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MISMATCHED HAEMATOPOIETIC STEM CELL TRANSPLANTATION AS A MODEL FOR TOLERANCE AND ENDOTHELIAL CELL DEVELOPMENT

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The phenomenon of tolerance provides a testing ground for theories of the immune response.

Peter Brian Medawar, 1961

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1. Summary

In contrast to solid organ transplantation, ABO blood group incompatibility is of minor importance for haematopoietic stem cell transplantation. Patients receiving ABO-incompatible haematopoietic stem cell transplantation do not have a worse overall survival or increased transplant-related mortality. In this thesis, ABO-incompatible haematopoietic stem cell transplantation was used as an *in vivo* model to study the immune mechanisms of antigen-mismatched transplantation. Because most patients are pretreated with intensive conditioning chemo-and radiotherapy, the recipient organs are consequently in high need of repair. Whether donor-derived cells contribute to such regenerative organ repair, was analysed with the focus on the endothelium. Furthermore, mismatched ABO histo-blood group antigens, expressed on endothelial cells, may represent a target for graft-versus-host disease (GvHD). However, in a multi-centre study involving over 3000 patients, no significant correlation between ABO incompatibility and the incidence of GvHD was found. Replacement of recipient-type endothelial cells by donor-type bone marrow-derived cells could thereby represent a potential mechanism of tolerance.

The aims of this thesis were 1) to investigate whether the full haematopoietic chimerism observed after haematopoietic stem cell transplantation extends to endothelial cells and whether donor-derived endothelial cells contribute to *in vivo* blood vessel formation and 2) analyse a putative B cell-tolerance after minor ABO-incompatible haematopoietic stem cell transplantation by measurement of the antibody production, determination of anti-host A/B antibody-producing B cells and finally, measurement of the antibody and complement deposition. Endothelial cells in skin biopsies, autopsy-derived heart, skin and bone marrow and tumour biopsies from over 50 recipients before and at various time points after haematopoietic stem cell transplantation were analysed in both, a prospective and retrospective manner. The results of this thesis provide clear evidence that endothelial cell replacement by bone marrow-derived donor cells did not occur routinely after allogeneic haematopoietic stem cell transplantation, donor bone marrow-derived endothelial cells did not contribute to *in vivo* blood vessel formation and could not explain ABO-tolerance after ABO-incompatible haematopoietic stem cell transplantation. Instead, the finding, that donor B lymphocytes do not produce anti-recipient antibodies after transplantation across the ABO barrier may be a more probable explanation for ABO-tolerance. Furthermore, these data are of general interest indicating local endothelial cell proliferation rather than replacement by circulating endothelial progenitor cells as responsible repair of endothelial damage.

2. Zusammenfassung

Im Gegensatz zur Organtransplantation spielt die Konstellation von ABO-Blutgruppen in der hämatopoetischen Stammzelltransplantation eine geringere Rolle. Die ABO-inkompatible hämatopoetische Stammzelltransplantation verzeichnet keine generelle und keine erhöhte Transplantat-assozierte Sterblichkeit. Die hämatopoietische Stammzelltransplantation diente in dieser Dissertation als *in vivo* Modell, um immunologische Mechanismen der Antigen-inkompatiblen Transplantation zu analysieren. Da die meisten Patienten vor der Transplantation mit einer intensiven Chemo- und/oder Radiotherapie vorbehandelt werden, sind die Organe des Empfängers in Folge auf eine Gewebsregeneration angewiesen. Es wurde analysiert, ob die Zellen des Spenders solche Reparationsmechanismen, insbesondere die Reparatur allfälliger Gefässchädigungen, übernehmen können. ABO inkompatible Gewebeantigene, die auf Endothelzellen exprimiert werden, könnten ein mögliches Ziel einer sogenannten Graft-versus-Host Erkrankung (GvHD) sein. In einer Multizenter-Studie mit über 3000 Patienten wurde jedoch kein signifikanter Zusammenhang zwischen ABO-Inkompatibilität und dem Auftreten einer GvHD gefunden. Der Austausch von Endothelzellen des Empfängers mit Zellen des Spenders könnte dabei ein möglicher Toleranzmechanismus darstellen. Die Ziele dieser Dissertation waren: 1) die Ermittlung des möglichen Vorhandenseins eines endothelialen Zellchimärismus neben dem vollständigen hämatopoetischen Zellchimärismus und die Analyse einer möglichen Rolle der transplantierten Spenderzellen bei der Blutgefässneubildung *in vivo* und 2) die Analyse einer möglichen B-Zell-vermittelten Toleranz nach minor ABO-inkompatibler hämatopoetischer Stammzelltransplantation mittels einer Messung der Antikörperproduktion und einer Bestimmung von Antiköper-produzierenden B-Zellen, die spezifisch gegen den Empfänger gerichtet sind und schlussendlich die Messung der Antikörper- und Komplement Ablagerung. Endothelzellen in normalen Hautbiopsien, aber auch im Autopsiegewebe von Herz, Haut und solidem Knochenmark und in Tumorbiopsien von total über 50 Patienten wurden in einer prospektiven wie auch retrospektiven Studie untersucht. Die Resultate dieser Dissertation weisen deutlich darauf hin, dass Endothelzellen nach einer hämatopoietischen Stammzelltransplantation nicht routinemässig durch Spenderzellen ersetzt werden, dass Endothelzellen aus dem Knochenmark des Spenders nicht zur *in vivo* Blutgefässneubildung beitragen und deshalb auch nicht verantwortlich für die ABO-Toleranz sein können. Stattdessen dürfte die Erkenntnis, dass Spender B-Lymphozyten nach einer Transplantation über die Blutgruppenschranke hinweg keine anti-Empfänger Antikörper produzieren eine

wahrscheinliche Erklärung für die ABO-Toleranz sein. Diese Daten sind von generellem Interesse, da sie darauf hinweisen, dass die lokale endotheliale Zellproliferation und nicht der Einbau von zirkulierenden endothelialen Progenitorzellen für die Reparatur beschädigter Gefässe verantwortlich ist.

3. General Introduction

The current organ shortage in transplantation medicine stimulates the exploration of new strategies to expand the donor pool including the utilisation of living donors, ABO-incompatible grafts and xenotransplantation. In ABO-incompatible transfusion, solid organ transplantation as well as in xenotransplantation, preformed immunoglobulins, alleged natural antibodies (Nab), lead to haemolytic transfusion reaction or hyperacute organ rejection and thus represent a major hurdle to the employment of such strategies. Corresponding to the rules of transfusion medicine, matching of the ABO blood groups has therefore been thought to be prerequisite for successful solid organ transplantation. In contrast to solid organ transplantation, haematopoietic stem cell transplantation is routinely performed across the ABO-blood group barrier in approximately one third of the patients [Lasky et al., 1983; Klumpp, 1991]. The terminology in transfusion medicine distinguishes minor, major and bidirectional ABO incompatibility. Minor ABO incompatibility is defined by recipient antigens, which are not present in the donor and therefore isoagglutinins are present in the donor serum (i.e. O in A, B or AB; AB in A or B). Major ABO-incompatibility is defined by the presence of preformed antibodies against donor ABO antigens and comprises major (i.e. A, B or AB in O; AB in A, B) and bidirectional (i.e. A in B; B in A) incompatibility. It has been confirmed in a large and homogeneous population including more than 3000 patients from the Center of International Blood and Marrow Transplant Research (CIBMTR) that there is no difference in the overall survival and the incidence of transplant related mortality, relapse or graft-versus-host disease (GvHD) in ABO-compatible and -incompatible haematopoietic stem cell transplantation [Seebach et al., 2005]. Levels of anti-donor anti-A/B antibodies in the serum of patients receiving major ABO-incompatible haematopoietic stem cell transplantation disappear rapidly and do not reappear in the further post-transplant course. Furthermore, donor-derived anti-host antibodies gradually decrease after minor ABO-incompatible haematopoietic stem cell transplantation despite compatible anti-A/B Ab remain positive [Stussi et al., 2005]. To date, research has focused mainly on cellular immunity, as alloreactive T cells were identified to play a key role in acute and chronic GvHD. In contrast, the humoral immune system and in particular the ABO blood group system has been studied less extensively in haematopoietic stem cell transplantation. Because inheritance of the ABO blood group antigens is independent of the human leukocyte antigen (HLA) gene complex, haematopoietic stem cell transplantations between ABO non-identical siblings are frequently considered. The goal of successful haematopoietic stem cell transplantation is primarily

engraftment of all haematopoietic cell lineages. Donor-derived haematopoietic stem cells have the capacity to replace the whole haematopoietic system, resulting in a full haematopoietic chimerism. This brought us to the idea to investigate whether this full haematopoietic chimerism can extend to other tissues. For example, grafted cells were shown to replace in recipient-type dendritic cells of the skin in addition to haematopoietic cell replacement [Auffermann-Gretzinger et al., 2006]. ABO-incompatible haematopoietic stem cell transplantation may be viewed as a human *in vivo* study model for the behaviour of anti-A/B Nab and the occurrence of B cell tolerance in humans. In addition, interaction between donor and recipient cells after transplantation has received great attention in an attempt to identify the mechanistic basis of rejection and GvHD. Blood vessels represent the primary interface between donor and recipient in a transplantation and it has been hypothesised that the acceptance of the graft may be the result of a gradual replacement of donor endothelial cells by those of the recipient [Brent et al., 1976; Medawar, 1965]. Nowadays, it is not clear which cells are responsible for blood vessel remodelling after damage. Historically, it has been assumed that new blood vessels originate from the pre-existing vasculature through the proliferation of endothelial cells, which maintain a high clonogenic potential throughout adulthood [De Palma and Naldini, L., 2006]. It has been proposed, however, that vascular healing and tumour angiogenesis are also mediated by the recruitment of bone marrow-derived endothelial progenitor cells. These endothelial progenitor cells are thought to reside in the bone marrow and can be mobilised to the peripheral circulation as circulating endothelial cells in response to angiogenic stimuli. Theoretically, bone marrow-derived circulating endothelial cells participate in organ and tumour neovascularisation by incorporating into newly-formed blood vessels [De Palma and Naldini, L., 2006].

3.1. Haematopoietic Stem Cell Transplantation

A novel protocol of radiation and chemotherapy followed by the intravenous infusion of bone marrow - a radical new approach to cancer treatment was delineated by Thomas et al. already in 1957 [Thomas et al., 1957]. Two decades later, Mathe *et al.* were the first to describe allogeneic bone marrow transfusion with transient engraftment of bone marrow cells in recipients, who were suffering from aplasia [Mathe and Schwarzenberg, L., 1979]. In the following years, bone marrow transplantation gained importance in the treatment of various haematologic diseases (Figure 1). To date, allogeneic haematopoietic stem cell transplantation

still is the only curative therapeutic option for leukaemia, malign lymphoma, severe aplastic anaemia and genetic diseases like Thalassaemia major and severe combined immune deficiency (SCID) [Gmür, 1991]. Until recently, haematopoietic stem cells have been harvested directly from the bone marrow by needle aspiration. But haematopoietic stem cells can also be recruited from the marrow into peripheral blood by treatment with granulocyte-colony stimulating factor (G-CSF) and harvested by the method of leukapheresis. This latter method is a good alternative to the bone marrow donation under complete anaesthesia and is correlated only with minor side-effects like headache, fatigue and nausea during the period G-CSF is given. Finally, a third method to obtain haematopoietic stem cells is the use of donated umbilical cord blood. The number of haematopoietic stem cells in cord blood is considerably lower than in classical marrow or peripheral blood haematopoietic stem cell donation. Therefore, it can only be used for the transplantation of children and small adolescents. The advantage of using cord blood is that the immature immune system of the newborn is less aggressive than that of an adult and as a result, the alloreactive T cells in the graft cause less GvHD. This allows transplantation with a higher degree of human leukocyte antigen (HLA)-mismatches. At present, umbilical cord blood banks have been established in many transplantation centres and more than 100'000 units are available.

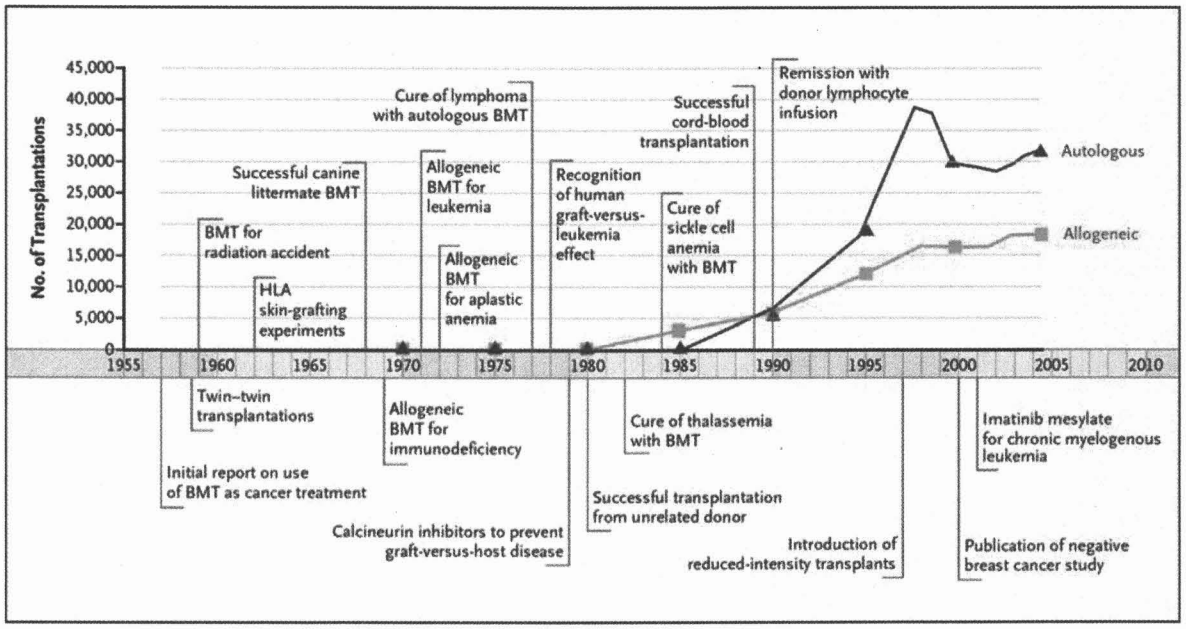

Figure 1: Timeline showing numbers of bone marrow transplantations and advances in the field, 1957-2006. BMT, Bone marrow transplantation. HLA, human leukocyte antigen. Data are from the Centre for International Blood and Marrow Transplant Research. © 2007 Massachusetts Medical Society, New England Journal of Medicine 357:1472-1475.

Autologous haematopoietic stem cell transplantation can be used for patients with non-Hodgkin lymphoma or as initial therapy for multiple myeloma, who will receive high-dose chemotherapy destroying their haematopoietic stem cells. Allogeneic haematopoietic stem cell transplantation from a donor are used to replace disease-affected haematopoietic stem cells of the patient mainly for leukaemias. In this thesis the focus will lay on the allogeneic haematopoietic stem cell transplantation.

3.1.2. Rejection and Tolerance in Haematopoietic Stem Cell Transplantation

The human immune system provides the crucial ability to distinguish between danger and non-danger or self and non-self. Unfortunately, exactly this ability can lead to severe problems in transplantation medicine. A variety of different cell types are involved in the immune response to rejection of allogeneic transplants. Preventing potentially minimising life-threatening immune mechanisms after haematopoietic stem cell transplantation is very difficult to achieve. Immunosuppressive treatment after haematopoietic stem cell transplantation leads to low post-transplantation anti-recipient antibody titres and to lowered vasculopathy, which is correlated with a better survival. But on the other hand, aggressive drug treatment also suppresses the natural presence of the so called graft-versus-tumour effect. This effect is established by infused allogeneic donor T cells and is needed to eradicate remaining leukaemic cells and to prevent relapse of the disease. Therefore, finding a compromise between high dose drug treatment and aiming a natural state of accommodation, a state where anti-recipient antibodies can persist without any adverse effects, is crucial for clinical treatment after haematopoietic stem cell transplantation. Since patients enter the treatment with a rigorous chemotherapy and drug pre-conditioning, that eradicates their own immune system to a great extent, immunological reactions as seen in solid organ transplantation, like hyperacute rejection or acute vascular rejection, immediately after the transplantation particularly do not occur in haematopoietic stem cell transplantation. However, there is a type of rejection called "hyperacute syndrome", that sometimes occurs during neutrophil recovery, usually 2-3 months after the transplantation [Spitzer, 2001]. This syndrome is often manifested by fever, erythrodermatous skin rash and/or cardiogenic pulmonary oedema. Hyperacute syndrome is mostly described after autologous haematopoietic stem cell transplantation, but can also occur in the allogeneic setting. In this case, it usually resembles a GvHD reaction by an extensive upregulation of pro-inflammatory cytokines. In some cases where reduced pre-conditioning is used, hyperacute syndrome is the

manifestation of a host-versus-graft (HvGD) reaction. Principally, cellular rejection causing GvHD is the most important cause of post-transplant morbidity and mortality after haematopoietic stem cell transplantation. Acute GvHD occurs in 20-50% of the patients. Mild GvHD may cause skin rash or gastrointestinal discomfort, more severe cases show blistering or peeling skin, serious pulmonary, liver, stomach or intestinal problems. The diagnosis and staging (I - IV) of acute GvHD is based upon criteria determined by Thomas et al. 1975 [Thomas et al., 1975a; Thomas et al., 1975b]. The pathogenesis of acute GvHD is complex and is now known to involve aspects of both the adaptive and the innate immune responses. Primarily donor immunogenic T cells and natural killer cells (NK) cells are reacting against foreign minor histocompatibility antigens on healthy endothelial cells of the recipient [Biedermann et al., 2002; Cooke et al., 2008]. Endothelial cells retract and start to express P-selectin and von Willebrand factor and secrete platelet activating factor. This initial phase is followed by a proper "storm" of pro-inflammatory cytokines and these mechanisms may lead to an initiation of the complement and coagulation cascades and may cause the actual endothelial cell damage. The histology of GvHD is marked by a T cell-mediated inflammation 1-3 days after antigen contact, microvascular lesions, pericapillary haemaorrhage, fibrinoid degeneration of the connective tissue and sometimes local deposition of antibodies or immunocomplexes can be observed. Furthermore, a rarification of microvessels has been described to occur in chronic GvHD [Biedermann et al., 2002].

