In addition, allogeneic cord blood and peripheral blood HSCs from unrelated donors are currently included in this category, even if only minimally manipulated. Products in this category will be subject not only to cGTPS, but also to the cGMP regulations (for drugs or biologics) or Quality System regulations (for devices), as well as the IND mechanism for clinical trials, and premarket approval procedures. The higher degree of risk associated with these products is the rationale for the higher level of regulation.

What Are the cGTP Requirements?

Table 3 provides an outline of the cGTP requirements as currently proposed [13]. The foundation of the cGTPs is the requirement that each establishment involved in any step of HCT/P manufacturing must have a quality program that is appropriate for the specific products and steps performed. The sections and actual requirements for the cGTPs are similar to those in the cGMPs for drugs or blood products. The cGTPs are less extensive in scope and contain fewer specific technical requirements, because they are largely focused on preventing

communicable disease transmission. However, the cGTPs clearly represent a higher level of expectation for all HCT/Ps. Of note is that for the more complex,

Table 3. Sections of FDA's Proposed cGTPs

Section	Title
1271.150	cGTP: general
1271.155	Exemptions and alternatives
1271.160	Establishment and maintenance of a quality program
1271.170	Organization and personnel
1271.180	Procedures
1271.190	Facilities
1271.195	Environmental control and monitoring
1271.200	Equipment
1271.210	Supplies and reagents
1271.220	Process controls
1271.225	Process changes
1271.230	Process validation
1271.250	Labeling controls
1271.260	Storage
1271.265	Receipt and distribution
1271.270	Records
1271.290	Tracking
1271.320	Complaint file

non-361 HCT/Ps, the cGTPs are considered to supplement, but not supersede, the cGMP or Quality System requirements.

Several features of the cGTPs are of special interest. The proposal has a strict requirement that the establishment releasing the product (the one that determines whether a product meets release criteria and makes the product available for distribution) must ensure that the product has met all cGTP requirements. Therefore, the manufacturer of the final product must ensure that the party procuring the cells or tissue has performed all appropriate donor screening and testing procedures, and cannot simply take it on faith that these procedures were done. There is also emphasis on establishing a product tracking method that enables tracking of all HCT/Ps from the donor to the recipient or final disposition, in a manner similar to what the blood manufacturing and service community is currently doing. There are specific criteria for documenting and reporting product deviations and adverse reactions. Finally, there is a specific statement requiring that "all personnel be made aware of the possible consequences of improper performance of their duties; e.g., the risk of transmission of communicable disease agents and diseases, and the hazards associated with those disease agents and diseases, and the risk of adversely affecting function and integrity of human cellular and tissue-based products."

While the overall format and substance of the proposed cGTPs will probably stand when they are finalised, some clarifications and changes are likely to be made, based on feedback from the formal comment period. One particularly controversial requirement is the prohibition against pooling of cells or tissue from two or more donors during manufacturing. Because this practice is already prevalent, and even essential for manufacture of certain products, it is unclear how this issue will be resolved.

Impact of cGTP Requirements

Although the latter two of the three cGTP proposed rules have not yet been finalised, it is anticipated that they will be in the coming year. FDA has already started requesting detailed written information from manufacturers – even those in academic-based programs like ours – as part of the annual reports for all investigational products. This has prompted our organisation to develop a comprehensive Master File for critical operational elements and quality systems, including all policies and procedures that address the cGTPs, as well as the cGMPs. This document, which will be continuously updated to incorporate new procedures and information, will be submitted to FDA and can be referenced by all IND holders whose clinical trials are supported by our facility.
Another recent initiative for our program is the planning of a formal validation study of two commercial blood culture methods for detection of bacteria and fungi in cellular therapy products. Although our facility has been using one of these methods (BacT/Alert™, BioMerieux)) for over 10 years, the FDA is requiring that we demonstrate the equivalence of the method to a method published in the Code of Federal Regulations almost 30 years ago [19]. This study will begin in December 2001 after FDA review of the protocol. Such validation studies are now a high priority with FDA since the March 2002 guidance document on validation procedures was published in response to a series of septic deaths caused by transplantation of bacterially contaminated allografts [20, 21].

Other ongoing quality program initiatives include development of a qualification and documentation system for ancillary reagents used in our manufacturing processes, and implementation of a computerised database to capture and track all key manufacturing data. While all of these projects will clearly improve our quality systems, the impact on resource requirements in our organisation has been dramatic. Not only is it difficult to see how smaller laboratories will survive in the current environment, but even larger manufacturers will need to find ways to meet the new expectations without driving costs to untenable levels.

Standards and guidance from other sources and an other countries

Several other organisations within the US have been involved with standard-setting activities relevant to cellular therapies. The US Pharmacopeia (USP) has published a chapter on Cell and Gene Therapy and more recently is working on a proposal that will offer guidance on Ancillary Products [22]. The American Association of Blood Banks (AABB), the American Association of Tissue Banks (AATB), and the Foundation for Accreditation in Cellular Therapies (FACT) have all published professional standards and have active accreditation programs [23-25]. While the AATB is concerned with tissue procurement and

banking, AABB and FACT have been primarily focused on haematopoietic stem cells for transplantation.

In the international community, there is wide variation on how these cellular therapy products are addressed in standards and regulations. In Europe, the Joint Accreditation Committee EBMT-EuroISHAGE (JACIE), is the FACT-affiliated body that administers an accreditation program that will eventually replace the accreditation system for the European Group for Blood and Marrow Transplantation (EBMT) [26]. These standards apply only to HSCs. Just this year, the Council of Europe has published "Guide to safety and quality assurance for organs, tissues, and cells," which was written by a Working Group of the European Health Committee (CDSP) [27]. This document is an impressive effort to unify standards for a wide variety of tissues and cells, and has some features in common with the cGTP proposals. It also includes an appendix with a long list of relevant standards and guidelines, which attests to the heterogeneity of current approaches for the array of products under discussion. Very recently, the EU's European Commission has begun to formulate a new framework for bioengineered tissues, and has submitted a consultation document entitled "Need for legislative framework for human tissue engineering and tissue-engineered products" [28]. The formal comment period ended on September 30, 2002. While it is likely that this will eventually result in a unified regulatory system for the more complex engineered tissues, the actual scope appears not to include the whole range of cell therapy products.

Disclaimer

The author is an employee of the US Department of Health and Human Services. However, the content of this publication does not necessarily reflect the views or policies of the US Department of Health and Human Services, nor does mention of trade names, commercial products, or organisations imply endorsement by the US Government.

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BIOLOGICAL MODIFICATION OF LYMPHOCYTES IN AUTO- AND ALLO-IMMUNE DISEASES[1]

J.W. Semple, J. Freedman[2]

Introduction

he term "biologic modification" can literally be applied to any molecule or drug derived from living things which has a clinical effect. Over the last 30 years, several peptides and proteins have been developed and have shown efficacy in treating both autoimmune and alloimmune disorders. The breadth of research into protein therapeutics has become huge and it is beyond the scope of this paper to fully analyze the literature. Thus, we will briefly review some of the major protein biologics either already used in transfusion/transplantation medicine or in development and will highlight their potential mechanisms of action. It will conclude with a brief discussion on transfusion-induced immunmodulation.

Cyclosporin (CsA) and Tacrolimus (FK506)

Cyclosporin is a cyclic polypeptide (Figure 1) originally discovered in the soil fungus Tolypocladium inflatum and since its introduction into transplantation medicine, it has significantly improved the survival of organ grafts [1]. CsA enters cells passively and binds to the cytoplasmic peptidyl-prolyl isomerase cyclophylin (CpN) forming a complex [1]. The cyclosporine–CpN complex binds and blocks the function of the enzyme calcineurin (CaN), which has a serine/threonine phosphatase activity [1]. As a result, CaN fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc) and thus the transport of NF-ATc to the nucleus [1]. This further inhibits NF-ATc binding to NF-ATn within the nucleus and binding to the promoter of the interleukin 2 (IL-2) gene to initiate IL-2 production. Consequently, T cells do not produce IL-2, which is necessary for full T-cell activation. Although development of CsA appears to be one of the major steps towards producing a more specific and selective agent for a subpopulation of lymphocytes, it side effects (e.g. nephrocytotoxicity) are considerable [1].

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2. Dept Lab Medicine and Pathobiology, St. Michael's Hospital, Depts Pharm, Med and Lab Medicine and Pathobiology, University of Toronto, The Canadian Blood Services and the Toronto Platelet Immunobiology Group, Toronto, ON, C.

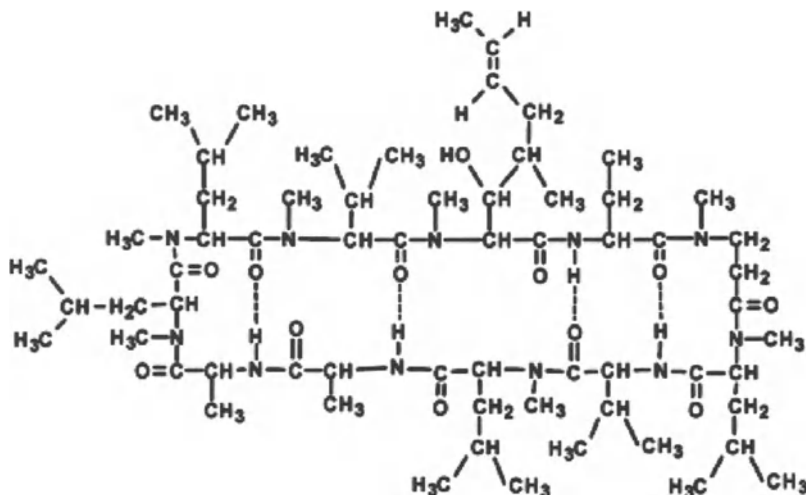

Figure 1. Structure of Cyclosporin A.

Tacrolimus (FK506) is derived from the fungus Streptomyces tsukubaensis and is a macrolide lactone antibiotic not chemically related to CsA [2]. The mechanism of action of FK506 is similar to CsA except that FK506 first intracellularly binds to FK506-binding protein (FKBP) and the complex then binds to and blocks Calcineurin [1]. On a weight basis, FK506 may be 10-100 fold more efficacious than CsA and there are less severe side effects noted with this drug [2]. These drugs have become the mainstay of treatment for transplant recipients.

Cytokines

Cytokines comprise a large and heterogenous group of proteins with diverse functions and play a critical role in the function of the immune system and hematopoiesis [3,4]. In most instances, cytokines act as coordinated intercellular protein messengers between various cell types and function by binding to their corresponding cell surface receptor. The shear number of different cytokines with different functions suggests that an intricate network of cytokine/ receptor-mediated events can take place during an immune response (Table 1). The first group of cytokines discovered were the interferons (IFN) followed by the discovery of the colony stimulating factors (CSF) and interleukins to which there is an increasing list of newly discovered molecules. Cytokines are playing an increasing role in immunopharmacology and will probably find many applications in the treatment of different autoimmune and alloimmune disorders [3,4]. For example, IFN-α has been approved by the FDA for clinical use in various neoplasms including hairy cell leukemia and malignant melanoma [4] whereas IFN-β has been approved for relapsing type multiple sclerosis [5]. Clinical investiga-

Table 1: Partial List of cytokines and their actions.

Interleukin	Principal Source	Primary Activity
IL1-α and -β	macrophages and other antigen presenting cells (APCs)	costimulation of APCs and T cells, inflammation and fever, acute phase response, hematopoiesis
IL-2	activated TH1 cells, NK cells	proliferation of B cells and activated T cells, NK functions
IL-3	activated T cells	growth of hematopoietic progenitor cells
IL-4	TH2 and mast cells	B cell proliferation, eosinophil and mast cell growth and function, IgE and class II MHC expression on B cells, inhibition of monokine production
IL-5	TH2 and mast cells	eosinophil growth and function
IL-6	activated TH2 cells, APCs, other somatic cells	acute phase response, B cell proliferation, thrombopoiesis, synergistic with IL-1 and TNF on T cells
IL-7	thymic and marrow stromal cells	T and B lymphopoiesis
IL-8	macrophages, other somatic cells	chemoattractant for neutrophils and T cells
IL-9	T cells	hematopoietic and thymopoietic effects
IL-10	activated TH2 cells, CD8+ T and B cells, macrophages	inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IL-11	stromal cells	synergistic hematopoietic and thrombopoietic effects
IL-12	B cells, macrophages	proliferation of NK cells, INF-g production, promotes cell-mediated immune functions
IL-13	TH2 cells	IL-4-like activities

Interferon	Principal Source	Primary Activity
IL1-α and -β	macrophages, neutrophils and some somatic cells	antiviral effects, induction of class I MHC on all somatic cells, activation of NK cells and macrophages
INF-γ	activated TH1 and NK cells	induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, antiviral effects

tions with many other cytokines such as IL1, IL3, IL4, IL6 and IL11 are in progress.

A more recent approach to immunomodulation involves the use of cytokine inhibitors for the treatment of inflammatory and autoimmune diseases. For example, the IL1-receptor antagonist (ra) occurs naturally in humans and has shown efficacy in animal models of septic shock and inflammatory diseases [6]. It was recently demonstrated in a phase III clinical trial that IL1ra effectively improved survival in patients undergoing septic shock [6]. Alternatively, recent research has indicated that in patients with active chronic autoimmune thrombocytopenic purpura (AITP), levels of soluble IL-2 receptor were significantly elevated [7-9]. During an immune response, the IL-2 receptor can be released from T cells and its soluble form is thought to control the levels of free IL-2 and, ultimately the immune response. The results in patients with AITP suggest that perhaps during active disease, increased IL-2 production leads to the release of the IL-2 receptor which may play a role in controlling the autoimmune response by binding and inactivating free IL-2. It thus appears that several of these cytokine inhibitors (e.g. IL1ra and TNF-receptor) may have benefit in manipulating cytokine responses in patients with autoimmunity and may be developed into an effective form of immunotherapy.

Transforming growth factor (TGF)-β is a potent immunosuppressive cytokine and it has recently been found that patients with chronic AITP in remission had significantly elevated levels of TGF-β [10, 11]. It was suggested that this cytokine might be part of bystander immunosuppression in patients with AITP in remission [11]. These reports suggest that TGF-β may also be a potential target cytokine to aid in the suppression of the autoimmune response in chronic AITP.

Monoclonal Antibodies

Over the last decade, several different monoclonal antibodies have been introduced into the therapeutic theatre and have shown efficacy in several forms of cancer [12]. Most of the monoclonal antibodies used in clinical medicine are humanized murine antibodies [12]. For example, several clinical studies have shown that a MoAb directed against CD3 is an effective treatment of renal allograft rejection [13-16]. On the other hand, in chronic AITP, the recognition of cellular immune defects has lead to the use of MoAb therapies directed at T and B cells [17-19]. Using a humanized monoclonal antibody against anti-CD40L (IDEC-131), Kuwana et al [17] followed 20 patients with chronic AITP after a single dose of the antibody. There were few side effects and a dose of 10mg/kg induced suppression of T and B cell responses to GPIIbIIIa resulting in blockade of autoantibody synthesis and an increase in platelet counts [17]. With respect to B cells in AITP, Stasi et al [19] treated 25 patients with refractory chronic AITP with RituxanTM (a monoclonal antibody specific for CD20) at a weekly dose of 375 mg/m2. Five of the patients had a complete response whereas 5 had a partial response and there were only mild side effects [19]. They concluded that rituximab therapy had a limited but valuable effect in patients with chronic AITP [19].

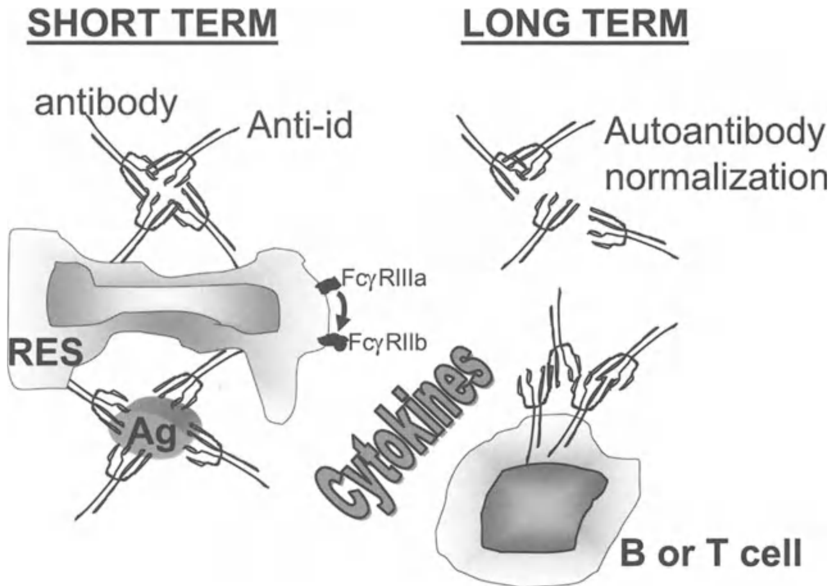

Figure 2. Potential mechanisms of action of IVIg or anti-D in AITP showing the inhibitory events (-). Both therapies appear to have short term (within 24 hours from infusion) and long term (beyond the biological half-life of the infused gammaglobulin) effects. Short term effects may be due to immune complex formation (IC, either by idiotype/antiidiotype or antibody/antigen interaction; the latter in the case of anti-D). The IC generated may physically block Fc receptors (FcR) or induce FcR inhibition (FcRIIIa to FcRIIb conversion) of the RES. Long term effects may be mediated by idiotype /antiidiotype interactions, which normalize the autoreactive antibody repertoire or directly suppress B and T cells. Superimposed on the short and long term effects is the stimulation of anti-inflammatory cytokines that may act to suppress the RES.

Polyclonal Gammaglobulins

Intravenous gammaglobulin (IVIg) therapy is effective in treating immune deficiency states, bacterial/viral infections and immune regulatory disorders, particularly immunohematologic disorders such as AITP, autoimmune neutropenia and autoimmune hemolytic anemia [20-22]. While the mechanism of action of IVIg is complex and not yet fully elucidated, several theories have been postulated. In AITP, for example, several experimentally supported theories of IVIg's mechanism of action have been proposed. These include reticuloendothelial Fc receptor blockade [23], downregulation of FcγRIIIa via FcγRIIb [24], antiidiotypic regulation [25-27], and/or cytokine alterations [28] (Figure 2). In contrast to the recognized efficacy of IVIg therapy in autoimmune disorders, there is controversy regarding its benefit in transfusion-induced HLA alloimmunization [29-32]. Several investigations have demonstrated that commercial IVIg preparations can inhibit anti-HLA in vitro [33-40], however, the inhibition was incomplete and may be the result of absence of the necessary anti-HLA specific antiidiotypes in commercial IVIg [41, 42].

Intravenous Anti-D (WinRhoSD) has also been shown to be effective in raising the platelet counts of patients with AITP [43] but little is known about the mechanisms of action of anti-D, although the above theories may apply; anti-D opsonizes the patient's D+ red blood cells (RBC) and blocks phagocytosis of platelets within the spleen [44]. Recently, a new theory of how IVIg and anti-D may function in vivo has been termed Fc-mediated inhibition [24]. It was shown in a murine model of ITP that IVIg changes the composition of Fcγ receptors on the surface of monocytes; it downregulates the phagocytic-stimulatory FcγRIIIa and upregulates the inhibitory FcγRIIb [24]. Alternatively, it has been suggested that anti-D may cause immunosuppression by either interacting with receptors other than FcγR or stimulating the production of immunosuppressive cytokines [45]. To examine the latter possibility, we designed a prospective anti-D dose crossover study in seven children with chronic AITP to compare the clinical findings with in vivo serum cytokine/chemokine levels. Our results showed that in all the children, anti-D administration induced a significant early (3 h post-treatment) modulation in inflammatory and anti-inflammatory cytokines [46]. The mechanism of how anti-D stimulates the production of the cytokines is unknown, however, several of the cytokines are interrelated and may be associated with anti-D's mechanism of action. For example, IVIg preparations have also been shown to significantly increase in vivo levels of IL1ra [46,47]. Since ILra is a potent inhibitor of macrophage function [6], its effects could culminate in a suppression of platelet phagocytosis. This possibility supports the need for further research into potential cytokine therapies in patients with AITP.

Antigen-specific Therapies

Currently, the major treatment modalities aimed at increasing platelet counts in patients with AITP are antigen non-specific immunosuppressive therapies (e.g. steroids, IVIg, cyclophosphamide etc.) [22]. These therapies have their limitations because of side effects, incomplete responses and generalized immunosuppression. With respect to autoimmunity, a therapeutic goal has been to develop antigen-specific therapies that target only the offending autoreactive T or B cells while leaving the rest of the immune system relatively intact. Several of these types of therapies have been examined, particularly within animal models and some clinical trials [48-50]. They include identification and modification of the offending autoantigen to suppress T cell activation (e.g. antigen-specific therapy targeting MHC binding peptides and T cell receptor interactions), antibody-mediated disruption of MHC-TcR interactions, costimulatory blockade of activated autoreactive T cells, oral tolerance induction and antibody-mediated blockade of costimulation or deletion of autoreactive B cells. These types of biological therapies have the potential to significantly alter the immune pathogenesis of disorders such as chronic AITP.

Donor-specific Transfusions (DST)

Transfusion therapy can be thought of as a special case of biological therapy. It is generally used to replace either a missing protein (e.g. haemoglobin in the case of RBC transfusions) or cell fragments expressing glycoproteins important

for coagulation (e.g., platelet transfusions). What is most intriguing about blood transfusions is that in addition to their detrimental side effects (e.g. alloimmunization, transfusion reactions etc.), they also appear to have side effects that may be beneficial to a recipient (e.g. transplantation tolerance, reduction in spontaneous abortions etc.). Understanding the molecular events in the blood product and the recipient that are responsible for these side effects may allow development of therapies that can exploit the beneficial effects while reducing the detrimental effects.

Most of the literature pertaining to animal models of transfusion has dealt with the recipient immunomodulatory effects of transfusion, the so-called 'transfusion effect'. The effect is associated with an apparent immunosuppression that occurs when allogeneic whole blood or leukocytes are transfused into a recipient. The effect is measured by observing, for example, the survival of a donor transfusion- specific allograft. Clinically, the immunosuppressive effect was first identified as an enhancement of renal graft survival following transfusion [51] and many experimental and clinical studies subsequently confirmed that transfusions containing viable APC could induce significant recipient immunomodulation [52-64]. These immunomodulatory effects have now been shown to also affect a number of other pathophysiologic processes, such as increased tumor growth [65-67] and increased infections [68,69], although the evidence supporting these effects remains controversial [70].

The immune mechanism(s) mediating the transfusion effect are not completely known. However, early studies by Quigley et al [56] identified a recipient splenic T suppressor activity during blood transfusions. Subsequently, Dallman et al [71] demonstrated a reduced production of interleukin (IL)-2 and of expression of the IL-2 receptor in recipients of whole blood (WB) transfusion, suggesting an IL-2 defect whereas Babcock et al [72] showed that WB transfusions induce increased IL-4 and IL-10 cytokine profiles in mice. Similar results have been reported in human recipients of WB [73]. Taken together, these results suggest that whole blood or leukocyte transfusions tend to shift the recipient's anti-donor T cell immune response toward what is termed a Th2 response. For the last decade there has been a trend in immunology to divide Th responses into categories termed Th1, Th2 and Th0 [74-77]. These responses are categorized based on the identification of cytokines elaborated. Th1-like responses produce IL-2 and interferon (IFN)-γ; they primarily mediate cell-mediated immunity and the synthesis of complement-fixing IgG antibodies [74-77]. Th2-like responses, on the other hand, generally produce IL-4,IL-5, IL-6, IL-10 and IL-13 and are superior at inducing non-complement-fixing IgG and IgE humoral immunity [74-77]. Th0-like responses are thought to be less differentiated than those mediating Th1 and Th2 responses because cytokines characteristic of both e.g. IL-4, IFN-γ and IL-10, etc, can be identified. What makes these patterns of cytokines so intriguing is that they appear to correlate with different immune functions. With respect to transplantation, for example, there is compelling evidence that Th1 responses are associated with graft rejection [78-82], whereas Th2 responses may be correlated with immune tolerance towards the graft [83-86], although several studies have not been consistent with this [87-89]. These cytokine patterns may have relevance to understanding the

role that whole blood transfusions play in immunomodulation of the recipient. However, very little is known of how component transfusions (e.g. RBC, platelets or plasma) might affect recipient immunity except that leukocytes appear to play a major role [90,91].

Summary and Future Considerations

It has become clear that protein therapeutics in transfusion/transplantation medicine has become a broad and successful discipline. Several different proteins have been developed and several are routinely used (e.g. cyclosporin and OKT3 in transplantation or IVIg/anti-D in AITP) while others are in earlier stages of development (e.g. cytokine therapy, anti-CD40L etc.). Perhaps the most important area of research for many of the protein therapeutics is to understand their mechanism of action. This will undoubtedly lead to the design of better therapeutic agents particularly with respect to polyclonal gammaglobulins. Modification of either the protein therapeutic itself or developing novel drugs which can mimic the effect of the protein are potential avenues of research.

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CELL HARVEST AND PURIFICATION TECHNOLOGY – STATE OF THE ART AND FUTURE DEVELOPMENT

P. Law[1]

Introduction

Different WBC populations in human blood can be collected for cancer therapy. Hematopoietic stem/progenitor cells (HSPC), originally harvested from bone marrow, are now increasingly collected from blood for autologous and allogeneic transplantations. Umbilical cord blood, discarded until recently, has become a source of HSPC [1-4]. Recently, HSPC have been shown to have the potential to develop into cells of other organ systems [5-21]. In the laboratory, it is possible to grow cells of neural, hepatic cells, and cardiac origins from the bone marrow [20,21]. Clinical protocols are being developed to test the hypothesis of the plasticity of the HSPC.

Other WBC populations, especially immune competent cells, are being used in therapy. Donor lymphocyte infusion (DLI) has been shown to be effective in treating leukemia relapse after allogeneic HSPC transplantation [22,23], and is the standard-of-care in the setting. Antigen presenting cells (APC), including dendritic cells (DC), are in different phases of clinical trials for treating various malignancies in the autologous setting [24-28]. Cytotoxic T lymphocytes (CTL), collected in the lymphocyte fractions as precursors, are being explored for treating viral infection and leukemia [29-43].

The majority of the WBC currently used in therapy can be collected from blood. Manipulations are sometimes necessary, as the presence of non-target cells can negatively influence the effectiveness of therapy (clumping during processing, release of undesirable factors, cause of harmful side-effects, etc). Since the cells are infused, the collection and separation systems must be designed to minimize the risk of infection. In-process monitoring may be necessary, as cells from different individuals, especially those from patients, may respond differently at different stages of manipulation. The goal of cell harvest and purification is to obtain the target cell population(s) with high purity, high yield, and without compromise of cell function.

Harvest

Leukapheresis is the standard procedure for collecting clinical quantities of WBC. The procedure separates cellular components by size and density. Most

1. Dendreon Corporation, Cell Process Development, Seattle, WA, USA.

of the therapeutically active cells are contained within the "mononuclear cell" (MNC) fraction, consisting mainly of lymphocytes and monocytes. The goal in improving harvest technology is to maximize collection of MNC while returning other components (RBC, platelets and granulocytes) to the donor/patient. Recent development focuses mainly on changes to (a) decrease the quantity of non-MNC components, and (b) automate the procedure for a more consistent collection [44-49]. Increasing the processed blood volume (from 8 to 12 liters, or 2x adult blood volume, to 20 liters or more) has increased the quantities of collected MNC [50-52]. This is especially important in HSPC transplantation as the dose of 2 to 5×10^6 CD34+ cells/kg can be achieved in fewer leukapheresis sessions during peak mobilization [53-56], leading to reduction in cost and inconvenience to patients/donors.

Platelet loss is a major complication of HSPC collection, especially in the autologous setting when patients are mobilized with growth factor after chemotherapy [53,54]. Platelet transfusion before or after collection is often required. Published results from recently developed protocols and new device(s) have shown that a decrease of platelet loss can be achieved without compromising CD34+ cell yield [44-49]. Concern for platelet loss is not as critical in MNC collection for immunotherapy applications, as the patients typically have normal or less perturbed hematological function. The need to improve collection procedure is not as important as in the HSPC setting, as relatively pure MNC (≥ 90%) can be procured in sufficient quantities with existing protocols and without little or no side effect to the patient/donor.

Purification

The purpose of purifying target cells is either for depletion of cell populations that can potentially cause unwanted clinical effects or removal of cell populations that can interfere with subsequent manipulations. Examples of the former include purging of tumor cells in autologous HSPC transplantations to decrease the probability of relapse 57-64] or T cell depletion in allogeneic HSPC transplantations to reduce the incidence and severity of graft versus host disease [65-68]. Examples of the latter include gene therapy using HSPC, where the quantity of vector will be reduced if CD34+ cells are enriched prior to transduction [57,58,69]; DC protocols that require enrichment of monocytes prior to activation [25,27]; or CTL applications that have a non-specific expansion phase [32,33,70] where the culture conditions promote growth of all lymphocytes.

Target cells can be enriched by removing unwanted cells (negative selection or purging) or selecting the desired cell population (positive selection). Choice of positive or negative selection is determined by whether distinctive selection marker(s) (size, density or surface phenotype) exist for the target cells and/or whether the target cells can be released from substrates (if used in the capture process). For example, while tumor cells can be removed from HSPC collections by purging techniques, the level of depletion may not be comparable to that achieved by positive selection of CD34+ cells because of the heterogeneity of marker expression on the tumor cells (due to multiplicity of clones, metabolic states, etc). In some applications, it may become necessary to combine purging

and positive selection to achieve the level of purity. Common selection systems are designed to enrich cells by physical or immunological parameters, as described below.

Physical separation

Density and cell size are two most common parameters to separate different WBC populations. Usually, physical separation of cells requires few steps and leads to high yield (target cell output divided by target cell input). However, because of the overlapping size and density of different WBC populations, it is more difficult to achieve high purity of final cell product [71].

A common density gradient material is Ficoll-Paque (density = 1.077 g/ml) and its variants. Centrifugation procedure using Ficoll-Paque removes RBC (after aggregation induced by Ficoll) and granulocytes from blood, marrow, cord blood, and/or leukapheresis units [66,72-75]. Despite its popularity, Ficoll-Paque is not approved for clinical use by regulatory agencies. Percoll, and similar material, is effective for the density gradient separation. At least one GMP reagent (approved in the US) (DACS, or Density Adjusted Cell Separation containing BDS, or Buoyant Density Solution, with density = 1.0600 ± 0.0005 g/ml) is commercially available for the enrichment of CD34+ cells from peripheral blood progenitor cells for autologous transplantation. After enrichment in a single centrifugation step, the proportion of CD34+ cells, assessed by flow cytometry, averaged to 2.5 ± 0.8 % (mean ± SD) (from 1.0 ± 0.4% before separation) with a yield of 68 ± 5%. T and B lymphocytes were reduced by 90% [71]. Cord blood units are typically sedimented in hydroxyethyl starch (Hetastarch) to remove RBC prior to banking [3,4]. Other density materials (eg, gelatin) have been used in cord blood processing [2,3].

Highly pure WBC populations can be separated by cell size through counterflow elutriation [76-79]. Granulocytes, monocytes and HSPC have been enriched in research applications or in clinical trial settings [77,79]. Current commercial system is intended for research only, as it is an open procedure that requires autoclave of individual components, aseptic assembly of the system, and sterilization of the fluid path prior to use in cell separation. It is possible that leukapheresis device(s) can be adapted through redesign of the separation chamber to achieve elutriation as a closed system.

Immunological separation

Different WBC populations can be separated by cell surface phenotypes. The procedure typically requires sensitization of target cells using monoclonal antibodies (a single antibody for positive selection or combination of antibodies for purging); capture of sensitized target cells using magnetic beads, high density particles or other solid substrates [80-86]; and release of captured target cells in positive selection (by enzymatic, competitive displacement, or mechanical means) [66,82,83]. Multiple washing steps (by centrifugation and/or flushing/mixing) are required after each step. Some device, such as the ISOLEX-300i, has integrated the washing procedures into system [83,84,87]. Multiple systems are now approved by regulatory agencies in US and Europe for the enrichment of CD34+ cells for autologous HSPC transplantation. For the

CliniMACS, release of CD34+ cells from substrate is not required, as the particles used to capture the cells are considered too small to cause clinical problems [66,88,89]. Other cell separation systems are in clinical trials or under development [85,86]. Earlier system such as the CellPro Ceprate has a reported purity of 50-80% CD34+ cells after selection with a yield of 30-60% [80-82,90]. Current systems, such ISOLEX-300i and CliniMACS, has demonstrated higher purity (75-95%) and yield (50-80%) [49,66,91]. Both purity and yield are important considerations in HSPC transplantation, as high purity implies a higher potential of depleting tumor cells in autologous setting [92], and high yield results in fewer required leukocytapheresis collections to achieve CD34+ cell dose of $\geq 5\times10^6$/kg [55,93].

The CD34+ cell selection systems can be adapted to enrich for CD8+ lymphocytes in CTL [29,32,70]. These applications are currently in clinical trials. For enrichment of CD8+ lymphocytes, purity ≥ 95% can be routinely achieved. The higher purity, compared to CD34+ cell selection, is not unexpected, as % CD8+ cells is typically ≥ 10x % CD34+ cells in starting material. High yield may not be as important, since the enriched CD8+ CTL precursor cells are typically cultured/activated and expanded into tumor or disease specific CTL [29,32,70]. It is possible to adjust and control cell dose during the culture step.

The same systems can be adapted for negative selection or purging. Monocytes can be effectively enriched by removal of CD2 + T cells and CD19+ B cells from leukocytapheresis units [78,94]. The monocytes are then used in culture activation of APC and/or DC. Purging or depletion is the method of choice because of the difficulty in releasing the captured monocytes from the magnetic beads or other substrates.

Culture/Selection

Enrichment of target cell populations can be achieved during culture/activation, especially in APC and CTL. For APC applications, the culture maturation/ activation process requires 2 to 14 days [24,25,27,78]. Selection of specific clones is not involved, but the antigen loading process increases expression cell surface markers necessary for potential in vivo interactions with effector T cells [24]. For monocyte-derived DC, some protocols require a plastic adhesion step that enriches for monocytes [25,27]. Enrichment of monocyte prior to culture may not be necessary to achieve the desirable clinical benefits [24]. For CTL applications, the culture process is typically longer (≥ 30days) since the CTL precursor clones specific for tumor or disease markers are first selected prior to non-specific expansion [29,31,33,70]. Initial selection of CD8+ cells is required, as other lymphocytes may be non-specifically expanded by the lengthy culture time and/or cytokine combinations.

Other Considerations

Approval of cell selection systems by regulatory agencies is a complicated process. Clinical trials must be well controlled and documented. All components have to be produced in GMP (good manufacturing practice) environment. Safety testing of all reagents, including wash buffer(s), antibodies and substrates used

in immunological approaches, has to be completed. The cost of developing and bringing a clinical cell selection system into market is high. Recent requirements of GTP (good tissue practice) by the Food and Drug Administration in the US may add to the cost of development and time of the approval process.

The cost of cell selection to a medical center includes purchase of system, reagents and disposable supplies, as well as labor expense. The price for a physical cell separation system is relatively low, but may not achieve the level of desired purity. For immunological separation, typical price for a disposable set (including antibody, substrate, and/or release reagent, as well as sterile tubing and mixing container) is ~5,000 USD. In autologous HSPC setting, multiple selections (one for each leukocytapheresis unit) may be required for each patient, bringing the total cost to 20,000 USD or more. In the current economical environment where health care expenditure is carefully scrutinized, clear and tangible clinical benefits (e.g., longer survival time, and/or replacing/improving treatment modalities leading to decreased side effects, etc) have to be demonstrated before insurance agencies (national or private) will consider reimbursement [95]. Both Ceprate and the ISOLEX-300i systems had been approved by most regulatory agencies in the world, but the use had not become wide-spread routine standard-of-care mainly because of the cost and benefit consideration. The systems, however, has become enabling technologies that can be integrated into other novel clinical trials, such as gene therapy.

Conclusion

Technology can produce highly pure target cell populations. Choice of an appropriate technology depends not only on performance of the system (processing capacity, time, purity and yield), but also on (a) requirements based on the clinical application, (b) environment (skill sets of personnel; laboratory equipment; and supporting facilities including regulatory compliance and clinical monitoring) [96] and (c) potential partnership with commercial enterprise (financial support, conflict of interest and intellectual properties). For developing and bringing new technologies into the market place, issues related to regulatory (GMP, GTP, centralized processing, etc), commercial (market size, competing therapeutic options, delivery of treatment, etc), and financial (development cost vs clinical benefits and reimbursement potential) must be fully considered.

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CELL MANIPULATION AND ENGINEERING – STATE OF THE ART AND FUTURE DEVELOPMENTS

S.J. Noga[1]

Introduction

Within the last 5 years, the new field of somatic cell therapy (SCRx) has gone through exponential growth. This term was coined by the U.S. Food and Drug Administration (FDA) in guidance documents to encompass all materials of cellular or tissue origin used for therapeutic purposes [1]. This included autologous, allogeneic and xenogeneic materials. Initially, human bone marrow was one of the only products other than blood that was processed or manipulated [2]. New sources of stem cells capable of more rapid engraftment with fewer side effects soon followed. During this time period, various regulatory agencies began focusing on these new products, with the growth of both commercial and academic cell processing facilities. Agencies such as the FDA developed practice guidelines for processing these products. When the initial set of guidelines were proposed, it seemed logical that guidelines used for current good manufacturing practice (cGMP) be applied. With the advent of embryonic stem cell research and the literal explosion in new cellular tissues and components, the cGMP format became nearly unobtainable by most academic centers due to cost, physical requirements and regulatory/administrative issues. Now, most researchers hope to process under more generic, good tissue practice (GTP) standards, but even these are quite restrictive. This manuscript will review the progress and problems experienced in the cellular therapy field and offer possible future approaches to cellular processing and manipulation in the new millennium of regulation.

Background

From the 1960's, harvested bone marrow used for BMT was the major driver for cell manipulation and processing [2]. With the discovery that T cells mediated graft-vs.-host disease (GvHD) and the growth of monoclonal antibodies during the same time period, it was natural to apply various anti-T cell antibodies to allogeneic bone marrow to reduce this morbid complication. These antibodies were potentiated using compliment or by conjugation to powerful immu

1. Associate Professor, Oncology and Pathology, The Johns Hopkins University Director, Hematology and Medical Oncology, Alvin & Lois Lapidus Cancer Institute, Sinai Hospital of Baltimore, Baltimore, MD, USA.

notoxins. Most of these studies were small phase I or II single institution studies. One of the few randomized clinical trials using an anti-CD2 monoclonal antibody showed that GvHD was significantly reduced but at the expense of high rates of hematopoietic graft failure, new onset EBV-associated lymphoproliferative disease and disease relapse [3]. Overall, the improvement in peritransplant mortality was offset by these complications resulting in no net improvement in overall survival. Other groups used e-rosettes along with soy bean agglutinin or other negative selection techniques such as elutriation to reduce the T cell burden [2]. By comparing the 2 diverse ways of processing the allogeneic grafts, it became clear that other graft factors, altered or destroyed by the processing step were vital to the integrity and long term outcome of the clinical trial. The later 2 methods, by virtue of their non-destructive negative selection methodology resulted in less long term problems such as those listed above. These techniques are still used today.

Residual tumor cell contamination was the major concern with using autologous marrow for transplantation. Non-specific pharmacologic agents such as 4-hyrdroperoxycyclophosphamde (4HC) or mefophosphamide (Asta Z) were used to effectively deplete tumors cells from the autologous graft [2]. There was a preferential destruction of tumors cells over that of human marrow progenitor cells. The destruction of up to 99% of the colony forming cells following pharmacologic purging resulted in delayed engraftment (> 6 weeks). Surprisingly, morbidity wasn't increased, but increased costs related to hospitalization was unpopular, despite the improved disease free survival shown on a phase II clinical trial in 2nd remission acute myelogenous leukemia [4]. More specific agents such as monoclonal antibodies, alone or as cocktails were used in similar manners as that listed for T cell depletion. Human variability in tumor antigens and their expression has hampered wide spread development of this approach with only a few centers continuing these trials.

Following the discovery of the CD34 epitope and its corresponding monoclonal antibody, researchers hoped to avoid the above issues for both allogeneic and autologous BMT by positively selecting out the stem cells leaving behind all contaminating cell populations. Initials successes on the "bench" showing high purity of stem cells was met with frustration in the clinical laboratories where low purity was the rule. It was learned that scale up from animal models or a few ml's of human bone marrow cells to full-scale marrow harvests was not a matter of simple multiplication. To the contrary, new methods, reaction vessels, instruments and validation techniques had to be worked out before human clinical trials could proceed.

Table 1. Graft Engineering: Definition in 1988

Manipulation of the haematopoietic graft as well as the host immune attributes for the purpose of improving graft performance
Continuous application of newly developed bio-technologies
Establish and translate animal and in-vitro models into clinical engineering trials
Long-term goal is to improve not only survival – but quality of life

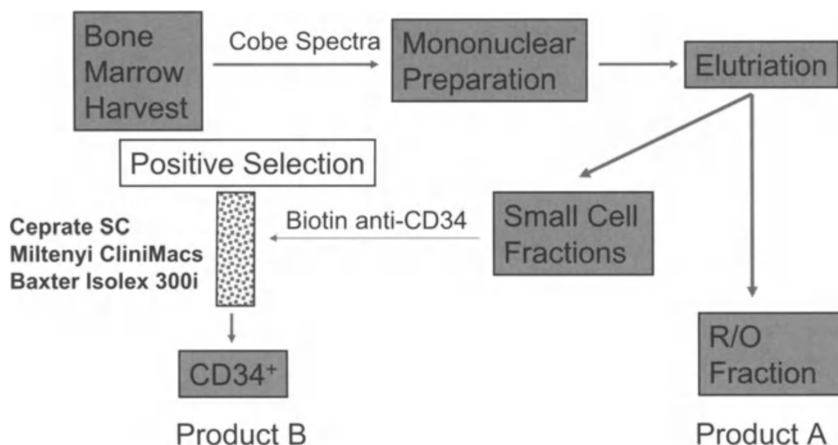

Figure 1. Cellular Engineering Using Elutriation and CD34+Selection for Modification of Allogeneic Bone Marrow. Bone marrow mononuclear cells are prepared and concentrated using a Cobe Spectra System. The product is lymphocyte depleted using counter flow centrifugation elutriation (CCE). The committed progenitor rich, lymphocyte depleted large cell fraction is immediately infused into the patient. The lymphocyte-rich small cell fractions are pooled, concentrated using a Cobe 2991 Cell Processor and additional CD34+ cells purified and used to augment the stem cell content of the engineered graft.

In the late 1980's, the concept of "graft engineering" was introduced to help guide investigators bringing new research methods forward in BMT to eventual use in clinical trials [5]. Essentially, this was an attempt to focus translational research to the final goals of improved quality of life and long-term outcome. As shown in Table 1, allowances were made for new biotechnological advances or success with animal models in shaping the final clinical trials. Test tubes, petri dishes, micro well culture plates and pipettes gave way to transfer and culture bags made of specialized plastics, sterile docking devices, automated cell washers and cell selection devices. By the early 1990's, fairly sophisticated graft engineering protocols were active at several cell processing centers, using multiple separation and manipulation steps. Given the investigational nature of many of the instruments, these studies were performed under investigational device exemptions (IDE's) under the control of the FDA in the US. Figure 1 shows a typical T cell depletion protocol using both negative (elutriation) positive (CD34+ section) processing techniques which resulted in highly reproducible "engineered" grafts which fulfilled the criteria of improved long term outcome and quality of life for graft engineering protocols [6]. The field of bone marrow transplantation has changed drastically in the last 5 years. The advent of mobilized peripheral blood stem cell (PBSC) collection from allogeneic donors has decreased the utilization of bone marrow – worldwide. Advantages include larger stem cell numbers, easier acceptance by donors and lack of dependence on bone marrow harvesters and operating rooms. Initial attempts at CD34+ selection of allogeneic PBSC products resulted in inordinately high rates of

GvHD [7]. This was considered unacceptable since investigators initially published that acute and chronic GvHD rates were lower with allogeneic PBSC than BM as the stem cell source. Recent analyses of larger studies along with the few randomized clinical trials by Cutler et al, which included follow up obtained from the authors since time of initial publication indeed show that the relative risk of acute (1.16) and especially of chronic (1.53 overall, 1.66 extensive) GvHD is probably higher for unmanipulated PBSC products than for bone marrow [8]. The large number of T cells in PBSC products no doubt contributed to this complication, but of course, other negative regulators of GvHD must be operational in PBSC grafts since even higher GvHD rates would be expected. It is also clear that chronic GvHD appears later in recipients (up to 2 years after transplant) of allogeneic PBSC grafts, thus accounting for initial reports of lower rates. In terms of CD34+ selection, initial studies were done with first generation devices which have now given way to instruments delivering much higher purity and yields [9]. Future research will show whether is will be more beneficial, or possibly, even more detrimental if highly purified cells results in the depletion of beneficial cell populations that down-regulate GvHD.

With the advent of non-myeloablative stem cell transplant (NST), a new set of problems confronts the cellular therapists. This approach capitalizes on the anti-tumor properties conferred from the allogeneic graft but uses increased immunosuppression, rather than myeloablation to ensure graft acceptance. It is now clear that although initial morbidity is low, an insidious, but relentless, form of mixed acute and chronic GvHD does occur in a significant group of patients that can be quite debilitating or lethal. Several centers are currently developing or enrolling patients on protocols using manipulated hematopoietic grafts for NST to improve overall engraftment rates and decrease the incidence of GvHD [10].

Impact of Regulation on Cellular Therapy

In the U.S. biopharmaceutical manufacturing occurs under a strict code managed and enforced by the FDA. These current good manufacturing practices (cGMP) are found in the Code of Federal Regulations (CFR) and start with the definition: "A set of current, scientifically sound methods, practices or principles that are implemented and documented during product development and production to ensure consistent manufacture of safe, pure and potent products" [10]. Large-scale production of monoclonal antibodies, blood derived products and pharmaceuticals fall under cGMP guidelines. Academic centers never had to consider such stringent process control systems. Under these guidelines, quality assurance programs had to be established, reagents and procedures validated, safety, sterility and potency assured, and tracking of products and possible consequences documented. As other authors have discussed, there was significant concern at academic centers in the US after the FDA published a series of "points to consider" and guidance documents on somatic cell therapy in the 1990's. This was in part prompted, not by academia, but due to a lack of systems control in industry concerning cellular products already being manufactured, some of which lead to deaths due to infection. As such, FDA wanted to

ensure that cellular manipulation and processing would not facilitate the transmission of communicable disease. This resulted in the proposed FDA guidelines and enforcement policies for current good tissue practice in 2001. They also finalized rules for the registration of all US facilities that process cells and tissues in an extensive manner and further defined minimal and extensive cellular manipulation. For whatever reason, many academic centers interpreted these rules as being akin to cGMP standards and many built state-of-the-art cGMP facilities to handle their cellular processing needs. As many centers have learned, having a physical plant is only one small hurdle in adapting cGTP. Most academic centers do not (and cannot afford) personnel whose primary function is that of regulatory officer or quality assurance manager. The technical teams must have particular expertise in an individual protocol, which may be quite diverse from other protocols being handled in the facility. With clinical processing laboratories averaging 3 full time technologist positions and a shortage of properly trained individuals, many facilities sat idle in the last few years. Again, having the facility did little to solve the problems of developing quality plans, validation procedures, tracking methods, etc. Even before the hysteria over these new regulatory requirements in the US, many cell processing laboratories were developing translational approaches for clinical trials to avoid all the errors and down-time experienced during the early years with hematopoietic stem cell processing. These incorporated many of the cGTP guidelines. The result was that simple feasibility studies for rather uncomplicated pre-clinical trials cost between 150,000 to 300,000 USD in 2001 [11]. More extensively manipulated or multi-stage studies could cost as much as 750,000 USD! Of course, the price tag was many folds higher for the actual clinical trials. With the virtual explosion in cell therapy protocols coming into the academic cell processing facilities, the formation of translational research boards and committees were necessary to prioritize projects and determine their financial security. The limitless possibilities of the new field of embryonic stem cell research has created further crisis in the cell processing laboratory as new discoveries offer possible cure for diseases afflicting large numbers of the general population.

The Future of Cellular Processing/Engineering

Clearly, a series of compromises will be needed to move the cellular therapy field forward in the new regulatory climate. While many of the above examples pertained to the US, Europe and a large part of the western world is also exploring cell and tissue regulations. Any possibility of sharing or exporting materials to other countries will have to consider the guidelines in effect in the respective countries. The impact on the cellular therapy trade could be considerable. First, there will be no compromise, in any country once their respective regulations are finalized. Outside of amendments and exemptions, the various processing facilities will have to work within the extant regulatory framework. In addition, the shear volume of expected cellular therapy protocols will far surpass the cGMP facility size of almost all current academic centers, if all aspects of processing are carried out under class 10,000 air handling conditions. There are at

least 3 major ways cellular processing facilities could cope with these increased demands:

- common processing pathways must be adapted;
- several of the processing steps could be done using commercial (and yet to be developed) kits; and
- portions of some processing protocols could be "outsourced" to commercial cGMP or cGTP facilities or strong industrial/ academic collaborations could be developed. In terms of common pathways, review by a translational research or cell processing protocol review board could aid in re-working protocols such that many common processing steps made redundant.

For example, several protocols using different media for washing and concentrating along with different protocols for washing may be reviewed to determine if the protocol could be modified to use already existent washing methods and materials. If not, what changes could be made to allow this to happen. This would decrease the number of active standard operating procedures (SOP's), validation steps, cost and personnel time and also speed up protocol implementation. In terms of the second point, corporations are already developing devices and instruments that are essentially closed and can be operated in < class 10,000 air handling facilities [12]. These systems would still meet all cGTP standards. Some of these systems will be single use, single product disposable items that could be used for a variety of applications including mononuclear cell separation, T cell depletion, CD34+ stem cell enrichment, monocyte/dendritic cell isolation, etc. Other systems would use validated kits that would carry out all necessary processes in a similarly closed system, with a permanent record of all steps and quality assurance maintained for GTP compliance [12]. The Aastrom Bioreactor system and the Cobe Spectra Purge System are emerging examples of this approach. Lastly, significant sub-contracting to commercial cGMP facilities specialized in specific categories (ie cellular vaccines) may be necessary to meet both cGTP demands and also the needs of investigators trying to bring clinical cellular trials forward. Some situations may call for the bulk manufacture of specified products under cGMP conditions, which could then be distributed to multiple centers participating in a cooperative trial. In other situations, a precursor material manufactured under cGMP conditions could be supplied to the cell processing facility for further manipulation and incorporation in the clinical trial.

Conclusion

In closing, it is impossible to predict which processing and manipulation steps will dominate cell therapy laboratories in the next 5 years. However, new and promising somatic cell therapies will supplant bone marrow as the major driver of cellular manipulation/processing technology in the next decade. While some successful academic centers will develop very large centralized facilities to support their investigators, most will depend on new alliances and collaboration with industry to supply some or all of the materials necessary to meet regulatory, protocol and investigator demands.

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CELLULAR IMMUNOTHERAPY – TOWARDS DIRECT DC ACTIVATION FOR LICENCE TO KILL.

F. Ossendorp, S.van der Burg, R.Toes, R. Offringa, C. Melief[1]

Introduction

In the case of cancer immunity, specific T cells against tumor associated antigens have been shown to be effective. In a bone marrow transplantation setting, adoptive transfer of Epstein Bar virus-specific T cells or infusion of donor lymphocytes, following relapse of chronic myelogenous leukemia, have proven their therapeutic potential. Theoretically, adoptive transfer therapy with highly purified and large quantities of specific CD8+ cytotoxic T lymphocytes (CTL) is desirable for many cancer patients, either in the allogeneic or autologous setting. Disadvantages of this approach include: 1) The high degree of difficulty to clone and expand the required numbers of specific CTL; 2) The patient-specific nature of this approach (MHC restriction); 3) The emergence of new escape variants that have lost the specific target antigens for these CTL; 4) The sophisticated and labor intensive infrastructure required, making this therapy extremely costly.

Therapy with ex vivo activated dendritic cells (DC) offers an alternative or preferably direct in vivo activation of DC. Combining well defined antigens with highly effective DC stimulatory agents will condition these antigen presenting cells to optimally prime the required tumor-specific T cells. We have investigated several of these DC stimuli in pre-clinical models. Molecular triggers are required for optimal dendritic cell (DC) activation sufficient for induction of CD8+ cytotoxic T lymphocyte (CTL) responses. In natural immune responses, specific CD4 cells reactive with peptide/MHC class II on DC can drive maturation of immature DC to the mature DC state required for CD8+ CTL response induction. CD4+ T helper cells operate through upregulation of CD40L which then interacts with CD40 on DC to cause the required DC activation. This signal can be efficiently mimicked by agonistic CD40 antibody [1, 2]. Independent of T helper cell specificity, microbial derived molecules can also activate DC [3]. Prime examples of such molecules are the ligands of Toll like receptors such as LPS (TLR4 ligand) or CpG (TLR9 ligand). We have studied these strong DC stimuli for their potency to induce CTL responses in vivo and their tumor therapeutic capacity.

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1. Dept of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, NL.

Dendritic Cell Transfer In Vivo

Peptide antigen

Dendritic cells can be matured by CD40 ligation, LPS or CpG treatment that generally leading towards a mature phenotype of the cells. This includes upregulation of co-stimulatory molecules like CD80, CD86 and CD40, MHC class II and class I, and a strong increase in the secretion of the IL-12 cytokine can be measured [4].

We made use of the mouse dendritic cell line D1 [5] that can be maintained in a stable immature state in culture and be matured with well-defined molecular triggers. Our experimental set-up was to mature these DC in vitro, pulse them with a synthetic peptide with the minimal sequence of an adenovirus encoded CTL epitope and inject them in a naïve B6 mouse [4]. These mice were previously depleted for CD4 T cells to avoid unwanted CD4-DC interactions in vivo. After 14 days these mice were analyzed for the presence of specific CTL using adenovirus epitope-specific MHC class I Db-tetramers and cytotoxicity assays. These studies showed that peptide-pulsed mature DC (either by CD40 ligations or LPS-mediated TLR4 triggering) can efficiently prime specific CTL responses while peptide-pulsed immature DC could not. This shows that maturation of DC results in qualitative changes resulting in priming capacity. We have further explored the magnitude of priming of CD8 cells by making use of purified naïve TCR transgenic CD8 T cells specific for the same epitope described above. These cells were CFSE labeled prior to adoptive transfer in a naïve animal that was subsequently challenged by either immature or matured D1 cells pulsed with the adenovirus peptide. By following the fluorescent T cells it was clear that peptide pulsed immature DC could induce divisions of the naive TCR transgenic T cells, but in contrast to interaction with mature DC no accumulation of specific CD8 T cells was observed. Mature DC induced not only division but also impressive expansion of the adenovirus-specific cells. These findings indicate that optimal maturation of DC is crucially related to the immunological outcome of the response.

Protein antigens

Antigen processing - Dendritic cells (DC) are specialized antigen-presenting cells that are able to present antigenic peptides in both MHC class II and I molecules for optimal priming of antigen-specific T lymphocytes. Efficient uptake of antigenic protein followed by processing to peptide fragments suited for presentation in MHC molecules, are crucial functions of these cells. DC can efficiently present antigen in MHC class II molecules utilizing the exogenous route of antigen uptake and degradation in the endosomal/lysosomal pathway (reviewed in [6]). In contrast, the precise MHC class I presentation route(s) of exogenous antigens in DC is less well understood, but is in general dependent on proteasome activity and therefore antigenic proteins need to enter the cytosol after uptake.

The immuno-modulatory cytokine IFN-γ, produced during an immune response by activated T helper 1 lymphocytes, CD8+ CTL and NK cells, enhances antigen presentation by up-regulation of MHC and TAP gene products as well

as proteasome subunits and regulators [7]. So far, IFN-γ appears to be the main regulator of the processing and presentation pathway. We have investigated the role of DC activation via Th dependent (CD40) and Th independent signals (LPS) on the MHC class I processing pathway. We have observed that such activation is required for efficient MHC class I presentation of a viral CTL epitope. We now report a detailed analysis of the changes in the molecular machinery of MHC class I antigen processing during maturation of D1 cells. Western analysis revealed enhanced expression of some components of the proteasome complex, in particular the PA28 γ chain of the PA28 γ γ heteromeric complex. Both LPS and CD40-mediated stimulation strongly induced RNA and protein expression of PA28 γ independently of IFN-γ The induced PA28 γ γ complexes were functionally active in mature D1 dendritic cells. In immature cells the assembly of PA28 γ γ complexes appeared to be limited by the low expression of PA28γ whereas PA28γ expression was already high. This study indicates differentially regulated gene expression of these two molecules [8].

These findings are compatible with the "licence to kill model" in which maturation of DC is a crucial step to initiate effective CTL immune responses. Our data show that maturation of DC not only improves co-stimulatory signals but also enhances the expression of ligands for the T cell receptor in a quantitative rather than a qualitative fashion.

Immune complexes - Dendritic cells can take up exogenous proteins for presentation in MHC class II and I. This so-called cross-presentation mechanism is especially well developed in DC and enables T cell priming against exo genous antigen sources. Since DC express several types of Fc receptors for IgG we investigated the possibility to use specific antibodies to target exogenous protein to DC and use these DC to prime CD8 responses in vivo. Therefore we used OVA protein incubated with OVA-specific rabbit IgG antibodies to produce preformed immune complexes (IC). These IC were very efficiently taken up by immature DC (as tested with FITC-labeled OVA) and the processed OVA-derived CTL epitope was optimally presented to specific CD8 T cells. In addition, IC could activate DC (via the FcR associated γ-chain) to express the co-stimulatory molecules CD80, CD86 and CD40, and to produce high levels of the cytokine IL-12. These IC-incubated DC were used to immunize mice and CTL priming capacity was measured. In contrast to DC incubated with OVA and control antibodies, the IC-DC were very efficient in priming CTL in vivo. Both against the CTL epitope processed from the intact OVA protein, as against an exogenously loaded synthetic peptide (comprising a CTL epitope non-related to the OVA protein), high numbers of specific CTL could be detected. These results show that IC by itself can optimally activate DC to acquire CTL priming potential [9]. Therefore, effective IC-mediated CTL priming is mediated both via efficient antigen targeting, followed by epitope processing, and via an optimal DC maturation signal.

Subsequently, we used these optimally IC-treated DC to immunize mice and challenge them with an OVA-expressing highly aggressive B16 melanoma cell line. The vaccinated animals were all (8/8) protected against tumor outgrowth and, in contrast to mock immunized animals, survived the lethal challenge (Schuurhuis, unpublished data). These results indicate that specific antibodies

can be applied for efficient tumor-antigen targeting and T cell priming in vivo. In the future, we will extend our studies by using tumor (cell)-derived antigens in therapeutic settings. These findings demonstrate that by optimal combination of both the B cell and the T cell arm of the immune system can lead to potent anti-tumor immunity.

Direct Dendritic Cell Activation In Vivo

Long peptides

We investigated the conditions for optimal therapeutic CD8+ CTL induction by using peptide vaccines against human papillomavirus type 16 induced mouse tumors. In contrast to the use of minimal CTL epitope comprising synthetic peptide, that can even lead to induction of CTL tolerance [10], we have used long synthetic peptides harboring CTL and potentially also tumor specific T helper epitopes.

The 35 amino acid long HPV peptides were given SC in IFA or combined with the CpG 1826 adjuvant. Powerful CTL induction by single peptide vaccination crucially depended on co-injection at the same site of the adjuvant and this effect of CpG was MHC class II independent. In prime-boost regimens, a second mechanism contributed to CTL induction namely CD4+ T helper cell mediated CD40L dependent activation of DC. Toll like receptor triggering is therefore very useful in CD8+ CTL priming, while CD40L activation appears to operate in the boosting phase.

In the case of HPV 16 induced mouse tumors, we observed that palpable established tumors can be completely and permanently eradicated by treatment with a combination of long (35 amino acids) HPV 16 peptide and the CpG adjuvant [11].

These data demonstrate a new generation of therapeutic anti-cancer vaccines consisting of completely molecularly defined synthetic compounds: specific synthetic peptides and synthetic adjuvants, that can both be fairly easily produced under GMP conditions. Clinical protocols to apply this for therapeutic vaccination of patients with cervical cancer are now developed.

Tumor-derived Antigens

Our main hypothesis is that local APC activation is crucially related to effective T cell-mediated anti-tumor immunity. We have tested this by experimental administration of agonistic anti-CD40 antibodies into the tumor site in vivo. Our previous data shown before that systemic treatment of mice with CD40 antibody can turn a preventive synthetic peptide vaccine into a therapeutic vaccine capable of clearing established HPV16-positive tumors [2]. We have now used this same antibody to treat animals that bear adenovirus E1A-expressing syngeneic tumors [12]. These tumors are essentially highly antigenic but are either not immunogenic enough to induce an effective CTL response or are able to escape CTL mediated immunity. Analysis of the presence of specific CTL in these tumor-bearing animals shows that the numbers are extremely low at the tumor site or the draining lymph nodes. Treatment of these animals with the agonistic

CD40 antibody, either peri-tumoral or systemically, might recruit DC to the tumor site or induce activation of DC that have sampled tumor antigens. Monitoring of the CTL responses showed an impressive systemic expansion of specific CTL both in blood, lymphoid organs and infiltrated in the tumor. CD40 treatment resulted in efficient tumor eradication and survival of the mice, even when antibody was administered peritumoral in one flank, tumor eradication occurred in the contra-lateral flank.

CD40 signaling can cause DC maturation and these provide signals to the CD8 T cells to expand or survive that otherwise are only present in non-effective low numbers. Subsequently, these CD8 T cells can develop into potent effector CTL that spread systemically and effectively kill the tumor cells.

Conclusions

The results described above illustrate the progress made in the elucidation of the mechanisms of CD8 CTL induction and the implications of these findings for the design of powerful anticancer vaccines. It is now possible to design entirely synthetic vaccines that contain both the relevant antigenic source and the required accessory signals for induction of a full scale CTL effector response. These signals employ molecularly well-defined innate immune receptors belonging of the TLR family, and/or receptors of the adaptive immune system such as CD40 or Fc receptors. In cancer, it is most likely that the triggering of these receptors is lacking. Therefore, despite the presence of tumor antigens, the resulting CTL responses are ineffective. By provision of the proper signals it appears possible to drastically enhance the responsiveness of the pre-existing anti-tumor CTL capable of eradicating established tumors.

Both for preclinical research and for the preparation and application of clinical grade vaccines, entirely synthetic formulations are of great advantage. Long synthetic peptides appear to provide a very good source of tumor antigens to enter both the MHC class I and II processing routes. We anticipate that optimal exploitation of various TLR ligands combined with long synthetic peptides for vaccine purposes, will allow major progress in the area of therapeutic vaccination against cancer. Such improved vaccines could well have a major advantage above ex vivo DC activation and antigen loading, because the synthetic vaccines will achieve directly in vivo what ex vivo prepared DC vaccines accomplish only in a far more laborious way.

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DISCUSSION

Paolo Rebulla, G.D. Martin Beun

B. Vandekerckhove (Gent, B): Dr. Read, what is the actual relation between the FDA standards, the GTP standards and the FACT standards?

E.J. Read (Bethesda, MD, USA): The FACT standards and accreditation program as they stand now are just for haematopoietic stem cells and related products. I believe that FACT has tried very hard to incorporate what would be in the FDA cGTP regulations, such that if you were FACT accredited you would be pretty close to cGTP. However, since the last edition of FACT standards was put out in 2001 and the cGTP regulations have not yet been finalised, a FACT-accredited lab may be close, but not completely in compliance with the cGTP regulations. On the other hand, if you are not in the US, the FDA does not have jurisdiction over your lab and would not be inspecting you. So cGTP is going to play out in the next few years. We are going to see, because the FDA will start inspecting and we will see if they are going to pick up on things that are not covered by FACT.

B. Vandekerckhove: So are you saying that we can still ship cord blood from Europe without having to meet FDA requirements?

E.J. Read: I am not sure what is going to happen with cord blood, because cord blood is being regulated in the area of the more complex, or "351", products. Technically if you are collecting cord blood you have to do it under an IND, which implies that you would file an IND, collect the data, submit it and get the product licensed by FDA. So I am just not sure what is going to happen, because obviously Europe is collecting cord bloods that might need to be shipped to the U.S. for transplant if found to be matched for certain recipients. Also, there are cord bloods in storage that were collected before the cGTP regulations were proposed. So this is a very tricky area that FDA will need to address.

C.Th. Smit Sibinga (Groningen, NL): Dr. Read, what struck me in the list of chapters in this cGTP, is that assessment is missing. Is that on purpose, are do you have any idea why there is nothing written up about internal and external assessment?

E.J. Read: Internal and external assessments are addressed as quality audits, which are required by FDA. This is included in the proposed cGTP rule in the section on Establishment and maintenance of a quality program.

J.J. Zwaginga (Amsterdam, NL): Dr. Semple, may I ask when you use donors, who have higher titres of HLA antibodies, as you described. Are you not afraid of the rare complication of transfusion related acute lung disease?

J.W. Semple (Toronto, C): No. What I did not mention is that if this is going to be used, those donors have to be screened for anti-HLA antibodies. You have to actually select those donors out. Because of course there was just a paper in Transfusion[1] from a Swedish group that showed that multiparous sera may cause increased TRALI. So those particularly anti- HLA molecules have to be screened out. Interestingly, if you screen multiparous women about a year after there last pregnancy, if they had a year to immune recover, only about 3 to 4 % actually have anti-HLA antibodies. It is a very low amount. They all seem to be deregulated or reduced. So it probably it is still feasible but you have to screen for those antibodies.

G.D.M. Beun (Groningen, NL): Dr. Semple, do you think that there is a role for the neonatal Fc receptor in endothelial cells, which can be blocked by excess IVIg and might speed up the catabolism of existing Ig[2].

J.W. Semple: That is one of the theories that have been proposed. That it is simply a catabolic balance. In the in vitro assays, at least with anti-D, you can flood this system with gammaglobulins and it does not seem to really cause a big reduction in the responses.

G.D.M. Beun: Is it in vitro?

J.W. Semple: That is in vitro. In vivo, we have not done those experiments. But that is another theory, that is certainly viable. There is literally ten or twelve out there that are potential possibilities for the mechanism of IVIg.

P. Rebulla (Milan, I): Can you comment on neonatal alloimmune thrombocytopenia.

J.W. Semple: In terms of neonatal alloimmunity, it is usually an HPA-A1 incompatibility. The immune response gammaglobulin has been used and seems to be is relatively successful in some of the patients anyway. Gammaglobulins, the way I look at it, probably works in those immune disorders where there is a limited repertoire that is been stimulated. So, in auto-immune diseases really not

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a lot of the repertoire is been stimulated. It is only usually a few variable regions that are selected and for allo point mutations, like the HPA-1a it is probably a limited repertoire also. But when you are talking about HLA, you can get up to 10 % of your repertoire stimulated. So it is such a huge response that the gammaglobumins probably do not have the right anti-idiotype to react with it. But in HPA-A1 incompatibilities it potentially can.