Naturally occurring antibodies for humoral responses develop in most healthy individuals soon after birth and remain throughout life. They are produced by pre-B lymphocytes which account for most of the B-cell repertoire in the fetus and neonate. Natural antibodies (NAb) are frequently polyreactive with low affinity for multiple self-antigens, and are generally encoded by rearranged antibody genes that have not undergone somatic mutation. In transplantation medicine, natural antibodies play an important role in the ABO blood group system. Despite the minor differences between the blood group antigens A and B, anti-A and anti-B antibodies have precisely defined specificities, *e.g.* donors of blood group A do not produce anti-A antibodies and conversely, donors of blood group B lack the expression of anti-B antibodies. Presumably, these natural antibodies are produced in response to the colonisation of the gut by bacteria and to the exposure to viruses, protozoa, or components in food carrying A or B antigens. Binding of natural antibodies to target cells can activate the complement cascade and induce antibody-dependent cell-mediated cytotoxicity. In contrast to solid organ transplantation, matching of ABO blood groups plays a minor role in allogeneic

haematopoietic stem cell transplantation. However, it is necessary to remove preformed anti-donor ABO antibodies (also called isoagglutinins) to permit the infusion and engraftment of major ABO-incompatible stem cells. Anti-ABO antibodies are successfully removed by large-volume plasma exchange and *in vivo* antibody adsorption with either A or B substance, donor-type erythrocytes or with commercially available anti-A or anti-B adsorbing columns [Gale et al., 1977; Thomas et al., 1975a; Thomas et al., 1975b; Tyden et al., 2005]. In most patients with major ABO-incompatible haematopoietic stem cell transplantation, isoagglutinin titres directed against the donor A/B type have been shown to decrease to undetectable levels, possibly due to either B-cell tolerance to the donor antigens or to elimination of recipient type B cells. In some patients, however, the titres were shown to persist without any adverse effects on the graft – a process called accommodation [West et al., 2001b; Soares et al., 1999; Fehr and Sykes, M., 2004]. The physiological mechanism of immunotolerance against antigens of the own blood group is still not fully understood [Mollison, 1970]. The overall survival is similar in patients receiving ABO-compatible and ABO-incompatible haematopoietic stem cells, however, several immunohaematological complications may arise in the ABO-incompatible setting. (I) Patients with major ABO incompatibility (i.e. A in O, B in O, AB in A, AB in B) have a delayed red blood cell engraftment and are at risk for pure red cell aplasia (PRCA). (II) Delayed neutrophil recovery was observed after major ABO incompatibility by several investigators. (III) ABO-incompatible haematopoietic stem cell transplantation increases the risk for transplant-associated microangiopathy. (IV) Patients with minor or major ABO-incompatible haematopoietic stem cell transplantation are at risk for post-transplant haemolysis. The results of all the latter studies was summarised in a recent review [Stussi et al., 2006a]. Furthermore, it was found that major ABO incompatibility did not delay engraftment and by this, prolong haemolysis in non-myeloablative haematopoietic stem cell transplantation. Patients with sustained engraftment experienced gradual declines of anti-donor isoagglutinins [Maciej et al., 2002; Stussi et al., 2005].

Several investigators have analysed the role of ABO blood groups in determining the outcome of haematopoietic stem cell transplantation. Overall, data show that ABO antigens are not clinically important targets of graft rejection or GvHD. The incidences of successful engraftment, graft rejection and GvHD were unaffected by ABO-compatibility [Gale et al., 1977; Seebach et al., 2005; Klumpp et al., 1994]. Nevertheless, the immunological mechanism that induces tolerance against ABH histo-blood group antigens is currently still not elucidated. It is well described, that polysaccharide epitopes can stimulate B cells through

pattern recognition receptors and the reaction to incompatible blood group antigens appears to be T cell-independent [Eiz-Vesper et al., 2005]. But since ABO antigens are mainly expressed on membrane glycoproteins [Finne, 1980], and T cell-dependent immune reponses can also be elicited when the carbohydrate determinant is coupled with a protein, both T cell-dependent and -independent antibody production may occur during ABO-incompatible transplantation [Wang et al., 1991; Ohdan et al., 2007].

3.2. The ABO Blood Group System

Although traditionally regarded as red cell antigens, A, B and H carbohydrate structures are expressed as well on a wide variety of human tissues, including sensory neurones, platelets, some epithelia and most importantly vascular endothelium [Eastlund, 1998]. ABH antigens are not restricted to humans and are widely found in bacteria, plants and animals. However, expression of ABH determinants on red blood cells is a feature only of humans and the higher anthropoid apes [Oriol et al., 1992].

The ABO blood group system was first described by Karl Landsteiner in 1900. He mixed the blood of members of his department and made the fundamental observation that some blood samples when mixed together with serum formed clumps whereas others did not. This led to the suggestion that there were three blood group antigens [Landsteiner K, 1901]. For his discovery of human blood groups he was awarded the Nobel Prize in Physiology or Medicine in 1930. Individuals who lack a certain antigen have natural antibodies that agglutinate cells carrying that antigen. The blood type AB was subsequently discovered by Decastello and Sturli, 1902. By the mid 1970s the pattern and mechanism of inheritance of the ABO groups, the relationship of the H antigen to the ABO-system, the chemical structures of ABH histo-blood group antigens in secretions and on the red blood cells and the primary products of the blood group genes was largely established [Watkins, 2001]. However, with the cloning of the ABO genes [Yamamoto, 2001] a new phase has opened up enabling further meaningful exploration of the association of blood groups and disease and the significance of the tissue-specific expression of ABH histo-blood group antigens.

3.2.1. Molecular structure and genetics of ABH histo-blood group antigens

Histo-blood group antigens are not protein antigens. Therefore, these antigens are not the primary gene products of A and B alleles at the ABO genetic locus. A and B antigens are

oligosaccharide structures synthesised through a series of enzymatic reactions, of which the final steps are catalysed by A and B glycosyltransferases, the primary products of A and B genes, respectively [Yamamoto, 2001]. The A and B genes differ in a few single-base substitutions, changing four amino-acid residues that may cause differences in A and B transferase specificity. A critical single-base deletion was found in the O gene, which results in an entirely different, inactive protein incapable of modifying the blood group antigen of O individuals, i.e. the H antigen. This implicates that A, B and AB individuals express glycosyltransferase activities converting the H antigen into A or B antigens, whereas O(H) individuals lack such activity [Yamamoto et al., 1990]. The H determinant forms part of the A and B blood group determinants and A and B antigens differ only with respect to the terminal sugar: in the case of the A determinant the immunodominant sugar is N-acetyl-D-galactosamine and in the case of the B determinant it is D-galactose [Greenwell, 1997]. The transferase adds the N-acetyl-D-galactosamine (GalNAc) residue of the nucleotide-sugar UDP-GalNAc to acceptor H substrates, the B transferase the galactose (Gal) residue of UDP-Gal to acceptor H substrates (Figure 2). ABH synthesis occurs in the endoplasmatic reticulum and maturation of the carbohydrate chains continues in the Golgi apparatus [Clausen et al., 1992]. The essential enzymes, GalNAc-transferase and Gal-transferase, are both located in the Golgi apparatus, but can also be present in the serum and therefore determine the tissue distribution of histo-blood group antigens [Mandel et al., 1992]. Individuals secreting a soluble form of blood group antigens into saliva and other body fluids are called "secretors" and are genetically distinct to so called "non-secretors". The Secretor gene (FUT2) regulates expression of ABH histo-blood group antigens and is present in 80% of the population [Costache et al., 1997].

Biosynthesis of H antigen, the immediate precursor of the A and B antigens, is dependent on a glycosyltransferase as well. H transferase constructs linkages of Fucα1 to 2Galβ, known as H determinants (Figure 2). Cloning of the H-gene (FUT1) and the assignment of this gene locus on chromosome 19 (19q13.3), revealed a clear independence of the Hh locus from the ABO locus on chromosome 9 [Larsen et al., 1990; Reguigne-Arnould et al., 1995]. Individuals of the very rare Bombay phenotype lack H transferases due to homozygosity of the rare inactive h allele, therefore fail to express H antigens and have anti-A, anti-B and anti H antibodies in their sera [Bhende et al., 1994]. Such people cannot synthesise A or B antigens regardless of their ABO blood group genotype.

Soluble ABH histo-blood group antigens get passively adsorbed onto platelets [Mollicone et al., 1988; Curtis et al., 2000; Cooling et al., 2005] and erythrocytes [Finne, 1980]. In addition, soluble forms of A and B antigens are usually bound to membrane glycoproteins, especially to PECAM-1 and von Willebrand factor (vWF), to a lesser extent they can also bind to glycosphingolipids or polyglycosylceramides [Curtis et al., 2000; O'Donnell and Laffan, M. A., 2003; Finne et al., 1980].

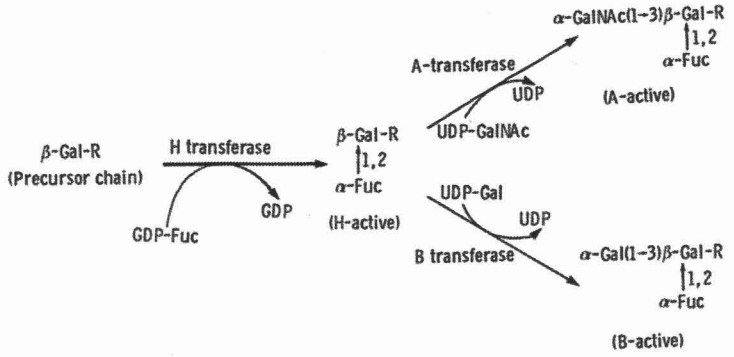

Figure 2: Biosynthetic pathways for the formation of ABH histo-blood group antigens. © 2001 Blackwell Science Ltd, Transfusion Medicine, 11:243-265

3.2.2. Histological location of ABH histo-blood group antigens

Landsteiner identified the antigens of the ABH system on erythrocytes by agglutination. But A, B and H antigens are also found on other cell types such as epithelial and endothelial cells [Ravn and Dabelsteen, E., 2000; Szulman, 1960]. ABH antigens are also ubiquitously expressed in bacteria. Specifically, in 1989 it was shown, that B antigen is present in the wall of the *E. coli O86* being part of its polysaccharides [Andersson et al., 1989]. Infection by such a strain of of bacteria could thus evoke an anti-B reponse in A and O blood group individuals. In humans, ABH histo-blood group antigens have been found on all epithelial cells, which stay in direct contact with the external environment, i.e. the higher respiratory tract, nasal epithelium and trachea, as well as the lower genitor-urinary tract, ureter and vagina. ABH histo-blood group antigens are expressed during the human embryonic development already 5 weeks post-fertilisation. All three blood group antigens were shown on cell surfaces of the endothelium throughout the cardiovascular system and all epithelia of most early organs such as the epithelia of the integument, the digestive tube and the mesonephric ducts [Szulman, 1964b; Szulman, 1964a; Szulman, 1960; Ek et al., 1994]. However, the strong expression of ABH histo-blood group antigens on epithelia decreases after about 9 weeks of development when the secretion of mucus begins. At 12-14 weeks of

embryonic life, the adult patterns of ABH histo-blood group antigen expression are established. Interestingly, a less pronounced expression of ABH blood group antigens was shown in infantile erythrocytes, chorionic vessels and in the distal part of the umbilical cord [Pedal et al., 1985; O'Donnell et al., 2000]. Whether this weak or even absent expression of ABH antigens may play a role in the immunological tolerance between mother and child remains to be elucidated. In skin tissue the ABH antigen expression pattern depends on the cellular differentiation as shown very nicely in the different cell layers [Clausen and Hakomori, S., 1989; Dabelsteen et al., 1984]. Rapidly growing basal cells near the basal membrane express an early carbohydrate precursor chain N-acetyllactosamine, the cells next to them are a more differentiated layer of cells, the stratum spinosum, express the H antigen and the final flattened, exfoliating layer at the surface, the stratum corneum, expresses A or B antigens [Eastlund, 1998].

Earlier studies used sensitized human sera for the staining of ABH histo-blood group antigens. Nowadays, the use of monoclonal antibodies show much better results. In this thesis monoclocal mouse anti-human ABH antigens, purchased from Dako, Carpintera CA, USA (anti-A A0581, clone 81 FR 2.2; anti-B A0582, clone 3E7 and anti-H type 2 A0583, clone 92FR-A2) were used to detect ABH histo-blood group antigens in tissue and on red blood cells (Figure 3).

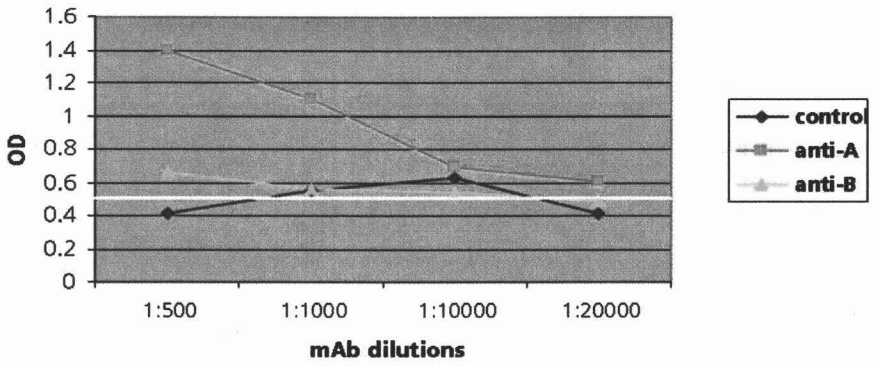

Figure 3: Enzyme-linked immunosorbent assay (ELISA) on a plate coated with red blood cells of blood group type A. Specificity of the monoclonal mouse antibodies anti-A antigen and anti-B antigen (Dako, Carpintera CA, USA). Control, supernanant of hybridoma cell culture (HE-14).

Unfortunately, the company has now withdrawn these well-working antibodies from their product list. We tried to reproduce the staining results with equivalent monoclonal antibodies from Abcam (anti-blood group H antigen ab8684, clone 92 FRA2) and from Zytomed (anti-

BG A Z-311-01-Y, clone T36; anti-BG B Z-312-01-Y, clone CLP-19B; anti-BG H type 1 chain Z-313-01 Y, clone 17-206) but did never obtain results of the same value. H antigen, in particular, was difficult to analyse, most likely due to its lower immunogenicity. Furthermore, we tried to use supernatant from cultured hybridoma cells HE-14 and HEB-20 (BioProbe, Indonesia) shown to produce IgM anti-A and B antigen, but these supernatants did not work in neither ELISA nor immunohistochemistry.

- **3.2.2.1. ABH histo-blood group expression in pathological situations**

The relationship between blood groups and cancer may at first appear much less complicated than their relationship with infectious disease, because it is generally accepted that tumour development goes paired with changes in cell surface carbohydrates. But still, there is much confusion in this area, partly because the natural tissue-specific expression of the antigens is not well established.

The loss of ABH histo-blood group antigens in malignant bladder and oral epithelia was accompanied by concordant loss of A/B glycosyltransferases [Mandel et al., 1992]. Downregulation of the A/B transferase gene might even cause de-blocking of certain growth factors which, by this, could cause a higher cell proliferation [Ichikawa et al., 1997; Ichikawa et al., 1998]. In pathological situations, such as cancer or wounds, an aberrant expression of ABH histo-blood group antigens was reported in several publications [David et al., 1993; Nosaka et al., 2007]. This aberrant expression may be due to genetic alteration of the ABO-gene and the following aberrant glycosyltransferases. These observations are undermined with the finding, that A and B transferases are highly homologous and only differ in four of their 354 amino acids [Yamamoto et al., 1990].

3.2.3. Function of ABH histo-blood group Antigens

In spite of the strong evidence of ABO gene conservation through evolution, there remains a gap of knowledge about the function for these polymorphic carbohydrate molecules. ABH histo-blood groups do not appear to have an essential function in cell physiology, since Bombay individuals (genotype hh) lacking ABH histo-blood group antigens develop normally. However, susceptibility to some diseases has been shown to be connected with the blood group of the patient. A and B antigens sometimes reappear in malignancy in tissues from which they have disappeared in early embryonal development [Hakomori, 1999; Hakomori, 1989]. In distal colon carcinoma, for example, A and B antigens were shown to be

re-expressed [Orntoft et al., 1991]. In contrast, deletion or downregulation of the ABH histo-blood group antigens has been discussed on erythrocytes of patients with various haematologic disorders [Rochant et al., 1980; Brody and Beizer, L. H., 1965; Hoogstraten et al., 1961; Salmon, 1976]. Deletion of A or B antigen in tumour cells was shown to be correlated to increased haptotactic cell motility [Ichikawa et al., 1997; Miyake et al., 1992]. In general, the degree of deletion of A and B activity in tumours has been associated with the degree of formation of metastasis and the level of malignancy. Continued expression of A and B antigens has been confirmed as a favourable prognostic factor in certain cancers [Matsumoto et al., 1993; Ichikawa et al., 1998; Dabelsteen, 2002]. The hypothesised protective role of ABH histo-blood group antigens in malignancy appears to fit another hypothesis, that ABH determinants are capping structures that cover up oligosaccharide chain endings, such as integrin receptors, that otherwise would interact with endogeneous lectins or cell surfaces. By this ABH histo-blood group antigens may protect skin and gastrointestinal tract from invasion of micro-organisms [Koscielak, 2001]. Furthermore, Le Pendu et al. reported, that glycosylation may affect the immune recognition of cancer cells by NK cells and that cells expressing the A antigen were much less susceptible to the actions of NK cells than cells expressing the H antigen [Blottiere et al., 1992; Labarriere et al., 1994].
Fucoslylated glycans, such as the H antigen are involved in cell-to-cell and cell-to-matrix interactions [Varki, 1993] and these glycans play an important role in permitting adhesive contacts between haematopoietic progenitor cells and bone marrow stromal cells [Schmitz et al., 1996]. H antigen is shown to be expressed on CD34+ haematopoietic progenitor cells, but is absent in more mature haematopoietic cells of myeloid and lymphoid lineage - during erythropoiesis CD34 is expressed until the orthochromatic erythroblastic stage [Southcott et al., 1999] – and, by this, may be important for the homing process of these cells to the bone marrow [Cao et al., 2001].

It is currently not clear whether ABH histo-blood group antigens clinically are non-essential targets of graft rejection or GvHD following bone marrow transplantation or if there is a mechanism to downregulate the expression or structurally modify the form of ABH antigens on haematopoietic stem cells and progenitor cells [Hershko et al., 1980; Gale et al., 1977]. In terms of stimulated blood group antigen expression versus deletion in pathological situations, ABH histo-blood group antigens were shown to persist strongly in human tissue samples from angiosarcomas of the skin [Ikegawa et al., 1992].

3.3. Endothelial Cells as a Barrier between Donor and Recipient

After allogeneic haematopoietic stem cell transplantation endothelial cells represent the primary barrier separating donor-derived leukocytes and allogeneic host tissue. The endothelial cell lining might be an exposed target tissue for cytotoxic T lymphocyte-mediated immune responses. Hereby, the severity of the immune response is determined by the amount of antigen presented and the amount of costimulatory signals delivered by the endothelial cells. Interstingly, endothelial cells are relatively resistant to GvHD reactions and do not undergo fatal destruction like it is seen in hyperacute rejection in solid organ transplantation [Biedermann, 2008]. However, a cutaneous microvessel loss late in the course of persistent chronic GvHD has been convincingly described [Biedermann et al., 2002]. Endothelial cells are poor presenters of both endogeneously processed antigenic proteins and exogeneously added antigenic peptides and therefore they often escape cytotoxic T lymphocyte-mediated killing [Kummer et al., 2005].

In ABO-incompatible solid organ transplantation, anti-A and B antibodies can bind to endothelial cells expressing A or B histo-blood group antigens and induce hyperacute humoral graft rejection. Therefore A/B antibodies need to be absorbed from recipient serum prior to transplantation in order to overcome anti-A/B antibody-mediated rejection and early graft loss in ABO-incompatible kidney transplantation [Puga Yung G* et al., 2007; Tyden et al., 2003; Takahashi and Saito, K., 2006]. Following minor ABO-incompatible haematopoietic stem cell transplantation, anti-recipient A and B antibodies appear in low numbers after haematopoietic engraftment. These low anti-recipient anti-A/B antibody levels might not be sufficient to cause endothelial cell activation. In addition, this time period might suffice endothelial cells to accommodate. The expression of histo-blood group antigens varies from cell to cell and from organ to organ. Human umbilical vein endothelial cells for example fail to express ABH histo-blood group antigens [O'Donnell et al., 2000]. In general, capillary endothelium is phenotypically different from endothelial cells lining large vessels [Page et al., 1992]. Endothelial cells derived from different vascular beds can demonstrate marked functional heterogeneicity (e.g. vWF and endothelial protein C receptor expression) [Cines et al., 1998].

3.3.1. Source of Endothelial Cells and Neoangiogenesis

It has become evident that endothelial progenitor cells (EPC) reside in the adult bone marrow and are mobilised into the circulation upon cytokine stimulation. It is not known whether adult endothelial cells differentiate from marrow-derived circulating EPC or whether they are repopulated directly from adjacent endothelial cells. It is estimated that EPC constitute approximately 1-5% of the total amount of bone marrow cells, whereas the amount of EPC in peripheral blood is only around 0.002% of total peripheral blood mononuclear cells (PBMC) [Peichev et al., 2000]. Theoretically, when endothelial cell are destroyed by transplant-related rejection mechanisms, they might be replaced by bone marrow-derived EPC. This kind of neoendothelialisation has been seen in a patient, that underwent haematopoietic stem cell transplantation for acute radiation syndrome after the Tokaimura nuclear accident [Suzuki et al., 2003]. Aortic endothelial cells of this patient were almost 25% replaced by donor-origin endothelial cells and provided that transplanted haematopoietic stem cells can be a source of EPC in humans. Whether such a repair mechanism occurs only in outmost urgent situations or whether EPC are also repairing damaged endothelium in the course of a physiological vessel remodelling is controversely discussed in the scientific literature. EPC were initially described by Asahara et al. as Flk-1+, CD34+ cells isolated from the peripheral blood and differentiating *in vitro* and *in vivo* into adult endothelial cells [Asahara et al., 1997]. Moreover, this report suggested, that the so called haemangioblast, a common haematopoietic and endothelial cell precursor might be present in the blood. In the following years, extensive data support the existence of EPC, their bone marrow origin and their ability to contribute to the formation of new blood vessels in adults. Gehling et al. demonstrated, that AC133+, VEGFR-2+ and CD34+ cells from G-CSF-mobilised peripheral blood differentiate into endothelial cells when cultured in the presence of VEGF and stem cell growth factor [Gehling et al., 2000]. In line with this report, it was later shown, that a small subset of CD34+ cells from different haematopoietic sources also expressing both AC133 and VEGFR-2 were capable to differentiate into adult $AC133^-$, VEGFR-2+ endothelial cells [Peichev et al., 2000]. Shi et al. used a canine transplantation model in which marrow cells from donors and recipients were distinguishable by a PCR assay. After 12 weeks, cells with endothelial morphology lining a vascular prosthesis were of donor-type. These results suggested, that a subset of CD34+ cells located in the bone marrow mobilise to the peripheral circulation and colonise the endothelium in the course of neoangiogenesis [Shi et al., 1998]. On the other hand, some investigators found that cells derived from the bone marrow, that attach to the culture dish, or PBMC did not differentiate into endothelial cells under culture conditions

with VEGF but stimulated angiogenesis in other ways [Ziegelhoeffer et al., 2004; Rehman et al., 2003]. Therefore the term EPC might not be an adequate definition of the total cell culture because not all cells might become true endothelial cells under the conditions used. These attaching cells with angiogenic capacity might be named more appropriately "angiogenic myeloid cells" [Loomans et al., 2006]. In support of this study, several other reports have described the myeloid character of endothelial cells [Nishimura and Asahara, T., 2005; Schmeisser et al., 2003; Fernandez et al., 2000; Romagnani et al., 2005]. At present, it is still not clear which progenitor cells are the best for stem cell infusion for tissue repair and whether bone marrow or peripheral blood is the better source. Endothelial progenitor cells are still not incorporated in schematic overviews of haematopoietic cell differentiation in the scientific literature (Figure 4).

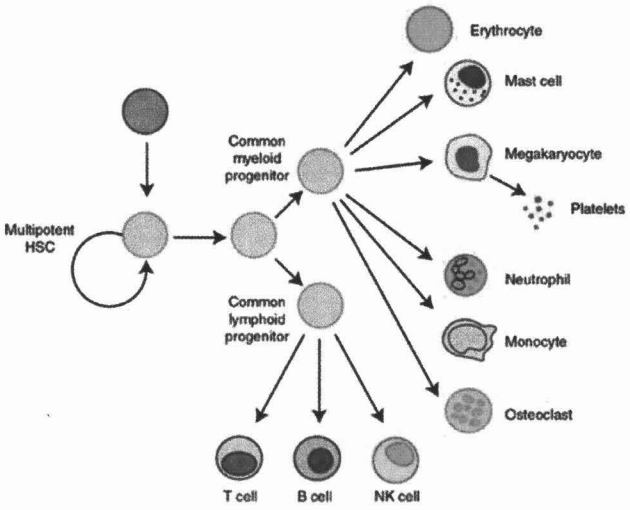

Figure 4: Normal haematopoiesis and the concept of stem cell transplantation.
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3.4. Possible Mechanisms leading to Immunological Tolerance in ABO-incompatible Haematopoietic Stem Cell Transplantation

Allograft acceptance involves a bidirectional immune reaction [Starzl and Demetris, A. J., 1995]. This "two-way-paradigm" describes the situation after solid organ transplantation, when two populations of immune cells manage to coexist in a stable state by becoming mutually non-reactive while retaining the ability to function collaboratively, which is contemporary GvHD and HvGD. In haematopoietic stem cell transplantation, this concept is valid as well, because complete destruction of host leukocytes is not possible with

conventional doses of cytoablation, the remaining cells stimulate an alloresponse by mature or maturing donor T cells. Half a century ago, Billingham, Brent and Medawar published a paper in Nature titled "Actively Acquired Tolerance of Foreign Cells" [Billingham et al., 1953]. This eight-page preliminary paper firmly established the basis of immunologic tolerance, seven years later, it led to the awarding of the Nobel Prize for Physiology and Medicine to Peter Medawar and his colleague Frank Macfarlane Burnet. Key-findings were that 1) inoculation of allogeneic cells into the peritoneal cavity of fetal mice facilitated the successful transplantation of skin grafts from the same donor in adults, 2) this "actively acquired tolerance" was highly strain-specific and third-party grafts were rejected normally by these tolerant mice and 3) the induction of tolerance depended on the implantation of living cells into young mice, which still were immunological immature (i.e. before birth). The conclusion from these studies was, that tolerance occurs when foreign antigens come in contact with the immature immune system and that tolerance is rather mediated by a central adaption of antibody-producing cells than by an active regulatory mechanism. From the late 1950s to the early 1960s it became apparent that tolerance can also be induced in adult animals by reducing their immune system to the level of the immature animal through whole-body irradiation or immunosuppressive drugs. This concept is still applied in modern haematopoietic stem cell transplantation. However, the exact mechanisms leading to tolerance after mismatched haematopoietic stem cell transplantation are still not fully understood.

ABH histo-blood group antigens are considered as major histocompatibility barrier for solid organ transplantation. Indeed, the first clinical tests resulted in accelerated rejection of ABO-incompatible skin grafts [Dausset and Rapaport, F. T., 1966]. Until few years ago, only few ABO-incompatible solid organ transplantations have been performed. Most of them have been accidental [Slapak et al., 1981]. Several mechanisms leading to a relative resistance of the endothelium after mismatched haematopoietic stem cell transplantation have been put forward. On one hand, donor-derived effector lymphocytes could become tolerant, and on the other hand there could be significant changes on the side of the endothelial cell. Alteration of the antigenic profiles on the endothelial cell surface is one possibility and a partial exchange of recipient-type endothelial cells by cells of the donor was the other one, hypothesised by Medawar and colleagues already in 1965 [Medawar, 1965].

3.4.1. Haematopoietic Chimerism

Establishment of a complete haematopoietic chimerism is the major goal of a haematopoietic stem cell transplantation. The leukaemic blood cells should be replaced by healthy, donor-derived cells. However, if complete donor haematopoietic chimerism is achieved very early after allogeneic haematopoietic stem cell transplantation, the risk for development of extensive chronic GvHD is strongly predicitive [Balon et al., 2005]. It seems that the most critical period with great influence on the whole post transplant course is the first 3 months after transplantation. Elimination of all detectable host cells during the early period after transplantation may be an indirect evidence of intense alloreactivity of donor cells and establishment of allogeneic tolerance. In contrast, coexistence of host and donor cells in transplant recipients leads to easier induction of immunological tolerance through a peripheral mechanism [Sykes and Sachs, D. H., 2001]. Partial or mixed haematopoietic chimerism occurs when milder forms of preconditioning are used, which initiately do not completely ablate the host haematopoietic system [Wekerle and Sykes, M., 1999; Sykes et al., 1999]. Recently, complete haematopoietic chimerism has also been described after liver transplantation in a 9-year-old girl. Severe haemolytic anaemia 10 months after gender-mismatched, Rhesus-incompatible liver transplantation was associated with a mixed chimerism and the initial studies by Rhesus-typing and fluorescence in-situ hybridisation for X and Y chromosomes showed the majority of lymphocytes to be of donor origin. Haemolysis resolved 14 months after the transplantation after the gradual withdrawal of all immunosuppressive therapy [Alexander et al., 2008].

The number of current methods to reliably analyse cell chimerism is limited [Bader et al., 2005; Ferrand et al., 2003]. To understand the engraftment mechanism of haematopoietic stem cells after haematopoietic stem cell transplantation, expression of ABH histo-blood group antigens on all haematopoietic stem cells and endothelial progenitor cells has been investigated. It was shown that ABH antigen expression is acquired with erythropoietic maturation [Karhi et al., 1981; Sieff et al., 1982] and that stem cells and progenitors are characterised with an increasing percentage of antigen expression during cell lineage development [Blacklock et al., 1984]. Therefore, the staining of donor- and recipient-specific ABH histo-blood group antigens can be used as a method to determine haematopoietic cell chimerism after haematopoietic stem cell transplantation. Other methods include determination of X and Y chromosome patterns in the blood or bone marrow of transplanted patients [Thiele et al., 2003]. However, this is only possible in gender-mismatched haematopoietic stem cell transplantations. The method, currently used in most routine

haematology laboratories is molecular analysis of highly polymorphic short tandem repeats in blood or bone marrow aspirates. In conclusion, mixed chimerism is a promising way to achieve transplantation tolerance. Recent progress in experimental models and in clinical stettings has dramatically reduced the potential toxicity of the conditioning required for allogeneic haematopoietic stem cell engraftment and offers a possibility that this approach to tolerance induction will soon be made from bench to bedside.

- **3.4.1.1. Feto-maternal Cell Transfer and Microchimerism**

Fetal cells transferred into maternal blood during pregnancy may persist at low levels in blood and tissue for decades post-partum. It is even hypothesised that fetal cells, termed pregnancy-associated progenitor cells (PAPCs), persist after delivery in a maternal stem cell niche and, in the case of tissue injury, home to the damaged organ and differentiate as part of the maternal repair response [Bianchi et al., 1996; Koopmans et al., 2005; O'Donoghue et al., 2004; Khosrotehrani and Bianchi, D. W., 2005; Johnson et al., 2002]. For these reasons determining endothelial cell chimerism in females after gender-mismatched transplantations only by the method of XY in-situ hybridisation may not be sufficient to prove the presence of donor-type endothelial cells.

Another indication for feto-maternal cell chimerism and tolerisation of stem cell donors was recently published. In a retrospective single-centre study over 300 patients were analysed for their overall survival, transplant-related mortality, relapse mortality and severity of GvHD after HLA-identical sibling haematopoietic stem cell transplantation. Patients who were born as the first child in a family and received haematopoietic stem cells from their younger sibling had the best survival and a significant reduction in acute GvHD and relapse mortality [Bucher et al., 2007].

3.4.2. Antibodies and Accommodation

ABH histo-blood group antigens are of outmost importance for solid organ transplantation. Yet, ABO-incompatible solid organ allo-transplantation has become a clinical reality for a small number of patients over the last two decades with the use of specific immunomodulatory protocols and various procedures to eliminate anti-donor antibodies prior to transplantation. ABO-incompatible adult kidney and infant heart transplants have nowadays similar patient and graft survivals as their ABO-compatible counterparts [West et al., 2001a; Tyden et al., 2005]. The protocol for ABO-incompatible renal transplantation includes early pre-transplant rituximab (anti-CD20 antibody) administration, routine pre-

transplant and if required post-transplant immunoadsorptions. If the first critical phase is successfully managed, ABO-incompatible grafts are no longer rejected. This resistance to anti-donor A and B antibodies and complement is referred to as graft accommodation. To achieve accommodation, it seems likely that a brief period in which anti-graft antibodies are relatively low and recover slowly, is necessary. Following minor ABO-incompatible haematopoietic stem cell transplantation, anti-recipient A and B antibodies appear in low numbers after haematopoietic engraftment [Stussi et al., 2005]. In addition, in a state of accommodation, cytokines produced by T-helper 2 cells can induce the expression of protective genes in endothelial cells [Soares et al., 1999; Hancock et al., 1998].

3.4.3. B cells

A study performed in our laboratory showed, that donor-directed anti-A/B antibodies disappeared rapidly after major ABO-incompatible haematopoietic stem cell transplantation and did not reappear in the further post-transplant course. Some of the results of this study have been published [Stussi et al., 2005]. In a majority of patients, recipient-directed anti-A/B antibodies were not detectable at any time point after minor ABO-incompatible haematopoietic stem cell transplantation despite of a complete donor-type haematopoietic chimerism. Anti-A/B antibodies not directed against a donor or recipient ABH antigen as well as anti-porcine antibodies remained stable after haematopoietic stem cell transplantation, excluding the possibility of unspecific immunosuppression. Moreover, neither antibodies nor complement deposition were detectable on recipient endothelial cells in skin biopsies of patients after minor ABO-incompatible haematopoietic stem cell transplantation excluding the possibility of antibody sequestration to ABH antigens expressed on endothelial cells (own unpublished observations). In contrast to ABO-incompatible solid organ transplantation, no anti-donor antibodies were measurable after minor incompatible haematopoietic stem cell transplantation [Stussi et al., 2005].

To date it is not clear, whether B cells are tolerised or anergised against these ABO antigens. We analysed the role of deletion or anergy of anti-host A/B producing B cells by ABO antibody-specific ELISPOT and flow cytometric assays according to a previously published protocol [Fan et al., 2004]. After numerous modifications of the cell culture protocol (i.e. using a mitogen stimulation with CpG, pokeweed mitogen and Staphylococcus Aureus Cowan instead of IL-2 and IL-4, see Figure 5), we obtained a nice number of antibody-secreting cells. Unfortunately, studying the specificity of these cells in the ELISPOT assay

was never possible due to a strong cross-reactivity making a distinction between A-specific and B-specific B cells impossible (Figure 6). A possible reason for this strong cross-reactivity might be the structural similarity of neo-glycoproteins (A-BSA and B-BSA, Dextra Laboratories, Reading UK) used for coating the ELISPOT plates.

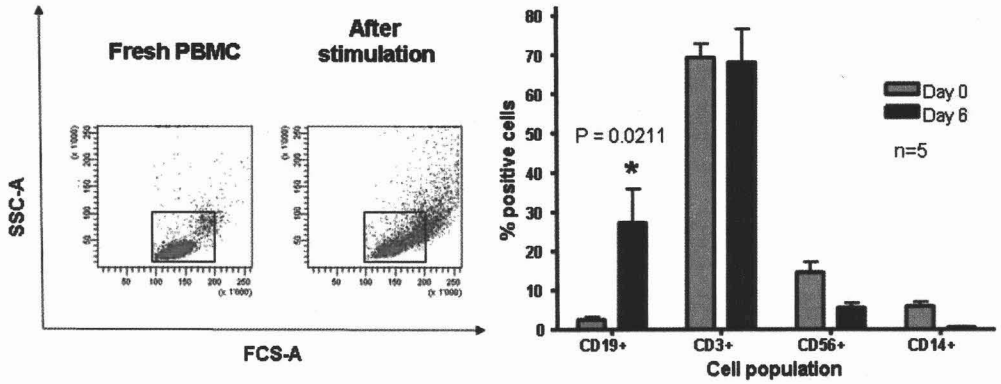

Figure 5: Flow cytometric phenotype analysis of peripheral blood mononuclear cells (PBMC) freshly isolated and stimulated in culture. *In vitro* mitogenic stimulation for 6 days with CpG 6µl/ml, pokeweed mitogen 1/100'000 and Staphylococcus Aureus Cowan 1/10'000.

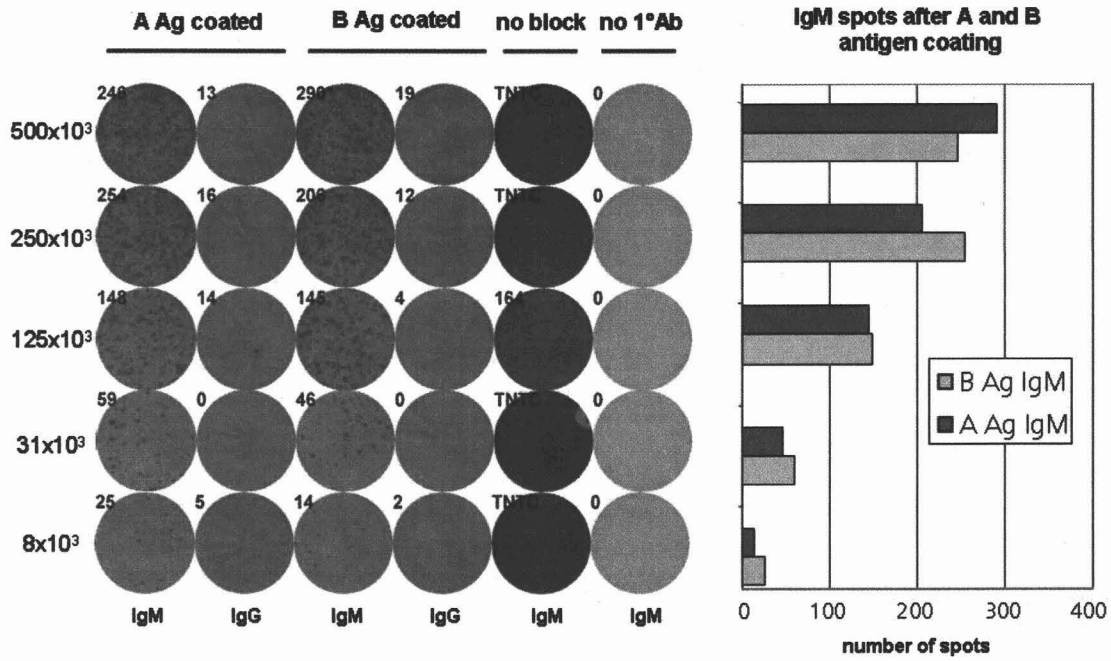

Figure 6: Enzyme-linked immunospot technique (ELISPOT) using stimulated PBMC of blood group type B after mitogenic stimulation. IgM spot detection in wells coated with either A antigen or B antigen shows the same amount of A- and B-specific antibody-producing cells.