I. Slaper-Cortenbach (Utrecht, NL): Dr. Law, you very nicely showed all the different systems. But in order to be able to culture you always have to use approved media, supplements and growth factors. What about that?

P. Law (Seattle, WA, USA): There are some media that are GMP produced with a drug master file in the US, though not approved for human use yet. The regulatory authorities would require cross-reference to the master files. Other requirements also ask the facility, if making cultured cells for infusion into patients, to qualify the reagents. Qualification of culture media may not be as difficult as qualifying cytokines since each cell product (after culture) requires a different combination. If you cannot find a single commercial company that has drug masterfile for all the cytokines, you would need to work with different companies with the accompanying legal and patent issues. In the US the FDA requirements for Phase I and Phase II trials are usually not as stringent as those in Phase III setting. So for Academic centres supporting few patients in small trials, the problems may be more manageable than for companies with the eventual goal for commercialisation. Cytokines could be a problem, just as antibodies is a problem for cell selection. There are some that can be used for selection. A lot of the antibodies really are not yet GMP produced.

P. Rebulla: Could you comment on the specific difficulty in separating cells from cryopreserved thawed samples as compared to fresh and small volume samples such as cord blood..

P. Law: It is good to start developing and practising the selection procedure in smaller bags or test tubes, and in a more controlled environment. Selection using frozen and thawed samples is a problem. We can select the desired cell populations after thawing, but not consistently. As I understand it, most laboratories can sometimes select and purify cells. But it works on one day, and will not work for other days with the same procedure. The inconsistency could be the result of the freezing process, the detail procedure of how the cells were cryopreserved. It could also depend on the composition of the cells prior to freezing. For example, if there is a lot of granulocytes in the leukocytapheresis unit, the granulocytes will deteriorate, leading to clumping and thus making the post-thaw selection almost impossible. If the start material has a reasonably pure level of mononuclear cells prior to cryopreservation, the potential for successful manipulation after thawing is quite good. The pre-freeze cell population has to be controlled more stringently if post thaw extensive manipulation is to be successful. That is my personal experience. Maybe other people have comments on that.

E.J. Read: We have been trying to work out some techniques to get some consistency. Just as Dr. Ping Law said, it is very inconsistent when you are starting out with cryopreserved cells, and you thaw and try to select them. We have been trying to do this with peripheral blood stem cells and bone marrow and we are going to try to work it out for cord blood as well. But we start with a pentastarch cryopreservation step. On the thaw side we actually have been trying to use DNAse and also add additional pentastarch and then we put through automated immunomagnetic cell selection. And we have been getting on some of them almost a 50% recovery, whereas before we used to get about 25 % at best. I think we are getting there, but again I think the pre-freeze procedures are as important as post-thaw.

A. Brand (Leiden, NL): Dr. Read, have you experience with holding these cells for a certain period in culture for instance to make them more comfortable?

P. Law: Maybe I can make a comment on that. Once you thaw the cells, they are in a fragile state. Culturing them would help to get rid of those that would die, and eliminate some problem at the front end before manipulation. The trouble is that you could loose a lot of target cells during the culture step due to non-specific clumping. Some culture/holding procedures requires the use of DNAse which requires calcium and magnesium. But if you put calcium and magnesium in the culture medium, you promote platelet aggregation and cell clumping. So the choice of whether you go to a DNAse situation to digest clumps or go to a clumping prevention situation is depending on what the starting cell populations are prior to freezing. The pre-freeze manipulation is as important as the post-thaw procedures, as we have commented on previously. For cell selection using frozen and thaw material, using fewer manipulation steps seems to be the better approach.

A. Brand: In the laboratory we have profits from the tetramer technology before expanding the CTLs. Did you come across any developments in that field for clinical practice?

P. Law: There are CD8 antibodies available for CTL selection. There are also release reagents available for CTL positive selection using Isolex® system, but those are manufactured by different companies for their own applications. You need to negotiate with them and see if they are willing to support your investigations..

G.D.M. Beun: During the recent congress in Barcelona[1] I was impressed by the Miltenyi performance in this field.

P. Law: When you are talking about the current regulation environment and the future of cellular engineering, on a small academic laboratory you are running

1. Annual meeting of the International Society for Cellular Therapy, Barcelona, Spain; May 25-28, 2002.

15 or 16 different processes and each of them apply to 3 or 4 patients. Compared to a commercial facility which runs 500, 600 processes that only apply to 2 or 3 protocols. How do you perform, how do you take care of the line clearance between production runs and other issues at the GMP and GTP level.

S.J. Noga (Baltimore, MD, USA): I think that is exactly the point. This is why in the US a whole series of laboratory directors actually left their facilities, because they could not reconcile with the academic institutions. The importance of the new ruling to the clinical investigators doing a handful of patients is not going to justify having a protocol through their facility, it is not possible. It is nice to show you can do a proof of principle and to get things rolling, obviously. Even if it costs you a little money it is worth showing that you can actually produce the material. But after that point somebody has to pay the bills. What a lot of them are finding in the US is that GMP facilities are "black holes". Millions and millions of dollars per facility is just flying through those places as they are trying to bring their laboratories up, as they are trying to bring their personnel up, as they are trying to meet their regulations, as they are bringing in consultants to help them meet the regulations and standards, and to work out the programs like you showed. They are going to bring in financial auditors who will tell them they cannot do this. They cannot do a handful of patients. They are going to have to find a different way to do this. This is where I think you are going to see the large consortiums come up, where one academic centre may pioneer a certain procedure, like a genetically engineered GMP component. They are going to pioneer that, and that is going to be their contribution. People are going to subcontract to these places, to get this material. Then they are going to have to downstream it from there.

R.M. v.d. Plas (Bilthoven, NL): It is difficult to say, but I think the situation in Europe is clearly different, because the European interpretation of the term GMP is so much different. I think for example things like qualified media are much more easier to obtain. So they are cheaper and hopefully at least not as strictly regulated. There are many things that are different, and the point of course is how much this will change in the near future, especially under the FDA influence.

S.J. Noga: I think that is the issue. I see the FDA flying to Europe and Australia all the time. They are invited there by the government and there is a reason for that. In some ways I look at my European colleagues and think how lucky they are, but then I wonder if somewhere along the line you are going to see that alligator come up from behind you. Unfortunately like in the US what it took was some accidents and that is usually the driving force for a lot of these things. Everything will be fine if your process controls are in line and no one has a problem. But when something happens, that is when everything always swings to the far right or left and things happen and that is costly for everybody. So I think you are going to see regulation come in, this is just me thinking ahead. You are going to see some different regulation come in. Hopefully it would not be as stringent right away, you have time to breathe and make the changes your-

self. Non of the processes as we do today are most of what we did twenty years ago. So possibly thinking ahead you may be actually making reagents and materials that are complying, that you do not have to worry about it.

R.M. v.d. Plas: It is difficult, of course the EU will probably issue some regulations in the very near future. But even then the problem is that if you go commercial and if you want to export your cellular products to the US, then the FDA might say, well we impose our standards. So you have to comply with both the European and the American standards.

S.J. Noga: There is one point of what Dr. Read brought up – the criteria for acceptance in a laboratory, excepting materials. Because then at that point you have to say that you guarantee that this product is useable under FDA stipulations. Just as if you made it yourself. So you are just taking total responsibility for that product. Many people do not want to accept a product from outside if they knew that the standards were not the same.

G.D.M. Beun: Dr. Ossendorp do you think that there is a place for artificial CD40 triggering, when you are preparing dendritic cells prior to administration in vivo, or would it be more logical to leave the CD40 interaction to the contact between the DC and T cells in vivo?

F.A. Ossendorp (Leiden, NL): I think both can be applied. We tried in mouse models C40 triggering in vitro, so ex vivo before injection and that also works very nicely. It is also feasible, at least in some situations, that you do it directly in vivo. That is a kind of surprising, we also did not expect. Apparently in some of our tumour models it works like that, so we have to do a lot of work. I agree that it probably is most feasible to use DC in vitro, manipulate them and then reinject them.

J.W. Semple: Dr. Ossendorp, two questions. The first is in your antigen-impulsed dendritic cell model, when you infuse them have you looked also for antibodies? The second is, how can you insure that the class I molecule, your cross presentation model, that is being recognised by the CD8 cells and driving them into CTL's. How can you be sure that that same class I molecule complex is actually on the tumour cell? Is it always the case that the same processing is going on within the tumour cells, so you can get the ligand for killing, or is there another mechanism?

F.A. Ossendorp: We are now looking into antibody responses as well. I cannot tell you too much about it, but that is a fair question. Of course that depends on the processing mechanisms in the tumour as well as the dendritic cell (DC). In this particular model we know that the immunoproteasome cells in the DC and the proteasomes in the tumour cells both express the epitope. But there are certainly cases where this does not happen. So this is one of the problems we have, and in that particular case it would, for instance if in the tumour proteasomes are present producing other epitopes than in the APC, minimal peptide loading on

the DCs might be advantage above one long peptide. The cases we have been studying so far, we have looked into the processing of these epitopes and these are indeed present on the tumour as well.

P. Rebulla: Dr. Ossendorp, you are discussing the relative effect of tolerising versus immunising of small versus larger peptides, is that right? Is this somehow due to the preferential presentation by class I and class II of smaller and larger peptides?

F.A. Ossendorp: That is a good question. First we have seen by minimal epitope, minimal peptide vaccination, tolerisation in some models, but not in all. We think that it depends on the physical-chemical properties of the peptide, how it behaves in the body. For instance if you inject an IFA when it distributes in the body and get present on all somatic cells, than you have a problem. But if it remains into the depot and slowly releases, than you get an immunisation. So that is the difficulty. The other thing is that there are two explanations why the long peptides work so good. First, it enholds an additional helper epitope. That is a possibility, but we have ruled it out. I ignored that part of the study. We also looked for helper responses in these mice, but there is a bit of complexity there. We see some helper responses in the patient studies – the helper cells do not play a major role here. In the induction or in the therapeutic phase. But the other thing is that we think that the long peptides are taken up by APC's and we think there might be a kind of depot in the DC's and slowly releases the epitope in a more physiological level on the dendritic cells. That provides it probably with a good immunogen. Because if it is too high on the DC than you might have tolerising effects.

P. Rebulla: Does this have anything to do with the working mechanism of the proteasome ?

F.A. Ossendorp: Not necessarily I think. It is clear that the proteasome epitope needs to be processed from a long peptide or protein by the proteasome. It is more like another delivery system and that it gets on this cell surface in more physiological concentrations.

M.K. Elias (Groningen, NL): If you use the cross-priming phenomenon to load the dendritic cells, that is to say allowing them to phagocytise the cell to present the antigen instead of the physical interaction with the antigen. Do you think we need the mature dendritic cells, because in contrast to cross-prime the dendritic cells we need the phagocytosis characteristic of the immature dendritic cells.

F.A. Ossendorp: We have not tried yet with already matured cells. But we know that matured cells cannot ingest antigen as easily as immature cells. So your point is well taken. In this model we like to work with immature cells. They give them the proper antigen and the signals and the readout. But in practice this is always a bit of a problem, because, for instance, monocyte derived dendritic cells in my view already look a kind of matured or sub-matured, because they

already express high levels of CD80, CD86, which is not the case in our immature cells we have in culture or in the very short term culture of bone marrow derived dendritic cells. They also look more immature, so we are testing whether mature cells are indeed worse then immature cells. Because that is an important issue.

L. de Leij (Groningen, NL): The protocols you described – breaking tolerance is one of the things you should be afraid of, so are you not afraid of just injecting in this protocols also some auto-antigens and then getting auto-immunity?

F.A. Ossendorp: You can envision that in two ways of course. Auto-immunity is bad, but in certain level it is good, because we need of course anti-tumour activity. So we need to break tolerance in a certain level, but take care that we do not go too far. But if we take specific antigens like we did for this virus model from HBV, that is fine. If you think about melanoma you might think of antigens that are not so bad.

L. de Leij: I know, but you must be very sure that the antigens which you give to the dendritic cells are completely pure. And if you give these back then you should be aware that those cells can pick up other antigens, auto-antigens when they are around. So that is what I was feeling.

F.A. Ossendorp: Of course therefore we are working on the long synthetic peptides, because we nicely produced these peptides in a very controlled way. So far we have not observed side effects of these treatments. So you could argue: these cells have auto-antigens anyway and if you break through that you get auto-immunity against unknown antigens. So far we have not observed that. Even then we try to really boost the system which I have been talking about.

C.Th. Smit Sibinga: A large part of the session dealt with the production and aspects where upscaling is definitely one of the major issues that we have to deal with. The message of Dr. Noga was clear. One millilitre is something different from thousand millilitres. There were two approaches that I have noticed in the presentations. One is trying to get to one set-up; one organisation with short lines; not much transportation; avoiding actually transportation of intermediate and finished products. Another one was having part of the production outsourced and part of the production in the harvesting institution itself. I think that if you get into the necessary venture with industry – which is needed – than you may have the problem of dealing with different set-ups of GMP facilities – inside and outside where actually the action will happen. How do we deal with that?

P. Law: Part of the answer would lie in how stable is your incoming cell product and how stable is your outgoing cell product. If you have a really short stability or time-limit, it becomes impossible to centralise operation which requires shipping. The scientific question is then how to prove whether you have a safe, pure and potent product at the time of harvest and a 24 hours harvest (when you have

a centralised facility) without doing expensive and complicated clinical trials. From a commercial point of view, centralised processing has the advantage that you are doing 400, 500 processes with one centre. This centre will only do few protocols. So they have the expertise, the repeatability and consistency. For them the difficulty is the transportation and control over the infusion site. The important issue is validation of all procedures.

S.J. Noga: It is actually the people who deal with GMP, and a lot of them are pharmaceutical people. It is a whole mindset. It is the process that may go through the exercise, that they go through where they are sure by the time they are finished with their product that they validated it. It is part of the internal quality system and what they come up with in terms of their standards and validation. That may be totally different than what another facility comes up with. But that is the process they are going through to ensure that indeed they have the product they say they do. Of course their end product and the reproducibility of their products is what stands for their products and that is what has to equal the product there as well as the product on the other side of the US, as well as the product in Singapore. So I mean that is the end result. Having said that, for an academic centre, that is an incredibly hard thing to swallow and a hard thing to produce. Some of you may have been involved in a little experiment that was done. You may wonder what is wrong when you send flowcytometry samples around Europe and get 30 different answers and they were all supposedly following the same protocol. There is a perfect example of how a application of GMP or GTP-type process would fix a lot of those problems. But indeed these are going to be big issues that one has to deal with when one decides what part of the project do they have to let somebody else handle. And will they accept their validation?

E.J. Read: I want to add just one thing. I think it is going to be very dependent on the particular product type, as sort of who does what and how much centralised processing will be done. I can give you an example of something that we have been working on. We were approached by our cardiologists who are working with a company in the US that is manufacturing and trying to commercialise mesenchymal stem cells. We were initially told that all we would be doing is storing and distributing the cellular products. But it turned out that in the process of working with the company and the cardiologists on the IND application to the FDA, the FDA in fact wanted us to do some additional assays after we thawed the product. It turned out that there were some dosing issues. So in fact, a lot of what the end-user does is going to have to be defined. The companies I think would like to create a finished product. But we still need knowledgeable laboratories on the receiving end that are treating the patient, perhaps to do some final manipulations and qualifications. So that is an example where something as minor as thawing may turn into doing additional final manufacturing steps. In fact we need to understand everything that went before. We even need to be concerned with the methods that were used in procuring the source material from, because we are issuing the product and we are responsible now for ensuring that all preceding manufacturing steps were followed. So we are going to be

creating some redundant systems of checking the donor suitability. By giving that example that is just one type of product, but depending on the company and the type of product, you are going to have to create different systems to ensure that manufacturing is being done as intended.

C.Th. Smit Sibinga: That is very true. The other issue that worries, I think not only me but many others, which has been discussed briefly in previous presentations is the matter of the consumables, the reagents getting into sufficient quantities and pharmaceutical grades. Because after all the GTP conditions as such are solvable. It needs a different approach and a different way of thinking coming from both the academic as well the industrial side. But the matter of the consumables, the reagents, is an entirely different ballgame. How are we going to deal with that?

S.J. Noga: About eight years ago, I was asked to show up in a meeting for a company. They went to see the people of the FDA to discuss the issue of the license of reagents. They took a rather interesting approach and this is something I did not know. They essentially presented data that shows only a small fraction of their tissue culture media is actually used by investigators in a clinical type setting. Much less then ten percent. The majority of their reagents do not go to academic centres. They go to large corporations where they use them for cell culture. So they proposed to the FDA that if they were liable for things done clinically with their ex-vivo reagents, then they would actually stop selling, the reagents to all academic centres. This is a rather interesting point. Alternatively, because they do understand the value of clinical research, what they would like to do is put out a drug master file on their product. In this way they would make the FDA in the United States responsible for making sure that everybody has their protocols on an IND. If someone does not they will actually turn them in to the FDA. Nothing ever happened of this, though it is interesting.

E.J. Read: I had a discussion with the director of part of the FDA that is looking at cell and gene therapies. She said that they have had a long discussion over the past few years. A lot of people have been approaching them about these ancillary products. Apparently the lawyers of the FDA have looked at it and there is not, within the legal structure of the FDA, an easy way for them to approve ancillary reagents without putting them through the whole system which is very cumbersome and expensive. However, she indicated that they might try to come up with some kind of new legal mechanism where perhaps they can do that maybe by using master files and having perhaps an approved list of ancillary reagents. But you know this is very preliminary. But it is a huge problem and I think it is really inhibiting a lot of the clinical investigations right now. You raise a good point, and there is not an easy answer.

P. Law: When planning these clinical trials even if still in the concept phase, it would be very helpful to build into the consideration that the reagents are licensed, have master files, or have been used in trials with lots of prior clinical experience. Or the reagents may have to be studied in your own lab to satisfy

regulatory requirements. It is good to plan them ahead of time, because it might cost a lot of money. The research investigator will get an early wake-up call and not be surprised by the time that the clinical trial is ready except the consumables.

E.J. Read: The one other thing I want to mention is that ancillary products technically include some of the devices and the bags, not just the reagents. One of the problems at least in the US you may all have noticed that the Isolex selection system as an example ís approved for use in Europe. We have to submit an IND to FDA in order to use it. However, some people would consider that system an ancillary product or device because it comes into contact with the product, but does not actually come into direct contact with the patient. The FDA, however, has required these devices to be tested in actual clinical trials for both safety and efficacy. This approach is incredibly cumbersome in the US and it is a real burden to companies, but I do not think it is going to change. In Europe, the regulatory pathway has not been as complex, and these systems can be made available more quickly.

L. de Leij: One of the points which was mentioned by Dr. Stephen Noga was the money problem. Who is going to pay for this, because if we as researchers want to do a lot of clinical trials, it is very difficult to to raise money for all these trials. If this is so expensive, than not a lot of science will be done.

S.J. Noga: First of all we saw this coming, because I run graft engineering laboratory at Johns Hopkins. We were submitting a program project where we actually asked for funding for a regulatory affairs officer and a quality assurance officer, as well as everything else. No one thought that it would go through. I was surprised that the National Institutes of Health actually recognised that as a real need in a cord cell laboratory and funded those positions. They did not fund the research nurse which was curious, but they did fund the regulatory piece. So whoever is working there is thinking about this at least in the US government, they pay for this. The other parts, I actually have had the funds from companies, corporations who I thought would be eventually very interested in this. This saves them a lot of the initial work and from committing to a project, because they too only have so much money and they are going to decide which project to do. If someone is doing this project for them then it only costs a tenth of what it will cost them. Well, than it is worth it to them. So it actually helps promote go out and pitched the companies if they are willing to help subsidise this. We may be interested in writing a license agreement with them. So, that is just a couple other things I have used. Than quite frankly there are others whom I just turned down because they had no ability to pay anything. We just thought that we should do it because we were a cell processing facility in the university.

E.J. Read: My institution, the National Institutes of Health, has a large budget and the pockets are very deep. So we were able to get off the ground and have been able to grow. We still hit certain limitations. Although we are not supposed to turn down requests from various institutes, we have had to put up some road-

blocks and to push back and than ask for more resources along the way. There has been a lot of resistance to really giving us everything that we think we need. We also have had to partner with companies. We had to, but in a way we wanted to also, because I think a lot of commercial companies bring in different kinds of expertise and have some different systems and products that we would like to use. So I think the way things are going to evolve is the larger academic places will partner with various companies and we will have more centralised facilities. But there has to be cooperation. It cannot be just pure competition, because you are going to end up with nothing. You are going to have to cooperate and try to be creative about how to evolve.

S.J. Noga: Another comment – when we were doing this, it was interesting, because we had the GMP facility which you know could hardly put anything through. We had the graft engineering laboratory, which actually handled the majority of cell processing, which was the license laboratory. Than we had investigators trying to make their own laboratories by trying to do lymphocyte work because they thought we were too expensive and finding out they are not licensed. In the middle of all this, it was decided that we actually put a committee together with people from all levels: technologists, academics, investigators and all projects for the whole system were brought through this committee. They had to be presented, they had to give concise report, they had to show what they had. Than the initial studies would go to a sub-committee of specialised individuals and that is where they work out the time frame, how long it would take and who is paying for it. They then came back to the committee after it went through the feasibility studies and was presented and moved forward from there. We had to do that. It is the only way we could control what was coming in. You know that Johns Hopkins had 3,000 clinical trials last year.

J.J. Zwaginga: I think the history of bone marrow transplantation learns us a lot. If we were to start now bone marrow transplantation and stem cell transplantation, we would probably not reach the current level as fast as we have reached now. Because now requirements that you need to initiate such therapies are much more laid down. The bone marrow transplantation, the stem cell transplantation was a black box and still is in some respects, but it could be implied while it worked. However, because the animal phase was almost skipped in the beginning, we now have to catch up to get more fundamental insights. So I think it should be an open door to implement and validate the therapies we are talking about now, it would be realistic to do more animal studies before we start very expensive facilities which will be black holes for our resource and be careful to frustrate ourselves because clinical trials cannot be paid for.

E.J. Read: Actually the FDA does require submission of the IND for the human trial. They have made the investigators get back and do a lot more of general work, but even the large animal work is incredibly expensive because they usually are using non-human primates or pigs for example. Even those are very expensive. But your point is very well taken.

S.J. Noga: If I can extend on that. I have heard the other day of that famous US researcher Bert Vogelstein who has worked on cancer genetics, went to his computer and from a section of sequenced human genome was able to find two new cancer genes[1]. Knowing what he knew in a laboratory that just going to the sequences found two new undiscovered cancer genes without ever lifting a test tube. We need to consider these things when we are moving forward. What can be done in other systems, not only in the animals, but possibly even on the bench, with the computer or whatever.

C.Th. Smit Sibinga: The need for more academic research as a foundation was already mentioned in the opening address by our chairman Prof. de Leij. The point, however, is would it not be more appropriate to bring in from the very beginning at least the basic principles of current Good Tissue Practices and quality principles with the ultimate aim to get where appropriate into this up-scaling. Because otherwise you keep bringing water to the see and find out that at a certain point in time the fascinating research leads to results, just to have to go over it all over again, because it does not match GTP principles. That is actually the question that you could raise for instance to Dr. Ossendorp: did you bring in already the concept of translational research of GTP from the very beginning on.

F.A. Ossendorp: In Leiden we have structurally already worked on that. Together with the Haematology Department there are certain facilities to make this possible. But of course in my group we are still on the academic side.

A. Brand: What Dr. Ossendorp has shown is that of course you need more models. But the problem is: what is a perfect read out in animal models (suitable for GMP) is not easy to translate into the clinic. There are two aspects: first, do we have to agree internationally for instance – maybe from the pressure of a FDA-like institute – that we should all use the same clinical model. Such as for instance the NOD/SCID for engraftment. In particular a humanised animal model for tolerance induction and a model for for tumour response. What is the idea about that, because there are many variables in all those laboratory animal models?.

You showed such a delicate balance between immature DCs and mature DCs which have totally different functions. We even do not know if that is simply a matter of quantity or whether it is a matter of quality. If you make mixtures of mature and immature DCs, will the immature DCs down regulate the mature DCs. All such aspects have to be addressed – and discussed and that is very complicated. Although I totally agree with Dr. Smit Sibinga that you should go as soon as possible to the idea that you can use it in a clinic, for the majority of developments it is too early. You are just preventing improvement when you go too early to GMP facilities. We should only do this when it is really planned in the phase I and II clinical trials. As Dr. Ossendorp says, the peptide can be GMP-made and we can use it for CTL expansions, but that is all. You cannot

1. Johns Hopkins Oncology Newsletter, Fall, 2003.

put everything already from the laboratory to the idea of GMP, at least not in my opinion.

S.J. Noga: One of the dangers of using a standardised animal model for answering a question is that you may miss things, so it is probably better to have many standardised assays that hopefully address the same issues so you compare one assay to the next. I think that is the important thing.

J.W. Semple: In Canada too in terms of GMP in at least government laboratories and laboratories such as those in the Canadian Blood Services it has been instituted that their laboratories go to GLP anyway. Basic research laboratories, however, I do not think could go to GMP, at least not the once that I know. But they certainly can go to something that is a little less. I think eventually the Canadian government at least will probably accept some of those weaker standards for some of the basic laboratories.

S.J. Noga: In the US we cannot let the basic researchers who are doing cellular therapy studies right now, be the ones to do the clinical trials. I mean that disconnect is necessary, and I think that is what GTP is all about. When you see people dying of Clostridium infection, because there was sloppy handling of connective tissue, you cannot have that. So these rules are to prevent infection. That is the idea. In other words the work must be done in the right place. You are mentioning the right thing, GLP laboratories. Most research laboratories are not GLP, we see people eating their lunch on top of counters. These cannot be what you use as you are working something through to clinical trials, so they have to be moved out of there into the proper environment. But I do not think in the end it is going to be strict science, which is the important point, because it is just a matter of who is doing it. The ego's are going to have to be brought down a little bit, they are going to have to realise that the clinical trial might have to moved out and they are going to have clinical investigators to help them.

J.W. Semple: Well just to let you know, our laboratories are absolute basic laboratories. I like to think that we always practice Good Laboratory Practice, and in fact it is not that bad. The biggest problem is the paperwork in the beginning, to do your entire SOP piece in that for various pieces of equipment in your laboratory. We are almost there and it has not been all that terrible.

III. QUALITY ASPECTS

FROM RESEARCH TO CLINICAL APPLICATION-THE QUALITY CONCEPT

P.H. Roddie[1]

Introduction

For those involved in clinical research in Europe there will be fundamental changes in the way clinical trials are regulated as a consequence of the European Clinical Trials Directive. EU member states have until 1 May 2003 to draw up legislation implementing the Directive, whose purpose is to ensure that quality is a mandatory component of clinical trials. Research sponsor, host institutions and investigators will be responsible for ensuring clinical trials fulfill the requirements of Good Clinical Practice (GCP) and, where studies involve the use of investigational medicinal products (IMP), Good Manufacturing Practice (GMP). The Licensing authorities will act to ensure compliance with standards, which will include performing inspection of trial and manufacturing sites. These changes offer particular challenges to those involved in translational research as the cost and resource implications are likely to be high.

Translational Research

In considering what are the quality issues relevant to translational research it is worthwhile reviewing the pathway from basic research to clinical application (Figure 1).

Following generation of a research hypothesis with therapeutic potential preclinical studies are performed to establish 'proof-of-principle', to identify possible toxicities and side-effects and to validate processes that will be used in the clinical trial. Based on these studies a clinical trial is designed and a trial protocol generated. The trial protocol is submitted for independent ethical review, to an external funding body and to the relevant national licensing authority. In the UK this involved application to the Medicines Control Agency (MCA) either for a clinical trials exemption certificate (CTX) for studies run by pharmaceutical companies or a doctors and dentists exemptions certificate (DDX) for trials initiated by doctors or dentists. Clinical trials have generally been conducted according to the principles of GCP [1] particularly in respect of ethical review and in matters relating to consent. Where investigational medicinal products were trialed these were manufactured in GMP or 'near' GMP facilities. How

1. Western General Hospital, Edinburgh, Scotland.

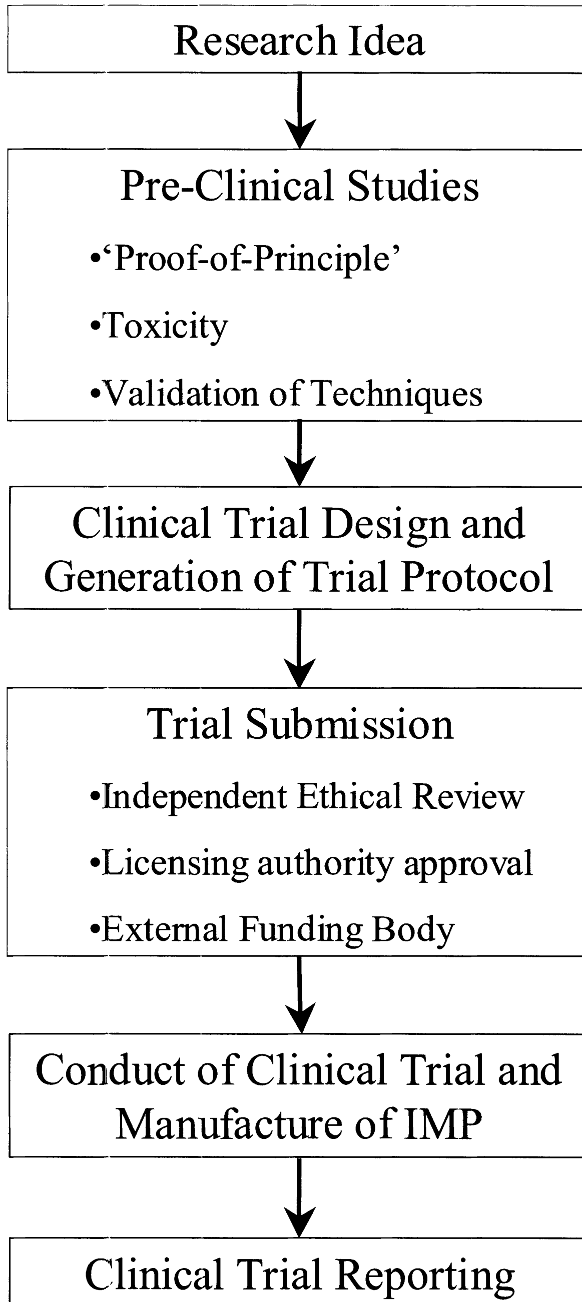

Figure 1. Pathway of translational research. IMP = investigational medicinal product.

quality was incorporated into clinical trial practice was variable and there were no mandatory requirements to meet the standards of GCP or GMP imposed on investigators or trial sponsors. Following the EU Directive there will be a legal requirement for trials to be run to the standards set for GCP/GMP.

The European Clinical Trial Directive

The Directive requires EU Member states to draw up legislation implementing the Directive by May 2003. The purpose of the Directive is to contribute to quality improvement in clinical research and to provide public assurance that the rights, integrity and confidentiality of trial subjects are protected and that the reported results are accurate and credible [3,4]. It seeks to achieve this aim by setting down standards for the protection of clinical trial subjects. There will also be a requirement for Member states to establish ethics committees on a legal basis and to impose legal obligations in relation to certain procedures, such as times within which an opinion must be given. In addition, the Directive covers certain Licensing Authority procedures for commencing a clinical trial. Standard have been set for the manufacture, import and labeling of IMPs and for providing quality assurance of clinical trials and IMPs. To ensure compliance with these standards, there will be a requirement for Member States to set up inspection systems for GMP and GCP. There is provision for the safety monitoring of patients in trials and the Directive sets out procedures for the reporting and recording of adverse drug reactions and events. In order to help with the exchange of information between Member States about approved clinical trials and pharmacovigilance, secure networks will be established, linked to European databases. The Directive will apply to all clinical trials involving medicinal products for human use, with the exception of non-interventional trials.

Good Clinical Practice

GCP is the cornerstone of good clinical trial practice. It can be defined as a standard for the design, conduct, performance, monitoring, audit, recording, analyses and reporting of clinical trials that provide assurance that the data and reported results are credible and accurate, and that the rights, integrity, and confidentiality of trial subjects are protected. The principles of GCP, as described in ICH Harmonised Tripartite Guideline for Good Clinical Practice [1], are as follows:

- Clinical trials should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and are consistent with GCP and the applicable regulatory requirements;
- Before a trial is initiated, foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial participant and society. A trial should only be initiated and continued only if the benefit justify the risks;
- The rights, safety and well-being of the trial participants are the most important consideration and should prevail over the interests of science and society;
- The available non-clinical and clinical information on an investigational product should be adequate to support the proposed trial;

- Clinical trials should be scientifically sound and described in a clear detailed protocol;
- A trial should be conducted in compliance with the protocol that has received prior Ethical Committee favourable opinion;
- The medical care given to, and medical decisions made on behalf of participants should always be the responsibility of a qualified physician.;
- Each individual involving in conducting a trial should be qualified by education, training, and experience to perform his or her respective tasks;
- Freely given informed consent should be obtained from every participant prior to clinical trial participation;
- All clinical trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification;
- The confidentiality of records that could identify participants should be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirements;
- Investigational products should be manufactured, handled and stored in accordance with Good Manufacturing Practice. They should be used in accordance with the approved protocol;
- Systems with procedures that ensure the quality of every aspect of the trial should be implemented.