3.4.4. Alterations on Endothelial Cells

Endothelial cells are particularly resistant to an attack by antigen-specific cytotoxic T lymphocytes [Kummer et al., 2005]. Comparison of antigen expression levels on vascular endothelial cells and fully susceptible leukocyte-derived target cells showed that the resistance of the former was the consequence of an altered major histocompatibiliy complex (MHC) class-I ligand profile expressed. This cell-type specific immune escape could be another mechanism of relative endothelial protection after allogeneic stem cell transplantation [Biedermann, 2008]. Furthermore, it was suggested in models of xenotransplantation that vascular endothelial cells can alter their gene expression as a regulatory response to injury [Bach et al., 1997b]. Endothelial cells, which normally react to antibodies and complement by generating a pro-inflammatory response, may react differently when accommodation is achieved. They may upregulate the expression of a series of anti-apoptotic genes (A20, Bcl-x_L and Bcl2), these genes can in turn block activation of NF-κB and thereby suppress induction of pro-inflammatory genes associated with endothelial cell activation [Bach et al., 1997a; Cooper et al., 1996; Badrichani and Ferran, C., 2001]. After ABO-incompatible haematopoietic stem cell transplantation, accommodation also occurs and could be due to complement regulatory proteins on the endothelial cell surface preventing activation despite the presence of sufficient numbers of antibodies [Stussi et al., 2006a].

3.4.5. Endothelial Cell Chimerism

Endothelial cell chimerism in transplanted organs is indicative for a potential mechanism by which recipient cells replace the donor endothelium. It has been hypothesised that this replacement could lead to a decrease in alloreactivity and thus would positively influence graft outcome [Medawar, 1965]. Exchanging donor endothelial cells with recipient endothelial cells may be an important "camouflage" mechanism to induce transplantation tolerance in solid organ transplantation. Nevertheless, a number of studies have shown using fluorescence in-situ hybridisation (FISH) or PCR to identify the Y chromosomes in gender-mismatched situations, that the amount of recipient-derived endothelial cells found in donor organs is relatively small. What effect on graft survival can we expect from such small numbers of chimeric endothelial cells? Can a chimerism at low levels be associated with allograft acceptance or rejection? The pathogenesis of vascular rejection is still poorly understood. Maybe, the consequence of intimal damage of graft endothelium is leading to an endothelial cell replacement by recipient type and/or transplant arteriosclerosis. Interestingly,

endothelial cell replacement and transplant arteriosclerosis does not occur after cyclosporine treatment [Hillebrands et al., 2001]. It has been shown in an animal models, that chronic rejection, also termed as graft arteriopathy, could be prevented by intensive immunosuppression by prohibiting the initial (vascular) acute rejection [Hayry, 1998]. Once the initial trauma has occured, there seems to be a „point of no return" and the process becomes self-sustaining and independent. Currently, there is no successful treatment of chronic graft arteriopathy. In conclusion, there are several lines of evidence that recipient type endothelial cells can replace damaged endothelial cells after solid organ transplantation. But the source of the recipient type endothelial cells replacing damaged graft vessels remains obscure.

- **3.4.5.1. Endothelial Cell Chimerism after Solid Organ Transplantation**

It has been shown by several research groups, that a small number of endothelial cells is replaced by recipient-derived endothelial cells after solid organ transplantation [Lagaaij et al., 2001; Quaini et al., 2002; Hove et al., 2003a; Tanaka et al., 2005]. Some reports even concluded a correlation to the grade of rejection. In one case report, accidental heart transplantation across the ABO blood group barrier resulted in a complete change to recipient-type ABH histo-blood group expressing endothelial cells, suggesting a full endothelial cell chimerism [Koestner et al., 2004a]. We analysed the endothelial cell chimerism after ABO-incompatible kidney transplantation by immunohistochemical staining of ABH histo-blood group antigens. In contrast to the results obtained by Lagaaij et al. we did not observe any recipient-derived cells in the endothelium of transplanted kidneys in 4 patients so far (Figure 7, own unpublished results).

Furthermore, in a cohort study the role of liver transplantation in the treatment of hereditary haemorrhagic teleangiectasia (HHT), was analysed. HHT is a systemic vascular dysplasia inherited as an autosomal dominant disease. Hepatic involvement is clinically evident in 8-31% of patients and orthotopic liver transplantation appears to be the optimum therapy for HHT with significant liver involvement. Relapse of HHT in transplanted livers was reported in several cases, indicating a renewed vascularisation by host endothelial cells [Lerut et al., 2006]. This study is particularly intersting, because here a possible endothelial cell chimerism was concluded by clinical parameters in contrast to the numerous reports analysing grafted organs for the presence of recipient-derived cells and many of these studies using FISH or molecular analysis to determine endothelial cell chimerism suffered from technical difficulties.

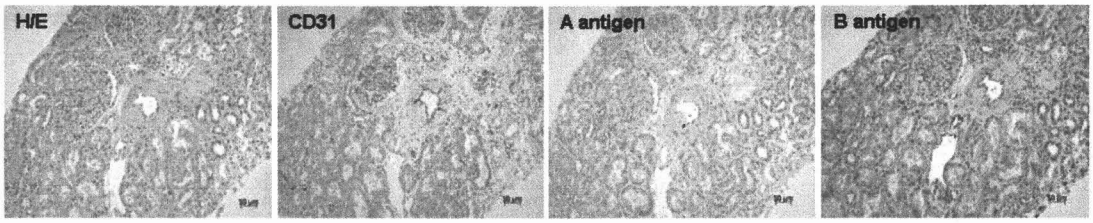

Figure 7: 1-year follow-up biopsy of an ABO-incompatible transplanted kidney. Transplantation performed at the University Hospital Zürich. Recipient of blood group type A. Donor kidney of blood group type B. Vascular endothelial cells in the biopsy show donor-specific B antigen.

- **3.4.5.2. Endothelial Cell Chimerism after Stem Cell Transplantation**

Only sparce and very controversial data is available on endothelial cell chimerism following allogeneic haematopoietic stem cell transplantation. Donor bone marrow-derived progenitor cells theoretically would have the capability to replace recipient endothelium and by this diminish GvHD. In line with this theory, donor-type endothelial chimerism has been described in the endothelium of skin, gut, heart, liver and bone marrow tissue after haematopoietic stem cell transplantation [Alison et al., 2000; Bittmann et al., 2003; Korbling et al., 2002; Thiele et al., 2004b; Jiang et al., 2004; Murata et al., 2007]. In contrast, no evidence for donor endothelial cell chimerism was found in gastrointestinal epithelium [Meignin et al., 2004] and in bone marrow [Athanasou et al., 1990] from patients receiving haematopoietic stem cells. In a rat bone marrow transplantation study, only 1-3% of the endothelial cells were originating from donor-bone marrow. This was measured in rats showing complete, haematopoietic peripheral bone marrow-donor chimerism (>98%) 3 months after transplantation [Hillebrands et al., 2002b]. Furthermore, very controversial results were published in animal models [Bailey et al., 2004; Ziegelhoeffer et al., 2004]. Recent studies have convincingly demonstrated that adult bone marrow contains cells, that are capable to differentiate into a variety of cell types. Haematopoietic stem cells have been described to differentiate into neurons and astrocytes [Mezey et al., 2000], into hepatocytes [Korbling et al., 2002], into buccal mucosa [Thiede et al., 2000] and into heart and muscle [Orlic et al., 2001; Ferrari et al., 1998; Palermo et al., 2005]. Donor haematopoietic stem cells have also been reported to contribute to the formation of secondary tumours after haematopoietic stem cell transplantation [Houghton et al., 2004; Soldini et al., 2008]. The potential ability of stem cells to differentiate across lineage barriers has been denominated

"plasticity" and refers specifically to transdifferentiation. The plasticity of cells belonging to the haematopoietic lineage into non-haematopoietic tissue was extensively studied and confirmed in an increasing amount of reports [Rovo and Gratwohl, A., 2008]. However, the concept of plasticity has been questioned by studies suggesting, that stem cells might fuse with other cells and mimic the appearance of differentiation [Wang et al., 2003; Vassilopoulos et al., 2003].

4. Objective of this Thesis

The overarching objective of the present study was to understand the mechanisms of tolerance induction after haematopoietic stem cell transplantation. Hereby, the focus lies on ABO-incompatible haematopoietic stem cell transplantation, that serves as *in vivo* model to study the effect of antigen-mismatched transplantation. The expression of ABH histo-blood group antigens on haematopoietic progenitor cell has poorly been described and most of the studies date back to the early 1960s. Therefore, evaluation of the kinetics of ABH histo-blood group antigen expression during erythropoiesis is an important point of investigation as well. Furthermore, the plasticity of bone marrow-derived stem cells and the contribution of bone marrow-derived endothelial cells for *in vivo* blood vessel formation is still debated with very controverse opinions in the scientific community and deserves a profound analysis before bone marrow-derived stem and progenitor cells are started to be used for clinical therapeutic approaches in regeneration medicine.

Aim #1: Establishment of methods to study ABH histo-blood group antigen expression during erythropoiesis and endothelial cell chimerism after haematopoietic stem cell transplantation. This includes i) immunohistochemistry of ABH histo-blood group antigens, ii) in-situ hybridisation for X and Y chromosomes and iii) molecular analysis of highly polymorphic short tandem repeats in DNA isolated from single vascular endothelial cells. Furthermore, analysis of possible mechanisms responsible for B cell tolerance after ABO-incompatible haematopoietic stem cell transplantation were addressed by flow cytometry (FACS) and enzyme-linked immunospot technique (ELISPOT).

Aim #2: Analysis of endothelial cell biology after allogeneic ABO- or gender-mismatched haematopoietic stem cell transplantation. Study of the *in vivo* development, engraftment and turn-over of bone marrow-derived endothelial progenitor cells (EPC) in bone marrow and in peripheral blood. Determination of the existence and degree of endothelial cell chimerism in skin biopsies, in bone marrow, in biopsies of secondary tumours and in autoptic tissue samples derived from patients who have undergone ABO- or gender-mismatched haematopoietic stem cell transplantation during the past 10 years at the University Hospitals of Zürich and Basel.

1.3. Aim #3: Investigation of B cell tolerance at different time points prior to and after minor ABO-incompatible haematopoietic stem cell transplantation by (1) measurement of the antibody production and (2) determination of anti-host A/B producing B cells *ex vivo* with regards to deletion or anergy by ELISPOT and FACS analysis. Measurement of the antibody and complement deposition on shock frozen skin biopsies.

The materials and methods used in this thesis are described in detail in each paper or manuscript. The experimental work is based on different *in vitro* models such as erythropoietic cell maturation, culturing of haematopoietic progenitor cells isolated from peripheral blood and bone marrow by CD133+ magnetic bead isolation (MACS, Milteny Biotec) and isolation of B cells from peripheral blood and donor-spleen tissue. A prospective study including 29 patients, that underwent haematopoietic stem cell transplantation at the University Hospital Zürich, has been conducted after approval by the local ethical committee (EK-951, Zürich 2003). Finally, various specimen derived from a total of 30 patients after haematopoietic stem cell transplantation at the University Hospital Zürich and Basel were analysed in a retrospective study. All patients gave written informed consent for this research study.

5. Results

5.1. PART I

Expression of ABH histo-blood group Antigens during Erythropoietic Stem Cell Differentiation

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Manuscript in preparation.

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Keywords: ABH histo-blood group antigen – erythropoiesis – erythroblast - pure red cell aplasia – bone marrow - methyl-methacrylate

5.1.1 Abstract

Background: ABH histo-blood group antigens (ABH Ag) are the immunological target for hyperacute rejection of solid organs transplanted across the ABO barrier and for pure red cell aplasia (PRCA) after ABO-incompatible haematopoietic stem cell transplantation (HSCT). To date, expression of ABH Ag during erythropoietic progenitor cells is only scarcely described. This study aims to determine the debut of ABH Ag during erythropoiesis and to elucidate the corresponding maturation stage of erythroblasts.

Materials and Methods: Haematopoietic CD34-positive progenitor cell were isolated from peripheral blood and cultured towards mature erythrocytes. Immunohistochemical examination was performed at different erythroblastic maturation stages. Furthermore, bone marrow aspiates and biopsies were obtained during routine bone marrow punction. Non-decalcified tissue specimens were embedded in methyl-methacrylate (Technovit 9100New) for the investigation of ABH Ag on erythropoietic cells. Cytospins from healthy blood donors of A, B or O blood group types served as controls.

Results: ABH Ag were present on all mature erythrocytes and on precursor cells with erythroid commitment onwards from the basophilic erythroblastic maturation stage. Haematopoietic and myeloid progenitor cells and pro-erythroblasts did not show ABH Ag. Morphological analysis of bone marrow from patients affected by PRCA revealed the existence of pre-erythroblasts but the lack of any other erythroblasts and erythrocytes.

Conclusion: Expression of ABH Ag on normal erythropoietic cells as early as the basophilic erythroblastic maturation stage and the eradication of all erythropoietic cells from bone marrow of patients with PRCA indicates a direct rejection mechanism of ABH Ag-expressing cells by anti-donor isoagglutinins in PRCA after major-incompatible HSCT.

5.1.2. Introduction

A, B and H histo-blood group antigens (ABO Ag) are not only found on erythrocytes but also other cell types such as epithelial and endothelial cells [Szulman, 1960; Ravn and Dabelsteen, E., 2000]. Expression of ABH histo-blood group antigens (ABH Ag) was found to be extensive on the cardiovascular endothelium and on epithelia of the integument, the digestive tube, the mesonephric and the Mullerian ducts during embryonal development from the 5th week after fertilisation. However, at about 9-weeks ovulation age, when the mucus-secreting apparatus begins to function, epithelial ABO Ag wane and only persist in the stratified epithelia of the integument, esophagus and lower urinary tract [Szulman, 1964a]. The strength of ABH Ag on red blood cells of the newborn ranges between 10% [Schenkel-Brunner, 1980] and 30% [Economidou et al., 1967] of adult values. A and B antigens sometimes reappear in maligancy in tissues from which they disappeared in early development. These antigens can also be classified as oncodevelopment antigens [Hakomori, 1999]. Expression of certain groups of antigens on the surface of haematopoietic cells changes either during the course of differentiation from pluripotent stem cells to mature functional cells or as a function of the proliferative state of the cells [Sieff, 1986]. *In vitro* studies revealed that antibodies to ABH Ag failed to inhibit the growth of progenitor cells committed to both granulocyte-macrophage (CFU-C) and erythroid (BFU-E) development [Hershko et al., 1980]. These findings indicate that ABH Ag are not operationally present on early haematopoietic stem cells [Hershko et al., 1980]. The pattern of antigen expression on haematopoietic progenitor and precursor populations has previously been described in bone marrow aspirates by flow cytometer (FACS) analysis [Sieff et al., 1982]. HLA-DR, defined by monoclonal anti-OKT10 antibody, is shown to be expressed on the earliest progenitors and lost during differentiation. HLA-DC1 is not expressed on progenitor cells. Transferrin receptor is expressed on the majority of erythroid progenitors, the bulk-forming units (BFU-E) and the colony-forming units (CFU-E), but only weakly on myeloid progenitors (CFU-GM). Expression of A antigen, defined by 2 monoclonal antibodies MAS 016 and N16, was shown to increase during differentiation from 7% on BFU-E to 38% on CFU-E cells [Sieff et al., 1982]. This result is correlating to the detection of A antigen using the specific lectin from Vicia cracca on cells of the erythroid series from the stage of basophilic erythroblasts forth (i.e. basophilic normoblast), pre-erythroblasts were negative [Karhi et al., 1981]. In contrast, another study using Helix pomatia and Dolichos biflorus lectins showed immunofluorescent labelling of the A antigen on the entire erythroid lineage, including pro-erythroblasts [Tulliez et al., 1987]. Finally, stem cells and progenitors were characterised with an increasing percentage of A and B antigen

expression by FACS sorting during cell lineage development from 5% in erythroid colony-forming cells (CFU-GEMM) to 49% BFU-E to 83% in CFU-E [Blacklock et al., 1984].

A and B antigens are oligosaccharide antigens synthesised through a series of enzymatic reactions, of which the final steps are catalysed by A and B glycosyltransferases, the primary products of A and B genes, respectively [Yamamoto, 2001]. The fucosylated H determinant forms part of the A and B blood group determinants and A and B antigens differ only with respect to the terminal sugar: in the case of the A determinant the immunodominant sugar is N-acetyl-D-galactosamine and in the case of the B determinant it is D-galactose [Greenwell, 1997]. In contrast to solid organ transplantation, ABH Ag do not represent a significant clinical barrier in allogeneic haematopoietic stem cell transplantation (HSCT). ABO-incompatible HSCT is routinely performed in a third of the patients without affecting overall survival and graft rejection in comparison to ABO-matched HSCT [Seebach et al., 2005]. Furthermore, ABH Ag are not clinically important targets in graft-versus-host-disease in HSCT [Gale et al., 1977]. Bone marrow cellularity is usually restored to normal after approximately one month after HSCT [Thiele et al., 2001]. After 3 months, T and B cells are present in normal numbers in the bone marrow and in the peripheral blood. However the quality and function of the lymphoid cells still remains underdeveloped, resulting in frequent impairment of immune reconstitution [Schulte and Beelen, D. W., 2005]. Furthermore, it is believed that persistent anti-donor isoagglutinins after major (i.e. A , B or AB in O) and bidirectional (i.e. A in B or B in A) ABO-incompatible HSCT may cause delayed red blood cell engraftment and posttransplant pure red cell aplasia (PRCA) [Griffith et al., 2005]. Posttransplant PRCA is characterised by the absence of erythroid precursor cells in the bone marrow, a lack of reticulocytes in the peripheral blood and often requires red blood cell transfusions for a prolonged period of time.

Therefore, the aim of this study was 1) to determine from which maturation stage of erythropoietic development ABH Ag are expressed and 2) to compare whether ABH Ag-expressing cells are prerequisitely eradicated in PRCA.

5.1.3. Materials and Methods

Mature erythrocytes

Fresh blood of blood group type A, B and O was obtained from healthy donors through the local blood bank. Red blood cells were fixed in ice cold Karnovsky buffer (Glutaraldehyde 25%, formaldehyde 36 %, pH 7.4) for 30 minutes at 4°C. Then washed once in 6% bovine serum albumin (BSA) diluted in phosphate buffered saline (PBS) and once in 0.6% BSA. After two additional washes in PBS, 4 blood smears for each blood group type were made on Superfrost glass slides (Menzel, Braunschweig, Germany) and dried at room temperature overnight.

In vitro cultured erythroblasts

Peripheral blood was obtained from totally six healthy donors with blood group type A, B and O after approval by the institutional Committee for Human Studies. CD34+ hematopoietic progenitor cells cells were purified by using a midi-MACS immunomagnetic separation system (Miltenyi Biotec) and directed into erythroid cell differentiation in culture as previously described [Zeuner et al., 2006; De Maria et al., 1999]. Serum-free medium was supplemented with 0.01 U/mL IL-3, 0.001 ng/mL GM-CSF, and 3 U/mL EPO (standard erythroid medium) to induce unilineage erythroid differentiation. Cytospins of 20'000 cells each were made at day 0, 3, 5, 6, 7, 9, 12, 15 and 18 of erythropoietic cell differentiation. The differentiation stage of erythroid precursor cells was evaluated by May-Grünwald-Giemsa staining and cytologic analysis.

Bone marrow aspirates

Bone marrow cells from a patient, who underwent major ABO-incompatible HSCT (i.e. A in O) were obtained at autopsy 102 days after HSCT. The cells were filtered by the use of a cell strainer (BD Falcon, San Jose, USA), washed in PBS and fixed in 4 % formalin for 20 hours. Followingly, cytospins with 20'000 cells each were prepared onto Superfrost slides and stored at 4°C until further processing. Cell morphology was analysed with a standard staining by the method of Pappenheim.

Bone marrow biopsies

Solid bone marrow biopsies were obtained by trephine needle biopsy during routine marrow punction from 6 patients before and after undertaking allogeneic hematopoietic stem cell transplantation. Two patients were transplanted ABO-compatible (A in A) and 4 patients received haematopoietic from an ABO-incompatible donor (B in O, n=1; O in A, n=1; A in O, n=2). Biopsies were fixed in 4% formalin for less than 24 hours and embedded in methyl-methacrylate (Technovit 9100New, Haeraeus Kulzer, Germany) without prior decalcification. Embedding was performed according to a previously published protocol [Yang et al., 2003; Mueller and Richards, R. G., 2004]. In brief, bone marrow biopsies were dehydrated in Xylene and gradually perfused thoroughly with methyl-methacrylate. Polymerisation of the plastic is achieved at a temperature of -20°C. Afterwards the blocks were store at room temperature until further use. Solid bone marrow sections were cut 4 mm thick with a microtome using a tungsten carbide-coated Histo d knife (Medite, Nunningen, Switzerland). In order to obtain good attachment to the used Superfrost slides (Menzel, Braunschweig, Germany), the tissue sections were flattened in 70% ethanol and pressed onto the slides with a polyethylene foil and a piece of filter paper and incubated at 37°C overnight. For morphological and immunohistochemical staining, the sections were de-plastified in 2-methoxyethyl-acetat (Merck, Darmstadt, Germany) two times 30 minutes and re-hydrated in a graded ethanol row (absolute, 96%, 80%, 70%, distilled water).

Immunohistochemical staining of ABH histo-blood group antigens

Immunohistochemical staining of mature erythrocytes, developing haematopoietic progenitor cells, bone marrow cell cytospins and non-decalcified solid bone marrow sections was performed according to a previously described protocol [Mueller et al., 2006]. Monoclonal anti-A, B and H antibodies (Dako, Carpintera CA, USA) were used for all analyses. Anti-A, clone 81FR2.2 was used in a dilution of 1:50 in PBS, anti-B, clone 3E7 in a dilution of 1:15 in PBS, and anti-H, clone 92FR-A2 in a dilution of 1:15 in PBS. Antibody binding was visualised with a secondary biotin-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) followed by immunoperoxidase staining. All slides were counterstained for 2-4 minutes in standard acidic hemalaun solution and cover slipped with aqueous mounting medium (Faramount, Dako, Glostrup, Denmark). The morphology of the tissues was analysed with an additional haematoxylin/eosin, Giemsa or standard Pappenheim staining.

5.1.4. Results

ABH histo-blood group antigen expression on normal erythrocytes

ABH Ag on standard blood smears of healthy, mature red blood cells confirmed a strong staining specificity of the monoclonal anti-ABH antibodies used in this study (Figure 1). Interestingly, the blood group antigen pattern on erythrocytes appeared to be very heterogeneous. ABH Ag were not expressed evenly on all erythrocytes. A antigen was present on 49 % of all cells, B antigen on 45 %, and H antigen only on 15 % of the erythrocytes.

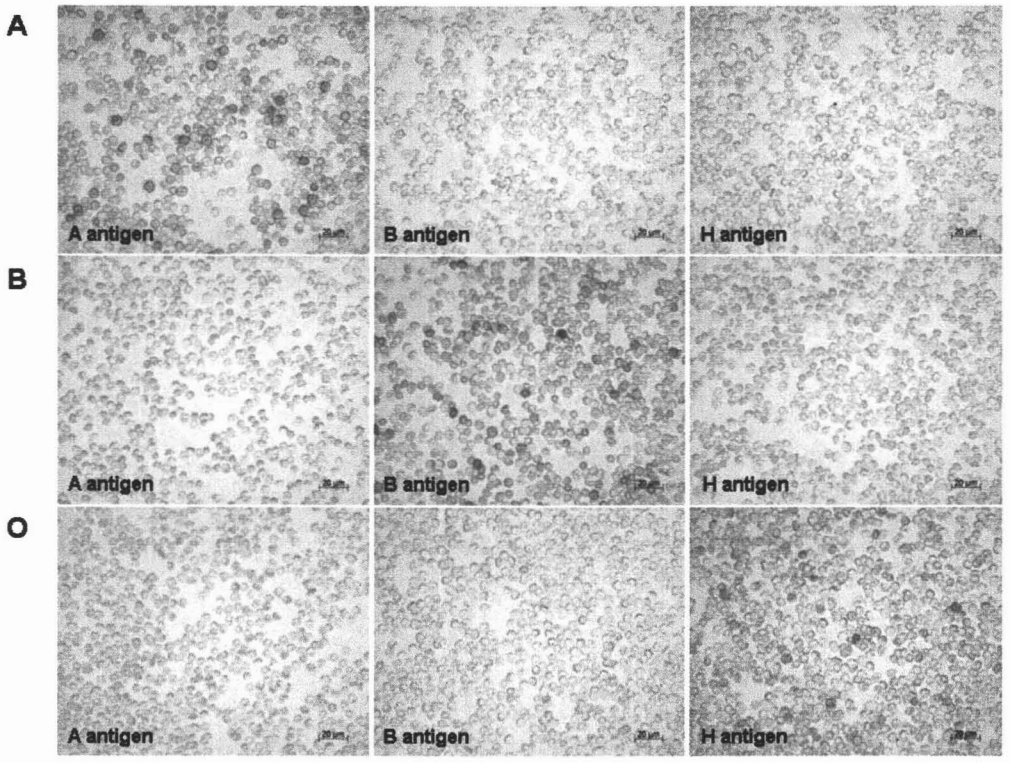

Figure 1: Specificity of the monoclonal anti-ABH antibodies. Fresh blood smear from donros with blood group type A (A), B (B) and O (O).

ABH histo-blood group antigen expression during *in vitro* erythropoiesis

A antigen expression debuted on developing eythroblasts on day 3 of *in vitro* culturing (Figure 2). Morphological analysis of the cells showed, that the earliest expression of A, B and H antigen correlated to the differentiation stage of basophilic erythroblasts (i.e. basophilic normoblasts). Figure 2c shows the percentage of A antigen-expressing cells during *in vitro*

erythropoiesis. Both, A and B antigen were very strongly expressed as from day 6 of *in vitro* differentiation on 30% - 40% of the cells. A antigen increased up to 84% on day 7 and 96% on day 9. However, B and H antigen expression stayed on a lower detection level than A antigen expression (average 30% and 11% respectively) during the whole period of investiagtion. On day 18 all cells reached full erythropoietic maturation and A antigen was present on 61%, B antigen on 20% of all erythrocytes.

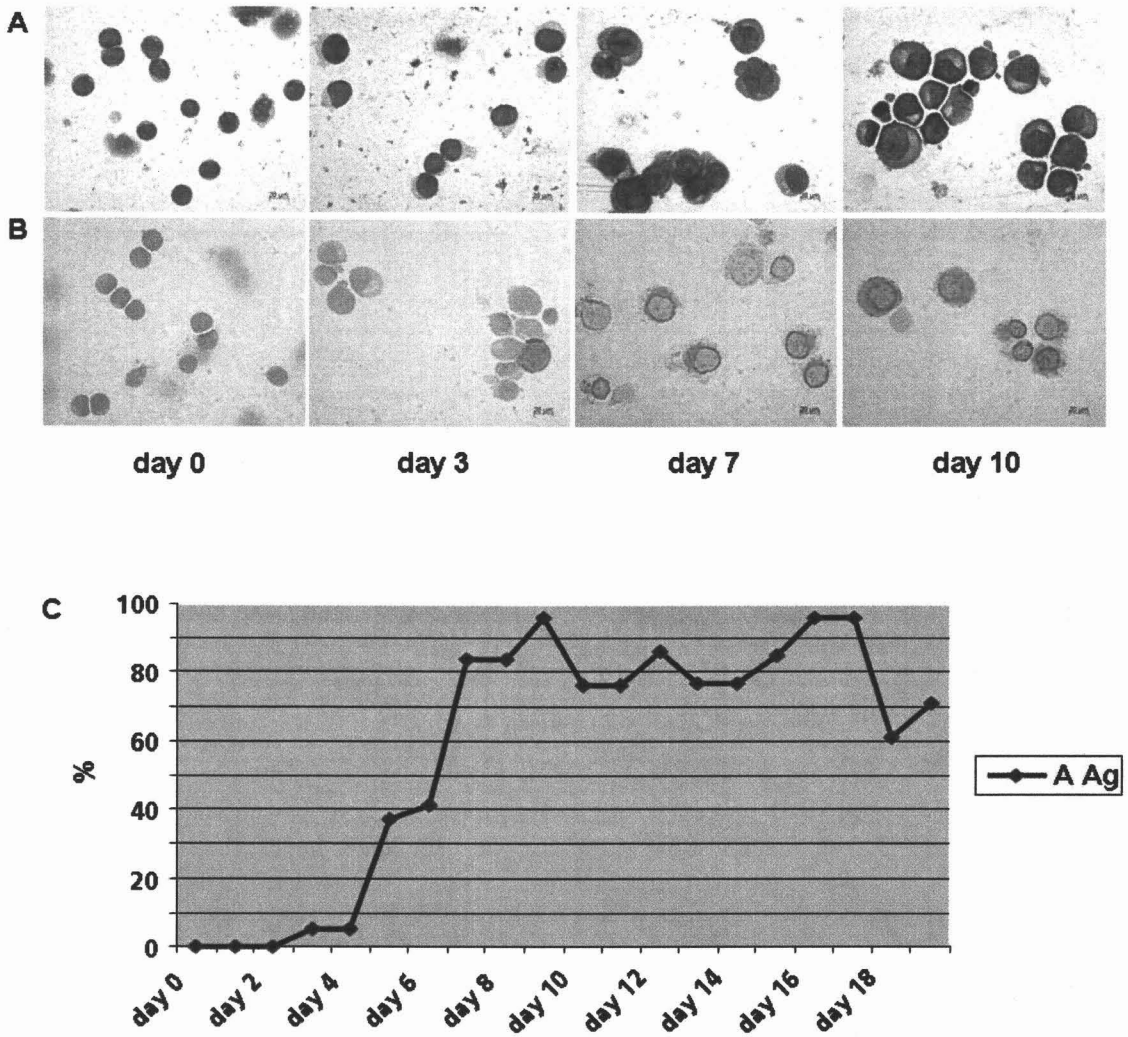

Figure 2: A antigen expression during *in vitro* erythropoietic cell maturation. Erythroblasts collected on cytospins on day 0, 3, 7 and 10 of *in vitro* erythropoiesis. Cytospins are stained for Giemsa (A) and for A antigen (B). Quantification of erythroblasts stained positively for A antigen indicated in percent of the total amount of erythroblasts on the corresponding cytospin (C).

ABH histo-blood group antigen on bone marrow erythroblasts

In line with the ABH Ag presence on cultured erythropoietic cells, the same erythropoietic maturation stages were detectable in bone marrow cytospins and biopsies of patients after they had undergone HSCT. Erythroblasts of different differentiation stages accumulated in erythroblastic islands were shown in the bone marrow as early as 102 days after ABO-incompatible HSCT (Figure 3). Generally, erythroblasts could easily be identified by the donor-specific blood group antigen staining (i.e. A antigen in Figure 3b). Neighbouring granulocytic cell islands did not show ABH Ag staining. Methyl-methacrylate appeared to be a good embedding solution for morphological techniques on non-decalcified bone marrow and cells of all maturation stages could be identified successfully by Giemsa. ABH Ag were strongly expressed in all investigated bone marrow biopsies. ABH Ag were detected on endothelial cells of all bone marrow blood vessels and on a variety of adult and progenitor cells. In line, megakaryocytes and thrombocytes were strongly positive for ABH Ag. On the other hand, all cells of the myelopoietic maturation, such as basophil granulocytes, monocytes and macrophages and all lymphocytes were shown to be negative fo ABH Ag.

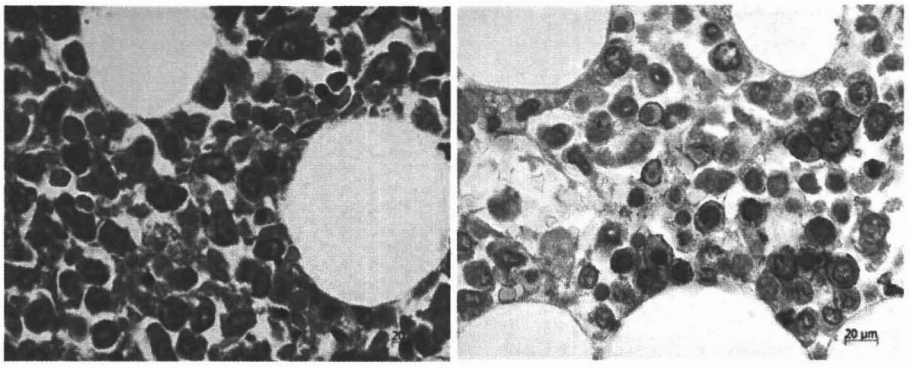

Figure 3: Bone marrow biopsy of a 60-year-old patient after ABO-incompatible haematopoietic stem cell transplantation. Transplantation performed at the University Hospital Zürich. Recipient of blood group type O. Donor of blood group type A. Erythropoietic cells in the biopsy show donor-specific B antigen. Pappenheim staining (A), A antigen staining (B).

5.1.5. Discussion

A and B histo-blood group antigens were detected on basophil eythroblasts and all following cell maturation stages during *in vitro* and *ex vivo* erythropoiesis in this study. *In vitro*, basophilic erythroblasts strikingly revealed the highest expression of A and B antigens, whereas the later differentiation stages polychromatic and orthochromatic erythroblasts showed a weaker expression. This can be explained by the loss of antigen due to increased cell apoptosis during prolonged cell culturing, but also may reflect results from two earlier reports analysing freshly isolated bone marrow cells. Yunis et al. found by direct agglutination assay with anti-A antibodies, that averaging 1% pro-erythroblasts, 10% basophilic erythroblasts and 48% polychromatic erythroblasts agglutinated, but further in the differentiation course only 40% orthochromatic erythroblasts agglutinated [Yunis and Yunis, E. J., 1963]. Karhi et al. confirmed this finding by showing a stronger staining of polychromatic erythroblasts than on adult erythrocytes with the blood group A-specific lectin from Vicia cracca [Karhi et al., 1981].

H histo-blood group antigen was also detectable from the basophilic erythroblastic stage on but its expression was much weaker than the A and B antigen. This finding may reflect a certain detection limit of the monoclonal antibodies used for immunohistochemistry or as well a lower immunogeneicity of H antigen despite a known presence of more ABH antigen sites on the surface of adult O blood-type erythrocytes in comparison with A and B blood-type erythrocytes [Mollison PL et al., 1987]. Since the H antigen forms the core structure of A and B antigens, it is very likely that it is present at the same time of A and B antigen expression. This is supported by reports demonstrating, that the H antigen type 2 (CD173) is expressed already on native CD34-positive haematopoietic progenitors (i.e. on erythroblasts until the orthochromatic differentiation stage) [Cao et al., 2001; Yunis and Yunis, E. J., 1963].

Expression of ABH Ag on mature erythrocytes appears to be very heterogeneous, with many cells staining weakly or not at all. Heterogeneous appearance of blood group antigens on erythrocytes has been described previously [Rochant et al., 1980]. But the underlying cause of this heterogeneity can only be hypothesised: it might be correlated to malignancy or the natural age of these mature cells. In studies of ABH Ag expression during *in vitro* megakaryocyte maturation, it has been hypothesised that this heterogeneous appearance of ABH Ag might be determined according to different colonies during cell proliferation [Dunstan, 1986]. This could indicate a similar mechanism in erythropoietic cell differentiation.

Bone marrow biopsies from patients who had normal cell engraftment after major ABO-incompatible HSCT were used in this study. For the analysis of ABH Ag expression in healthy bone marrow, samples between 97 and 296 days after the transplantation were used. Pre-transplant biopsies were excluded because leukaemia and chemotherapy can influence physiological differentiation of cells in the bone marrow [Zeuner et al., 2007]. In contrast to cells of the erythroblastic and megakaryocytic lineages, lymphocytes and their progenitors did not express ABH Ag. This result is in line with earlier reports, that concluded an intrinsic ABH Ag expression on erythrocytes and thrombocytes under control of the H gene, but not on lymphocytes [Mollicone, Blood 1988] or neutrophils [Dunstan et al., 1985; Dunstan, 1986]. In a study using the same monoclonal antibodies (Dako, Carpentaria CA, USA) it has been shown that decalcification does not have adverse effects on immunohistochemical expression of the A and B Ag in B5-fixed, paraffin-embedded bone marrow tissue sections, but may nullify the expression of H Ag [Adegboyega and Gokhale, S., 2003]. In our study, H Ag could be displayed strongly in several tissue sections derived from donors with O-blood group type. We therefore recommend the use of hard plastic, such as methyl-methacrylate for embedding of bone marrow biopsies for downstream analysis by immunohistochemistry.

We hypothesise, that myeloid cells and pro-erythroblasts are not harmed in PRCA after major-ABO-incompatible HSCT due to the fact that these cells do not express ABH Ag and by this are not attacked by anti-donor isoagglutinins. It was previously reported, that some blood group components may have clinical implications for immune-mediated disorders causing PRCA in recipients of incompatible haematopoietic stem cells [Bony et al., 1999].
In conclusion, in this study ABH Ag were detected on cell surfaces during erythropoiesis from the early erythroblastic differentiation stage on. This finding is crucial for future clinical approaches using stem cells for therapeutic purposes. It is important to carefully exclude mismatched settings using progenitor cells in patients and to respect the same laws as routinely applied in transfusion medicine.

5.1.6. Acknowledgements

We would like to thank Regula Rüegg for the excellent help with the morphological anlysis.

5.2. PART II

Major ABO-incompatible Haematopoietic Stem Cell Transplantation: Study of post-transplant Pure Red Cell Aplasia and Endothelial Cell Chimerism

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Keywords: ABO blood group – chimerism – endothelial cells – haematopoietic stem cell transplantation – isoagglutinins – pure red cell aplasia.

Abbreviations used in this paper: CISH, chromogen in-situ hybridisation; EC, endothelial cells; FISH, fluorescence in-situ hybridisation; GVHD, graft-versus-host reaction; HSCT, haematopoietic stem cell transplantation; PCR, polymerase chain reaction; PRCA pure red cell aplasia

5.2.1. Abstract

Background: In contrast to HLA matching, ABO-blood group incompatibility plays a minor role for the success of allogeneic haematopoietic stem cell transplantation (HSCT). Incompatible ABH histo-blood group antigens, expressed on recipient endothelial cells (EC) and donor erythroid progenitor cells, may represent targets for graft-versus-host disease (GvHD) and host-versus-graft reactions, respectively. The aims of the current study were to investigate: (1) red blood cell engraftment and (2) EC chimerism as a potential result of replacement of recipient EC by donor bone marrow-derived EC in a patient following major ABO-incompatible (A to O) and gender-mismatched HSCT who died at day 350 of severe acute GvHD.