It can be seen that to ensure compliance with the principles of GCP that there should be a quality system that addresses all aspects of clinical trial design, conduct, recording and reporting of clinical trials. The quality system should include a quality policy, clinical standard operating procedures (SOPs) for each individual trial procedure, training requirements for staff involved in research, systems for monitoring quality and compliance with auditing being performed on the basis of documented risk assessments and inspection of research quality systems should be performed. For studies involving the use of investigational medicinal products, which includes cell-based therapies, the Directive states that manufacture of IMPs must be performed to GMP standards.

Good Manufacturing Practice

The manufacture of IMPs will require prior authorisation from the Licensing Authority for trials performed in EU member states. Essentially, the preparation of IMPs will require compliance with standards equivalent to those for manufactured products at licensed sites, authorised via a manufacturer's license. The manufacturer must follow GMP requirements and have available a Qualified Person (QP) who is responsible for ensuring that the IMP fulfils the necessary requirements prior to its release. When developing a quality system to ensure manufacture of IMPs is performed to GMP standards the following categories need to be considered:
- Personnel
- Premises
- Processes
- Production
- Quality system

Personnel
Ideally personnel should be dedicated to the manufacture of a particular IMP although this is usually not practicable in smaller facilities. There should however be separate individuals responsible for production and quality control. Personnel should undergo appropriate training and be certified as competent to perform the procedures necessary in the manufacture of the IMP.

Premises
Again premises should ideally be dedicated to the manufacture of a single IMP in order to prevent the risk of cross-contamination although this is usually not possible in smaller facilities. In place of dedicated self-contained facilities it is acceptable to perform campaign working. Cleaning of premises and equipment between different products is of particular importance. Cleaning should be very stringent with procedures designed in light of the incomplete knowledge of the IMP.

Processes
Production processes for IMP may not be validated to the extent necessary for routine production but premises and equipment are expected to be fully qualified. In the manufacture of cell-based therapies open and manual procedures are often used and so enhanced attention should be given to operator training and validating the aseptic technique of individual operators.

Production
A comprehensive specification file should be produced for the IMP including information on starting material, manufacturing methods, microbiological testing and in-process testing. If the IMP is in the development phase the documentation needs to be continually updated whilst ensuring appropriate traceability to previous versions.

Quality systems
There should be an individual(s) responsible for quality assurance that is not involved in the manufacture of the IMP. There should be a quality system that covers all aspects of the manufacture, packaging, labelling and release of IMP. Audit and monitoring should be performed on a regular basis. The Clinical Trial Directive also states a requirement for a qualified person who is responsible for ensuring that products meet the specification approved for the trial and that they have made accordance with existing EU guidance on GMP [5]

Good Manufacturing Practice for Cell-based Therapies

For manufacturers of cell based therapies there are particular issues pertaining to the production process. The starting material is human in origin and as such is subject to guidance provided in Annex 14 of the EU guide to Good Manufacturing Practice [6]. Quality assurance programmes need to cover all stages leading to the finished product that includes collection, storage, transport, processing, quality control and delivery. Whilst fully respecting confidentiality there must be a system in place that enables the path taken from each donation to be traced, both forward from the donor and back from the IMP. Before any cell-based

product is released it must have been tested using a validated method of suitable sensitivity and specificity for the following markers of specific disease-transmitting agents: HbsAg, antibodies to HIV-1 and HIV-2 and antibodies to HCV. In the UK the Code of Practice for Tissue Banks specifies the requirements for the activities of tissue banks that store and/or process human tissues for therapeutic use including use in clinical trials [7]. The code of Practice is based on a quality system approach and will form the basis by which the UK Licensing Authority, the MCA, inspect facilities involved in the production of cell based IMP for clinical trial purposes. Given the expertise that exists within the National Blood Services in the area of cell collection, processing and storage there is a strong argument for their involvement in providing advice or facilities for the manufacture of cell based IMPs [8]. There is also an opportunity for extending and adapting the quality systems that exist for the manufacture of standard blood components to novel cell therapies.

Inspections

Within the Directive there are provisions for inspection arrangements and appointment of inspectors by competent authorities. Inspections will include the trial sites, manufacturing site any laboratory used for analysis, and/or the sponsor's premises. Statutory GCP inspections are likely to be of two types:

- Cyclical, systems-based inspections of sponsors (commercial and non-commercial) and contract research organisations involved in clinical trials in the UK;
- Triggered inspections as required.

The sponsor/contract research organisation will be expected to meet the fee for cyclical systems-based inspections.

How will the Directive impact on translational research

It can be seen that the Directive will have a significant impact on the ways clinical trials are designed and conducted and how IMPs are manufactured. Within the academic community there are concerns over the implications for academic research. The cost and resource implications for running trials to the requirements of GCP and GMP are very high. One potential negative outcome of GCP/GMP compliance is that it will only be possible for Research Charities to fund smaller numbers of clinical trials. Phase I/II trials of IMPs are subject to a high attrition rate with only a small proportion of IMPs tested finally reaching clinical practice. Smaller numbers of Phase I/II trials on smaller numbers of IMP may mean that potentially beneficially new therapeutic agents may not be realised. There is therefore a concern that the Directive will stifle academic research that will ultimately be to society's detriment. Whilst it is indisputable that the principles of GCP and GMP should apply to Phase I/II trials the standards of practice need to be appropriate to these types of studies. Above all the safety of patients and quality of data is paramount. This can be achieved in non-commercial research by applying of appropriate levels of documentation and quality control/assurance systems whilst recognising the role of academic clinical research and the need to balance good practice with cost.

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FACTORS CONTROLLING EXPANSION AND MATURATION OF HAEMATOPOIETIC PROGENITOR CELLS

A.L. Drayer[1], E. Vellenga[1]

Introduction

In cancer patients receiving high-dose chemotherapy and haematopoietic stem cell (HSC) transplantation the period of profound cytopenia generally lasts between 1 to 6 weeks, depending on the number and source of HSCs infused [1-4]. Experimental transplantation models in mice have demonstrated that long-term engraftment is supported by undifferentiated stem cells, while short-term (transient) engraftment is mediated by more differentiated progenitor cells. Therefore, supplementing stem cell transplants with ex vivo expanded progenitor cells may be an approach to accelerate the haematopoietic recovery. The principle of this cell-based therapy has been demonstrated in mice and non-human primates, although the recovery is slower than predicted from the large number of progenitors infused [5-7]. In the human setting, multiple studies have now demonstrated that the ex vivo expansion process can be used to generate large quantities of more mature progenitor cells, and a number of clinical studies have shown promising results [8-11]. During culture with cytokines, cells go through different stages of the cell cycle and the expression of cell surface adhesion molecules is altered; both factors appear to influence the capacity of stem cells to migrate through the circulation and back to a supportive haematopoietic microenvironment in the bone marrow, a process referred to as "homing". Therefore, optimised culture conditions for expanded cells showing improved homing abilities are required before ex vivo expansion-based cellular therapies can be used efficiently in the future.

Dissecting the interaction (cross talk) between cytokines involved in proliferation (to generate many progeny) and differentiation (to generate cells at a specific maturation stage with the appropriate homing properties) should prove to be valuable in uncoupling the proliferation and differentiation processes during expansion culture. In this paper we will discuss how cytokines interact at the cellular and molecular level by investigating their effects on proliferation, differentiation and signal transduction processes. Two examples of cross-talk between signalling pathways will be presented: the effect of transforming growth factor beta (TGF-ß) and thrombopoietin (Tpo) in megakaryocyte progenitor

1. Sanquin Blood Bank Noordoost, Groningen, NL.
2. Department of Haematology, University Hospital Groningen, NL.

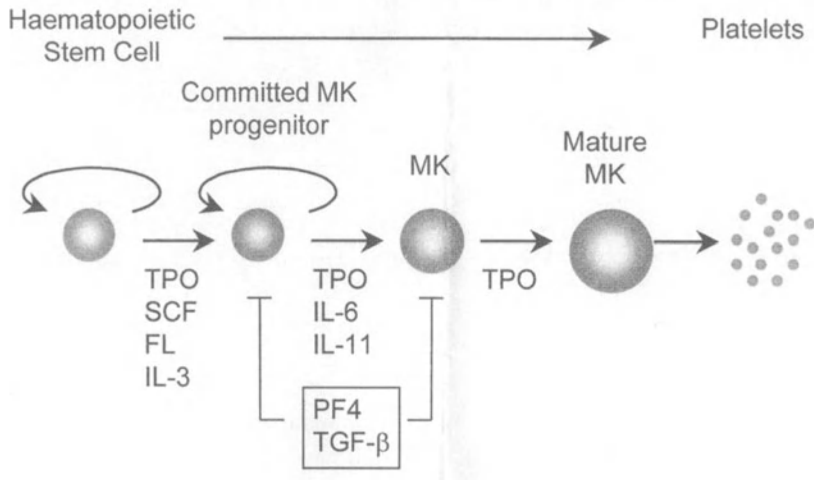

Figure 1. Cytokines controlling megakaryocytopoiesis: Tpo is the major cytokine influencing megakaryopoiesis, however, interactions with other cytokines such as stem cell factor (SCF), Flt3 ligand (FL) and interleukin (IL)-3, IL-6 and IL-11 stimulate proliferation and differentiation of megakaryocytes, whereas factors such as platelet factor 4 (PF4) and transforming growth factor ß (TGF- ß) negatively influence megakaryocytopoiesis.

cells and the interaction between prostaglandin-E2 (PGE2) and erythropoietin (Epo) signalling in erythroid progenitor cells.

Ex Vivo Expansion of Megakaryocytic Progenitor Cells

In our ex vivo expansion studies we have focused on expansion of the megakaryocytic lineage, as the period of thrombocytopenia following stem cell transplantation is a major problem for many patients. During the ex vivo expansion process, CD34+ cells are stimulated with recombinant cytokines in vitro to generate partially differentiated progenitor cells. A variety of growth factors influence the proliferation of megakaryocyte precursor cells and the development to mature megakaryocytes, as illustrated in Figure 1. Full-length Tpo and its truncated, pegylated form termed megakaryocyte growth and development factor (MGDF), are the most potent stimulators of megakaryopoiesis, inducing both proliferation and maturation of megakaryocyte progenitors [12-15]. Tpo is not essential for megakaryopoiesis as mice deficient in Tpo production or the Tpo receptor still produce platelets, although at a strongly reduced rate [16-19]. Combinations of TPO and other cytokines including stem cell factor (SCF), Flt3-ligand (FL), and interleukin (IL)-3, IL-6 and IL-11 have been shown to induce high levels of proliferation of megakaryocyte progenitors in suspension cultures [10,20-22]. Using serum-free culture conditions we evaluated the effect of three different cytokine combinations on expansion, with special emphasis on the type of colony formation and migration of megakaryocytic cells [23]. We made a distinction between two types of clonogenic megakaryocytic colonies (CFU-Mk); those which consisted of 5-20 cells per colony and colonies of more

than 20 megakaryocytic cells. The latter are the high-proliferative colonies that were generated exclusively from the CD34+/CD61- cell fraction, whereas the small colonies with a low-proliferative potential originate from both the CD61+ and CD34+/CD61- cell fraction [23]. Selected CD34+ cells were cultured in the presence of two cytokines (MGDF plus SCF), 3 cytokines (MGDF, SCF plus IL-3) or with a combination of 6 cytokines (MGDF, SCF, IL-3, IL-6, IL-11 and FL). It appeared that the growth factor combination has a significant effect on the type of progenitor that is expanded. The number of megakaryocytic (CD61+) cells and CFU-Mk with high- (>20 cells/colony) and low-proliferative capacity (5-20 cells/colony) were significantly increased by including IL-3 to cultures containing MGDF plus SCF. In the combination of 6 cytokines the number of CD34+/CD61- cells and CFU-Mk with a high-proliferative capacity were further increased compared to the combination with 3 cytokines, without a further increase in the number of CD61+ cells and CFU-Mk with low-proliferative capacity. Although the megakaryocyte progenitors generated under all cytokine combinations studied were able to differentiate into mature, pro-platelet forming megakaryocytes, we observed that in the cultures with 3 and 6 cytokines the in vitro migration towards stromal cell-derived factor 1 (SDF-1) was diminished. In addition, the reduced migration was related to a lowered expression of CXCR4, the receptor for SDF-1, on megakaryocytes from the cultures with 3 or 6 cytokines compared to culture in MGDF plus SCF alone. This could be of clinical importance as the chemokine stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 have been shown to mediate migration of human CD34+ cells and engraftment in a NOD/SCID mouse model [24-26].

Ex Vivo Expansion in Patients with Delayed Platelet Engraftment

A prerequisite for ex vivo expansion protocols to be feasible in the autologous setting, is that patients who could potentially benefit from this treatment do supply stem cell grafts which can be expanded successfully. Therefore, we questioned if patients with a delayed platelet reconstitution in vivo are able to generate megakaryocyte progenitors in vitro. The megakaryocyte expansion of CD34+ cells selected from stem cell grafts of patients with strongly delayed platelet engraftment after autologous transplantation was examined. A homogeneous group of patients was selected [27]: with the same disease (multiple myeloma), all received high amounts of CD34+ cells (>2×106 c/kg) so that this was not a limiting factor, and all showed rapid myeloid engraftment indicating there was no general engraftment defect. However, the time to platelet recovery (>20×109 /L) varied widely from 7 to 115 days. Our results demonstrate that patients with a strongly delayed platelet recovery did not show impaired in vitro expansion of CD61+ cells when compared to the rest of the study group and compared to normal bone marrow samples (28). In the patient group studied, four of the five patients with strongly delayed platelet recovery received CFU-Mk doses in the lower range and might benefit from infusion of additional megakaryocytic progenitors; however, a similar CFU-Mk dose gave rapid platelet recovery in other patients. It is possible that the delayed platelet recovery is not caused by insufficient megakaryocyte progenitors in the graft, but e.g. by a

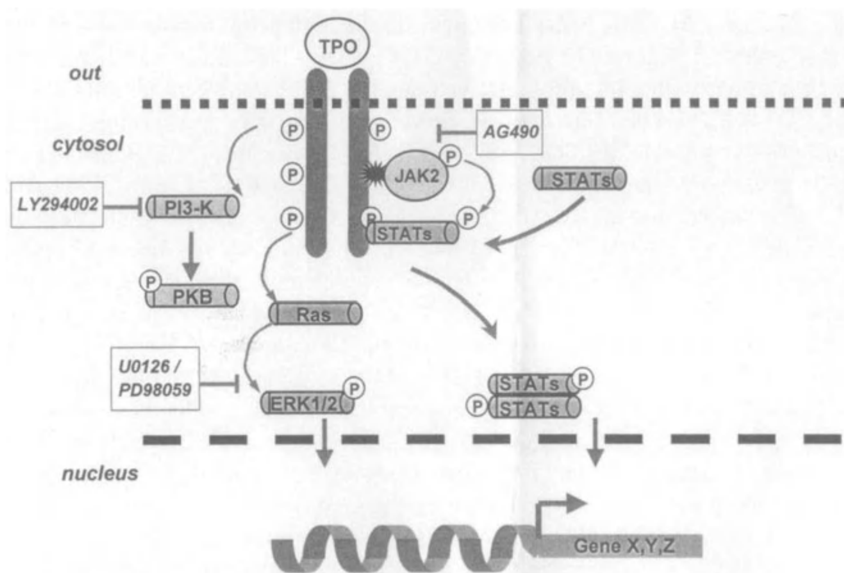

Figure 2. TPO stimulation leads to activation of multiple signal transduction pathways: binding of Tpo to its receptor results in activation of the tyrosine kinase JAK2, and phosphorylation of the Tpo-receptor. The activated receptor binds and activates other signalling molecules by means of its phosphorylation sites, including STATs (signal transducer and activator of transcription), Ras/MAPK (mitogen-activated protein kinase) and PI (phosphatidylinositol) 3-kinase, which are involved in megakaryocytic survival, proliferation and differentiation. Pharmacological inhibitors inactivate specific pathways: LY294002 inhibits PI 3-kinase activity, U0126 and PD98059 block the activation of ERK1/2 by inhibiting the upstream kinase MEK, whereas the JAK2 inhibitor AG490 blocks downstream STAT signalling.

defect in expression of adhesion molecules on the progenitors or by a suboptimal microenvironment in the myeloablated bone marrow.

Tpo-mediated Signal Transduction

Binding of Tpo to its cell surface receptor Mpl results in the activation of the associated Janus family of tyrosine kinases Jak2 and Tyk2 by transphosphorylation [29-31]. Subsequently, the Tpo receptor is phosphorylated on tyrosine residues which act as docking sites for SH2-containing signalling proteins. Signal transducer and activator of transcription (Stat) 5 is recruited to the activated receptor complex and phosphorylated on tyrosine residues Y695/Y699 (Stat5A/B, respectively) [32]. The phosphorylated Stat proteins dimerise and translocate into the nucleus, where they increase the transcription of target genes, including Bcl-xL, cyclin D, and the cyclin-dependent kinase inhibitors p27 and p21. As illustrated in Figure 2, at least two other pathways are of importance in Tpo signalling. The activated Tpo receptor contains docking sites for Shc [33], which in turn is phosphorylated and then recruits Grb2/Sos, leading to activation of the classic Ras/Raf/mitogen-activated protein (MAP) kinase path-

way (reviewed in [34]). Once activated, the MAP kinases Extracellular Regulated Kinase (ERK) 1 and ERK2 can phosphorylate p90 (Rsk) which regulates the transcription factors ATF1 and CREB, or translocate to the nucleus where they directly phosphorylate transcription factors such as Elk1. Alternatively, it has recently been shown that in megakaryocytic cells ERK can be activated by Rap1/B-Raf, and through the phosphatidylinositol (PI) 3-kinase pathway [29,35]. The p85 adapter subunit of PI 3-kinase forms a signalling complex with Gab2, Shp2 and c-Cbl in response to Tpo stimulation, although its direct interaction with the Tpo receptor remains unclear [36]. An important downstream effector of PI 3-kinase is Protein Kinase B (PKB) which regulates proteins involved in cell survival and cell cycling, such as Bad and Forkhead [37,38].

A number of studies using Tpo-responsive cell lines and primary murine megakaryocytes have now established that activation of the Jak/Stat and PI 3-kinase pathways play an important role in cell survival and proliferation, while activation of the MAP kinase/ERK pathway is critical for differentiation and endomitosis in megakaryocytes [31,39-41]. In vitro, these Tpo-activated signalling routes can be positively and negatively modulated by additional growth factors and inhibitors. Pharmacological inhibitors modulate the signalling pathways in a specific way, as illustrated in Figure 2: the PI 3-kinase inhibitor Ly294002 inhibits Tpo-induced proliferation in megakaryocyte progenitors, the compound U0126 and PD98059 block the activation of the ERK1/2 by inhibiting the upstream kinase MEK and thereby down-regulate megakaryocytic maturation, whereas the JAK2 inhibitor AG490 blocks cell survival. At a more physiological level, the growth factors SCF and TGF-ß are known to affect Tpo-regulated processes in, respectively, a stimulating and a down-regulating way. As SCF is widely used in expansion protocols and TGF-ß is synthesised, stored and secreted by megakaryocytes themselves, it will be of interest to study the effects of these growth factors on activation or down-regulation of specific signalling pathways linked to proliferation or differentiation. The cross talk between TGF-ß and Tpo-activated signalling routes is discussed below.

Cross-talk Between TGF-ß and TPO-Mediated Signal transduction Pathways

Transforming growth factor beta is a member of the TGF superfamily of proteins, also comprising bone morphogenetic proteins (BMPs), activins and inhibins, which exert control over many cellular responses [42]. Megakaryocyte progenitors are highly responsive to negative regulation by TGF-ß [43]. Treatment with exogenous TGF-ß in vitro has been shown to reduce the number of CFU-Mk, whereas neutralisation of autocrine TGF-ß by addition of anti-TGF-ß antibody increased CFU-Mk formation [44-47]. During megakaryopoiesis TGF-ß furthermore negatively regulates differentiation to mature megakaryocytes by inhibiting endomitosis [45]. TGF-ß exerts its effects by stimulating heterodimerisation of type I and type II serine/threonine kinase receptors [48]. The TGF-ß receptor type II contains the ligand binding domain, whereas the type I receptor is responsible for the specificity of downstream signalling. The Smad proteins

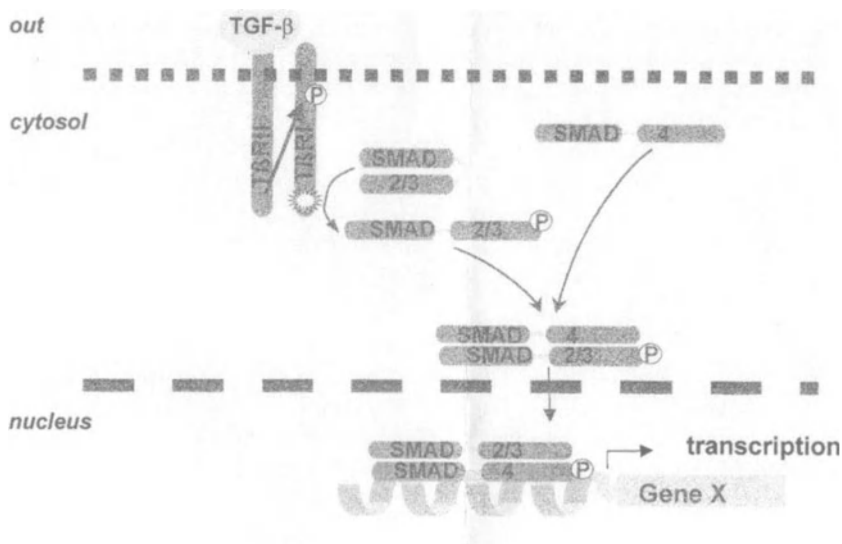

Figure 3. TGF-ß signalling: TGF-ß binding to its receptor TβRII (type II receptor for TGF-β) results in heterodimerisation with the type I receptor TβRI and phosphorylation of Smad2 and Smad3 proteins. The phosphorylated Smad proteins associate with Smad4, translocate to the nucleus where they act as transcription factors by binding to specific DNA sequences..

Smad2 and Smad3 are phosphorylated by the activated TGF-ß receptor type I, leading to their association with Smad4 and translocation to the nucleus where they control the transcription of target genes [49], as is illustrated in Figure 3. The amino-terminal domains of Smad3 and Smad4 have intrinsic DNA binding activity to specific DNA sequences, termed Smad-binding elements (SBEs). The Smad7 protein has been suggested to antagonise TGF-ß signalling. In addition, other proteins have been reported to interact with TGF-ß receptor type I, which might account for the Smad-independent pathway, which have been described.

We investigated the effects of TGF-ß on TPO-induced activation of signalling pathways in relation to proliferation and differentiation in the megakaryoblastic cell line MO7e [50,51]. TPO stimulation resulted in proliferation and maturation of MO7e cells, whereas IL-3 only supported the proliferation pathway. By comparing the signalling routes activated by TPO or IL-3, we could identify specific cross talk between the TPO-induced ERK1/2 pathway and the TGF-ß signalling pathway. Down-regulation of TPO-induced differentiation by TGF-ß correlated specifically with reduced activation of the ERK pathway. TGF-ß did not down-regulate the JAK2 or PI3-kinase signalling pathways, which are involved in survival and proliferation, indicating other additional routes involved in proliferation are modulated by TGF-ß.

Cross-talk Between PGE2 and Epo-mediated Signal Transduction Pathways

Erythropoietin (Epo) is crucial for proliferation and differentiation of erythroid progenitor cells. Mice with defects in the Epo or Epo-receptor (EpoR) gene die in utero of anaemia due to impaired foetal liver erythrocyte generation [52]. Similar to the activation by Tpo, binding of Epo to the EpoR results in a conformational change of the receptor leading to JAK activation and phosphorylation of the receptor, followed by recruitment of Src homology 2 (SH2) domain-containing proteins [53]. Several signalling cascades are activated upon Epo-stimulation, including the PI 3-kinase pathway, the MAP kinase ERK pathway and STAT proteins. In vitro erythroid colony assays have shown that PGE2 increases the number of colony-forming units of erythroid cells (CFU-e) [54, 55], and up-regulates erythroid differentiation by increasing haemoglobin synthesis [56]. In a recent study we investigated the cross talk between Epo- and PGE2-activated signal transduction pathways by analysing their effects on STAT5 signalling [57]. STAT5 is ubiquitously expressed in haematopoietic cells and is involved in erythroid differentiation and survival [58-60]. Two isoforms of STAT5 have been identified - STAT5A and STAT5B [61,62]. These isoforms share 90% homology and are rapidly tyrosine phosphorylated on Epo stimulation on tyrosine residues 694 (STAT5A) and 699 (STAT5B). STAT5 can also be phosphorylated on serine residue 726/731 (STAT5A/STAT5B) and 780 (STAT5A) [63,64]. The role of serine phosphorylation in STAT5-signaling is not fully understood, although some studies have indicated a role for STAT5 serine phosphorylation in maintaining STAT5-DNA binding [63-65]. Epo-mediated STAT5 transcriptional activity in erythroid AS-E2 cells was enhanced 6-fold by PGE2, without modulating the STAT5 tyrosine or serine phosphorylation, or STAT5 DNA binding. One of the signalling pathways that are activated by PGE2 is the cAMP pathway, involving activation of adenylyl cyclase and protein kinase A (PKA). We demonstrated that the co-stimulatory effect of PGE2 on Epo-mediated STAT5 transactivation was mediated by PKA and its downstream effector cAMP-responsive element binding (CREB) protein, as illustrated in Figure 4. In addition, the CREB-binding protein (CBP) was shown to play an essential role in Epo-mediated STAT5 transcriptional activity. As a result of PGE2 co-stimulation, the STAT5 target genes Bcl-X, SOCS2 and SOCS3 were upregulated in vivo [57]. These results indicate that the stimulatory effects of PGE2 on erythroid proliferation and differentiation might in part be regulated by STAT5 and are mediated by activation of the PKA/CREB pathway.

Conclusions and future perspectives

These studies demonstrate that combinations of growth factors modulate the proliferation and differentiation status of cells, which can influence the properties of ex vivo expanded cells. Inclusion of IL-3 had a positive effect on the number of megakaryocyte progenitors generated, but down regulated the chemokine receptor CXCR4. TGF-ß was shown to down-regulate certain signalling pathways in a highly specific manner, while not affecting others. On the

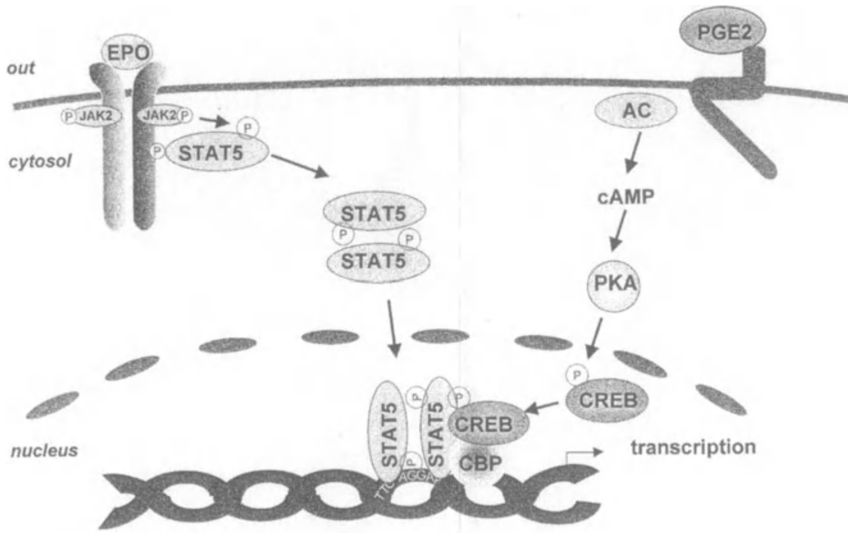

Figure 4. Cross talk between growth factors: PGE2 co-stimulation enhances STAT5 transcriptional activity by activating the PKA/CREB pathway. Binding of Epo to its receptor results in activation of the tyrosine kinase JAK2, and phosphorylation of the Epo-receptor. The activated receptor binds and activates other signalling molecules by means of its phosphorylation sites, including STAT5. The phosphorylated STAT proteins dimerise and translocate to the nucleus where they act as transcription factors. PGE2 binding to its G-protein coupled receptor results in the activation of the cAMP pathway involving adenylyl cyclase (AC), protein kinase A (PKA) and phosphorylation of the cAMP-responsive binding protein CREB. Phosphorylated CREB binds to the CREB-binding protein (CBP) and STAT5 and thereby upregulates STAT5 transcriptional activity.

other hand, activation of the cAMP pathway as demonstrated in erythroid progenitor cells could result in increased survival during expansion in the presence of Epo. A greater understanding of the specific signalling pathways activated following growth factor stimulation will allow us to make an optimal choice which growth factors to use during a specific phase of the expansion process and dissect the process of proliferation and differentiation.

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CELL PROCESSING FOR GENE AND CELL THERAPY PROTOCOLS: *LIMITATIONS AND OPPORTUNITIES!*

I. Slaper-Cortenbach[1,2], E. Simonetti[2], C. Arts[2], Ph.G. de Groot[2], A. van Dijk[1,3], S. Ebeling[2], A. Hagenbeek[1,2]

Introduction

At the UMC Utrecht, a new Gene and Cell Therapy Facility (GCT-F) for gene and cellular therapy protocols has recently been opened and is now fully operational. The main objective for building this expensive facility was to enable translation of preclinical research into clinical protocols, with an emphasis on the preparation of genetically modified cellular products.

In the Netherlands, the preparation of genetically modified cellular products is considered to be regulated under the law for pharmaceutical drugs under GMP conditions. The facility has three separate laboratories, each equipped with agrade A biohazard Laminar Air Flow cabinet and fulfilling grade B background environment specifications. The facility has also a CI biological safety level, allowing the handling of genetically modified organisms. Each individual laboratory has been equipped with incubators, a bag centrifuge, microscopes, refrigerators, freezer, and a place for daily storage of disposables. There is also a fully equipped QA/QC laboratory inside the facility. Furthermore, at the moment several different cell selection systems are available, like the CliniMACS^{plus} system from Miltenyi and the Isolex 300SA and 300i from Baxter allowing different kind of cell separation procedures. There is a general storage room for disposables and a large refrigerator and 2 freezers (-20°C and -80°C) for storage of clinical grade materials. A controlled rate freezing system for cryopreservation and liquid nitrogen storage containers are located in the vicinity of the facility, also continuously monitored and operated by authorized personnel only. To ensure that the facility maintains it status, it is permanently monitored for all relevant cleanroom parameters such as: particle counts; pressure hierarchy system; temperature, humidity and CO_2 in the incubators; temperature in coolers and freezers; room temperature etc. by means of a certified monitoring system. Microbiological evaluations are routinely being done at specified time intervals and during the procedures.

Taken together, this facility has all the major systems in place to perform gene and cell therapy studies at the UMC Utrecht, by the presence of a validated, classified GCT-F with multiple cell selection systems in place and a qual-

1. Gene and Cell Therapy Facility, University Medical Center Utrecht, NL.
2. Department of Hematology, University Medical Center Utrecht, NL.
3. Hospital Pharmacy, University Medical Center Utrecht, NL.

ity management system to ensure the quality of the endproducts. Processing conditions are currently being established for therapeutic application of a wide variety of gene and cellular therapeutic products. Below, we will discuss examples of preclinical translational studies in each type of protocol.

Suicide Gene Therapy Protocol

Allogeneic stem cell transplantation for patients with hematological malignancies is performed using high dose therapy in combination with T cell reduced stem cell transplants, which contain a low number of T cells (1×10^5 T cells/kg body weight) [1]. This number of T cells causes only minor Graft versus Host Disease (GvHD grade I/II) and exerts also an anti-leukaemia effect (GvL). Unfortunately, relapses are still occurring, so increasing doses of T cells are necessary to 'cure' the leukemia in vivo, thereby increasing the risk and severity of GvHD. Herpus Simplex Virus-Thymidine kinase (HSV-Tk) gene transduction in T cells allows the effective and selective kill of these T cells in vivo by Ganciclovir (GCV). So, HSV-Tk gene-expressing donor T-cells can be added to enhance the graft-versus-leukemia effect and can be eliminated by GCV in vivo when the GvHD increases [2].

In our first gene therapy protocol, donor T cells will be modified with clinical grade retrovirus, which encodes the HSV-Tk and ΔLNGF-R genes (SFCMM-3). The expression of the ΔLNGF-R gene allows selection of transduced T cells via a biotinylated LNGF-R antibody and anti-biotin Microbeads and the Tk-gene allows selective 'in vivo' elimination with Ganciclovir. Clinical administration of these cells necessitates a cell population consisting of >95% pure transduced T cells and a transduction procedure in a closed system [3].

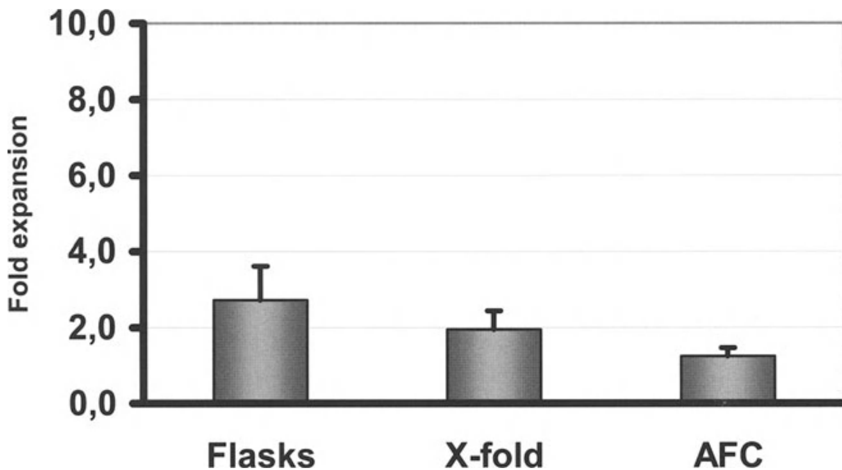

Figure 1. Comparison of the expansion of T cells during the transduction performed in flasks and in two types of transduction bags.