Methods: Blood counts and anti-A/B isoagglutinin titres were analysed repeatedly. Heart and bone marrow specimens were obtained at autopsy. The expression of ABH histo-blood group antigens was examined by immunhistochemistry, X/Y chromosomes were detected by chromogen in situ hybridisation (CISH).

Results: Red blood cell engraftment defined as appearance of 1% reticulocytes in the peripheral blood was delayed and correlated with anti-donor isoagglutinin titres. Circulating haematopoietic cells were exclusively of donor origin demonstrating full donor haematopoietic chimerism, whereas EC in heart and bone-marrow blood vessels were exclusively of recipient-type.

Conclusions: Pure red cell aplasia after major ABO-incompatible HSCT was caused by anti-A/B isoagglutinins produced by recipient-type plasma cells. Using ABO- and gender-mismatch for discrimination, heart and bone marrow blood vessels demonstrated no evidence for EC chimerism 11 months after ABO incompatible HSCT. These findings suggest that EC replacement and chimerism do not represent major mechanisms responsible for tolerance-induction after HSCT.

5.2.2. Introduction

ABO-incompatibility, in contrast to HLA matching, seems to play a minor role in HSCT. Under special consideration of pretransplant isoagglutinin removal and post-transplant transfusion policies the outcome including survival of ABO-incompatible transplanted patients is not affected [Seebach et al., 2005], possibly with the exception of bidirectional ABO-incompatibility (A to B, B to A). Therefore, HSCT donors are selected primarily according to HLA type, which is inherited independently from the ABO-blood group system. Consequently, about 30% of all HSCT are ABO-incompatible [Seebach et al., 2005].

Major, including bidirectional, ABO incompatibility is defined by the presence of anti-donor isoagglutinins in the recipient. To avoid immediate post-transplant haemolysis, donor red blood cells (RBC) are removed from the graft or anti-donor isoagglutinins titres are reduced in the recipient [Lasky et al., 1983; Warkentin et al., 1985; Nussbaumer et al., 1995]. However, there is still a risk for delayed RBC engraftment and post-transplant pure red cell aplasia (PRCA) characterised by the absence of RBC precursors in the bone marrow and the lack of reticulocytes in the peripheral blood [Gmür et al., 1990; Sniecinski et al., 1988]. The reason(s) for the occurrence of PRCA after some major ABO incompatible HSCT is not fully understood, possibly donor RBC precursors are destroyed by anti-donor isoagglutinins produced by persisting host B or plasma cells.

ABH histo-blood group antigens are not only expressed on RBC but also on other cell types including endothelial cells (EC) [Ravn and Dabelsteen, E., 2000; Le Pendu et al., 1988]. The latter represent an important interface between donor and recipient after transplantation and therefore may elicit graft-versus-host-reactions (GvHD). Hypothetically, following such a graft-versus-endothelium reaction leading to endothelial damage, recipient EC might be replaced by bone marrow-derived donor endothelial progenitor cells. Whereas full haematopoietic chimerism is expected after HSCT using standard (myeloablative) conditioning regimens, the potential occurrence of EC chimerism following HSCT has not been elucidated. Moreover, EC chimerism may represent a mechanism leading to tolerance after HSCT as well as solid organ transplantation. This notion was supported by the observation of EC chimerism following solid organ transplantation both in animal models as well as in clinical transplantation [Hove et al., 2003b; Lagaaij et al., 2001; Quaini et al., 2002; Gao et al., 2001; Koestner et al., 2004b; Tanaka et al., 2005]. However the issue remains controversial since several other authors failed to demonstrate recipient-type EC chimerism in transplanted organs [Fogt et al., 2002b; Hillebrands et al., 2000; Hruban et al., 1993a].

Only limited data is available on EC chimerism following allogeneic HSCT. In analogy to solid organ transplantation, bone marrow-derived donor EC may replace damaged recipient endothelium as well as normal endothelium as part of the physiological turnover. In support of this hypothesis donor-type EC chimerism has been described in heart, liver and bone marrow tissue following HSCT [Alison et al., 2000; Korbling et al., 2002; Thiele et al., 2004b]. In contrast, no evidence for donor chimerism was found in gastrointestinal epithelium and controversial results were recently published in animal models [Meignin et al., 2004; Bailey et al., 2004; Ziegelhoeffer et al., 2004]. The observed discrepancies might be explained by different detection methods, pretransplant male chimerism in female recipients after giving birth to a male child [Bianchi et al., 1996], and physiologic differences in the extent of chimerism in various organs and cell types. Currently, chimerism is assessed by fluorescence or chromogen in situ hybridisation (FISH/CISH) techniques aimed at the detection of X/Y chromosomes in gender-mismatched situations [Ferrand et al., 2003; Bader et al., 2005]. A major limitation of these methods is that they are time-consuming and only applicable for a subset of patients. The advent of PCR techniques in clinical practice allows the determination of molecular DNA polymorphisms by means of restricted fragment-lengths, however, this method has not been applied for the detection of EC chimerism so far. Alternatively, variable numbers of tandem repeat markers can be amplified to establish haematopoietic chimerism after HSCT and new real-time quantitative PCR amplification methods for chimerism analysis in the peripheral blood have been described recently [Alizadeh et al., 2002]. In contrast, few studies used immunohistological staining of ABH antigens as an alternative approach to analyse EC chimerism after ABO-incompatible HSCT.

The aims of the current study were therefore (1) to correlate the occurrence of PRCA with anti-donor A/B isoagglutinin titres and (2) to establish two different methods to study EC chimerism in a patient after major ABO-incompatible and gender–mismatched HSCT.

5.2.3. Materials and Methods

Laboratory evaluation

During hospitalisation haemoglobin was determined daily, reticulocytes counts were performed at least twice weekly. After discharge these values were assessed weekly. ABO-blood groups and isoagglutinin titres were determined by standard methods. Post-transplant PRCA was defined as reticulocytopenia (<1%) in the peripheral blood for more than 100 days

with normal white blood cell and platelets counts. RBC engraftment was documented by the appearance of 1% reticulocytes in the peripheral blood.

Immunohistochemistry for ABH histo-blood group antigens

Tissue samples were fixed in 4% formalin and embedded in paraffin. Sections of 4 µm were placed onto Superfrost glass slides (Menzel-Gläser, Germany, Prod. No. 041300). Bone marrow was decalcified with EDTA prior to paraffin embedding. Following deparaffinization the sections were incubated in methanol containing 0.3% H2O2 for 20 minutes in order to bind endogenous peroxidase. The specimens were then incubated with monoclonal mouse anti-blood group A/B/H-antigen primary antibodies (Dako, Carpintera CA, USA). For antigen A, clone 81FR2.2 was used in a dilution of 1:50 in PBS, for antigen B, clone 3E7 in a dilution of 1:15 in PBS, and for antigen H, clone 92FR-A2 in a dilution of 1:50 in PBS for 1 hour in a humidified chamber. After 6 washes with PBS, the sections were stained for 45 minutes with a secondary biotin-conjugated rabbit anti-mouse antibody diluted 1:200 in PBS (DakoCytomation, Glostrup, Denmark, Code No. E0354, Lot No. 042). The slides were then washed 6 times in PBS and incubated for additional 30 minutes with avidin-biotin ABComplex/HRP (DakoCytomation, Glostrup, Denmark, Code No. K 0377, Lot No. 101). For detection 45 µl amino-ethyl-carbazole (AEC+) substrate-chromogen was placed onto each tissue section after additional 6 washes with PBS. Colour development was observed under a bright field microscope and stopped in PBS after an average of 2-3 min. Finally, the slides were washed in distilled water, counterstained for 2-4 min in standard acidic haemalaun solution and mounted with aqueous Faramount mounting medium (DakoCytomation, Glostrup, Denmark, Code No. S3025).

All additional immunohistochemical stainings shown in this study were performed using the Ventana benchmark automated staining system (Ventana Medicals, Tucson, Arizona, USA). CD31 (platelet/endothelial cell adhesion molecule, PECAM-1) was detected using a monoclonal mouse anti-human CD31 primary antibody (Dako, Glostrup, Denmark, Product No. M0823, Lot No. 079) diluted 1:10, CD34 using a monoclonal mouse anti-human CD34 primary antibody (Serotec Ltd, UK, Clone No. QBEND 10, Batch No. 130900), diluted 1:20 and von Willebrand factor by a polyclonal rabbit anti-human von Willebrand factor (FVIII) primary antibody (Dako, Glostrup, Denmark, Product No. A0082, Lot No. 096) diluted 1:1000 in Ventana buffer. To rule out the possibility of donor leukocytes located within or close to the endothelial wall lining, staining of the pan-leukocyte marker CD45 was

performed in parallel to every ABH histo-blood group antigen staining on sequential sections. For morphological analysis a standard haematoxylin/eosin staining was used.

Chromogen in situ hybridisation (CISH)

X and Y chromosomes of interphase nuclei were stained by a double-stranded DNA probe containing a digoxygenin-labeled chromosome X centromere probe and a biotinylated chromosome Yq probe (SPOT-Light Probe Cocktail, Zymed Laboratories Inc., San Francisco CA, USA). Alkaline phosphatase (AP)/ Fast Red was used to detect the digoxygenin-labeled X probe and horseradish peroxidase diaminobenzidine (HRP/DAB) to detect the biotinylated Yq probe (SPOT-light CISH detection kit, Zymed Laboratories Inc., San Francisco CA, USA). After staining of the sex chromosomes, the samples were counterstained with standard haematoxylin for maximally 6 seconds. Male and female skin tissue sections were always analyzed in parallel as positive control. Evaluation was performed by light microscopy.

Quantification

Tissue sections were analysed by counting all A, B or H histo-blood group antigens positive EC located in cross-sectioned blood vessel displaying a clearly recognizable cell nucleus. Secondarily, the number of cross-sectioned blood vessels on each section was counted and used for normalization of the sections with different sizes by calculating ratios of the number of positive EC per blood vessel. For the analysis of the CISH method, only EC which displayed a clear signal of two chromosomes in their nucleus, i.e. X and X for female or X and Y for male. Cells showing only one of the sex chromosomes by in situ hybridization due to the fact that the nucleus has been cross-sectioned, were excluded from further analysis.

5.2.4. Results

Patient history and isoagglutinin titres

A 40-year-old male patient with a low grade B-cell non-Hodgkin lymphoma had progressive disease after having achieved a partial remission using conventional radiochemotherapy. Following reduced intensity conditioning with Fludarabin, Busulfan, and Antithymocyte Globulin (Flu/Bu/ATG-Fresenius) he received a major ABO-incompatible peripheral blood HSCT from his HLA-identical sister (A into O) with 6.45×10^6 CD34+cells/kg. Prompt engraftment occurred with PMN counts >500/ul on day 14 and platelet counts > 50'000/ul on day 15. The course of anti-donor isoagglutinin titers and the reticulocyte counts are shown in

Figure 1. The pretransplant anti-donor titre was 1:64 and was not reduced prior to transplantation. Cyclosporin A and a short course of methotrexate were given as GvHD prophylaxis. Initially, post-transplant anti-donor isoagglutinin titres decreased without haemolysis. RBC engraftment with increasing levels of reticulocytes was first detected 20 days after HSCT. However, 30 days after HSCT anti-donor isoagglutinin titres increased again. The emerging erythropoiesis was suppressed and the patient remained RBC transfusion-dependent. Several bone marrow specimens showed normal myelo- and megakaryopoiesis in the absence of erythroid precursors. Third party anti-B and anti-porcine antibody titres remained measurable after HSCT. Severe acute GVHD grade IV of the skin, liver and gut occurred on day 193 after withdrawal of immunosuppression. Strikingly, GvHD was associated with a rapid disappearance of anti-donor isoagglutinins.

Immunosuppression was restarted at a higher level due to severe GVHD. Consequently, invasive aspergillosis and colitis due to cytomegalovirus developed. One year after HSCT the patient died from infectious complications and GvHD without evidence of relapse. Heart- and bone marrow tissue was obtained at autopsy.

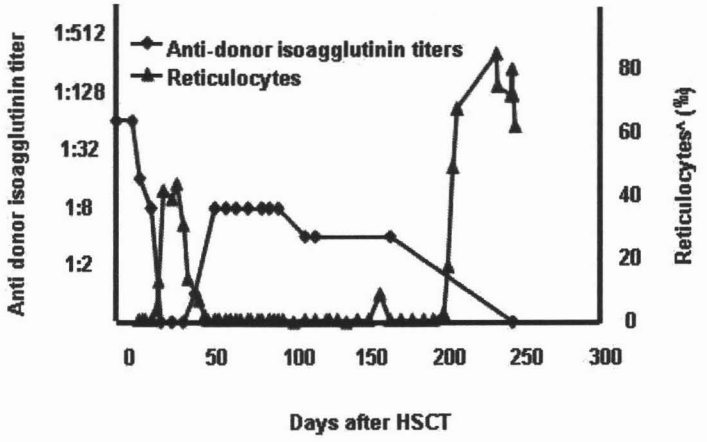

Figure 1: Course of anti-donor isoagglutinintitres and reticulocyte counts. The patient received major (A into O) ABO-incompatible HSCT for advanced stage non-Hodgkin lymphoma, no anti-donor isoagglutinin titer reduction was performed. Shortly after HSCT the anti-donor isoagglutinins decreased without signs of hemolysis and RBC engraftment. Increasing levels of reticulocytes were first detected 20 days after HSCT. Thirty days after HSCT, however, anti-donor isoagglutinins increased again, the emerging erythropoiesis was suppressed and the patient remained transfusion-dependent. On day 193 he developed severe GvHD (grade IV) leading to a rapid disappearance of the anti-donor isoagglutinins due to a graft-versus-plasma cell effect.

Evaluation of EC chimerism

Whereas all intravascular RBC expressed the A blood group antigen of the donor at day 350 after major ABO-incompatible HSCT, all vascular EC in 2 different heart and 1 solid bone marrow tissue specimens exclusively expressed the recipient O blood group antigen H (A to O) (Fig. 2).

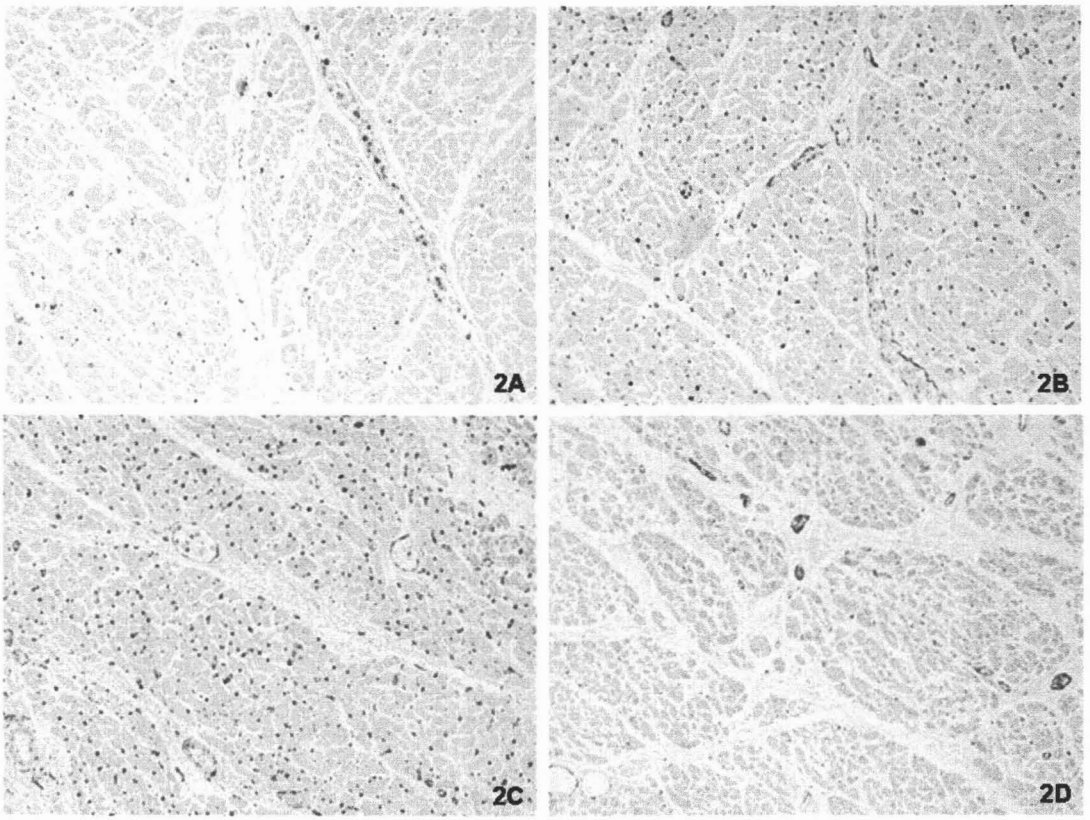

Figure 2: Immunohistochemical staining in heart tissue after ABO-incompatible HSCT. A histo-blood group antigen (2A), H histo-blood group antigen (2B) and the EC markers CD34 (2C) and von Willebrand factor (2D). Positive H antigen staining is seen on all EC of capillaries, arterioles and venules.

Figure 3 shows a representative example of the A antigen expression on haematopoietic cells and the H antigen staining on EC (Fig. 3A and B). Also shown are CD34 (Fig. 3C) and von Willebrand factor staining (Fig. 3D), other commonly used immunohistochemical markers for EC, which were always used on corresponding serial sections. The H antigen was strongly expressed on EC of all capillaries, arterioles and venules in the investigated heart tissue (Fig. 3D, arrows), whereas the donor-type A could not be detected in any EC of the heart. In contrast, the donor-type antigen A was seen on blood cells present on the same tissue

specimen (Fig. 3B) indicating a haematopoietic donor-chimerism at the time of autopsy. Complete donor-type haematopoietic chimerism was confirmed by VNTR analysis. Similar results were seen in the bone marrow analysis (Fig. 3H, arrow). The donor-type A antigen was present in some cell groups that represent most likely the engrafted donor haematopoietic stem cells (Fig. 3F).

As expected in this A to O major ABO-incompatible situation, antigen B was not present on any cell of the investigated tissue. Occasionally, staining artifacts in the tissue stained for antigen B were observed (Fig. 3C and G, asterix). The clear results of the ABH histo-blood group antigen staining on fresh erythrocytes in standard blood smears confirmed the specificity and reliability of the immunohistochemical staining protocol which was used (data not shown).