Aim of this preclinical study was to investigate the usage of different transduction and culture bags in combination with the performance of the newly developed CliniMACS^{plus} 5.1 enrichment protocol for cells with high antigen expression. Peripheral blood mononuclear cells from different donors were isolated on day 0 and stimulated with CD3 McAb and IL2. Retroviral transduction was performed on day 2 and 3 (after coating with CH-296 (Retronectin^{TM}) in the Baxter X-fold or Cellgenix AFC bags. Both bags were designed for optimal gene transduction. Control experiments were performed in T25 flasks. The results are shown in Figure 1.

These data show that optimal expansion is still reached during transduction (day 2-4) in the flasks, and that more T cells are recovered from the X-fold bag than from the AFC bags.

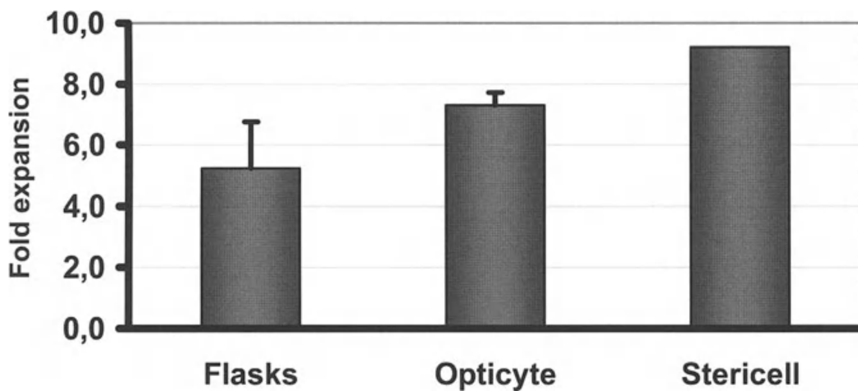

Figure 2. Comparison of the expansion of T cells after the transduction (day 4-7) in flasks and in two types of expansion bags.

After culturing the T cells for an additional culture period of 3 days in flasks or in 2 different types of expansion bags (Opticyte and Stericell, both obtained from Baxter), the results show that the bags allow a more rapid expansion of T cells than measured in flasks (Fig. 2). The transduction efficiency, mostly depending on the number of dividing T cells, was best in the cell population that has been transduced in the flasks, and somewhat lower in the X-fold and AFC bags. The overall procedures resulted in equivalent numbers of transduced T cells.

The immunomagnetic selection of the transduced T cells was done on day 7, and the Ganciclovir (GCV)-sensitivity was measured on day 8 by ^{3}H-Thymidine incorporation. Aim of this clinical protocol was to reach > 80% kill of the selected and transduced T cell population by 10^{-6} M GCV in vitro, an equivalent to the GCV concentration which can be detected in vivo. Earlier experiments using the VarioMACS cell seperation device for research purposes only, showed that the combination of biotin-labeled clinical grade 20.4 McAb and anti-biotin Microbeads resulted in a 95% pure transduced T cell population. Unfortunately, this selected population contained 25% weakly positive LNGF-R-positive cells.

Flowcytometric cell sorting showed that this population had a reduced GCV-sensitivity (54% survival compared to 10% for the high expressing cells at 10^{-6} M). Therefore, in this study, we used the CliniMACS^{plus} and the new 5.1 enrichment protocol for cells with high antigen expression and frequency, in combination with the newly developed CliniMACS tubing set 150 (Fig.3).

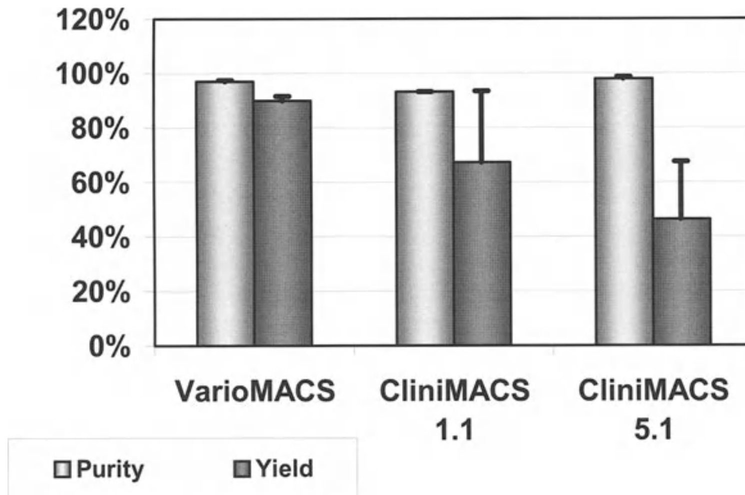

Figure 3. Comparison of the yield and purity of transduced T cells different selection methods.

These data show that we have set up a closed culture and selection system for the generation of HSV-Tk$^+$ T cells. Good transduction efficiencies were obtained in the X-fold and AFC transduction bags (40-50%). Expansion of cells during the entire transduction procedure is sufficient to generate the required number of HSV-Tk$^+$ T cells. The selection procedure 5.1 using the CliniMACS results in purity of 98.0 ± 0.5% and yield of 46.3 ± 21.2% (n=7). In the GCV assay, 10^{-6} M GCV results in 87.8 ± 3.9% T cell kill (n=4). Overall results showed that the procedure is feasible and fulfills the clinical relevant criteria.

Cellular therapy

In one of our cellular therapy protocols, microvascular endothelial cells will be isolated from subcutaneous fat and seeded on vascular prosthetic grafts to improve patency. In bypass surgery, the patency of prosthetic grafts is less than that of vein grafts, but unfortunately, not all patients have suitable veins. Prosthetic grafts lack an endothelial cell (EC) layer, so an isolation protocol was developed to obtain large numbers of EC from fat tissue. Main aim of this study was to purify the EC, because contaminating cells (mainly fibroblasts) enhanced the thrombogenicity and the formation of intimal hyperplasia [4]. We compared different strategies: depletion of fibroblasts/macrophages using the monoclonal antibodies Fibrau-11 and CD14 versus two different CD 34 selection procedures

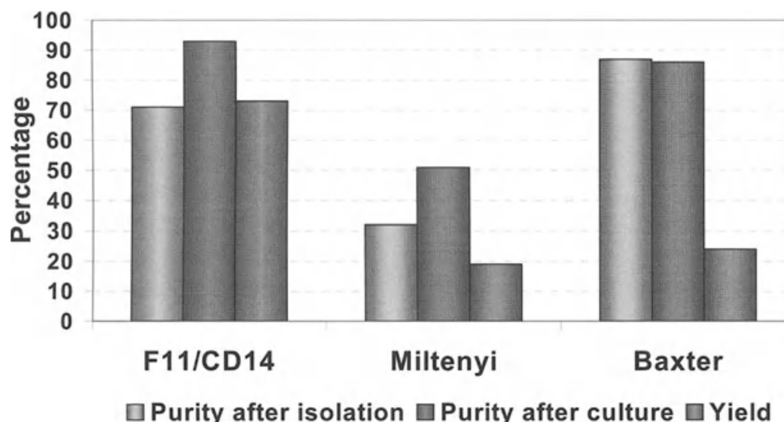

Figure 4. Comparison of the yield and purity of endothelial cells using different methods.

(Baxter & Miltenyi). The latter two immunoselection techniques are currently being used for the isolation of haematopoietic stem cells. The Baxter kit uses CD 34 McAs, Dynabeads and a peptide releasing agent, and the Miltenyi kit uses CD34 labeled Microbeads. The highest yield (73%) and purity (>90%) of EC was obtained with the depletion protocol, but unfortunately the antibodies used are not approved for clinical trials. Testing the two different CD 34 immunoselection methods, we showed that the Baxter method resulted also in a highly purified cell population (87%), but with a lower recovery (24%). The Miltenyi approach using CD 34-Microbeads did not enrich the endothelial cells under the conditions tested [5]. This protocol will be upscaled and soon open for patients with end stage renal failure requiring dialysis. The prosthetic haemodialysis shunts will be coated with autologous fat tissue derived microvascular endothelial cells and the in vivo performance will be studied.

Concluding remarks.

In the UMC Utrecht, several research protocols were developed using routine laboratory equipment. However, the translation from research bench to clinical application required additional experiments. In the Netherlands, the preparation of genetically modified cellular products are considered as drugs, and thus require pharmaceutical conditions for aseptic production. So, by establishing the Gene and Cell Therapy Facility in Utrecht, we have overcome these limitations.
We have tested different processing media, selection kits, disposables and the handling in closed systems and currently two protocols are ready to be tested in clinical protocols.

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DISCUSSION

P. Law, H.C. Kluin-Nelemans

P. Law (Seattle, WA, USA): Dr. .Roddie, you mentioned that classical validation may not be appropriate for some of these cell therapy products. What is your thought and how do you propose to integrate the validation into the early phases of development, so that it does not become an issue when the clinical trials have been started

P.H. Roddie (Edinburgh, UK): I think it is an important but difficult point, things do change. We heard already that things change, so when you do the clinical research and find that things do change politically, there seems to be a sense that has to be understood. Certainly within the EU guidance to GMP there is sense that they understand that it is difficult to perform classical validation. However, there are certain critical things that you must ensure, which include aseptic techniques, and microbiological control, which is absolute mandatory. That does not mean that you should not try to validate, but that you should ensure these things are of paramount importance and the others maybe are slightly less important.

R.M. v.d. Plas (Bilthoven, NL): Under the European GMP regulation, which is different indeed from the American regulation, the starting point of manufacture is sometimes ill defined. Which means that it is not very clear always when the start of medicinal product manufacture commences. For classical chemical medicines for example this is more or less at the moment that you are actually making tablets. So if you are only making active substance the GMP rules MAY not ALWAYS apply.

On a very different level I like to note that currently in the Netherlands it is not decided, at least to my knowledge, in which way the clinical trial directive will be implemented. There has not yet been made a political choice which competent authority will control the clinical trials. This may be the CCMO or it may be the Inspectorate, but there are other options.

P.H. Roddie: I am not so familiar with this situation. I thought there were co-rapporters on the EU clinical directive. I think it is up to the EU Member States how they implement the legislation. But I think the EU clinical trial is relatively clear about what the standards are. However, it is true to say that there will be a fair amount of interpretation when it comes to cellular therapies. A lot of re-

sponsibility I think is going to fall on the licensing authorities to decide what they feel is appropriate level of GMP and that is a phrase that other people have used in the UK. There maybe a level to GMP appropriate to phase 1 and phase 2 clinical trial investigation of medicinal products less stringent than would be applied to a phase 3 trial: pharmaceutical product.

P. Rebulla (Milan, I): Dr. Roddie how do you determine how many units must be tested to validate a clinical cell therapy protocol?. We were discussing this with Dr. Read some time ago and she told me that usually they try to make it three times. As we were newer to the field, we decided we would make it five times. I wonder whether there is any rationale. For example, if in five runs you show that some parameters, such as purity and sterility, are not met by one unit, what do you do? Do you re-run another five, another three?. What is the rational for this?

P.H. Roddie: To be honest I do not think we have been as stringent in that regard in terms of purity and sterility. As we are running the clinical trial in terms of sterility, the product is tested for sort sterility during manufacturing process. Obviously it would not be used if it did not match the sterility criteria. In terms of the validation of what the product was, there were certain criteria set designed on the basis of doing patient samples. We are doing 40 patient samples to decide what were these specific criteria that made our product what it said it was. I think it is slightly difficult over the years because the definitions of what you are producing change al the time. So your set of validation criteria at one point it may be different sometimes.

J. Power (Cork, Ireland): Just a note of encouragement. To let the audience know that the first tissue bank accredited in the UK under the new regulations was actually a bone bank in a Welsh hospital. So it can be done.

J.C. Kluin-Nelemans (Groningen, NL): Dr. Roddie, in fact you were in an ideal situation. You have your own pre-clinical laboratory and your own final end product. Suppose you were at the clinical part and there was a pre-clinical laboratory somewhere else, would you follow than the official rules or would you have your own standards, perhaps even more stringent then the NIH or the EU rules? How are you planning to deal with that?

P.H. Roddie: I think I would be confident if it was produced according to the EU guidance on GMP. I think one of the sort of critical aspects which is probably changed to what has happened previously, is that it has to be someone who looks at the product once it is being produced and could say it does not meet the product specifications which will include safety testing, microbiological testing. So provided that person is doing his job and that is what they are for, than that product should be relatively safe. The other side of the coin, if the trials are being conducted and all patients with leukaemia in phase 1 phase 2 trial will die, there is a need to do a research. I think that we do need to ensure that what we

are giving is safe and effective, but it is in the context of doing something for many patients..

J.C. Kluin-Nelemans: Dr. Drayer, it is very complicated work and very clever how you explained all the signalling routes. I think it is very encouraging that you could test in those patients who were slow responders, slow recoverers after the transplant or high dose chemotherapy, that you indeed succeeded in expanding the megakaryocytic precursors also from these patients. This indeed might give possibilities for the future. We know that for GMP regulations you need to have the products also produced according to good manufacturing rules. You have been working with TPO, TGF-β and so on. Are these products suitable for the future real in vivo studies?

A.L. Drayer (Groningen, NL): Obviously not yet. There are a number of cytokines which can be used. But I think that it is a limiting factor, the cytokines which are clinical grades. What I propose is to use pharmacological inhibitors or other interactants within the cell. But these at least still have to be investigated and they or not up to clinical grade at all.

L. de Leij (Groningen, NL): Dr. Drayer, you have focused now on chemoattractants for the homing behaviour of progenitor cells. Have you also looked into adhesion molecules, just the normal adhesion molecules which all these cells need if they want to adhere to endothelial cells and do go into the bone marrow?

A.L. Drayer: No, that would be the thing to do. But it has been shown now that the adhesion molecules VLA 4, VLA 5 e.g. ICAM-1 and LFA-1 are essential for the homing ability of stem cells and progenitor cells. So these would be obvious markers to look at in the ex vivo expanded product to see if they express these adhesion molecules.

L. de Leij: So you think these adhesion molecules will be missing or do you think that they will not be expressed, so they home into other tissues than bone marrow?

A.L. Drayer: It is known that if you give CD34 positive cells, 90 or 95 % of the cells do not home to the bone marrow. Maybe only a very small amount of cells express the right receptor. That could be the case.

L. de Leij: Could you also inject these cells directly into the bone marrow?

A.L. Drayer: I don’t know if that has been done.

C.Th. Smit Sibinga (Groningen, NL): After all the work done I feel a bit guilty, because I was one of the stimulating factors in initiating the project, which took much more time than we anticipated in the beginning. What, having arrived at this point in time, is a much better understanding of what actually is going on in

getting the cells to that progenitor stage and to the ultimate maturation. What do you think at this point in time are the key factors to address to bring it into the real translational stage?

A.L. Drayer: I think you could start with the ex vivo expanded cells and see which stage is the most efficient to transplant to get the more rapid recovery. But on the other hand you could look more basic, it is still largely unknown how to megakaryocytic process develops in vivo, how these megakaryocytes are generated. We know in vitro that it goes to the colony stage, then the megakaryocyte precursor stage, but in vivo in the bone marrow, we do not even really know were the platelets are produced. So I think in vivo a lot of work is still unknown. You would have to know those kind of things. You have megakaryocytes of course in the bone marrow, but where are really the platelets produced. I think you would have to look at those aspects too. At the ex vivo expansion level, look at generating the most optimal conditions and maybe then these protocols would have a good chance of giving a good result.

C.Th. Smit Sibinga: The conditioning factors, so the consistency of the environment and all the other aspects that we looked into, now developed to such an extent, that we may have reached a point to start translating it into something that would be really applicable for clinical trials?

A.L Drayer: Yes, I think the first trials were of course looking at generating large numbers of cells and now we should focus more at marker studies of these cells; first in animal models, are these good in homing and than go back to the clinical trials. That would be my opinion.

J.C. Kluin-Nelemans: Suppose you would get one million EUROs, but only if you would proceed with the protocol within one year. Where would you start?

A.L. Drayer: I think in two ways – looking in vivo how platelets are produced, and on the other side making the best kind of cell with all the adhesion fields and CD64 receptor, which could home very well to the bone marrow and develop into a progenitor cell.

A. Brand (Leiden, NL): The TGF beta that is produced by the megakaryocytes in the platelet stage can be in several forms. Have you looked whether that interferes with TPO and magnitude of maturation? The second question – in your model can you overcome the inhibitory effect by simply increasing the dose of TPO?

A.L. Drayer: With preliminary experiments, which we did not present here, we were using anti-TGF beta antibody. TGF beta is produced in latent forms and activated form. When we add the anti-TGF beta antibody to CD34 positive cells you see an increase in colony formation and increase in the size of these colonies. So obviously these cells are producing endogenous TGF beta in an active form. I did not do the measurements, others have looked at that. So they are

producing it in an active form. I would not increase the dose of TPO, because that is already chosen to be optimal. I do not think increasing the dose would stimulate the down regulatory effect induced by TGF beta, because that is a separate receptor.

A. Brand: Have you tried simply to remove the medium to remove the TGF beta?

A.L. Drayer: I do not think that would be the approach for the ex vivo expansion protocol.

G. de Haan (Groningen, NL): Dr. Drayer, have you ever made calculations on the fold expansion you would require to have a clinical impact?

A.L. Drayer: I would like to point out that the way I presented is per CD34 positive cell. So, with TPO we see a small expansion, one or two fold. That it is much higher if you relate it to the number of CD61 positive cells within this CD34 population which you started with. If you express it as that you have a 300 to thousand fold expansion of CD34/CD61 positive cells.

G. de Haan: Even if it were a million fold, is that enough? You know the half life of platelets, you know how many platelets are required to reduce thrombocytopenia, so you know how many platelets are made per CD61 positive cell and so on. Than you can make a calculation and model it and see how many progenitors you need.

A.L. Drayer: I think I would have to do that calculation. But on the other side we would also have to look at what stage are these megakaryocytic progenitor cells still able to home to the bone marrow, because obviously if it is only the more immature cells you would still have the time lag in the haematopoietic recovery.

P. Law (Seattle, WA, USA): We have a separate QC and QA laboratory within the manufacturing laboratories. Do you have separate staffing and review process on the between manufacturing and QC/QA?

I. Slaper-Cortenbach (Utrecht, NL): We hope to have separate staffing, but so far we we only did a trial to validate all the systems. If the number of protocols allows us we will have separate staff in doing the QA/QC.

L. de Leij: Dr. Slaper-Cortenbach, for the selection of the endothelial cells, the fact that you only have 25% of the CD34 cells in your yield, are you sure that all endothelial cells do express CD34 after isolation from the fat? Have you ever considered to use cyclic RGD peptides for this kind of isolations?

I. Slaper-Cortenbach: To answer your second question first: no, we did not. Answering your first question, we monitor the yield of endothelial cells by using

CD34 in combination with CD31. Most of the endothelial cells we lose, because they still stick beads together with the fribroblasts. So you can manipulate that, but than you also release fibroblasts. That is one of the problems. We intend to harvest more fat and than have more cells.

C.Th. Smit Sibinga: Three processing clean rooms. What are they actually used for one, two and three?

I. Slaper-Cortenbach: That depends on the protocols which are going to run, because first of all we have the suicide gene protocol, which is ready for us to start now. That would be a protocol which is taking place in one unit. The other cellular therapy protocols can take place in the other two units, but there are also industries very interested in using these clean-rooms, because we have this high GMP level. So we will try to work batch-wise. When one procedure is closed, we will cleanup the whole laboratory and use it for another option. Because in the suicide gene therapy protocol if we going to do that, we have to infuse TK positive cells in the patient and wait for an other month before we can start another procedure. So, there is a lot time laps in uses of the laboratory. So, in between, we can use it for other purposes.

C.Th. Smit Sibinga: That means that through a time lag between the protocols that you run you solve the problem of crossing lines.

I. Slaper-Cortenbach: Yes

C.Th. Smit Sibinga: Where do you do the preclinical work?

I. Slaper-Cortenbach: The original work was done in a separate research laboratory. By now ten separate runs on buffy coats were done to study the whole procedure we did in the laboratory. The only thing we did in the facility so far were the trial runs.

C.Th. Smit Sibinga: How do you handle the responsibilities and the legal aspects when you also host one of your clean rooms for industry?

I. Slaper-Cortenbach: Very good question. We are about to discuss whether they want to come in. That is a firm, which also wants to bring in their own personnel and their own media. So that is an easy way of discussing things, because they have their own liability, they only use our facility as class B facility. But when you go further in this direction and your are going to do things for other industries, that is one of the main problems. We have to think that over, more than just intentions.

C.Th. Smit Sibinga: Is it actually the same room, with three different cabinets…

I. Slaper-Cortenbach: No, they are totally separated. So you bring in your material in a closed system and all three laboratories are totally isolated.

C.Th. Smit Sibinga: But they are in the same area, in the same 150 m2 and you enter through one or two lockers you kept in the current corridor to have access to the three rooms.

I. Slaper-Cortenbach: Yes, that is true. We have a central corridor.

P. Rebulla (Milan, I): When we started our cell factory a couple of years ago, we considered how we could document on a daily basis what the people were doing in the cell factory. We have not started yet a clinical protocol, but we are close to. I think Dr. Read mentioned that it is very useful to have a master file and that she designed something together with stem cell technology people. Do you have something similar? How do you keep records of the critical information, which may be important to know, for example, in ten years? What are the formal requirements for this at present time?

I. Slaper-Cortenbach: I do not know the formal requirements at this time in Europe, but certainly NIH have a lot more protocols running. We have our own control management system for the facility. So we describe all the criteria for the facility by itself and we are also principle investigators for the protocols to write our own SOP's and we keep them also in the laboratory. We might end up with the master file when we have many protocols. But this is still limited at this time.

P. Rebulla: When we started our exercises on the new protocols, on one occasion we discovered that the air-controlling machine was not working properly. So we were concerned if this could happen in a clinical procedure. Is there any idea of what sort of backup system should be available for clinical cell factories ? Should this be physically separate from the main facility?

P.H. Roddie: Our facility is of relative small scale. But I have also spoken to people in London at the Royal Free Hospital, they built recently a GMP facility. I do not remember if they have a separate building, but I think they have got two power plants, so if one fails then the other one still operates. They have that sort of safety built into their facility.

I. Slaper-Cortenbach: we have a backup in the ventilation system. So we have two systems in place. We monitor continuously, so people will be alarmed.

E.J. Read: We do not have backup ventilation systems. Heating, the ventilation and air-conditioning systems are incredibly expensive and it is even more expensive to monitor them. I wish we could just get everything in the closed systems, because than we would not have to worry. But in the mean time we do. I want to make a plea for these protocols. The first thing you try to do is get it into a closed system if you possibly can, because it makes your life so much easier. But there are necessarily things now that you start going into closed systems. Even the gene transduction you can actually do closed, because you have bags and you have sterile-connecting devises. But there are still some transfers when

you sample the product, where you really should not be working in the open air unless you have a really controlled environment. So, my first comment is that hopefully we would not have to build such fancy facilities in the future, because we will have closed systems.

It think the monitoring facility is really a continuous problem. When things do happen you document them and you try to make an assessment about whether it is really going to infect the product. Than ultimately you do some release assays on the product and make sure it is not contaminated. So we sort of have a monitoring of the facility, but even if that is perfect you still could contaminate the product, and visa versa. So I think you try to have as many backups as you can, but you can never be perfect.

I. Slaper-Cortenbach: Is it good to have a medical director who signs off your product?

E.J. Read: We have realistic release criteria for every product, especially the IND one's. We have different release criteria on every product. There are some continuous monitoring systems that will document everything on the computers, but they are really expensive. You may need that if you build a fancy facility, but you have to maintain it.

C.Th. Smit Sibinga: There is indeed a lot of experience in pharmaceutical industries, which we could easily tap on. It is also very critical that you have a separate power circuit that runs the machinery that takes care for the filtration of the air and that also secures any power dips that could occur.

E.J. Read: We do have backup power, but it does not take into account a fact that somebody would manually turn off the whole system. The other thing is that they would not let us have uninterrupted power for everything in our facility, that is going to cost too much money, so we got it for critical systems. But in the pharmaceutical industry you are right. They probably got it a lot better then we have it right now.

C.Th. Smit Sibinga: It is good having a validated contingency plan as part of your quality system, so that you could run at the moment something really seriously is going to happen

I. Slaper-Cortenbach: Absolutely, the main difference with the pharmaceutical companies is that they make money, and we do not. Of course they have very nice equipment and very nice procedures, but they are too expensive.

C.Th. Smit Sibinga: That is right, but that does not necessarily prevent you from tapping on their experience.

E.J. Read: I really think we should take advantage of the pharmaceutical industry's experience, they can make very good consultancy. The big difference that I see is that in the pharmaceutical companies if they have a problem with there air

handling they just shut and they do not go into production or if they are in production they just can throw the whole lot out. Most of the time we can not do that, because there are patient specific products. They are coming in and we cannot control when they are coming in are going out, because the patients have already been mobilised or the donors have already been mobilised or the pancreas is on the way or whatever. So there is those kind of unpredictables that we have to deal with. In some ways that makes us more obligated to set up better quality systems.

P. Law: I am speaking as an individual going from a stem cell transplant environment to an antigen presenting cell infusion environment. The patients who receive stem cell transplantation are really certainly in a lot more critical situation than patients who need antigen presenting cell products. The opportunity to recollect a patient is a lot better in the APC environment compared to a stem cell environment. So the criticality of the patient is really sometimes has to drive these risk management thoughts and discussions.

P. Rebulla: I have a question concerning the positive and negative pressures. When we built our two rooms we set one on negative and one on positive pressure with the assumption that in the negative pressure room we would do procedures like gene marking, because we would in those cases use a vector. Is it expected that a regulation can be set requiring positive pressures surrounding an area with a negative sink. Is that right?

I. Slaper-Cortenbach: I don't know. We have a negative sink in our lock. So the lock is based on a negative pressure where the laboratories are zero pressure.

P. Law: What you bring up is a good point. Do you have a procedure in place to document the line clearance and how do you make sure that you have cleared all the vectors from all of your laminar flow hoods, for example?

P. H. Roddie: We are not doing any gene therapy work. We did it in preclinical stages, but for various reasons it did not work. So we have not yet translated that.

P. Law: Line clearance is also very much related to GMP. Product segregation and product identity actually come under the same GMP language. So after you run, for example you have three rooms, room number one you run a factor transduction on Monday, do you clear it Tuesday morning to do some expansion studies or do you have to do some documentation on Tuesday morning?

I. Slaper-Cortenbach: Yes, of course. We do not intend to use the unit on two separate procedures on two separate protocols in one time period. So we are going to use it for gene therapy and then after the gene therapy procedure clean out the whole laboratory, do our monitoring and than start with another protocol. So we are not going to switch gene therapy and cellular therapy protocols in one unit.

P. Law: Would they carry over to your equipment such as centrifuges and other equipment and bench tops that get in contact with cells that were modified by a transgene for example?

I. Slaper-Cortenbach: Yes, then you have to clean out and proof that they are clean. I do not want to take the risk.

E.J. Read: I just want to make a comment about the positive and negative pressure, because I have been a little confused about what the FDA wants. Just for the audience not that familiar with this, the reason you want positive pressure in the laboratories, is you really try to protect the product. You do not care what goes out in the hall. You are dealing with high concentration HEPA, so it is not the same as the biological safety containment issue, which is where you want negative pressure. Now, my understanding with the FDA was, they wanted to create negative sink around the positive pressures, because of the capability just for gene therapy. But for the other types of products I do not think that negative sink around the positive was particularly an issue. Our laboratory was obviously built back in 1997 so we are 5 years old already and we have a negative sink in our freezer room and in our office base, but it sort of goes out into the hall. So it is not like negative pressure with containment. We do gene therapy in those positive pressure rooms, so we are obvious not doing what the FDA is now saying, but I do not think we are going to go back. So, I think this whole thing about how to design the facility is really evolved of the past, five, six, seven years. Anyway, if people understand the principle, so that is what is important about the positive and the negative pressure.

P. Rebulla: For the easiness of constructions and operational convenience several of these rooms have been built at the end of corridors or building wings. Therefore, in many cases an extra area surrounding the negative sink may not be available.

S.J. Noga: I do not believe any of these scenarios have been tested by the FDA. It really is their opinion at the time. They found out the government facilities were not put together very well in Western Virginia when they had some of the Semian viruses lead through the system.

Dr. Read, another comment on your system – the people in your laboratory told me that actually the exhaust that goes into your office.

C. Th. Smit Sibinga: The question of the negative pressure is kind of a semantic, because you start with a higher pressure and all what is outside that is negative relative to the higher pressure. You really do not go into a negative pressure, below zero atmosphere. The normal air pressure is just one atmosphere, and what you build up is a pressure higher and than cascade wise go down.

I. Slaper-Cortenbach: Yes, you talk about pressure differences instead of negative and positive pressures.

P. Rebulla: I wonder if anyone is using fumigations with formalin. This may damage the incubators.

P. Law: Dr. Drayer and Dr. Slaper-Cortenbach, you use a lot of stem cell expansion technology. If you have to bring your protocol into a GMP facility, how would you try to establish this stem cell expansion in the GMP facility?

A.L. Drayer: Our expansion protocols would be done at the blood bank. We have most of the facilities available for doing this, it could be applied tomorrow if you want to. Except for the media and growth factors, but the technical equipment and the environment are there.

I. Slaper-Cortenbach: Well, it is probably much more difficult than you think it is, because expansion in bags is not as easy as we thought and that is what I tried to show you in my presentation. Everyone thinks it is only a matter of multiplying your system, but it is not as easy as you think it is. So usually, the people come to me in an early stage and say: well we want to do this. Than you can really co-operate with each other in order to get their clinical protocol.

P. Law: Actually, when Dr. Noga was referring to the 18 to 24 months delay, he was referring to commercial development like us in terms of picking up the large-scale experiments.

M.K. Elias (Groningen, NL): Dr Drayer, you demonstrated that the migration potential of the expanded cells is affected by additional cytokines. It would be very interesting to know the migration behaviour of these cells in vivo as well. Using animal models, as you intend to do, would you be able to differentiate whether an eventual migration defect is due to a proliferation defect or a migration defect or damage to the micro environments – myelodestruction, which is not uncommon with chemotherapy?

A.L. Drayer: Yes, we could distinguish in an in vivo model between proliferation defect and a homing defect, if we use marked expanded cells so that you can distinguish from the recipient's own cells. So, in that way you could see where the cells home, if they home properly and if they expand properly. So there are techniques to distinguish between these different aspects.

IV. CLINICAL EXPERIENCES AND PROSPECT FOR TREATMENT

STATE OF THE ART IN DENDRITIC CELL VACCINATION

J. de Vries, G.J. Adema, C.J. A. Punt, C.G. Figdor[1]

Dendritic cells (DC) constitute a family of antigen-presenting cells defined by their morphology and their unique capacity to initiate a primary immune response [1]. It has been shown that DC pulsed with MHC class I peptides are potent inducers of a cytotoxic T lymphocyte (CTL) response *in vitro* [2]. Furthermore, the presence of DC in tumor tissue has been correlated with a favorable clinical prognosis. Therefore DC are thought to play a pivotal role in the induction of T cell-mediated anti-tumor responses *in vivo* [3-5]. We, and several other laboratories in the world have initiated clinical studies in which DC vaccines are used to treat cancer.

The ability to grow large numbers of DC from monocytes, the availability of class I-restricted peptides derived from tumor-associated antigens, such as gp100, tyrosinase, MAGEs, and NY-ESO-1, and the experience of CTL induction *in vitro*, led to the use of peptide-pulsed DC in anti-tumor vaccination trials [2,6-8]. Recent studies have demonstrated the safety and immunogenicity of DC vaccinations in humans [9-11]. It is becoming clear that the type of DC and the route of administration and subsequent migration may play a critical role in determining the quality and quantity of the immune response [12]. Therefore, DC vaccination strategies need further investigation to optimize clinical efficacy.

We have compared the efficacy of immature and mature DC in inducing an immune response in advanced stage IV melanoma patients (Figure 1, vaccination protocol). Melanoma is well suited to explore vaccine strategies because it is one of the more immunogenic tumors in which melanoma-associated antigens, like gp100 and tyrosinase, and specific T cell responses towards these antigens have been identified [1,13] (Figure 2). DC were pulsed with HLA-A2.1-binding peptides derived from gp100 and tyrosinase, and keyhole limpet hemocyanin (KLH) [9] (figure 1). The latter was used to provide T cell help and to verify the immunogenicity of the two DC populations as well as competence of the patients' immune system to mount an immune response.