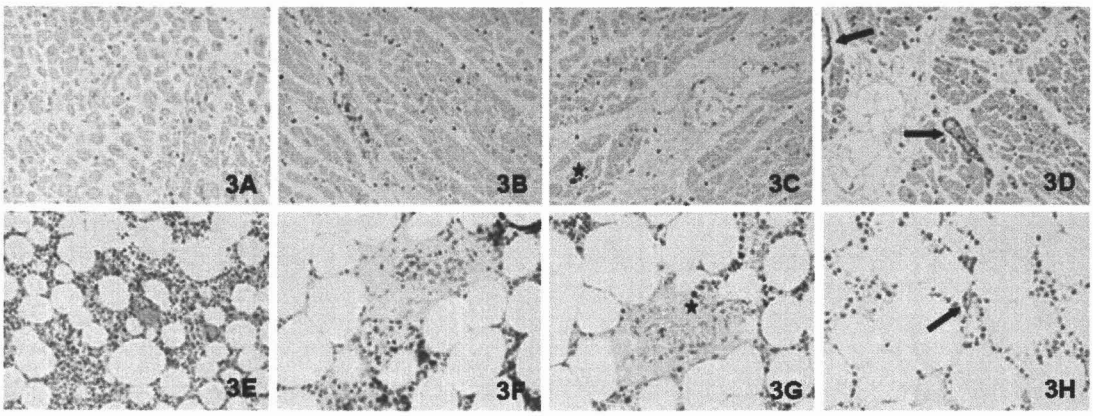

Figure 3: Immunohistochemical staining in heart and solid bone marrow tissue after ABO-incompatible HSCT. Heart (3A-D) and solid bone marrow (3E-H) tissue 350 days after ABO-incompatible HSCT. H/E morphology staining (3A, 3E). Donor-type antigen A is present on haematopoietic cells inside the depicted blood vessels (3B, 3F). Antigen B is not present on any cell (3C, 3G). Recipient-type antigen H is expressed on EC (arrows) of all blood vessels in the heart and in the bone marrow (3D, 3H).

To confirm the observed lack of EC chimerism after HSCT additional CISH analysis of sex chromosomes was performed. Technically, it was not possible to evaluate all EC present in the examined tissue by CISH analysis. In several cases only one of the two sex chromosomes was visible on 4 μm sections precluding reliable conclusions of the cellular origin. However, in accordance with the results obtained by immunohistochemistry, none of the analyzed EC with two stained sex chromosomes was of donor-type. Exclusively EC with one red and one brown dot (of the male XY caryotype) in the nucleus were detected. If the morphology of the cells was badly impaired due to the intense tissue pre-treatment protocol, cells which could

not be clearly determined as to be EC were omitted from further evaluation. Furthermore, out of 50 cells in post-transplant heart tissue showing the recipient X/Y characteristics (Fig. 4A and C) 24 were clearly EC whereas the remaining cells were excluded from further analysis because they were not unambiguously attributable to the endothelium. Additionally, 18 haematopoietic cells located in the lumen of blood vessels showing clear donor X/X characteristics were detected (Fig. 4A and B).

In summary, donor-type EC was observed neither by ABH immunohistochemistry nor by CISH. Regarding the very long CISH protocol that may result in serious damage of the tissue morphology, immunhistochemical staining of ABH histo-blood group antigens is by far the more effective method for studies of EC chimerism.

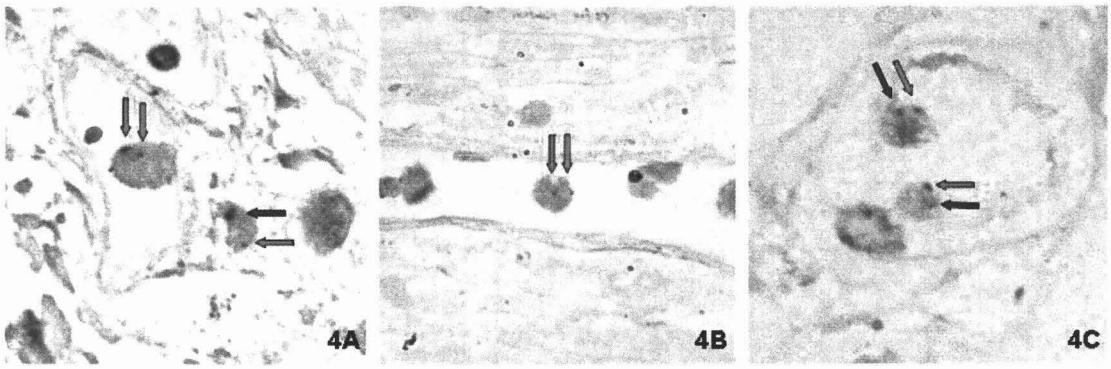

Figure 4: Chromogen in-situ hybridisation for the X and Y sex-chromosomes in heart tissue after gender-mismatched HSCT. Pink arrows do indicate the positively stained X chromosomes and brown arrows do point onto positively stained Y chromosomes. In the EC layer all cells are of the recipient XY-type (4A, 4C), but within some blood vessels donor XX-type cells can be seen (4A, 4B).

5.2.5. Discussion

In support of the importance of anti-donor isoagglutinin levels for the development of PRCA after major ABO incompatible HSCT, we observed a clear association between the recovery of reticulocytes and the decrease of anti-donor isoagglutinins. We could confirm this finding in a retrospective analysis of HSCT recipients showing that recovery from PRCA was associated with a decrease of anti-donor isoagglutinin titres in almost all cases of PRCA and that the incidence of PRCA depended on the pretransplant reduction of anti-donors isoagglutinin titres (own unpublished observation).

In this study, a reliable immunohistochemical staining protocol for the analysis of ABH histo-blood group antigens in human tissue was established to investigate EC chimerism after ABO-incompatible HSCT. To exclude the possibility that EC were mistaken for leukocytes adhering to the endothelium, additional stainings using a pan-leukocyte (CD45) and 3 EC-specific markers (CD31, CD34 and von Willebrand factor) were carried out in adjacent sections of all specimens. All EC showed positive labelling of the recipient type blood group antigens by immunohistochemistry and these results were confirmed by a second method, CISH.

In heart tissue the circular positive antigen labelling of a single capillary was difficult to distinguish from the surface staining of a RBC regarding the close structural association. Using two colour stainings, additional RBC and EC markers and confocal microscopy in future studies might further improve the described technique of ABH immunohistochemistry. Moreover, in any body organ tissue the expression of histo-blood group antigens varies in relation to the individual's blood group, i.e. genetic factors (ABO-, Lewis- and Secretor type), to the presence of glycosyltransferases, to epithelial type and to cellular differentiation within the epithelium [Clausen and Hakomori, S., 1989; Le Pendu et al., 1988; Ravn and Dabelsteen, E., 2000]. Whether environmental factors play an additional role in this individual expression pattern, remains questionable. Keeping the possibility in mind that (i) the EC surface is covered by a protective coat of host antibodies early after major ABH-incompatible transplantation [Kanetsuna et al., 2004; Shiozawa et al., 2002], that (ii) ABH antigens may be shed by EC and that (iii) soluble ABH antigens may be absorbed by EC the observed lack of immunohistochemical ABH antigen staining on EC after HSCT is not a final proof. For these reasons, other methods such as an indirect immunofluorescence staining for deposition of IgG and IgM, DNA typing and/or X/Y chromosome detection are needed to confirm the finding of a lack of EC chimerism after HSCT.

In concern of the described unspecific blood group staining in autoptic tissue which most likely can be attributed to the partial autolysis process, that already had occurred before fixation of autoptic tissue specimen, another possible explanation should be discussed here as well. The presence of ABH blood group antigens could also be due to the bacterial colonization of the autoptic tissue, since bacteria are reported to possess ABH blood group antigens [Andersson et al., 1989]. It is further of note that in this study none of the cells evaluated by CISH had more than two sex chromosomes. This finding is meaningful addressing the issue of potential cell fusions following stem cell transplantation [Nygren et al., 2004].

In conclusion, there was no evidence for donor-type EC chimerism following ABO-incompatible HSCT. Improved understanding of the basic mechanism of EC replacement and angiogenesis may help to develop new transplantation protocols. Further clinical applications could be envisioned in arteriosclerosis or the treatment of other organ damages. These data indicate that EC replacement *in vivo* occurs predominantly due to local EC proliferation rather than replacement by circulating bone marrow-derived EC progenitor cells.

5.3. PART III

Persistence of recipient-type Endothelium after Allogeneic Haematopoietic Stem Cell Transplantation

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Keywords: Endothelial cells – chimerism – haematopoietic stem cell transplantation – ABO-incompatible – tolerance - ABH histo-blood group antigen – FISH – short tandem repeats

Abbreviations used in this paper: HSCT, haematopoietic stem cell transplantation; GvHD, graft-versus-host disease; FISH, fluorescence in-situ hybridisation; CISH, chromogen in-situ hybridisation; STR, short tandem repeat; PCR, polymerase chain reaction; AML, acute myelogeneous leukaemia, CML, chronic myelogeneous leukaemia; ALL, acute lymphoblastic leukaemia; MM, multiple myeloma; CLL, chronic lymphocytic leukaemia; MDS, myelodysplastic syndrome; MPS, myeloproliferative syndrome; AA, aplastic anaemia.

5.3.1. Abstract

Background: ABO blood group antigens are expressed on endothelial cells and represent a potential target for graft-versus-host disease (GvHD). Still, allogeneic haematopoietic stem cell transplantation (HSCT) can be performed successfully without GvHD despite an ABO-barrier. Endothelial replacement by donor bone marrow-derived cells has been postulated to explain this state of tolerance. This study aimed to investigate the level of endothelial cell chimerism after allogeneic HSCT thereby to delineate the plasticity of haematopoietic stem cells.

Methods: The frequency of donor-derived endothelial cells was prospectively analysed in normal skin biopsies obtained from 22 HSCT recipients by ABO immunohistochemistry, fluorescence in-situ hybridisation and short tandem repeat (STR) analysis of laser captured endothelial cells. In addition, endothelial cell chimerism was retrospectively analysed in 21 GvHD affected skin biopsies, various tissues from 5 autopsies and 4 secondary solid tumours biopsies.

Results: Skin biopsy samples from two minor ABO-incompatible (i.e. O in A) transplanted patients showed a low level H antigen-positive (3.3% and 0.9% respectively), donor-derived endothelial cells by ABO immunohistochemistry. All other tests, in 23 additional skin samples, in heart, liver or solid bone-marrow tissue and tumour biopsies failed to reveal donor-type endothelial cells up to several years after ABO-incompatible HSCT.

Conclusions: Endothelial cell replacement by bone marrow-derived donor cells does not occur routinely after allogeneic HSCT and therefore cannot explain ABO-tolerance. In addition, donor bone marrow-derived endothelial cells do not seem to contribute to *in vivo* blood vessel formation in a physiological course and in tumour neoangiogenesis after HSCT.

5.3.2. Introduction

For many years endothelial cell chimerism has received great attention in the field of solid organ transplantation as a potential mechanism leading to tolerance. In 1965, Medawar hypothesised that long-term acceptance of organ transplants may be the result of a replacement of their vascular endothelial cells by recipient-type cells leading to an endothelial cell chimerism [Medawar, 1965]. In particular, bone marrow-derived endothelial progenitor cells may replace damaged endothelium in the graft after rejection episodes. An increasing body of evidence has been put forward in favour of this hypothesis both in animal models as well as in human transplantation, but there are also equivocal results [Lagaaij et al., 2001; Quaini et al., 2002; Xu et al., 2002; Tanaka et al., 2005; Hove et al., 2003a; Hruban et al., 1993b; Fogt et al., 2002a; Hillebrands et al., 2002a]. The reported discrepancies might be due to the different methods used to detect chimerism, preexisting male chimerism in female donors after giving birth to male children or receiving male blood transfusions and physiologic differences in the extent of chimerism in various organs and cell types [Bianchi et al., 1996; Koopmans et al., 2005]. In general, endothelial replacement by recipient-type cells is a phenomenon observed after organ transplantation, but it has not been proven to be a mechanism responsible for tolerance.

Endothelial cell chimerism following haematopoietic stem cell transplantation (HSCT) is likewise controversial. In theory, bone marrow-derived donor-type endothelial cells may replace damaged host endothelium in graft-versus-host disease (GvHD) affected tissues as well as normal endothelium during physiological cell replacement. In support of this hypothesis donor chimerism has been described in the endothelium of skin, gut, heart and bone marrow in patients after HSCT [Jiang et al., 2004; Kvasnicka et al., 2003; Thiele et al., 2004a]. Furthermore, it was recently suggested that donor-derived endothelial cells are participating in endothelial repair during the effector phase of acute GvHD, but there was no evidence of chimeric endothelial cells in patients without GvHD [Murata et al., 2007]. Among others, ABO histo-blood group antigens (ABO Ag) expressed on endothelial cells represent a potential target for GvHD. However, allogeneic HSCT is performed across the ABO-blood group barrier in one third of the patients without affecting overall survival, transplant-related mortality and relapse rates [Lasky et al., 1983; Seebach et al., 2005]. Whether endothelial replacement and chimerism by donor bone marrow-derived cells is responsible for this apparent ABO tolerance remains unclear.

Since previous studies often suffered from technical difficulties to reliably distinguish between donor and recipient endothelial cells, we aimed to meticulously investigate the level

of endothelial cell chimerism after allogeneic HSCT by using three different methods. After ABO-incompatible HSCT, ABO Ag expression on the vascular endothelium was analysed by ABO immunohistochemistry [Mueller et al., 2006; Ravn and Dabelsteen, E., 2000; Le Pendu et al., 1988]. In addition, tissues from gender-mismatched HSCT recipients, representing approximately 50% of the patients undergoing allogeneic HSCT, were analysed by fluorescence in-situ hybridisation (FISH) for X and Y chromosomes. Finally, PCR based short tandem repeat (STR) analyses were performed on selected samples using single laser-capture microdissected endothelial cells to identify their donor or recipient origin. Physiological endothelial cell turnover was analysed in normal skin and autopsy tissue in addition to GvHD-affected tissues and secondary tumour biopsies.

5.3.3. Materials and Methods

Patients and tissue samples

A total of 52 patients were included in this analysis. Skin punch biopsies were obtained from 22 patients in a prospective manner during routine bone marrow punctures before and after HSCT. The study protocol was approved by the local ethical committee and all patients provided written informed consent. In addition, diagnostic skin biopsies derived from 21 patients suspected of having GvHD, various tissues (skin, heart, bone marrow and liver) from 5 autopsies and 4 secondary tumour biopsies were analysed retrospectively. In total, 10 patients (19%) had acute GVHD of grade III-IV, 32 (62%) had acute GVHD of grade I-II and 10 (19%) had no signs of acute GvHD. At the time of biopsy 12 patients (23%) had extensive chronic GvHD, 11 (21%) had limited chronic GvHD and 20 (39%) did not have any signs of chronic GvHD (table 1).

Analysis included 29 ABO-incompatible and 23 ABO-compatible HSCT, 28 gender-mismatched and 24 gender-matched HSCT. Seventeen patients received a minor (O in A 9; O in B 4; A in AB 1; B in AB 3), 10 patients a major (A in O 7; B in O 3) and 2 patients a bidirectional ABO-incompatible HSCT (A in B 1; B in A 1).

The study included 13 patients (25%) with acute myelogeneous leukaemia (AML), 17 (33%) with chronic myelogeneous leukaemia (CML), 6 (12%) with acute lymphoblastic leukaemia (ALL), 6 (12%) with multiple myeloma (MM), 3 (6%) with chronic lymphocytic leukaemia (CLL), 2 (4%) with myelodysplastic syndrome (MDS), 3 (6%) with myeloproliferative syndrome (MPS) and 1 (2%) patient with aplastic anaemia (AA). The median age at HSCT

was 41 (range 14-63) years and 34 of 48 (71%) recipients were male. The biopsies were taken after a median of 194 days (range 7-3476 days). The average size of the skin biopsies was 0.0025 m3.

Immunohistochemistry

Immunohistochemical staining of ABO Ag and endothelial cell markers were performed according to a previously published protocol [Mueller et al., 2006]. Briefly, serial 4 µm sections were incubated with monoclonal anti-A, B and H antibodies (Dako, Carpintera CA, USA) and monoclonal antibodies against CD45, CD31 (PECAM-1), CD34 and von Willebrand factor. Antibody binding was visualised with a secondary biotin-conjugated rabbit anti-mouse antibody followed by immunoperoxidase staining. Counterstaining was done for 2-4 minutes in standard acidic haemalaun solution. Morphology of the tissues was analysed with an additional haematoxylin/eosin staining. Endothelial cell chimerism was quantified by counting cells in the endothelial lining with a clearly recognisable nucleus and well-defined endothelial morphology that stained positively for donor ABO Ag. In addition, the cell count was normalised for variable section sizes by calculating the ratio between the number of endothelial cells and the number of cross-sectioned blood vessels on every section. Results were verified by a second observer unaware of the donor/recipient blood groups.

Combined fluorescence in-situ hybridisation (FISH) for X and Y chromosomes and immunohistochemical staining for vWF

Tissue samples after gender-mismatched HSCT were analysed by combined FISH and immunohistochemical staining for the endothelial cell-specific marker vWF [Page et al., 1992]. The sections were deparaffinised in Xylol, dehydrated in ethanol and incubated in 0.2 mol/L HCl at room temperature for 20 minutes. Thereafter, they were immersed in Pretreatment Solution (Vysis®, Downer's Grove IL, USA) for 10 minutes at 80°C and digested with Protease I (Vysis®, Downer's Grove IL, USA) for 15-20 minutes at 37°C. After dehydration in ethanol, CEP Y Spectrum Red probe and CEP X Spectrum Green (Vysis®, Downer's Grove IL, USA), which cover classical satellite III sequences on the X and Y chromosomes, were co-incubated simultaneously at 80°C for 6 minutes and allowed to hybridise overnight at 42°C. The samples were then washed in 2xSSC/0.3 NP40 at 72°C for 5 minutes and 2xSSC for 2 minutes at room temperature. For subsequent immunohistochemical staining, the slides were incubated overnight at 4°C with monoclonal mouse anti-vWF

antibodies (Dako, Glostrup, Denmark, Product No. M0616) followed by a secondary fluorescent goat anti-mouse antibody (Alexa Fluor 647, Molecular Probes®). Finally, 10 µl 4'6-diamidino-2-phenylindole (DAPI II, Vysis®, Downer's Grove IL, USA) was applied to the samples for chromatin counterstaining. Isotype controls and omission of the primary antibody were used as negative controls. FISH signals and fluorescent immunostaining were evaluated with a Leica DM Fluorescence Microscope (with Z-stack analysis) and the corresponding LAS Leica Fluorescence Software AF 6000 DFC. For the quantification only morphologically clearly distinguishable vWF-positive endothelial cells which displayed a signal of two chromosomes in their nucleus were counted, i.e. X and X for female or X and Y for male. Cells with only one visible signal were counted separately. In some samples chromogen in-situ hybridisation (CISH) was performed to confirm the results obtained with FISH. SPOT-Light® chromosome X/Y probe cocktail and SPOT-Light® CISH™ detection kit (Zymed® Laboratories Inc., San Francisco CA, USA) were used according to a previously described protocol [Mueller et al., 2006].

Short tandem repeat analysis (STR)

Single endothelial cells were captured out of 6 µm cryostat sections previously stained either for CD45 (negative selection) or for recipient-type ABO Ag (positive selection) following careful microscopic evaluation with a laser capture microdissector (LCM Arcturus VERITAS™, Molecular Devices, USA). Nuclei from 20-40 endothelial cells per section were needed for complete STR amplification. The thermoplastic polymer film of the LCM caps (Arcturus CapSure® HS LCM caps, Molecular Devices, USA) containing the isolated cells was added directly to the Amp FℓSTR® Profiler® STR multiplex PCR amplification kit (Applied Biosystems, Rotkreuz, Switzerland), amplifying 9 different STR loci and the Amelogenin locus, discriminating X and Y chromosomes. DNA amplification was performed with a multiplex STR polymerase chain reaction (PCR) and PCR fragments were separated by capillary electrophoresis on an ABI Prism 3130 Genetic Analyser (Applied Biosystems). Fragment size and peaks were analysed using the GeneScan analysis software (Applied Biosystems). To determine donor- and recipient-specific patterns of STR loci, peripheral blood mononuclear cells (PBMC) of the donor and the recipient were analysed prior to transplantation.