All patients vaccinated with mature DC, showed a pronounced proliferative T cell and humoral response against KLH (Figure 3). In these patients, the presence of IgG2 antibodies against KLH and the absence of IgG4, indicate that DC

1. Dept. of Tumor Immunology and Medicine, University Medical Center Nijmegen, Nijmegen, NL.

Vaccination protocol Trial; Mature/ Immature DC

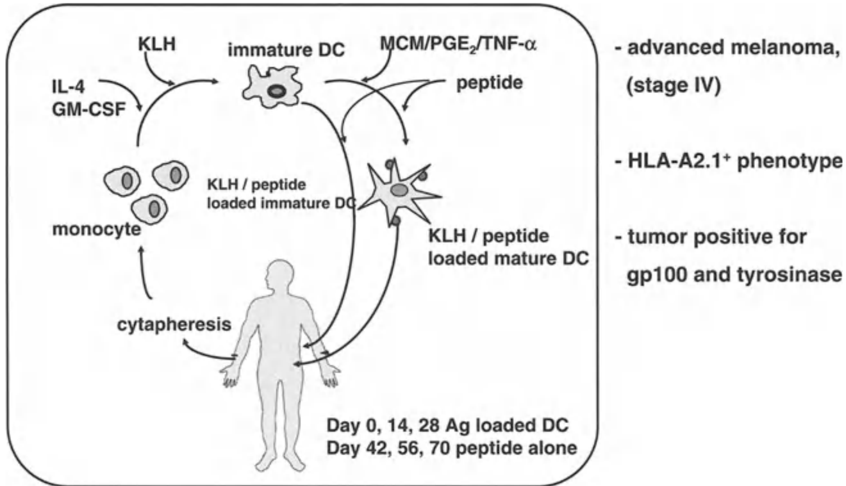
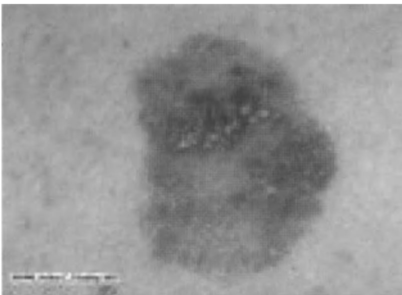


Figure1. Treatment of Melanoma patients (HLA A2.1+) with monocyte derived peptide loaded DC. Immature or mature DC (maturation by cytokine cocktail) are loaded with melanoma associated peptides and KLH. Cells are injected intradermally and intravenously.



Malignant melanoma

*** tumor originating from melanocytes, pigment producing cells**
*** aggressive tumor, high metastatic potential**
*** one of the most immunogenic tumors in man**
- **spontaneous tumor regression does occur**
- **mAbs reactive with melanoma cells can be detected in serum of melanoma patients**
- **CTL reactive with melanoma cells can be obtained**

Figure 2. Melanoma lesion. Melanoma is a very aggressive skin tumor. Several reports indicate that it is one of the most immunogenic tumors.

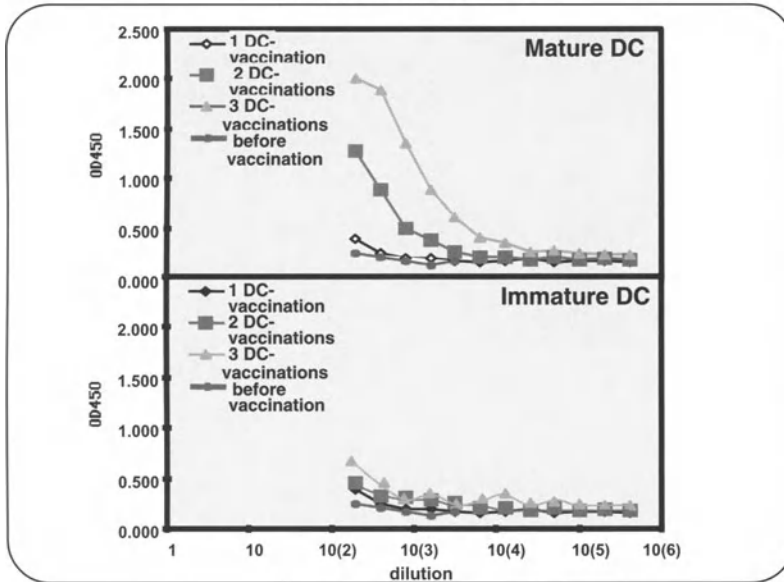


Figure 3. Patients injected with mature DC but not with immature DC develop an antibody response against KLH as measured by Elisa.

induced T helper 1 responses. This was confirmed by the observation that KLH-stimulated PBMC derived from patients vaccinated with mature DC produced IFN-gamma. By contrast, in most of the patients vaccinated with immature DC no strong KLH responses could be detected.

The notion that mature DC are required for effective vaccination was further substained by the finding that DTH reactions against antigen-loaded DC were only observed in patients vaccinated with mature DC and not in patients vaccinated with immature DC. In some patients, MHC-peptide tetramer staining of delayed type hypersensitivity (DTH)-derived T cells revealed the presence of specific T cells recognizing the melanoma-associated peptides (Figure 4). The clinical outcome was somewhat improved for patients vaccinated with mature DC, although the number of patients in both groups is still too small to draw definitive conclusions. Patients vaccinated with immature DC had a median time to progression of 3.5 months whereas patients vaccinated with mature DC showed a median time to progression of 6.5 months. Interestingly, antigen-reactive T cells infiltrating the skin after a DTH reaction with peptide-loaded DC were only observed in patients with clinical responses underscoring the hypothesis that monitoring via DTH-infiltrated lymphocytes is a useful tool. It becomes clear that mature DC are superior to immature DC in the induction of immunological responses in melanoma patients, which may translate into improved clinical results.

Cytotoxic lymphocytes isolated from DTH

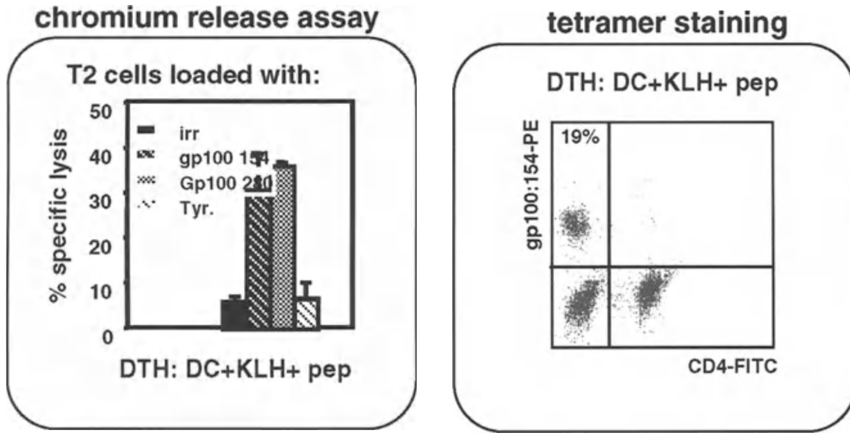


Figure 4. Lymphocytes isolated from a DTH of some patients that were vaccinated are cytotoxic as measured by a chromium release assay. Only target cells (T2) loaded with gp100 peptides are lysed, indicating that this patient developed activity against the gp100 protein. Similarly, 19% of the T cells isolated from the DTH of this patient bound gp100/ MHC tetramer complexes as measured by flowcytometry.

Biodistribution of ^{111}Indium-labeled mature DC

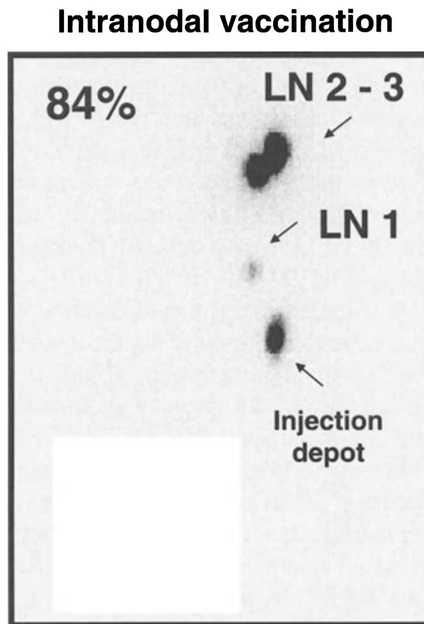


Figure 5. An example of a patient injected with radio-labelled mature DC in the lymph node. A significant amount of the DC migrates to adjacent lymph nodes.

In another clinical study, we investigated the absence of T- and B cell responses to KLH after vaccination with KLH-pulsed immature DC. The incapacity of immature DC to induce an immune response is at least in part due to a low expression of antigen presenting- and co-stimulatory- molecules. In addition, monocyte-derived immature DC lack the chemokine receptor CCR7, required for migration into the T cell areas of lymph nodes [14,15] Hence, immature DC generated *in vitro* may not co-localize and interact with naïve T cells in the lymph nodes, a prerequisite for the induction of an effective immune response. Here we compare the migratory capacity of immature and mature DC after radiolabeling with Indium. We investigated not only the effects of the maturation state, but also the route of administration on DC migration *in vivo* [16].

DC injected into patients must actively migrate into the T cell areas of lymph nodes. We unequivocally demonstrate that mature DC are migratory *in vivo*, irrespective whether they are administered into the skin or intra-nodular. By contrast, *in vitro*-generated immature DC are unable to leave the skin after intraermal injection. Moreover, when migrating to distinct lymph nodes after direct lymph node application, immature DC do not invade the T cell areas, which precludes effective interactions with naïve T cells.

Intra-nodular application of immature or mature DC leads to a substantial migration to several distant lymph nodes, already one hour after vaccination. Although only the mature DC reach the T cell areas (Figure 5), after intranodular injection of immature- and mature DC little or no difference is observed between the migration to lymph nodes. Intra-nodular injection results in a rather variable migration in both cell populations. Figure 6 shows an example of mature

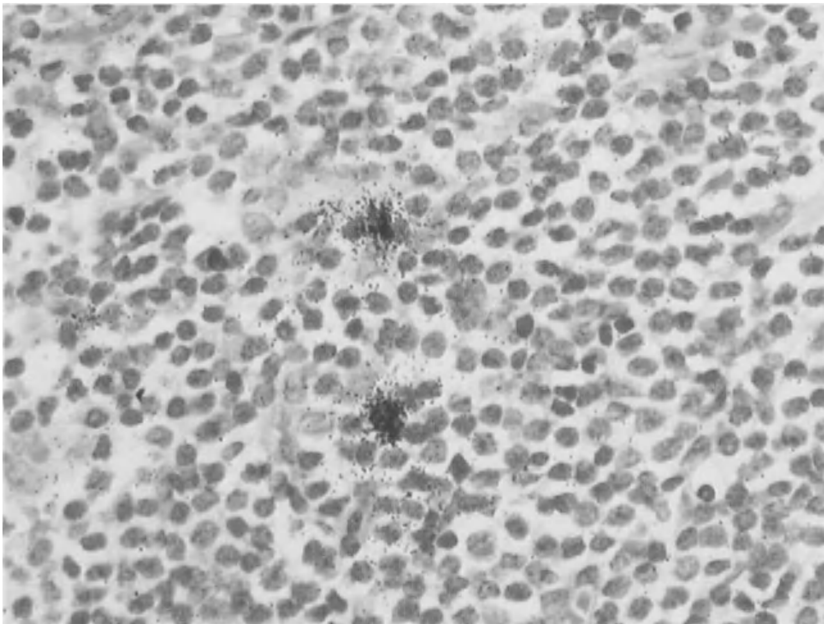


Figure 6. An example of a radio-labelled DC (black) that has migrated into the T cell area of a lymph node.

DC injected intra-nodular. Cells injected in the node reach adjacent lymph nodes. Whether this is due to active migration or to passive transport with the lymph flow is currently under investigation. This might be due to the fact that injection of DC directly into a lymph node leads to a partial destruction of the lymph node architecture, resulting in migration of DC to distant lymph nodes, which would otherwise reside in the injected node. Likewise, during intra-nodular administration a significant proportion of DC may be transported by the flow of lymphatic vessels to nearby lymph nodes. A major advantage of intranodular over intra-dermal vaccination is therefore that an increased number of DC are getting to the lymph nodes, e.g. the site where the initiation of the immune response occurs. Especially when peptide-loaded DC are used as a vaccine, the time required to reach the T cell areas of the lymph nodes is of great importance, since the half life of DC and of a MHC-peptide complex they express is limited [17]. In the first clinical study in melanoma patients, reporting the efficacy of peptide-loaded DC vaccines, mature DC were injected intranodular. Our results may provide a mechanistic explanation for the effectiveness of the protocol used in that study [10].

Conclusion

Given the previously described tolerising capacity of immature DC, the recent results from mature DC vaccination studies, and the migration data presented in this study, we conclude that mature DC are preferred over immature DC in clinical vaccination studies in cancer patients. These results of early clinical trials show that DC based vaccines can be effective in the treatment of cancer. Further investigations are required to optimise this treatment modality, since a large number of parameters (antigen loading and dose, DC subsets, DC activation, route of vaccination) still has to be determined.

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RECONSTITUTING T CELL IMMUNITY FOLLOWING HEMATOPOIETIC STEM CELL TRANSPLANTATION

S.R. Solomon, A.J. Barrett[1]

Introduction

Impaired recovery of T cell immune function is a major source of morbidity and mortality following allogeneic haematopoietic stem cell transplantation (HSCT). Although natural immunity (NK cells, monocytes, neutrophils) function normally within weeks after transplant, helper T cell activity and T cell-dependent B cell responses remain impaired for months to years. The etiology of the immune defect is multifactorial. Attempts to reduce graft-versus-host disease (GvHD) through T cell depletion or immunosuppressive drug therapy significantly impair immune reconstitution. GvHD itself has a negative impact on immune function due to both decreased thymopoiesis [1,2] and increased apoptosis of peripheral blood T cells [3,4]. Furthermore, tissue damage from total body irradiation (TBI) and other intensive conditioning regimens damages important stromal elements of the thymus, as well as the BM, secondary lymphoid organs, and other tissues in which T cell generation and proliferation take place. The composite impact of these factors leads to T cell immunodeficiency following allogeneic HSCT, which can contribute to unacceptably high rates of opportunistic infection and relapse. Improving the results of allogeneic HSCT requires better understanding of the mechanisms involved in post-transplant T cell reconstitution.

Pathways to T Cell Reconstitution

Following bone marrow transplantation (BMT) in mice, T cell recovery depends on two separate mechanisms, one involves thymic-dependent T cell generation from haematopoietic progenitors and can be considered a recapitulation of ontogeny; the other involves repopulation by peripheral expansion of grafted mature T cells. The relative importance of these two pathways is dynamic and dependent on the degree of thymic function – the contribution of peripheral expansion is only significant when the function of the thymus is impaired, and its contribution in euthymic mice is negligible (Figure 1). In humans, the same correlation seems to exist between insufficient thymic function and the prevalence of peripheral expansion as a mechanism to restore the T cell compartment.

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1. Stem Cell Allotransplantation Section, Hematology Branch, NHLBI, National Institutes of Health, Bethesda, MD, USA.

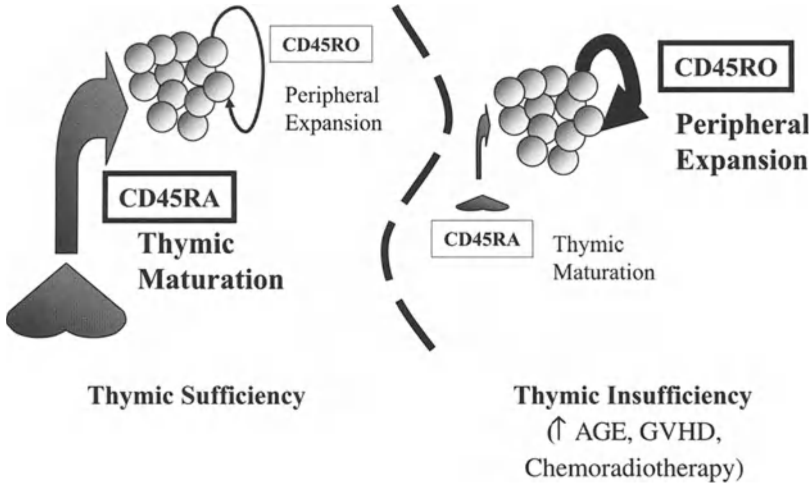

Figure 1. Pathways to T cell regeneration post HSCT. The generation of the T lymphocyte compartment following allogeneic HSCT follows two distinct pathways: expansion of post-thymic memory cells from the transplant inoculum and later thymus-dependent regeneration of naïve cells derived from donor-derived hematopoietic progenitor cells. The relative contributions of each depend on the degree of thymic sufficiency – age-related involution, radiation, cytotoxic drugs, and GvHD all negatively impact the thymic-dependent pathway of T cell regeneration.

Disease, therapy-related toxicity, and age-related changes all negatively impact the thymic-dependent contribution to T cell reconstitution post-transplant.

Thymic-dependent T cell neogenesis

Although the thymus is the primary site of T cell generation during fetal and early post-natal life, its role in reconstituting the adult immune system after HSCT has, until recently, remained controversial. Studies suggest that the thymus-dependent pathway is age-related [5,6] and becomes drastically reduced in young adulthood. It is known that the T cell neogenesis decreases with advancing age-related involution of the thymus and with thymic damage from chemoradiotherapy and GvHD. Direct damage to thymic epithelial cells may lead to loss of thymic IL-7 production bringing a decreased ability to support post-HSCT thymopoiesis, as has been shown in mice [7].

Until recently, studies of post-transplant T cell reconstitution have been hampered by the lack of a good assay to study thymopoiesis. Although the phenotypic marker, CD45RA, has been utilized to quantitate naïve T cells, there are several limitations to such an approach. CD45RA+ naïve T cells can have a long quiescent life span [8] or may rapidly convert to CD45RO+ phenotype memory/effector phenotype T cell upon antigen stimulation, and thus may not reflect recent thymic output. Furthermore, memory T cells may acquire CD45RA expression or have an overlapping phenotype, especially among CD8+ cells [9].

As progenitor cells undergo T cell receptor (TCR) rearrangement in the thymus, chromosomal sequences are excised to produce episomal DNA byproducts

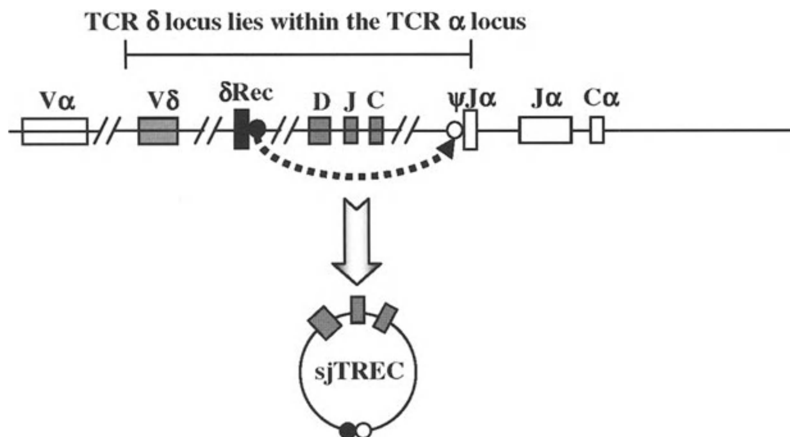

Figure 2. Generation of signal-joint TCR rearrangement excisional circles (TRECs). Simplified representation of the TCRD locus flanked by portions of the TCRA locus (labelled as Vα, Jα and Cα). End-to-end ligation of the recombination signal sequences flanking the δ-rec locus and the ψ-Jα locus removes most of the TCRD gene region, forming a single TREC containing a unique signal joint (sj) sequence.

termed TCR rearrangement excisional circles (TRECs) (Figure 2). As a result, the determination of TREC levels in the peripheral blood becomes an excellent surrogate marker for the measurement of recent thymic emigrants (RTEs). TRECs are only lost by cell death or dilution through division and thus represent the composite production, proliferation, and death of RTEs [10].

Several recent studies have utilized TREC levels to monitor the contribution of thymopoiesis to T cell recovery following allogeneic HSCT. Most have monitored thymic activity in recipients of T cell depleted (TCD) grafts, to avoid the confounding problem of transfusing TREC+ naïve T cells with the allograft. These studies have consistently shown low TREC levels at 3 months following myeloablative conditioning, with T cell neogenesis occurring 6-12 months, and age-appropriate levels reached by the second year following transplant [1, 6, 11]. This increased thymic output has been associated with an overall improvement in T cell repertoire diversity, as measured by the CDR3 size spectratyping assay [12], and the appearance of phenotypically naïve T cells in the peripheral blood [2,6].

Peripheral expansion of post-thymic "Memory" T Cells

In the absence of active thymopoiesis early post-transplant, the T cell pool is reconstituted by peripheral expansion of mature "post-thymic" T cells infused from the allograft. Termed peripheral homeostatic expansion, this highly regulated mechanism of T cell repopulation occurs regardless of the size of the initial T cell inoculum, resulting in rapid reconstitution of the T lymphocyte compartment in both mice [13] and humans [14]. In our institution, a comparison of immune recovery following TCD and unmanipulated transplants, demonstrated an equivalent number of circulating T cells by 6 weeks post-transplant [29].

This concords with other reports [15] and emphasizes the enormous potential of even a small T cell inoculum to fill an empty peripheral lymphocyte compartment.

Recent data shows that antigen drives peripheral homeostatic expansion, following both TCD and T cell replete BMT [16,17]. When limited numbers of T cells are transplanted, however, interactions with immunodominant antigens (minor histocompatibility antigens, viral proteins, leukemic cells, etc.) can lead to massive oligoclonal expansions of T cell clones, skewing of the T cell repertoire, and limiting T cell diversity [12,18,19]. In other words, the T cell repertoire is limited both by the size and diversity of the starting T cell inoculum and the diversity of antigens present within the host at the time of transplant.

Many of the immune defects seen in adult BMT recipients can be directly attributed to the limitations inherent in peripheral expansion mechanisms. For example, the inversed CD4/CD8 ratio and prolonged abnormalities in CD4+ T cell numbers reflect the more limited ability of CD4+ T cells for homeostatic proliferation [20]. Rapidly proliferating T cells often demonstrate qualitative defects using in vitro assays of lymphocyte function [21] and tend to have an activated phenotype, as well as a propensity for activation-induced cell death [4].

Recent evidence suggests that the cytokine, interleukin-7 (IL-7) can play an important role in peripheral T cell expansion, in addition to its known role in supporting thymopoiesis. IL-7 is a critical regulator for the homeostatic proliferation and survival of naïve T cells [22,23]. In addition, exogenous IL-7 potently enhances T cell regeneration post-transplant in athymic mice by increasing antigen-driven T cell expansion [24]. IL-7 is produced by stromal cells in the thymus, BM, and secondary lymphoid organs. Myeloablative conditioning may damage IL-7 producing stromal cells [7], as well as destroying the microenvironment necessary for peripheral T cell expansion. Dendritic cells are another important source of IL-7, which are of particular interest given their importance in the survival of mature CD4 T lymphocytes in mice [25].

Clinical Results: Effects of T Cell Dose and Impact of the Preparative Regimen

Given that adult transplant recipients reconstitute early T cell immunity almost exclusively from post-thymic T cells delivered with the allograft, it is important to understand the impact of the various components of the transplant regimen (T cell dose, stem cell source, preparative regimen, etc) on immune recovery. It is now clear that the use of mobilized peripheral blood, instead of bone marrow, as the stem cell source has significant effects on T cell reconstitution. Peripheral blood stem cell (PBSC) apheresis products contain approximately 10-fold more T cells than BM allografts, as well as a higher percentage of T cells expressing a naïve phenotype [26]. In comparison to BM, PBSC transplants result in more rapid T cell recovery, including striking improvements in CD4+ T cell recovery [27, 28]. Early reconstitution of naïve T cell subsets has also been demonstrated; this appears to correlate with the number of infused T cells, pointing to the allograft as the source of these cells [28]. Infusion of higher T cell doses may limit

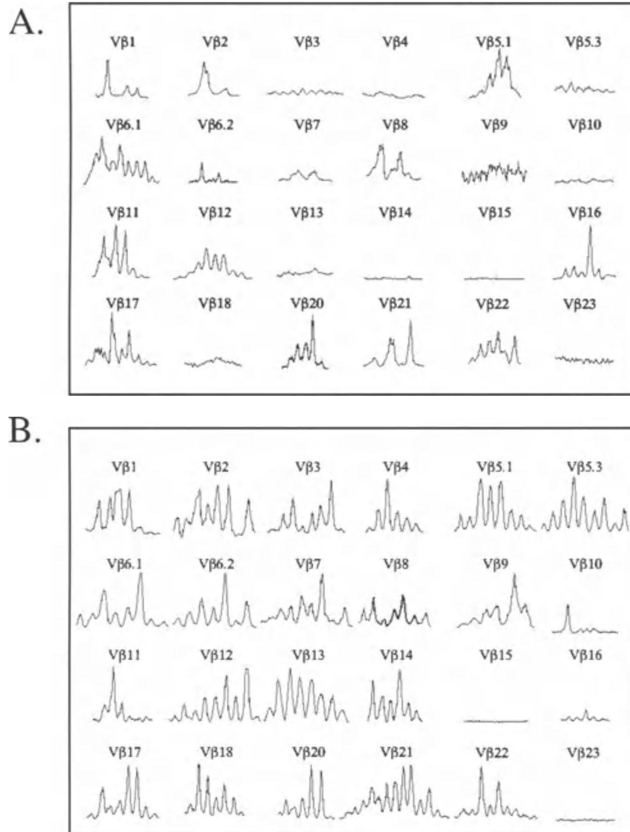

Figure 3. Analysis of T cell diversity post-HSCT. TCR Vβ spectratyping was performed on day 45 post-transplant in patients following (A) myeloablative conditioning and T cell-depleted PBSC infusion and (B) nonmyeloablative conditioning and unmanipulated PBSC infusion. Representative examples are shown above, highlighting the almost complete normalization of the TCR repertoire following nonmyeloablative PBSC transplantation.

their post-transplant expansion, make them less susceptible to antigen-induced cell death, and maintain a more naïve and less restricted T cell repertoire.

Over the last several years, nonmyeloablative preparative regimens, containing purine analogs and an alkylating agent or low-dose irradiation, have been used with success to achieve hematopoietic stem cell engraftment. Avoidance of radiation and reduced intensity conditioning may limit the degree of damage to important stromal elements of the thymus, bone marrow, and peripheral lymphoid organs, thus impacting on T cell reconstitution post-transplant. Studies from our institution [29] and others [30] demonstrate superior and rapid acquisition of T cell diversity following nonmyeloablative stem cell transplant (NST). NST recipients regenerate an almost complete T cell repertoire within the first 3 months post-transplant (Figure 3). This result appears to be independent of the

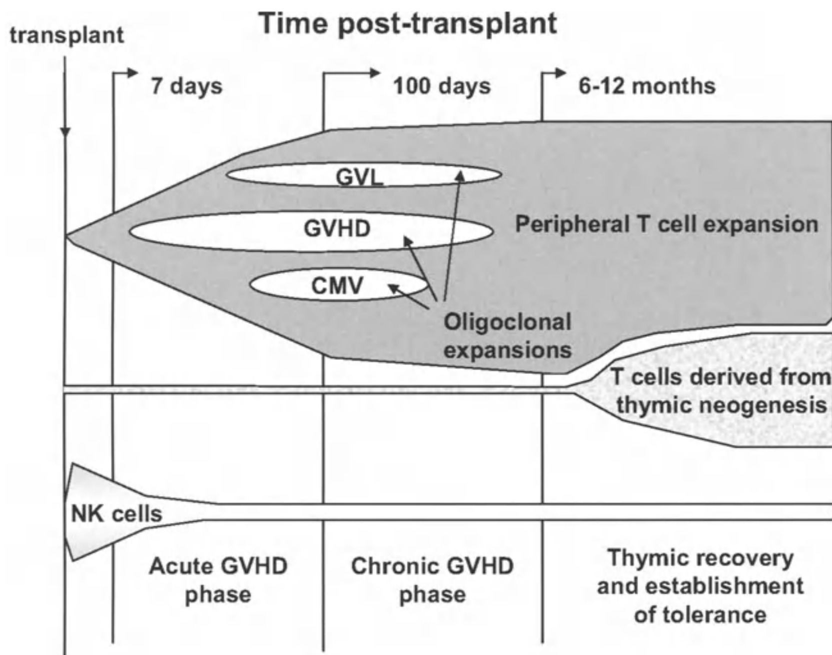

Figure 4. Pattern of immune recovery following allogeneic HSCT. Early T cell recovery following allogeneic HSCT is almost exclusively generated through peripheral expansion of transfused post-thymic T cells. Thymic recovery is much slower and has little impact on the early events post–transplant that determine the success or failure of treatment (engraftment, lethal GvHD, opportunistic infections, and GVL).

stem cell source (BM vs. PBSCs) [30], suggesting that the preparative regimen, rather than T cell dose, was the major reason for this finding.

Conclusions

In adult recipients of HSCT, T cell recovery is predominantly driven by peripheral expansion of post-thymic, memory T cells infused with the allograft. Even in the optimal situation of a pediatric transplant recipient without GvHD, thymic recovery is slow and has little impact on the early events post-transplant that determine the success or failure of treatment (opportunistic infections, lethal GvHD, or a favorable GVL effect) (Figure 4). The homeostatic mechanisms controlling peripheral T cell expansion result in rapid reconstitution of circulating T cell numbers, even in the setting of limiting T cell doses, however this often occurs at the expense of persistent immunological defects (depressed numbers of CD4+ and naïve T cells, restricted T cell diversity, and an increased susceptibility to activation-induced cell death). Newer reduced-intensity conditioning regimens and the use of mobilized peripheral blood have helped to improve the quality of T cell recovery post-transplant. In the future, immune reconstitution may be further improved through the use of T cell growth factors such as IL-7 [31], radioprotectants such as keratinocyte growth factor [32] or

new GvHD prevention strategies, specifically targeting host-reactive T cells, allowing the infusion of large T cell doses in the absence of immunosuppression [33].

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STATE OF THE ART IN GENE THERAPY

H.J. Haisma, M.G. Rots[1]

Where Are We Now in Clinical Application of Gene Therapy

The elucidation of the complete human genome with approximately 30.000 different genes will lead to new possibilities for diagnosis and prevention of a wide variety of diseases. In addition, this knowledge may be used for the design of new therapeutics, based on the DNA sequence information. One of these approaches is gene therapy, where new genes are introduced in a patient to correct a specific disease. For many years, gene transfer techniques have been used in the laboratory setting to introduce altered or foreign genes into cells in order to produce a specific, desired outcome. Since the 1990s, gene therapy has been studied as a possible means of modifying the genetic makeup of patients to treat a specific disease.

In the laboratory, gene transfer is a powerful tool that can be readily adapted to meet the needs of an individual investigator. In the clinical arena, however, gene therapy is more complex and requires consideration of the interplay between the disease and the patient, along with any other conventional treatments being administered.

The results of clinical gene therapy trials began to appear in the 1990s, and today gene therapy is being actively explored as treatment for a wide variety of diseases. The first targets were monogenetic diseases such as haemophilia and certain immunodeficiency diseases. Later, other diseases without an underlying genetic disorder were treated including cardiovascular diseases, cancer and infectious diseases.

Severe Combined Immunodeficiency

In 1990, the first gene therapy clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA- SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remained to be perfected, this first clinical gene therapy study showed that this form of therapy can be a safe and

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1. Dept of Therapeutic Gene Modulation, University Centre for Pharmacy, University of Groningen, Groningen, NL.

Table 1. Clinical trials overview

Disease	Gene	Vector	Reference
SCID	ADA	retrovirus	Blease, 1990
SCID	ADA	retrovirus	Aiuti, 2002
SCID	gamma cytokine receptor	retrovirus	Cavazzana-Calvo
Haemophilia	Factor IX	Adeno-associated virus	Kay, 2000
Haemophilia	Factor VIII	Fibroblasts	Roth, 2001
cystic fibrosis	cystic fibrosis transmembrane conductance regula-tor	Adenovirus	Crystal, 1994 Harvey, 2001
chronic myocardial ischemia	VEGF	DNA	Losordo, 2002
critical limb ischemia	VEGF	DNA	Isner, 1996 Baumgartner, 1998
Glioblastoma	Thymidine kinase, ganciclovir	Retrovirus	Rainov, 2000
Cancer	multidrug resistance-1	Retrovirus	Abonour, 2000
non-small-cell lung cancer	P53	Adenovirus	Swisher, 2000
melanoma			Dummer, 2000
Head & Neck cancer	Onyx-015	Adenovirus	Khuri, 2000

Table 2. Preclinical studies

Disease	Gene	Vector	Reference
Mucopolysaccharido sis	Glucuronidase	retrovirus	Ponder, 2002
Azoospermia	c-kit ligand	lentivirus	Ikawa, 2002
Sickle cell disease	betaA globin	Lentivirus	Pawliuk, 2001
Blindness	RPE65	Adeno-associated virus	Ackland, 2001
muscular dystrophy	dystrophin	Adenovirus	DelloRusso, 2002
Parkinson's disease	tyrosine hydroxylase	Adeno-associated virus	Muramatsu, 2002
Diabetes	single-chain insulin	Adeno-associated virus	Lee, 2000
Ebola	Viral proteins	DNA, Adenovirus	Sullivan, 2000
HIV	Gag protein	DNA, Adenovirus	Shiver, 2002

effective addition to treatment for some patients with this severe immunodeficiency disease [1]. In a recently reported similar study in two patients for whom enzyme replacement therapy was not available, sustained engraftment of engineered hematopoietic stem cells with differentiation into multiple lineages resulted in increased lymphocyte counts, improved immune functions (including antigen-specific responses), and lower toxic metabolites. Both patients are clinically well, with normal growth and development [2].

Another recent clinical trial showed encouraging gene therapy results in patients with x-linked severe combined immunodeficiency disease. Severe combined immunodeficiency-X1 (SCID-X1) is an X-linked inherited disorder characterized by an early block in T and natural killer (NK) lymphocyte differentiation. This block is caused by mutations of the gene encoding the gamma cytokine receptor subunit of interleukin receptors. After a 10-month follow-up period,. T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit [3].

Haemophilia

Another genetic disease targeted for gene therapy is haemophilia. Pre-clinical studies in mice and hemophilic dogs have shown that introduction of an adeno-associated viral (AAV) vector encoding blood coagulation factor IX (FIX) into skeletal muscle results in sustained expression of F.IX at levels sufficient to correct the hemophilic phenotype. A clinical study of intramuscular injection of an AAV vector expressing human F.IX was performed in adults with severe hemophilia B. Assessment in the first three patients of safety and gene transfer and expression show no evidence of germline transmission of vector sequences or formation of inhibitory antibodies against F.IX. In muscle biopsies expression of F.IX was demonstrated by immunohistochemistry. Modest changes in clinical endpoints including circulating levels of F.IX and frequency of FIX protein infusion were observed. This approach seems to offer the possibility of converting severe hemophilia B to a milder form of the disease [4]. Another phase 1 trial was conducted in six patients with severe hemophilia A. Dermal fibroblasts obtained from each patient by skin biopsy were transfected with a plasmid containing sequences of the gene that encodes factor VIII. The cells were then harvested and administered to the patients by laparoscopic injection into the omentum. The patients were followed for 12 months after the implantation of the genetically altered cells. There were no serious adverse events related to the use of factor VIII-producing fibroblasts or the implantation procedure. In four of the six patients, plasma levels of factor VIII activity rose above the levels observed before the procedure and coincided with a decrease in bleeding, a reduction in the use of exogenous factor VIII, or both. This form of gene therapy seems promising for the treatment patients with severe hemophilia A [5].

Cystic Fibrosis

Cystic fibrosis is a recessive hereditary disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene. This results in a thick and sticky mucus of the airways leading to recurrent infections, inflammation and a decrease in lung function. A recombinant adenovirus vector (AdCFTR) containing the normal human CFTR cDNA was administered to the nasal and bronchial epithelium of four individuals with cystic fibrosis (CF) [6]. This vector expressed the CFTR cDNA in the CF respiratory epithelium in vivo with no long term adverse effects. Levels of CTFR thought to be sufficient to protect against development of the respiratory manifestations of the disease were demonstrated. However, expression is transient and cannot be maintained by repeat injections, limiting the application of this approach [7].

Cardiovascular Disease

In addition, to these monogenetic diseases, successful clinical trials have been performed in cardiovascular disease. This comprises angiogenic gene therapy with vectors expressing the human vascular endothelial growth factor. After successful preclinical studies in pigs [8] a phase 1/2 study was initiated to investigated the safety of percutaneous catheter-based gene transfer of naked plasmid DNA encoding for vascular endothelial growth factor 2 (phVEGF2) to left ventricular (LV) myocardium in 19 patients with chronic myocardial ischemia who were not candidates for conventional bypass surgery. Patients were randomized in a double- blind fashion to receive placebo or phVEGF2. End-point analysis at 12 weeks disclosed a statistically significant improvement in angina class in phVEGF2-treated versus placebo-treated patients. This phase 1/2, double-blind, randomized trial provides preliminary data that support safety and efficacy of phVEGF2 catheter-mediated myocardial gene transfer [9]. In another study angiogenic growth factors were investigated for the simulation of the development of collateral arteries. Naked plasmid DNA encoding VEGF was used in patients with critical limb ischemia in a phase 1 clinical trial Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers and/or rest pain due to peripheral arterial disease. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved significantly; newly visible collateral blood vessels were directly documented by contrast angiography in 7 limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in 8 limbs. Ischemic ulcers healed or markedly improved in 4 of 7 limbs, including successful limb salvage in 3 patients recommended for below-knee amputation. Complications were limited to transient lower-extremity edema in 6 patients, consistent with VEGF enhancement of vascular permeability. These findings may be cautiously interpreted to indicate that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia [10,11].

Cancer

Gene therapy studies for the treatment of cancer have been less successful. One approach is suicide gene therapy, in which an enzyme capable of activating a non-toxic pro-drug into an active anti-cancer drug is selectively expressed in cancer cells. Clinical trials have shown the effective transduction of glioblastoma cells with the thymidine kinase gene and subsequent systemic treatment with the prodrug ganciclovir to be feasible and well tolerated. A phase III trial was performed in 248 patients with newly diagnosed, previously untreated glioblastoma multiforme. Patients received, either standard therapy (surgical resection and radiotherapy) or standard therapy plus adjuvant gene therapy during surgery. Progression-free median survival in the gene therapy group was 180 days compared with 183 days in control subjects. These differences were not significant. The feasibility and good biosafety profile of this gene therapy strategy were further supported. The adjuvant treatment improved neither time to tumor progression nor overall survival time probably due to low transduction rates obtained with the retroviral gene transfer system used [12].

Another approach used in cancer gene therapy is the introduction of a drug resistance gene into bone marrow stem cells in order to protect these cells from toxic effects of chemotherapy. Pre-clinical studies indicated that efficient retrovirus-mediated gene transfer into haematopoietic stem cells and progenitor cells can be achieved. In a pilot study, this technique was used to transfer the human multi-drug resistance 1 gene into stem and progenitor cells of patients with germ cell tumors undergoing autologous transplantation. There was efficient gene transfer into these cells and the infusion of these cells back into patients was associated with no harmful effects and led to prompt hematopoietic recovery. Gene marking has persisted more than a year [13].

p53 Mutations are common genetic alterations in human cancer. Gene transfer of a wild-type (wt) p53 gene reverses the loss of normal p53 function. Preclinical studies in animal models demonstrated tumor regression following intratumoral administration of an adenovirus vector containing wild-type p53 complementary DNA (Ad-p53). In a phase I clinical trial, Ad-p53 was administered to 28 patients with non-small-cell lung cancer (NSCLC) whose cancers had progressed on conventional treatments. Patients received up to six, monthly intratumoral injections of Ad-p53. Polymerase chain reaction (PCR) analysis showed the presence of adenovirus vector DNA in 18 (86%) of 21 patients with evaluable post-treatment biopsy specimens; vector-specific p53 messenger RNA was detected by means of reverse transcription-PCR analysis in 12 (46%) of 26 patients. Apoptosis (programmed cell death) was demonstrated by increased terminal deoxynucleotide transferase-mediated biotin uridine triphosphate nick-end labeling (TUNEL) staining in posttreatment biopsy specimens from 11 patients. Vector-related toxicity was minimal in 84 courses of treatment, despite repeated injections (up to six) in 23 patients. Therapeutic activity in 25 evaluable patients included partial responses in two patients (8%) and disease stabilization (range, 2-14 months) in 16 patients (64%); the remaining seven patients (28%) exhibited disease progression. Repeated intratumoral injections of Ad-p53 appear to be well tolerated, result in transgene expression of wild-type p53, and seem to mediate antitumor activity in a subset of patients with

and seem to mediate antitumor activity in a subset of patients with advanced NSCLC [14]. A similar phase I study of single intratumoral (i.t.) injection of a replication-defective adenoviral expression vector containing wt p53 was carried out in patients with metastatic melanoma or breast cancer. A total of six (five melanoma and one breast adenocarcinoma) patients were treated at dose levels dependent upon tumor size/dose escalation sequence. Five of six patients became positive for the transfer of wt p53 into tumor tissue 2 days after injection of the vector. Of the four patients assayed, all developed anti-adenoviral antibodies. Adverse reactions associated with i.t. injection were mild, with no obvious correlation between the incidence, severity, or relationship of the events and drug dose. p53 gene therapy by i.t. injection of a replication-defective adenoviral expression vector is safe, feasible, and biologically effective (with respect to transduction frequency) in patients with either metastatic melanoma or breast cancer [15].

Yet another cancer gene therapy approach is the use of oncolytic viruses, capable of replicating and thus destroying cancer cells. ONYX-015 is an adenovirus with the E1B 55-kDa gene deleted, engineered to selectively replicate in and lyse p53-deficient cancer cells while sparing normal cells. A phase II trial of a combination of intratumoral ONYX-015 injection with cisplatin and 5-fluorouracil in patients with recurrent squamous cell cancer of the head and neck was performed. There were substantial objective responses, including a high proportion of complete responses. By 6 months, none of the responding tumors had progressed, whereas all non-injected tumors treated with chemotherapy alone had progressed. The toxic effects that occurred were acceptable. Tumor biopsies obtained after treatment showed tumor-selective viral replication and necrosis induction [16].

Which approach is the most promising and potential for which group of disorders. The studies discussed above indicate that great progress has been made in several diseases and that gene therapy may be a relevant addition to the treatment of several diseases. Apart from these clinical studies, many preclinical studies have been performed which indicate that gene therapy holds promise for the treatment of some other diseases.

Monogenetic Diseases

Mucopolysaccharidosis is a lysosomal storage disease that results from deficient activity of enzymes that degrade glycosaminoglycans. Deficiency in beta-glucuronidase results in growth retardation, mobility problems, mental retardation and other abnormalities in patients. The features of MPS dogs resemble those of in humans. They cannot walk or stand after 6 months, and progressively develop eye and heart disease. Dogs with mucopolysaccharidosis VII were injected intravenously at 2-3 days of age with a retroviral vector expressing canine beta-glucuronidase (cGUSB). Transduced hepatocytes expanded clonally during normal liver growth and secreted enzyme. Serum GUSB activity was stable for several months. Treated animals could run at all times of evaluation for 6-17 months because of improvements in bone and joint abnormalities, and had little or no corneal clouding and no mitral valve thickening. This is the first success-

ful application of gene therapy in preventing the clinical manifestations of a lysosomal storage disease in a large animal [17].

Azoospermia and oligozoospermia is thought to be of primarily genetic origin, caused by disruption of spermatogenesis. Sl/Sl(d) mutant mice offer a model system in which lack of transmembrane type c-kit ligand (KL2) expression on the somatic Sertoli cell surface results in disruption of spermatogenesis. The ability of different gene transfer vectors to transduce Sertoli cells was investigated and it was found that transduction with either adeno- or lentiviral vectors led to reporter gene expression for more than 2 mo after testicular tubule injection. Because adenoviral vectors showed toxicity, lentiviral vectors were used to express the c-kit ligand in Sl/Sl(d) Sertoli cells. Restoration of spermatogenesis was observed in all recipient testes. Furthermore, the sperm collected from recipient testes were able to generate normal pups after intracytoplasmic sperm injection. None of the offspring carried the transgene, suggesting the inability of lentiviral vectors to infect spermatogenic cells in vivo. This study shows that lentiviral vectors can be used for gene therapy of male infertility in animal models without the risk of germ-line transmission [18].

Sickle cell disease (SCD) is caused by a single point mutation in the human betaA globin gene that results in the formation of an abnormal hemoglobin [HbS (alpha2betaS2)]. A betaA globin gene variant was designed that prevents HbS polymerization and introduced it into a lentiviral vector which was optimized for transfer to hematopoietic stem cells and gene expression in the adult red blood cell lineage. Long-term expression (up to 10 months) was achieved, in all transplanted mice with erythroid-specific accumulation of the antisickling protein in up to 52% of total hemoglobin and 99% of circulating red blood cells. In two mouse SCD models, inhibition of red blood cell dehydration and sickling was achieved with correction of hematological parameters, splenomegaly, and prevention of the characteristic urine concentration defect [19].

Leber congenital amaurosis (LCA) causes near total blindness in infancy and can result from mutations in the RPE65 gene. A naturally occurring animal model, the RPE65-/- dog, suffers from early and severe visual impairment similar to that seen in human LCA. A recombinant adeno-associated virus (AAV) carrying wild-type RPE65 (AAV-RPE65) was used to test the efficacy of gene therapy in this model. After treatment, visual function was restored in this large animal model of childhood blindness [20].

Duchenne muscular dystrophy is a lethal X-linked recessive disorder caused by mutations in the dystrophin gene. Delivery of functionally effective levels of dystrophin to immunocompetent, adult dystrophin-deficient mice has been challenging because of the size of the gene, immune responses against viral vectors, and inefficient gene transfer in mature muscle. Gutted adenoviral vectors carrying full-length, muscle- specific, dystrophin expression cassettes were able to efficiently transduce muscles of 1-yr-old dystrophin-deficient mice. Single i.m. injections of viral vector restored dystrophin production to 25-30% of mouse limb muscle 1 mo after injection. Furthermore, functional tests of virally transduced muscles revealed almost 40% correction of their high susceptibility to contraction-induced injury. These results show that functional abnormalities of dystrophic muscle can be corrected by delivery of full- length dystrophin to

adult, immunocompetent mice, raising the prospects for gene therapy of muscular dystrophies [21].

Parkinson's disease is caused by a severe decrease in the dopamine content of the striatum secondary to a progressive loss of nigrostriatal dopamine neurons resulting in characteristic motor symptoms including tremor and muscular rigidity. One potential strategy for gene therapy of Parkinson's disease (PD) is the local production of dopamine (DA) in the striatum induced by restoring DA-synthesizing enzymes. In addition to tyrosine hydroxylase (TH) and aromatic-L-amino-acid decarboxylase (AADC), GTP cyclohydrolase I (GCH) is necessary for efficient DA production. Mixtures of three separate Adeno Associated Virus vectors expressing TH, AADC, and GCH, respectively, were stereotaxically injected into the unilateral putamen of the brain in a monkey model of Parkinson's disease. Coexpression of the enzymes in the unilateral putamen resulted in remarkable improvement in manual dexterity on the contralateral to the AAV-TH/-AADC/-GCH-injected side. Behavioral recovery persisted for months. These results show that AAV vectors efficiently introduce DA-synthesizing enzyme genes into the striatum of primates with restoration of motor functions. This triple transduction method may offer a potential therapeutic strategy for PD [22].

The development of type 1 diabetes results from the almost total destruction of insulin- producing pancreatic beta cells by autoimmune responses specific to beta cells. A cure for diabetes has long been sought using several different approaches, including islet transplantation, regeneration of beta cells and insulin gene therapy. However, permanent remission of type 1 diabetes has not yet been satisfactorily achieved. A recombinant adeno- associated virus (rAAV) that expresses a single-chain insulin analogue was constructed, which possesses biologically active insulin activity without enzymatic conversion, under the control of hepatocyte-specific L-type pyruvate kinase (LPK) promoter, which regulates insulin expression in response to blood glucose levels. The insulin produced from the rAAV gene construct caused remission of diabetes in diabetic rats and autoimmune diabetic mice for a prolonged time without any apparent side effects. This new insulin analogue gene therapy may have potential therapeutic value for the cure of autoimmune diabetes in humans [23].

Vaccines

Vaccines are produced from attenuated pathogens or proteins derived from these pathogens. Gene therapy may be used as an alternative to introduce the genes encoding for these proteins.

The Ebola virus causes outbreaks of haemorrhagic fever which are associated with high mortality rates. Its rapid progression allows little opportunity to develop natural immunity, and there is currently no effective anti-viral therapy. Therefore, vaccination offers a promising intervention to prevent infection and limit spread. A combination of DNA immunization and boosting with adenoviral vectors that encode viral proteins generated cellular and humoral immunity in non-human primates. Challenge with a lethal dose of Ebola virus resulted in uniform infection in controls, who progressed to a moribund state and death in

less than one week. In contrast, all vaccinated animals were asymptomatic for more than six months, with no detectable virus after the initial challenge. These findings demonstrated that it is possible to develop a genetic preventive vaccine against Ebola virus infection in primates [24].

Similar studies were performed to prepare a preventive vaccine against HIV. Studies of human immunodeficiency virus type 1 (HIV-1) infection in humans and of simian immunodeficiency virus (SIV) in rhesus monkeys have shown that resolution of the acute viral infection and control of the subsequent persistent infection are mediated by the antiviral cellular immune response. To elicit such immune responses in monkeys, a replication-incompetent Ad5 vector expressing the SIV gag protein was used either alone or as a booster inoculation after priming with a DNA vector. After challenge with a pathogenic HIV-SIV hybrid virus, the animals immunized with Ad5 vector exhibited the most pronounced attenuation of the virus infection. Thus, the replication-defective adenovirus seems a promising vaccine vector for development of an HIV-1 vaccine [25].

Cancer

Diphtheria toxin (DT) is a potent inhibitor of protein synthesis. As little as a single molecule of DT can result in cell-cycle independent cell death. This profound potency has led to difficulties in the development of DT as a suicide gene in cancer gene therapy, because toxicity appears to be related primarily to the fidelity of basal gene expression and the yield of viral titer. The feasibility of prostate-specific DT gene therapy was evaluated by cloning the catalytic domain (A chain) of DT under the control of the prostate-specific antigen (PSA) promoter, in an Adenoviral vector. In vitro studies showed that the viral constructs preferentially killed PSA-positive prostate cancer cells. In vivo studies showed that the nu/nu mice with PSA-positive cancer cell LNCaP xenograft treated with wild-type DT-A virus had a rapid regression of tumors and survived over a year without tumor progression. The same constructs had no significant effect on the non-PSA-secreting cell line DU-145. These encouraging results suggest that DT-A viral gene transfer may ultimately have a therapeutic role in the treatment of advanced human prostate cancer [26].

New Techniques

One of the difficulties in advancing gene therapy technology from the laboratory to the clinic is that the perfect vector system has yet to be created. In clinical gene therapy, the ideal vector would be administered through a noninvasive route, transducing only the desired cells within the target tissue. This vector would then allow for expression of therapeutic amounts of the transgene product with desired regulation for a defined length of time. By definition, gene therapy should be able to replace, augment, or block gene expression toward a specific therapeutic goal.

Regulation of gene expression was investigated in an experimental model relevant to gene therapy. Mouse primary myogenic cells were engineered for doxycycline-inducible and skeletal muscle- specific expression of the mouse

erythropoietin (Epo) cDNA by using two retrovirus vectors. Gene expression increased 200-fold in response to both myogenic cell differentiation and doxycycline stimulation. After transplantation of transduced cells into mouse skeletal muscles, Epo secretion could be iteratively switched on and off over a five-month period, depending on the presence or the absence of doxycycline in the drinking water. These studies show that tetracycline regulation provides a suitable control system for adjusting the delivery of therapeutic proteins from engineered tissues in animal models over long periods of time [24,27].

Improving Gene Transfer Vectors

The utility of current gene therapy vectors is limited by 1) their lack of specificity which results in an adverse balance of gene transfer of non-target cells and 2) lack of efficient penetration of target tissues and limiting the number of target cells transferred. For effective therapy it is therefore necessary to use an amplification mechanism which targets not only the transduced cells itself, but the neighbouring cells as well.

Liposomes and other nonviral delivery systems are also under investigation for use in gene therapy. At present, the efficacy of these systems is limited largely by poor transduction efficiencies. Though the opportunity for systemic delivery with predictable pharmacodynamics is attractive, long-term gene expression is not possible, and the need for repeated delivery greatly increases the costs associated with manufacture of the vehicle and the therapeutic transgene. Liposomal formulations are now common drug delivery systems for conventional drugs in medicine, and it is hoped that lessons learned from the use of liposomes in other diseases may advance liposome use for gene therapy.

Retroviral vectors are used if the integration of transgenetic material into the cellular DNA is required. Retroviruses infect only replicating cells and have relatively low transduction efficiency (measured as the percentage of cells expressing the transgene). For clinical application, retroviruses are best suited for diseases that require lifetime production of a specific gene that has been lost because of a hereditary disorder. An excellent example of this approach was recently described, which demonstrated that ex vivo gene therapy with a retroviral vector containing the gene encoding for the common g chain corrected the immune deficiency of patients with X-linked severe combined immunodeficiency. Follow-up observation showed that sustained production of the transgenic protein lasted up to 30 months [3]. Unfortunately, these studies have recently been suspended because one of the boys treated has developed a leukemia-like side effect. It's unclear if the gene therapy actually caused the boy's new illness and, if so, how often such a side effect would occur. In cancer trials retroviral vectors have been quit unsuccessful. A phase III clinical trial of retroviral delivery of the herpes simplex virus thymidine kinase (HSV-tk) gene to patients with glioblastoma showed no marked benefit [12]. Enzyme-prodrug combination strategies such as HSV-tk plus the prodrug ganciclovir have been commonly used in suicide gene therapy approaches. This same enzyme-prodrug combination has been used with adenovirus vectors with more promising results in patients with malignant brain tumors [28] and, prostate cancer [29].

What New Directions Are To Be Chosen to Realize the Full Potential of Gene Therapy?

Recombinant adenoviral vectors are currently the most promising reagents for gene transfer because of their superior in vivo gene transfer efficiency on a wide spectrum of both dividing and non-dividing cell types. They also have low pathogenicity in humans, can accommodate relatively large segments of foreign DNA and its genome is easy to manipulate. Adenoviral infection is initiated by binding of the C-terminal part of the fiber protein, termed knob, to the primary cellular receptor (the coxsackie B virus and adenovirus receptor (CAR)) [30]. After binding, entry of the virus occurs via interaction of the viral penton base with cellular integrins [31]. CAR and integrins are expressed on a broad spectrum of cell types, representing an important limitation for the use of adenoviruses in therapeutic applications where specific gene transfer is required. Selective targeting of adenoviral vectors should be exploited to achieve transgene expression in target cells only. This would allow systemic administration, while immune responses against the vector will be reduced since uptake into antigen-presenting cells will be less. Toxicity may even be further decreased as the dose of virus can be reduced since infection efficiency is increased, Targeting can be achieved at the level of cell recognition (transductional targeting), at the level of trangene expression (transcriptional targeting) or at the level of the transgene product.

Transductional targeting

Many cell type specific cell surface proteins have been defined by (monoclonal) antibodies which subsequently can be emploied in adenoviral retargeting strategies. We succesfully demonstrated tumor targeting by complexing the adenovirus to neutralizing anti-fiber antibodies conjugated to antibodies against the Epithelial Cell Adhesion Molecule (Egp-2) [32,33]. The Egp-2 antigen was chosen as the target because this antigen is highly expressed on a variety of adenocarcinomas whereas Egp-2 expression is limited in normal tissues. Gene transfer was dramatically reduced in Egp-2 negative cell lines, thus showing the specificity of the Egp-2-targeted adenovirus. The bispecific antibody conjugate could also successfully mediate gene transfer to primary human colon cancer cells, whereas it almost completely abolished infection of liver cells.

To allow the production of bispecific molecules in a single step, constructs can be made encoding neutralizing anti-adenovirus fiber single-chain Fv (scFv) Abs fused to scFv Abs directed against tumor antigens. Such approach employing a bispecific scFv directed against the epidermal growth factor receptor (EGFR) markedly enhanced the infection efficiency of adenoviral vectors in EGFR-expressing cell lines, such as gliomas [34], meningioma [35], osteosarcomas [36] and bladder cancer [37]. Combining targeting of adenoviruses to integrins and EGFRs further increased gene transfer into primary glioma cells and spheroids in an additive manner (3-56 times), while gene transfer into normal brain explants was reduced dramatically [38]. Targeting to integrins was performed by insertion of an integrin-binding sequence, RGD-4C, in the HI-loop of the Ad [39]. Targeted doubly-ablated adenoviral vectors, lacking cox-

sackie-adenovirus receptor and alpha v beta 3 integrines binding capacities [40], complexed with bispecific single-chain antibodies targeted towards human EGFR or Egp-2 [41] also showed very efficient and specific gene transfer. On primary glioma cell cultures and normal brain explants derived from the same patient, EGFR-targeted doubly-ablated adenoviral vectors had a 5- to 38-fold improved tumor over normal brain targeting index compared to native vectors. From these studies, we conclude that adenovirus may be retargeted to alternate receptors on (cancer) cells and that cell entry can take place independent from CAR receptor and penton base RGD interactions.

Transcriptional targeting

Target cell specific gene expression has been accomplished through restriction of gene expression by tumor or tissue specific promoters (reviewed by Haviv et al [42]. Several groups, including ours [43,44], have reported on reduction of liver toxicity observed for adenoviral vectors encoding for the prodrug-converting enzyme HSV-tk under CMV promoter, by introduction of tissue-specific promoters restricting the expression of HSV-tk to the cells of interest. However, also for tissue-specific suicide gene therapy, the low transduction efficiency of currently available vectors is hampering good therapeutic responses. To augment the initial infection of adenoviruses, replicative systems have been developed in which replication of the virus is restricted by encoding the early gene E1 under the control of tumor specific promoters. These so-called conditionally replicative adenoviruses (CRAds) will replicate in tumor cells only, resulting in oncolysis and the release of viral progeny in the tumor area, The new viruses will subsequently infect and replicate in neighboring tumor cells. This approach could therefore dramatically increase the therapeutic index of cancer gene therapy through both amplification of initial infection and lowering of toxicity.

For melanoma, the tyrosinase promoter has been demonstrated to retain its specificity in adenoviral context [45] and has subsequently been used to restrict E1 expression in a CRAd. Although replication was shown to be specific for melanoma cells at low infectivity units, replication was also demonstrated in non-melanoma cells at a higher dosage [46]. These data indicate that specificity of promoters might get lost either through weak promoter-like activities of upstream sequences in the adenoviral genome or through presence of El-like proteins. We are therefore exploring the possibilities of double controlled CRAds in which not one, but two replication essential genes are controlled by two different tumor specific promoters.

The approach of double controlled CRAds preferentially includes a more general promoter which shows specificity for a broad range of tumors. In this respect, we investigated the use of the human Telomerase Reverse Transcriptase or the Egp-2 promoter. The latter promoter is active in all epithelial cancers and not in normal cells, especially not liver cells [47]. This tumor selectivity was retained in the context of the adenovirus genome with high CMV-like expression levels of the reporter gene in tumor cells, and a 3-log reduction in expression in the liver {SvE manucript in preparation}. Future CRAd research will include the combination of transductional and transcriptional retargeting.

Transgene targeting

Current gene therapy vectors have a limited tissue penetration and therefore gene transfer is limited to the first cell layers. Improved transgene penetration may be accomplished by secretion of the transgene product like HSV-tk, which is a widely used "suicide" gene converting the non-toxic prodrug gancyclovir in toxic metabolites [48]. In theory, all tumor cells have to be transfected with the HSV-tk gene for an adequate treatment with suicide gene therapy. However, animal experiments showed that complete tumor regression can occur when as few as 10% of the implanted tumor cells express the HSV-tk gene [48]. This is thought to be due to transport of toxic metabolites from the transfected cells into neighboring non-transfected cells via the gap junctions [49]. The first clinical results with HSV-tk, however, show that the adjuvant gene therapy treatment improved neither time to tumor progression nor overall survival time presumably due to the poor delivery of the HSV-tk gene to tumor cells [50]. From these first clinical trials it has become evident that further improvements in HSV-tk gene therapy are needed. To this end, we constructed fusion proteins of HSV-tk and the HIV-1 TAT protein transduction domain (TatPTD). TatPTD has the ability to rapidly and indiscriminately migrate across cell membranes and has been shown to mediate the uptake of various proteins [51]. Constructed fusion-proteins including a secretion signal were secreted by the cells and all proteins were functional in activating the prodrug and causing cell-death.

To avoid immune responses, however, it is desirable to use human enzymes for suicide gene therapy. Weyel et al. [52] used a secreted form of the normally lysosomal human beta-glucuronidase to convert inactivated glucuronidated derivatives of doxorubicin to active drugs. Secreted glucuronidase in combination with doxorubicin prodrug efficiently induced tumor cell killing mediated through a pronounced bystander effect of the generated cytotoxic drug. Similarly, we constructed a fully human tumor targeted glucuronidase for specific doxorubicin prodrug activation [53]. The secreted fusion protein was capable of binding to nearby antigen-positive tumor cells in vitro. Specific pro-drug conversion was seen both in the transduced as well as in the targeted tumor cells. When delivered in a gene therapeutic approach, constructs like these may prove superior over intracellularly expressed proteins.

Conclusions

Although some clinical successes have been reported for gene therapy, this exciting new treatment approach has not yet lived up to its promises. Especially in cancer gene therapy, clinical results are disappointing presumably since all tumor cells need to be destroyed. Current gene therapy vectors, however, are not sufficiently efficient nor specific and research should focus on improving the way of transferring DNA into specific target cells. Increasing the specificity and efficiency of gene therapy through incorporation of tissue specific promoters combined with transductional targeting of the vector will increase the therapeutic index of gene therapy. Target cell specific gene expression may be accomplished by specific gene expression driven by a tissue specific promoter. The

combination of transductional and transcriptional targeting with transmitted proteins should lead to an improvement of current gene therapy approaches.

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NEW HORIZONS IN CELLULAR THERAPIES

P. Rebulla, R. Pacchiana, T. Montemurro, R. Giordano, L. Lecchi, L. Porretti, L. Lazzari[1]

Introduction

The term 'cellular therapy' identifies a modality of medical treatment in which drugs are replaced with cells. Examples of classical cellular therapies that have been successfully used for decades include bone marrow or peripheral blood stem cell transplantation and red blood cell or platelet transfusion. Currently, the terms 'cellular therapy' are mostly used to identify rather sophisticated procedures in which well defined cell sub-populations undergo some degree of manipulation or engineering, such as positive and negative cell selection, ex-vivo expansion, gene modification, etc., under strict environmental control ensured in laboratory facilities known as 'cell factories'.

The evolution of conceptually relatively simple therapeutic approaches mainly aimed at cell replacement, such as whole blood transfusion and unprocessed bone marrow transplantation (BMT), into the growing list of cellular therapies currently in use or under advanced development (Table 1), has been mainly prompted by a deeper knowledge of the physiology and pathology of the haematopoietic and immune systems, in particular of T lymphocytes and dendritic cells, and a number of recent observations challenging the classical dogma negating the possibility for cells to transdifferentiate across tissues originated by different embryonic layers.

Table 1. An incomplete list of recently developed or emerging cellular therapies

T-cell depleted bone marrow transplantation
Umbilical cord blood transplantation
Donor leukocyte infusion
Dendritic cell vaccine
Gene therapy
Myocardial repair
Mesenchymal stem cell expansion

1. Cell Factory 'Franco Calori', Milano Cord Blood Bank, Centro Trasfusionale e di Immunologia dei Trapianti, IRCCS Ospedale Maggiore, Milan, Italy.

Table 2. Examples of current research projects which may be relevant for a Cell Factory

Examples of current research projects
Ex-vivo expansion of cord blood
Selection and expansion of dendritic cells
Gene marking of ex-vivo expanded cells
Clinical grade CD34+ cell selection from fresh and cryopreserved samples
Preparation of anti-viral T cell clones
Preparation of anti-leukemic T cell clones
Clinical grade expansion of muscle stem cells
Use of bone marrow cells for myocardial repair
Purification and culture of hepatocytes
Trans-differentiation of CD133+ cells
Trans-differentiation of neural stem cells
Development of rapid assays for the release of clinical grade manipulated cells
Development of animal models suitable to investigate human cell therapies
Cost analysis of cell therapies
Defining the curricula for translational research professionals

Figure 1. Cord blood processing laboratory at the Milano Cord Blood Bank.

Figure 2. The cryogenic area of the Milano Cord Blood Bank.

The former has been exploited to select and expand T cell clones with anti-tumor or anti-viral activity and to develop cancer vaccines based on dendritic cells. The latter have generated a nascent field of interest for physiologists and cell biologists known as cell 'plasticity', which in turn opened new therapeutic perspectives related to the repair of the nervous system, muscle, liver, gut and other tissues, known as 'regenerative medicine'. In view of the desire to ensure a long term positive effect with these therapies, investigators interested in plasticity and regenerative medicine focussed their attention on stem cells or very early progenitor cells, which display longer potential survival and higher clonogenic and proliferative capacity as compared to more mature cell types. Both embryonic and adult stem cells have been used in such investigations, although the clinical use of the former, which seem to display higher transdifferentiative and proliferative potentials, poses a number of ethical problems for human applications. For this reason, many countries have banned or limited the use of human embryonic stem cells and several research groups have concentrated their efforts on adult stem cells. It is now possible that the opposing views expressed in the debate on embryonic versus adult stem cells can be reconciled by novel observations suggesting that heterogeneous stem cell populations from the early embryonic developmental stage of the organism may home into tissues and reside there maintaining their embryonic potential during adult life [1].

In this article we review some recent discoveries in the field of cellular therapy, with particular attention to ex-vivo expansion of haematopoietic progenitor cells (HPC) from cord blood (CB) and to the relation of HPC with regenerative medicine. Our perspective derives from the experience gathered during the

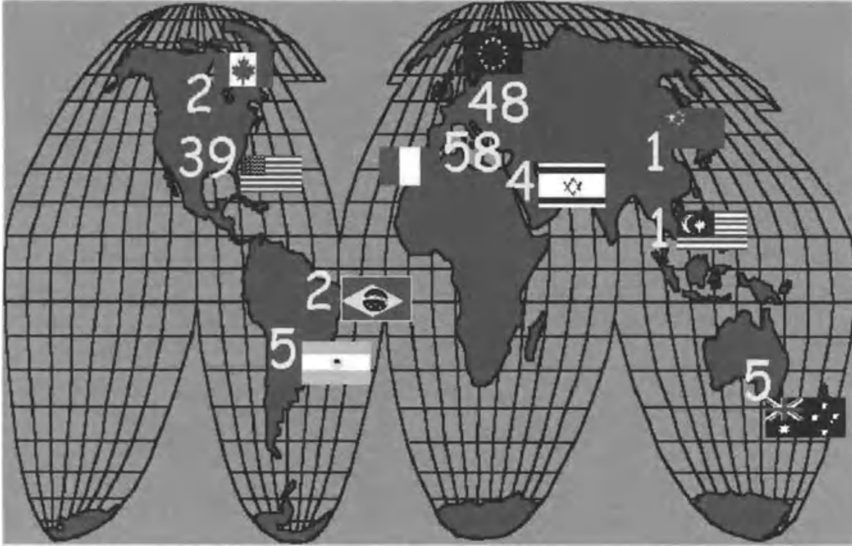

Figure 3. Number and location of cord blood transplants performed with units released by the Milano Cord Blood Bank during 1995 – Sep 2002.

Figure 4. Clinical-grade CD34+ cell purification from cryopreserved/thawed cord blood at the 'Franco Calori' Cell Factory.

10-year activity of the Milano Cord Blood Bank (Fig. 1-3) and of the 'Franco Calori' Cell Factory, a facility for clinical grade cell manipulation which was developed in our institution in year 2000 (Fig. 4).

Ex-vivo Expansion of Hematopoietic Progenitors from Cord Blood

BMT from HLA-matched related and unrelated donors has been successfully used for the treatment of malignant and non-malignant hematological diseases. The major limitation of BMT is the availability of a suitable donor. In particular, the availability of an unrelated donor is limited by the length of time for the donor search and the suitability of the donor at the time requested. For that reason, less than 40% of patients who need a BMT have a donor. Over the last decades CB has been used as an alternative source of HPC for transplantation. Moreover, CB gives some immunological advantages since it contains fewer and more naive T cells as compared to BM. These characteristics allow a wider degree of HLA mismatch and a much powerful prevention of *graft-versus-host disease* (GvHD). Since the first transplant performed by Gluckman and coworkers in 1988 [2], more than 2,300 patients worldwide have been transplanted with CB . About 80% of them are children. In fact, although CB compares favourably to other HPCs sources as regards long term repopulating ability, its use in large patients is limited by the low number of cells available, that affects the time to neutrophil and platelet engraftment. In this regard, data from the EUROCORD Group show that a CB nucleated cell dose greater than 0.37×10^8/kg increased the speed and probability of engraftment [3]. To overcome the limitation of small cell content, ex vivo expansion of CB HPCs has been explored.

Our group developed a system for ex-vivo expansion of HPCs from CB based on the use of a cytokine cocktail including interleukin (IL)-6, IL-11, Flt3-ligand (FL) and thrombopoietin (TPO) in association with a serum-free medium [4]. Initial experiments showed that expansion could be slightly improved using serum, but we chose to use serum-free medium in the subsequent investigations, to apply culture conditions suitable for clinical use. 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 [5]. 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 [6] and Dr. W. Piacibello, Turin.

The final objective of our work on ex-vivo expansion of CB cells is the development of a clinical protocol able to improve neutrophil and platelet engraftment after transplantation. In this regard, we designed a clinical trial in co-

operation with pediatric hemato-oncologists (Dr. Franco Locatelli, University of Pavia and Prof. Enrico Madon, University of Turin, Italy), in which an aliquot of the selected CB unit, corresponding to the recommended cell dose, will be infused unmanipulated while the rest of the unit will be thawed and CD34+ cells will be selected and cultured under clinical grade conditions for 14 days.

As regards the culture conditions capable of sustaining an efficient expansion of CB HPCs, an interesting approach was suggested by McNiece and coworkers [7]. Based on the correlation between rapid neutrophil engraftment and the number of total nucleated cells (and not CD34+ cells) contained in the graft, they obtained an increased expansion of CB cell content by applying a modified, two-step expansion culture system. In their system the cells were seeded in teflon bags, first in a defined volume supplemented with SCF, G-CSF and MGDF and after 7 days they were transferred in larger bags containing a double volume of the medium.

Despite the large number of in-vitro and in-vivo preclinical studies on ex-vivo expansion of CB HPCs, few clinical experiences have been reported. In particular, Shpall and co-workers at the University of Colorado have recently reported the results obtained from 37 patients (25 adults and 12 children) affected by hematological malignancies and breast cancer, transplanted with expanded and non-expanded aliquots of CB [8]. Their reported median times to engraftment of neutrophils and platelets of 28 and 106 days, respectively. Another approach to evaluate the contribution of expanded cells to the engraftment potential was followed by Fernandez and coworkers [9]. In their work, five patients were transplanted with the best available CB units and cells from a second donor simultaneously, CB in two patients and highly purified CD34+ haploidentical peripheral blood cells in the other three patients). Unfortunately, the culture conditions used in this work (6 days-culture in serum free medium with SCF, FLT3, TPO and IL-3) did not result in early neutrophil recovery. Moreover, this work showed just a transient engraftment of expanded CB cells. The available evidence indicates that further studies are required to better understand what cells are responsible for short and long term engraftment and which are the best culture conditions to promote their expansion.

Regenerative Medicine

Growing evidence has been recently reported that stringent environmental control – both in the laboratory and in vivo – may induce stem cells to generate cell lineages belonging to embryonic layers different from their own.

In this regard, Ferrari et al [10] found that lac-Z-marked cells derived from the bone marrow of donor mice could be incorporated into regenerating skeletal muscle of the recipient animals. Moreover, Bittner et al [11] and Gussoni et al [12] showed that murine cells from muscle tissue could reconstitute hematopoiesis in lethally irradiated recipients.

Extensive investigations from Verfaillie et al [13] indicate that mesenchymal stem cells obtained from the bone marrow can be expanded under stringent culture conditions and differentiated into cells of limb-bud mesoderm, osteoblasts, chondrocytes, adipocytes, stroma cells and skeletal myoblasts, as well

as visceral mesoderm and endothelial cells. The cells able to undergo the above differentiations were named Mesodermal Progenitor Cells (MPCs) or Multipotent Adult Progenitor Cells (MAPCs). They were selected by depleting bone marrow mononuclear cells of cells showing known lineage markers and culturing the lineage-negative fraction under essential media conditions.

More recent work by Verfaillie and coworkers [14] indicates that cells from human bone marrow show neural phenotypes after grafting into ischemic rat brains and determine clinical improvements in the treated animals.

Similar examples of 'non-orthodox' differentiation have been documented for hepatocytes [15], neurons, astrocytes [16], oligodendrocytes [17], endothelial cells [18,19], and cardiomyocytes [20-22]. In a number of differentiation studies, cord blood derived cells have been successfully used as the starting material [23,24]. A brief summary of the most recent findings which may be relevant for cardiac and nervous repair is reported below.

Cardiac repair

The injection of cardiomyocytes or skeletal myoblasts [21,22,25,26] into myocardial scar tissue represents a new approach to restore impaired heart function. Orlic et al. injected Lin-/c-kit+ BM cells in the contracting myocardium borders of acute infarct [20]. Their data suggest that tissue damage attracts stem cells from distant areas, which migrate in the damaged area and differentiate into the local phenotype. This group used transgenic mice expressing the EGFP marker, in which a temporary ligation of the coronary artery induced a myocardial infarct. To verify the contractile capability of the new cells, the presence of specific markers was determined: the cells have shown phenotypical characteristics of functional myocardium. Hemodynamic studies have also shown the functionality of the repaired cardiac muscle, though it is not clear if the cells fully maintain their functions after definitive differentiation. These studies have also demonstrated the presence in the adult heart of a subpopulation of cardiac muscle cells, which are able to re-enter the cell cycle after infarction. It is supposed that these precursors derive from cells residing in the myocardium or from circulating stem cells, which reach the damaged site attracted by different cytokines. Further in vitro studies demonstrated that a sufficient number of BM cells mobilized by stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF) can home to the infarcted heart and promote cardiac repair [27].

Besides the myogenic potential of the precursor cells, other in vivo and in vitro studies have supported the hypothesis that the latter contain a subpopulation able to generate endothelial cells [28,29]. Moreover, additional investigations have elucidated the potential role of endothelial cells and growth factors such as Epidermal Growth Factor (EGF) and Vascular Endothelial Growth Factor (VEGF) in myocardial repair [29]. In particular, some evidence has been collected which indicates that endothelial precursors contrast the reduction of angiogenic function associated to high mortality of ischaemic disease of old age in animal models [30].

An important problem is the characterization of cells responsible of cardiac regeneration. In this regard, Toma et al. [31] transplanted mesenchymal cells from human BM in immunodeficient mice and found that these cells were able

to integrate in the myocardial tissue, by generating striate muscular tissue. Findings from Orlic et al suggest that this potentiality is a feature of Lin-/c-kit+ cells [20].

Recently, Jackson et al. have presented preliminary results on the potentiality of a subpopulation named "side population", characterized by the phenotype CD34-/low, c-kit+, Sca-1+. These cells seem to be able to give not only complete hematopoietic reconstitution in a myeloablated animal, but also to migrate to infarct areas of the myocardium [28].

Nervous system -

The adult central nervous system is considered a stable structure in which the genesis, migration and differentiation of neurons end in the perinatal periods. However, there is some evidence that, the mechanisms of neurogenesis remain active in adult life in specific areas of the brain such as the hyppocampus and olfactory bulb. Based on this evidence, cell replacement has been investigated in some neurological diseases. Fetal mesencephalic cells have been transplanted into patients with Parkinson's disease [32] and fetal striatal grafts have been reported in a cohort of patients with Huntington's disease. One study showed the efficient integration of donor cells into the host tissue [33].

However, in spite of preliminary encouraging results, significant constraints hamper the use of fetal cells for neural transplantation and other cell sources have been investigation. Priller et al. [34] transplanted cells from the BM, marked with Green Fluorescent Protein (GFP), into irradiated mice and found that initially these cells localized in the haematopoietic system, but after 12 months it was possible to find GFP-positive Purkinje cells. The latter cells could survive for at last 3 months. Other investigators reported that BM stromal cells can differentiate into astrocytes [16].

The group of Vescovi et al. [35] transplanted a lac-Z marked clone of neuronal stem cells into irradiated mice and found that the neuronal stem cells could reconstitute hematopoiesis in recipient mice. In view of the great interest of these observations, the need of replicating the data by different groups has been stressed [36]. More recently, the same group showed that clonally derived neural stem cells from mice and humans can produce skeletal myotubes in vitro and in vivo. Myogenic conversion in vitro required direct exposure to myoblast. The authors concluded that neural stem cells, which can generate neurons, glia and blood cells, can also produce skeletal muscle cells, and can undergo various patterns of differentiation depending on exposure to appropriate epigenetic signals in mature tissues [37].

Cord blood has been used as a starting material to select a subpopulation of stem cells able to generate cells with neuronal phenotype. In this regard, Buzanska et al. [23] have shown that CD34-/CD45- CB-derived precursor cells, cultured in the presence of defined media and growth factors such as trans-retinoic acid, Epidermal Growth Factor and Brain-Derived Neurotrophic Factor, can produce nestin-expressing clones that are able to differentiate toward neuronal/

Year	1999			2000			2001			2002		
	1-4	5-8	9-12	1-4	5-8	9-12	1-4	5-8	9-12	1-4	5-8	9-12
1. Laboratory studies: ex vivo expansion and gene marking												
1.1 Purification	■	■										
1.2 Culture		■	■	■	■	■						
1.3 Characterization		■	■	■	■	■						
2. Animal studies												
2.1 NOD/SCID: ex vivo expansion					■	■	■					
2.1 NOD/SCID: gene marking									■	■	■	▨
3. Regulatory issues												
3.1 Facilities					■	■	■					
3.2 Staff training							■	■	■			
3.3 Quality control							■	■	■			
3.4 Approval by regulatory body								■				
4. Translation and validation										■	■	▨
5. Transplant												▨

Figure 5. A time chart showing type and duration of phases from idea to transplant of ex-vivo expanded hematopoietic progenitor cells from cord blood.

astrocytic or neuronal/oligodendrocytic phenotypes, thus displaying a bipotentiality. Other investigators cultured CB monocytes with Nerve Growth Factor, trans-retinoic acid and other factors. These conditions induced the cells to express markers of neural lineages such as GFAP, beta-tubulin III, neurogenin [24].

In spite of these encouraging results, debate is still open on the real existence of adult stem cell plasticity [38].

Facilities

Cell therapy products have raised specific issues at the regulatory, ethical and quality control levels, which have been addressed in a number of documents published by the regulatory bodies of various countries [39-42].

For a multi-disciplinary team in charge of designing a phase I/II somatic cell therapy clinical trial, it is mandatory to operate in a dedicated facility, so as to avoid microbial contamination of the product and to protect the operators and the environment. Current guidelines for somatic cell therapy prescribe that all the operations are performed in biosafety level 3 (BL3) laboratories, with controlled access through a series of inter-locked doors and HEPA filtered air. Moreover, specific training of the personnel must be documented and standard operating procedures must be rigorously followed.

Another fundamental issue concerning the design of 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. In this regard, close interaction with the reagent manufacturers may help to obtain appropriately characterized materials. In addition, to fulfil 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, etc).

Another difficulty is generated by the fact that 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 is 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 share with the laboratory 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.

All the actions aimed at developing a novel, clinical grade cell therapy procedure usually require considerable time and efforts, as shown in the example reported in Figure 5.

Conclusions

Cellular therapies represent an expanding and promising field for the treatment of a number of diseases in which the current therapeutic tools show limited efficacy. Despite the understandable enthusiasm and hope generated in the public and in politicians by the astounding discoveries concerning cellular therapies in general and stem cell plasticity or their developmental heterogeneity in particular, straightforward scientific and organizational efforts need to be developed by the scientific community to transform cellular therapies from the current experimental protocols into standard procedures. Of special interest and value in this regard are stem cells from the bone marrow and umbilical cord blood, because of their large availability, easy procurement and relatively simple processing.

Before the newer cell therapies can become standard procedures, it is necessary not only to resolve a number of pending scientific and ethical issues, but also to develop new professional skills in the area of translational research, the specific branch of cellular therapy in charge of transforming small-scale, laboratory-bench procedures into large scale protocols for human use. Finally, a number of regulatory matters concerning the procurement and banking of stem cells and the certification, licensing and accreditation of clinical grade cell manipulation both at the national and international levels need to be addressed and resolved. All the above will need significant integration and mutual understanding of cell biologists, physicians, engineers, administrators, veterinaries, clinical trial specialists, ethicists and lawyers from both the academic and industrial leading communities.

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CELLULAR ENGINEERING AND CELLULAR THERAPIES – TRANSFUSION MEDICINE AND THE ACADEMIC WORLD

C.Th. Smit Sibinga[1], J.P. Jansen van Galen[1]

Introduction

Cellular engineering and cellular therapies hold promise for the treatment of a variety of both acquired and genetic disorders. An engineered cell or a transgene can be introduced to augment or to restore a cellular function. Such function is of great importance to an increasing variety of disease conditions, both acquired as well as inherited. For the future of clinical medicine it could positively effect the disease burden and help restore quality of life.

Cellular engineering and gene transfer can be accomplished using a variety of methods, either by introducing new genetic material directly in vivo or by modifying or engineering cells first ex vivo followed by transfusion or re-implantation. These approaches require an academic environment for research, development and clinical implementation. The necessary infrastructure should be based on a well-developed quality system and management, fueled by a motivation and preparedness to contribute to the advancement of clinical transfusion medicine as an integral part of clinical medicine.

Currently virtually all tissues are studied as potential targets for cellular therapies or therapeutic genetic modification, including the bone marrow, lymphocytes, stroma, hepatocytes, skeletal and cardiac muscle, respiratory epithelium, vascular smooth muscle and dopaminic and other cells from the central nervous system. Today in the first place due to their plasticity pluripotent stem cells are in focus as extremely potential cells for manipulation and transplantation. These embryonic stem cells may be derived from the bone marrow or the peripheral blood, whether collected from the umbilical cord or a peripheral vein and have at least in vitro a specific capacity to differentiation into visceral mesoderm, neuroectoderm and endoderm.

Transfusion Medicine

Transfusion medicine is the bridging science involved in both the processing and engineering of harvested cells and the response to potential application of cellular therapies – in practice 'vein to vein' but also academically 'brain to

1. Academic Institute for International Development of Transfusion Medicine, Faculty of Medical Sciences, University of Groningen, WHO Collaborating Center and Sanquin Consulting Services, Groningen, NL.
2. Sanquin Blood Bank Northeast, Groningen, NL.

brain'. For a considerable period of time the focus has been on the basic immunohaematology aspects and the development of fundamental quality principles such as Good Manufacturing Practice or GMP. Additionally, due to the traditional small scale operations linked to hospital laboratories, the opportunities for extended and spear head oriented research and development have been limited and largely depending on hospital pathology rather than transfusion medicine priorities. The procurement part of the 'vein to vein' chain for an equally long period of time has been focused on the production of the conventional products or components such as red cells, platelets and plasma. While largely ignoring the potential of the pluripotent stem cell and the white cell population, technologies for specific cell harvesting have been developed and introduced in the procurement field.

Specifically harvested cells are attractive targets for cellular therapies and gene transfer, because

- cells can be easily obtained and manipulated,
- stem cell transplantation has already been successfully applied for treating a number of genetic disorders.
- experience with lymphoid cells for immune therapies is rapidly increasing.

These experiences have almost exclusively been encountered in academic settings. However, the basic cell harvesting technology for transfusion or transplantation purposes is a specific competence of the procurement facilities, known as blood banks or blood establishments. The extension of the basic technologies to the harvest of specific cell populations such as stem cells and mononucleated white cells has been developed over the past two to three decades and introduced in the procurement institutions. However, related to these harvesting technologies are the methodologies needed for purification, preservation and manipulation to allow the specific cells harvested to be used to the specific purpose or target based on their functional potential.

These methodologies require a complex and well-developed infrastructure - specific selection and collection criteria as well as an environment for the harvesting, processing and preservation of specific cells, the development of specific quality criteria and documentation as captured in the principles of Good Tissue Practice or GTP. To accomplish this a complex of dedicated research facilities needs to be developed and implemented, not only focused on the fundamental aspects of the research, but more importantly on the translational aspects in order to be able to apply at the bedside what has been developed at the bench. As a consequence, the established quality system with its core documentation system and management needs to be extended into integrated laboratory facilities. Stepwise development and arrangement of the physical lay out such as dedicated conditioned and clean room facilities are of paramount importance to allow appropriate resource management.

Leadership and the awareness of an optimal and justified division of authorities, responsibilities and accountability structures are to be defined based on clear and concise project definition.

In practice it means the development and maintenance of knowledge and expertise in specific cell harvesting and processing, including the dedicated pres-

ervation of intermediate and finished cellular products. Also it means the development and maintenance of knowledge and expertise in appropriate and applicable quality systems and quality management. However, the most important element probably is in the development and maintenance of knowledge and expertise in 'bridging' between the disciplines and fields required and involved in cellular engineering and cellular therapies.

The Academic World

Thus the transfusion medicine centres of excellence should no longer be regarded as simple production facilities but should be academically integrated and become bridging centres for translational research. Since cellular therapies including gene transfer entail both cell processing and genetic modification under strict pharmaceutical conditions securing and guaranteeing optimal quality, there is a need for integrated processing and clinical laboratories where these activities can be carried out. Such an infrastructure allows the development and maturation of proper resource and quality management. It is no longer a condition of importance whether the facilities, such as the specific research and development laboratories are physically located in the academic hospital or the blood establishment, important is the integration of developmental and implementation activities that are paramount for translational research.

It is evident that a complex of conditioning requirements need to be fulfilled, including a highly developed infrastructure, cost containment and above all team spirit and leadership. Whether transfusion medicine should play the first or the second violin is not the question, as there is also the viola and the cello in the string quartet equally important. Ultimo is the clinical outcome of a joint effort in an academic setting based on strictly controlled and well governed production processes – *ex pluribus unum.*

DISCUSSION

Stephen J. Noga, Peter L.M. Jansen

P. Law (Seattle, WA, USA): Dr. Figdor, what would be your specific target cell population in relation to efficacy of the dendritic cell. How many cells are you infusing at this point per dose and what is the total cell dose?

C.G. Figdor (Nijmegen, NL): The last question first: how many cells are we infusing? We started with a relatively low dose, because we did not know how much to inject. At this point, we inject per vaccination approximate 30 million dendritic cells. We are now capable of getting at least 500 million DC's from each of these cytapheresis. Than we prepare the cells and freeze them at the mature state and we load them after thawing just before injection.

Regarding the first question, this is a myeloid dendritic cell and the phenotype is really a mature dendritic cell expressing CD83 and high numbers of class I, class II and co-stimulatory molecules.

P. Law: So in the GMP language, what would be your product release specifications?

C.F. Figdor: Our release specification is that they must have high co-stimulatory molecule activity at their cell surface and that they must show a uniform peak of CD83 positive cells.

P. Law: On your immune monitoring, you showed that some patients did demonstrate immune response and those patients tended to be responding to the dendritic cell treatment. Do you have a baseline measurement of their immune response of those patients before dendritic cell infusion?

C.F. Figdor: Yes, we do. There we do not see responses to these antigens, and specifically the KLH which they have never seen before there is a baseline response which is negative.

F.A. Ossendorp (Leiden, NL): Dr. Figdor, I think your data fit very nicely our mouse studies, where we also showed that the mature cells are the much better cells to induce T cells. But I was wondering, in your clinical study did you also look in the tumours of the patients that did not respond? Can you give an explanation why you see actually only two patients really responding?

C.F. Figdor: It is not so easy to get access to tumour cells from these patients. You must imagine that these are stage 4 patients, and they all do not have the primary tumours anymore. They are excised a long time ago, so we are dealing with metastases. It is very well known that these patients can lose these antigens while they are progressing and that the tumour can become heterogeneous in this respect. However, one of the inclusion criteria for these patients is that their tumours are positive for these antigens before they enter into the study. So we are definitely sure that the tumours of these patients express these antigens at the cell surface and that they also express their appropriate HLA type on the tumour cell.

A. Brand (Leiden, NL): Dr. Figdor, the mature cells migrate compared to the immatures, and you had a few immature cells in the lymphnodes. Have you been able to look with what cells they interact, because we assume they are down regulating or tolerising.

C.F. Figdor: Yes, everybody is now very afraid that when you treat these patients with immature dendritic cells that you are tolerising in contrast to getting a Th1 response. Currently we are looking in more detail at these sections and staining of course these cells with all kinds of antibodies to see what type of cells are surrounding these dendritic cells. Although that may not still give a conclusive answer, because you do not know what is happening really there. That is what we analyse at the moment.

A. Brand: Fatigue was the main side effect, and you showed that non-responding patients had no TNF alpha release and the responding patients had. You assume that fatigue is the result of TNF alpha?.

C.F. Figdor: I do not know whether that is the case. You must remember these are T cells that are not found at detectable levels in the peripheral blood.

S.J. Noga (Baltimore, MD, USA): Dr. Haisma, almost ten years ago I was told that I had to stop all my cell processing work, because gene therapy was here. It would be the future and it would start now. Why do you think it has taken so long to get successful trials?

H. Haisma (Groningen, NL): If you look at the field of gene therapy it started out with monogenetic diseases. Everybody thought, we just fix the gene and than it is done. Than there is one success but all the other studies basically fail. One of the failures is that if we put a corrective gene into a diseased patient, we lose gene expression. The reason that some of the trials now are successful is that there is some benefit for the cells that have this transferred gene. If you do not have that benefit you will lose the gene entirely. So that is one major thing why I think that gene therapy has not been that successful. As in the studies for specially SCID patients but also FIX deficient patients we see that we can indeed have gene expression for years in these patients. That will be a treatment, I think that will survive, but only in a selective number of diseases. I think cancer

is one of the hardest to tackle. There are now numerous cancer trials for gene therapy that focus on vaccination studies. We will have to see whether a gene therapy vaccination is better than all sorts of other vaccination studies. I have been watching the vaccination studies for cancer patients for a long time. I think more than one hundred years we are trying to cure patients with vaccinations, and now maybe we have one or two patients that show some promising results. So we have a long way to go.

S.J. Noga: Dr. Read, I am struck by the differences. We did not have track assays or spectra typing. We started to work with bone marrow as compared to peripheral blood. Now we have the comparison between allogeneic peripheral blood and allogeneic peripheral blood of non-myeloablative transplants. It may be the initial lymphocytes that are the most important.

E.J. Read (Bethesda, MD, USA): So I guess that argues for very careful engineering of the graft up front, and perhaps doing something a whole lot earlier rather then just adding the T cells post-transplant on day 45 and day 100.

B. Vandekerckhove (Gent, B): Dr. Read, you showed that after the T cell depletion, when you do DLI the T cells do not expand anymore? Do they not expand on antigens? So can you predict whether the patients do develop Graft-versus-Host after DLI?

E.J. Read: They definitely do develop graft-versus-host disease after the DLI. I cannot answer whether you see it in the spectratype or not. But, the one thing I did not mention is in the T cell depleted transplants all the graft-versus-host happens after the DLI. None of it happens until day 45. But I am not sure whether or not this spectratype specifically changed at that point.

B. Vandekerckhove: So the vector cells should be in the T cells of the DLI?

E.J. Read: Yes. They should be.

Dr. Rebulla, I think you are aware of a recent case in Minnesota where it is was discovered that there was a cord blood that had been mislabelled and almost was not discovered. But they discovered it as they were getting ready to give the cord blood to a child. There has been some talk about requiring a sample such as a segment on a blood bag. A sample being attached to the actually cord blood unit. Maybe you could give your impression of how all of these cord bloods that have been collecting by all these different bags and all different ways, how we should perhaps go back and create some standards to qualify those cord blood units in retrospect? Should that be done, and if so, how should that be done? Because clearly these cord bloods are going to go all around the world. They are going to be used just as the cord bloods but also perhaps in some of the more complex protocols that you are talking about. It is a really important raw material, so how do you think that should be done?

P. Rebulla (Milan, I): I agree that this is an important issue. In our bank we have taken as a standard rule to check the HLA from a segment before shipping a unit. The segment is integrally attached to the bag during storage. Segment collection may be tricky because of the very low temperatures and the need to avoid stem cell exposure to high temperature variations, but skilled technologists can do it properly. Other banks may have chosen different systems. I would say that this is an important step that should become a standard. Another important measure is to ensure that technicians never process two units at the same time, as this can cause sample exchange or mislabelling. To address and resolve such difficulties, a group of Italian banks chose to work together with a common quality system and set of banking standards. This group is known as the GRACE (Gruppo per la Raccolta e l'Amplificazione delle Celle Emopoietiche – Group for the Collection and Expansion of Hemopoietic Cells) network of cord blood banks. Your question also raises the issue of the possible uses that will be made in the future of cord blood units collected some years ago, when international standards were not available. I believe that the ultimate responsibility of unit selection and use goes to the clinician, who is in the best position to balance risks and potential benefits for the patient.

P. Law: Let me be the materialistic person and ask that if your costs are120 thousand dollars just in material to qualify your expansion procedure and then you have to account for the construction costs, maintenance costs and costs with GMP facility. How do you see the financial or commercial reward in doing an expansion program with cord blood?

P. Rebulla: I cannot give you a detailed analysis aimed at determining the cost effectiveness of cord blood expansion, mainly because the clinical experience is very limited. We were lucky enough to be able to get economic support for our expansion protocol, but I do agree that clinical research in cell therapy is very expensive.

L. de Leij (Groningen, NL): Dr Rebulla, in your clinical protocol where you did the expansion of the cord blood, in the end you give back to the patient one third of expanded material and two third of original. Could you explain why?

P. Rebulla: In developing a new procedure we have to ensure that we do not do any harm to the patient. So our initial aim is to show the safety of the procedure. For ethical reasons it was considered unacceptable to expand one full unit, but rather to transplant a part expanded and a part non expanded. In this protocol we will select a very large unit, in which the number of cells per kg would exceed the currently recommended minimum dose of nucleated cells.

M.K. Elias (Groningen, NL): Dr. Rebulla, do you collect cord blood exclusively extra uterine_as you illustrated and if so do you prefer it above the in utero method in spite of the low yield and the contamination hazard?

P. Rebulla: No, in fact, we prefer collection in utero. Most of our collections are done before placenta delivery, because this is preferred by the midwives. They have some ten minutes intermission time before placenta delivery and they find it very convenient to perform their collection during that phase. Your question raises also to the issue of who should do the collection. If you can rely on the midwives, you do not need to employ your own staff.

M.K. Elias: Is cord blood cryopreserved as such or processed?

P. Rebulla: From the whole cord blood, we collect 40 ml of buffy coat after centrifugation. We use a procedure which differs from that developed by Dr. Rubinstein. We do not sediment the cells with hydroxyethylstarch, but we just perform a gentle centrifugation and we collect the 40 millilitres buffy coat at the interface between red cells and plasma with the automated blood component separator named Compomat G4.

G.D.M. Beun (Groningen, NL): I have a remark and some questions to the panel members about the quality requirements for dendritic cells. Generally these are only phenotypically characterised, the standard criteria, whereas while we are still in the experimental phase, we should characterise them more fully. Especially with respect to migration capacity and functional abilities. That is the ability to induce in vitro a primary response against the relevant antigen. I would like to know if the panel agrees.

E.J. Read: Dendritic cells have been difficult. You are raising exactly the biggest problem with dendritic cells. They are so complicated and there are so many different groups who have identified different phenotypic markers as well as different functions. I would absolutely agree that even though various groups are going into phase 1, 2 and even into phase 3 clinical trials, there is a difference between the release assays and the assays that you really need to do to advance the science and figure out what you are doing and correlate those with what is happening clinically. So, in our dendritic cell trials we have had many discussions with the FDA. The FDA absolutely requires us to have a release assay that involves a phenotypic characterisation. But we also have our investigators work on the functional assays and in retrospect those are being analysed. But ultimately there is a problem I guess in the international community on agreeing what the best assays are. So I do not know if that answers your question, I think it raises as many questions as it does answer right now.

S.J. Noga: I just want to add something to that. It is interesting, because the last time I saw Dr. Figdor was in 1980 and that was The Reticuloendothelial Society in Savos, Switzerland where they were discussing monocyte heterogeneity. Now of course we are talking about heterogeneity of dendritic cells. The point you address is – is it possible that a subset is more important, should we try to push without subset? So now we are working on the same principles with dendritic cells. Probably those initial results just show sporadic successes, probably buried in some other logic that we don’t know about yet.

E.J. Read: Also the dendritic cell area is a really good example of what several of the speakers have talked about in terms of once you go into production you cannot ignore what the basic scientists are doing. It really speaks to a need for continued communication and interaction between the basic scientists and the translational scientists and the people in production and those doing the clinical trials. Once you have a dendritic cell product that works in a particular application you cannot stop there, the science has to go on. It is not like a drug product where the drug is approved for specific disease and you may move on to a different drug. I think that in this particular situation it is much more complex and really speaks to continued communication among the various parties involved in the cellular therapy.

P. Law: I like to make some comment on the phenotype of the antigen presenting cell and also pick up on what Dr. Read has said of release assay versus functional assay, because the release assays are really an important part of the GMP. On the day the cells come off the GMP facility you would have to tell your quality assurance unit that the cells are pure, safe and potent. That particular assay may not have anything to do with science as required for an academic institution. The release assays may not really detect detailed or specific cell functions, the measurement of which may not be possible or even necessary on the day of release. Currently the antigen presenting dendritic cells have diverse phenotypes and no one has come up with what is the best phenotype. One of the ideas is that it is not the phenotype that matters, it is the number of certain molecules on the cell surface. For example, co-stimulatory molecules are always on monocytes. That difference in cell surface density (or number of molecules on cell surface) relates to activation or maturation of these dendritic cells. There is a tremendous increase in the cell surface co-stimulatory molecular density and there is the possibility to use that as release criteria. These are possibilities that should be explored in a more standardised fashion by the national or international organisations, such as ISCT and other societies such as AABB.

L. de Leij: No comment. I think you are perfectly right.

I have a question or remark about the storage and shipping of for instance cord blood. Is it possible to get a DNA fingerprint of what is really in that bag, and than ship that kind of description with the bag, so everybody somewhere in the world can just check if what is in the bag is really what it says it is. Do you think that is a possibility?

S.J. Noga: DNA fingerprinting is an interesting point. I am sure that technology is improving. They keep using it for all these crimes, which they are trying to solve. So it must be getting down to a point where we can bring this into the laboratory.

P.L.M. Jansen (Groningen, NL): Dr. Haisma, you answered a question about gene therapy. The question was why does it take all that long. In your answer you addressed the point that many people in the gene therapy field addressed monogenetic diseases. This gives me an opportunity to make a remark. Mono-

genetic diseases, especially monogenetic liver diseases, stil are a very important goal for gene therapy. However, it is very important that correction of the gene that is defective, gives the cell a clear growth advantage over non-corrected cells. The corrected cell will then start to replicate and in this way amplify the efficiency of gene therapy. Maybe this is something that was not realised enough in the beginning of gene therapy.

H. Haisma: I am glad that you get back to this issue. It does not mean that I want to say that it is wrong to treat monogenetic diseases. It is just that apparently the issue you raise about survival of these treated cells is a most important issue. I think for any disease, and we now know with the SCID patients, that these cells need to have an advantage. That is the reason that these patients survive and are cured. I think for other diseases it is much more difficult. Especially if we look for instance at Haemophilia. If you transfer muscle cells there may even be a disadvantage. If the muscle cells turn over relatively slow you keep expression for months, and maybe a couple of years, and eventually you know that you lose gene expression. Of course there is no benefit for these cells to make this factor. If there are other diseases, especially liver diseases I think it will be a good idea to collaborate on this to see if we can find some treatments.

S.J. Noga: Another comment: What the organising committee has done putting this symposium together, is a good example of how things have changed and how things are going forward. Maybe this is my ignorance, but when I used to go to these meetings where they would have a talk about different approaches for cancer like transplantation and gene therapy, the gene therapists usually arrive in a group. Their meetings were a kind of separated because we did not understand what they were talking about, they did not want to know what we were talking about either. So they were separated. Here we see the merging between the two. Neither was understanding totally the other ones technology, but yet this is how you bridge together. So this type of a meeting shows that we are moving in the right direction. We are going to work out common pathways thanks to the organisers of this special symposium.

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