5.3.4. Results

Persistence of recipient-type ABO Ag expression in skin endothelial cells after HSCT

Skin biopsies from 25 patients were evaluated for endothelial cell chimerism by immunohistochemistry for ABH Ag in the context of ABO-mismatched HSCT. Normal skin was analysed in 10 patients participating in the prospective study and 15 GvHD-affected skin samples stemmed from the retrospective analysis. ABO Ag were found on endothelial cells and erythrocytes of the epidermal and dermal layers. In general, the expression of ABO Ag was comparable in all types of blood vessels, with the exception of H antigen staining in biopsies from type O recipients, where capillaries showed a stronger expression than arterioles and venules. The granular layer of the epidermis, the sweat glands and hair follicles were positively stained in about 80% most likely reflecting the genetically determined percentage of human ABO blood group secretor type [Szulman, 1966].

Representative examples of ABO Ag staining in skin biopsies and the average quantification of each recipient group according to the different blood groups (i.e. recipients of type A, B, O and AB) are shown in Figure 1. Staining for the pan-leukocyte marker CD45, the endothelial cell markers CD31, CD34 and vWF and H/E on serial sections was always evaluated in parallel to analyse the presence of leukocytes and the number of blood vessels in the tissue sample. A median of 34 vessels (range 2-257) and 108 endothelial cells (range 18-2906) on every skin biopsy section were analysed for the expression of ABO Ag. In 23 out of 25 patients, the skin biopsies exclusively showed recipient-derived vascular endothelial cells (figure 1E-H, Y-axis). The remaining two skin biopsies, derived from minor ABO-incompatible HSCT (patients #25 and #28), exhibited low levels of donor-type endothelial cells. In proportion to the total amount of evaluated endothelial cells on the sections, the 2 patients showed 3.3% and 0.9% of H antigen-positive cells in the endothelial lining, respectively (table 1).

Moreover, 3 out of 10 patients after ABO-identical HSCT (O in O 1; A in A 2) showed, in addition to their own blood group type, an aberrant B antigen (O in O and A in A) or H antigen (A in A) expression in skin biopsies.

					ABO			Gender			
Patient	Age	Disease	Biopsy*	GvHD	R/D	R	D	R/D	R	D	STR
					Prospective skin biopsy study						
1	32	CML	102	0/no	A/O	46/21	0/14	m/m	NA	NA	R
2	46	AML	151	I/no	A/O	257/71	0/45	f/m	46	0	
3	43	CML	245	II/no	A/O	84/29	0/27	f/m	ND	ND	
4	39	CML	615	IV/no	A/A	75/39	NA	m/f	ND	ND	
5	40	CML	103	0/no	A/A	79/28	NA	m/m	NA	NA	
6	40	CML	102	I/no	A/A	40/15	NA	m/m	NA	NA	
7	41	AML	381	I/ext.	A/A	91/33	NA	m/m	NA	NA	
8	41	AML	192	0/ext.	A/A	116/27	NA	m/m	NA	NA	
9	48	ALL	420	I/ext.	A/A	74/23	NA	m/f	ND	ND	
10	51	ALL	181	0/no	A/A	109/35	NA	m/m	NA	NA	
11	56	AML	264	I/no	A/A	21/8	NA	f/m	ND	ND	
12	34	CML	94	II	B/O	203/95	0/50	m/m	NA	NA	
13	45	AML	547	0/no	B/O	74/38	0/31	f/m	25	0	
14	52	AML	193	I/lim.	B/O	49/13	0/15	m/m	NA	NA	
15	41	AML	849	IV/no	O/A	28/14	0/12	m/m	NA	NA	R
16	27	MPS	100	0/no	O/A	34/16	0/18	f/m	6	0	R
17	26	CML	718	II/ext.	O/B	114/56	0/51	f/m	29	0	R
18	57	MPS	132	I/lim.	O/B	59/49	0/45	m/f	7(8)**	0	R
19	20	ALL	189	I/ext.	O/O	22/17	NA	m/m	NA	NA	
20	28	AML	100	I/lim.	O/O	64/24	NA	f/m	ND	ND	
21	52	AML	101	II/ext.	O/O	92/61	NA	f/m	ND	ND	
22	58	MM	51	II	O/O	2906/781	NA	m/f	21**	0**	
					Retrospective skin biopsy study and autopsies						
23	41	CML	1915	III/no	A/B	106/29	0/25	m/f	5**	0**	
24	15	AML	1928	II/lim.	A/O	293/45	0/33	m/f	4(9)	0	
25	28	ALL	1552	I/lim.	A/O	148/72	5/68	m/f	11**	0**	
26	57	CLL	83	II	A/O	273/87	0/41	f/m	19**	0**	
27	31	CML	7	III	A/O	23/3	0/3	m/f	10(12)	0	
28	48	MM	1001	II/lim.	A/O	432/164	4/82	m/m	NA	NA	
29	57	AML	96	IV	A/O	577/267[I]	0/228[I]	m/m	NA	NA	
30	14	CML	613	II/ext.	A/A	123/46	NA	f/m	8**	0**	
31	35	AML	918	II/ext.	A/A	110/42	NA	f/f	NA	NA	
32	63	MM	94	III	A/A	327/103	NA	f/f	NA	NA	
			903	III/lim.	A/A	1111/463	NA	f/f	NA	NA	
33	61	MM	145	IV/no	B/A	1950/665[I]	0/461[I]	m/m	NA	NA	
			145	IV/no	B/A	18734/134[II]	0/14[II]	m/m	NA	NA	
			145	IV/no	B/A	974/78[III]	0/13[III]	m/m	NA	NA	
34	35	ALL	1412	II/lim.	B/O	418/142	0/35	m/f	29(12)**	0**	
35	42	CML	195	I/no	B/B	221/58	NA	m/f	3**	0**	
36	53	MPS	489	0/lim.	O/A	44/26	0/22	m/m	NA	NA	
37	30	ALL	999	II/ext.	O/A	266/91	0/65	f/f	NA	NA	
38	39	NHL	350	IV/no	O/A	14256/94[II]	0/13[II]	m/f	26[II] **	0[II] **	
			350	IV/no	O/A	41/18[III]	0/20[III]	m/f	ND	ND	
39	44	CML	115	IV/no	O/A	ND	ND	m/f	20(20)[II]	0[II]	
40	60	AML	102	II	O/A	12456/81[II]	0/3[II]	m/m	NA	NA	
			102	II	O/A	246/27[IV]	0/25[IV]	m/m	NA	NA	
41	45	CML	34	I	O/O	35/18	NA	m/m	NA	NA	
42	24	CML	1121	I/no	O/O	92/69	NA	f/m	ND	ND	
43	44	CLL	866	0/lim.	O/O	18/92	NA	m/m	NA	NA	
44	54	CLL	89	II	O/O	117/19	NA	m/m	NA	NA	
45	28	CML	3476	II/ext.	AB/A	579A/158	0/66	m/m	NA	NA	
			3476	II/ext.	AB/A	442B/126	0/66	m/m	NA	NA	
46	38	MDS	35	III	AB/B	69A/33	0/12	m/m	NA	NA	
			35	III	AB/B	35B/19	0/12	m/m	NA	NA	
47	59	MDS	188	I/lim.	AB/B	290A/84	0/50	m/f	19(24)	0	
			188	I/lim.	AB/B	336B/101	0/50	m/f	NA	NA	
48	32	MM	1548	I/no	AB/B	444A/106	0/73	m/m	NA	NA	
			1548	I/no	AB/B	433B/109	0/73	m/m	NA	NA	
					Tumour tissues						
49	47	MM	181	II/ext.	O/B	74/37	0/28	m/f	ND	ND	
50	14	CML	12 years	II/no	A/A	ND	ND	f/m	34	2	
51	12	AA	13 years	0/no	A/A	ND	ND	f/m	31	4	
52	41	CML	21 years	0/no	O/O	ND	ND	f/m	13	0	

Table 1: **Patient's characteristics and evaluation of endothelial cell chimerism.** Grade of GvHD (acute 0-IV; chronic lim.=limited; chronic ext.=extensive). Number of ABO Ag expressing endothelial cells is shown as ratio to the number of counted blood vessels on the same tissue section. Counted numbers of vWF^+ endothelial cells showing either XX (f=female), XY (m=male) karyotype or only the Y chromosome (indicated in brackets) are shown. *Days after HSCT. **Results confirmed by CISH. Autopsy-derived tissue: [I]skin, [II]heart, [III]bone marrow, [IV]liver. D=donor. R=recipient. AML=acute myelogenous leukaemia. ALL=acute lymphatic leukaemia. CML=chronic myelogenous leukaemia. MPS=myeloproliferative syndrome. MDS=myelodysplastic syndrome. MM=multiple myeloma. NHL=non-Hodgkin-lymphoma. A= antigen A^+. B=antigen B^+. NA=not applicable. ND=not determined due to technical limitations.

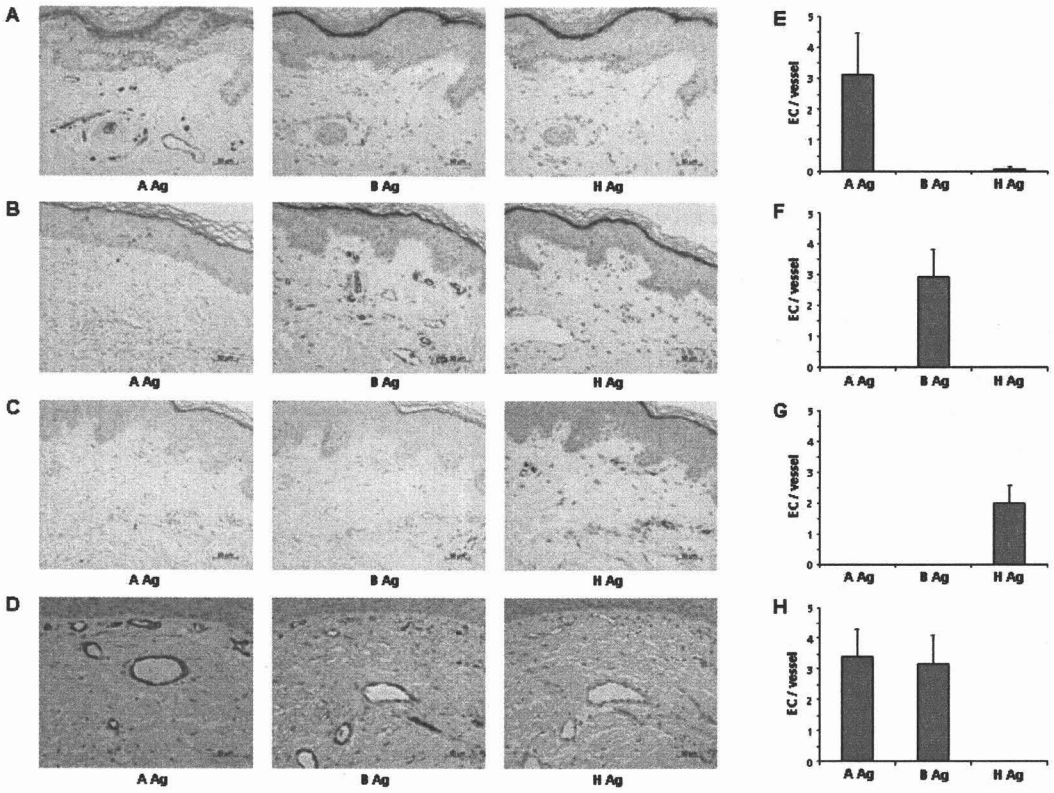

Figure 1: ABO Ag expression in skin biopsies. (A-D) Representative pictures from ABO Ag-stained skin biopsy sections. (A) 46-year-old patient #2 151 days after minor ABO-incompatible HSCT (O in A). (B) 34-year-old patient #12 94 days after minor ABO-incompatible HSCT (O in B). (C) 26-year-old patient #17 718 days after major ABO-incompatible HSCT (B in O). (D) 28-year-old patient #45 3476 days after minor ABO-incompatible HSCT (A in AB). (E-H) Quantification of ABO Ag-positive endothelial cells in biopsies derived from 25 patients after ABO-incompatible HSCT. The number of ABO Ag expressing endothelial cells is shown as the ratio to the number of counted blood vessels on the same tissue section (Y axis). (E) B, O in A, n=10. (F) A, O in B, n=5. (G) A, B in O, n=6. (H) A, B in AB, n=4.

Persistence of recipient-type X and Y chromosome karyotype in skin endothelial cells after HSCT

In-situ hybridisation for X and Y chromosomes confirmed the findings of ABO immunohistochemistry in patients with gender-mismatched HSCT (table 1 and figure 2A). Skin biopsies of 22 patients after gender-mismatched HSCT were available. Chimerism was analysed in 15 patients by combined immunohistochemistry/FISH and was confirmed in 8 patients by CISH analysis. Skin biopsies derived from 7 patients could not be analysed due to severely impaired tissue morphology. In the analysed skin biopsies, a total of 242 endothelial cells were counted with a signal of two chromosomes in their nucleus in relation to 204

endothelial cells which only displayed one X or the Y chromosome due to a cross-sectioned nucleus.

Recipient-type endothelial cells persisted in all skin biopsies both in the 6 samples from the prospective analysis and the 10 samples from the retrospective analysis. In contrast, donor leukocytes were frequently found in the perivascular areas most likely representing infiltrating donor leukocytes. The HSCT of patient #25 that showed 3.3% donor-type endothelial cell chimerism by ABO Ag staining was gender-mismatched. Evidence for donor-derived endothelial cells could not be found, neither with immunohistochemistry/FISH nor with CISH. None of the endothelial cells analysed in this study had more than two sex chromosomes in the nucleus, making cell fusion as potential repair mechanism unlikely.

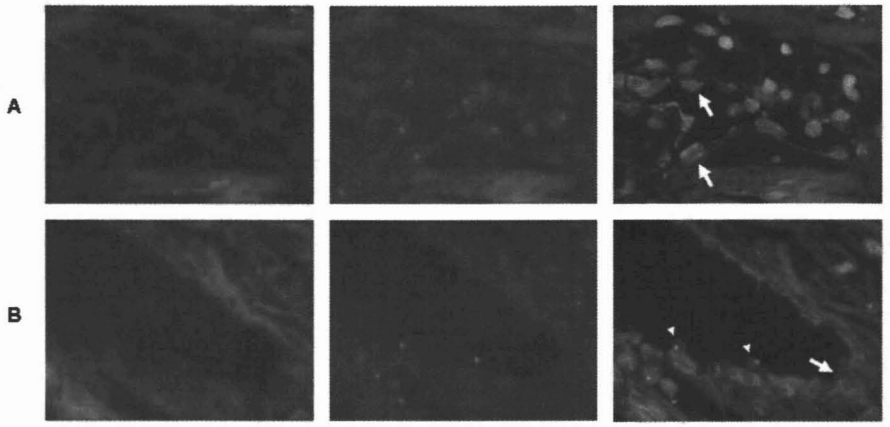

Figure 2. FISH in endothelial cells after gender-mismatched HSCT. In-situ hybridisation shows the X chromosome marked with CEP X Spectrum Green (green) and the Y chromosome with CEP Y Spectrum Red (red). Endothelial cells are stained for vWF (blue). (A) Skin biopsy from a 59-year-old male patient, who was treated for myelodysplastic syndrome with haematopoietic stem cells from a female donor. Biopsy 188 days post HSCT. Endothelial cells show recipient-specific XY caryotype (arrows). (B) Mucoepidermoid carcinoma of the parotid gland from a 26-year-ol female patient, who was treated for chronic myeloid leukaemia with haematopoietic stem cells from a male donor 12 years earlier. Haematopoietic cells show donor-specific XY caryotype (arrowheads), whereas endothelial cells show recipient-specific XX caryotype (arrow).

Persistence of recipient-type short tandem repeat patterns in skin endothelial cells after HSCT

STR analysis was performed on DNA extracted from vascular endothelial cells in skin biopsies from 5 patients. In each case 7 or more highly polymorphic STR loci, including the Amelogenin locus, were successfully analysed. All of the captured endothelial cells contained DNA with a STR pattern matching the previously analysed STR pattern of the recipient and no donor repeats could be detected (table 1 and figure 3).

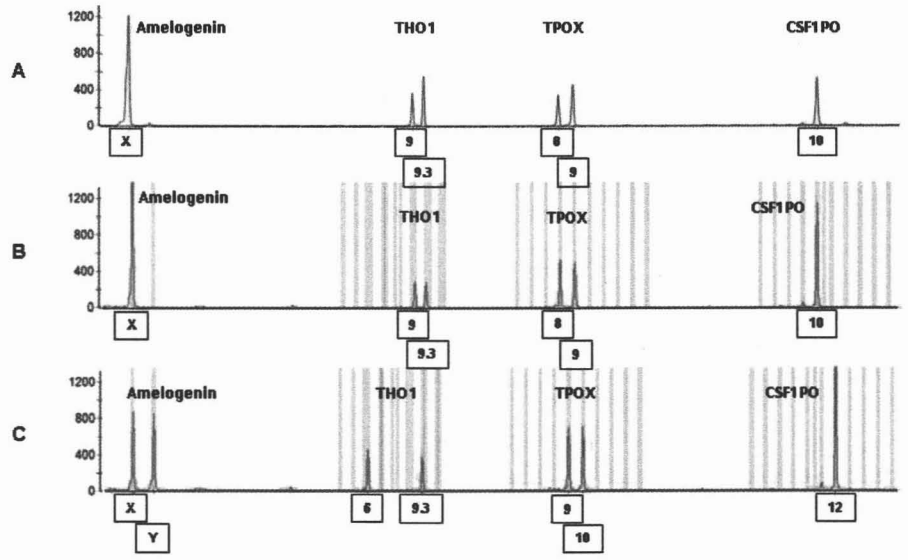

Figure 3. STR analysis. (A) Representative picture of the STR analysis of DNA isolated from 40 endothelial cells from a skin biopsy derived from a 27-year-old female patient #16, who was treated for osteomyelofibrosis with haematopoietic stem cells from her brother. Skin biopsy 100 days post HSCT. Four STR loci (THO1, TPOX, CSF1PO and Amelogenin) are shown. (B) STR pattern of the recipient determined pre-HSCT. (C) STR pattern of the donor determined pre-HSCT.

No evidence for donor-type endothelial cell chimerism in autopsy-derived tissues after HSCT

Necroscopy tissues were obtained from 5 patients. Three patients died from grade IV acute GvHD (patients #29, #33 and #38), one patient due to CMV-ileitis (patient #40), one patient had a general pancytopenia and died subsequently of bronchiolitis obliterans and subarachnoidal haemorrhage (patient #39). Using ABO immunohistochemistry in skin (4 patients), heart (3 patients), bone marrow (2 patients) and liver (1 patient) and additional in-situ hybridisation for X and Y chromosomes in heart tissue of 2 patients, endothelial cells

from blood vessels in skin, heart, liver and solid bone marrow showed no evidence for donor-type endothelial cell chimerism (table 1 and figure 4).

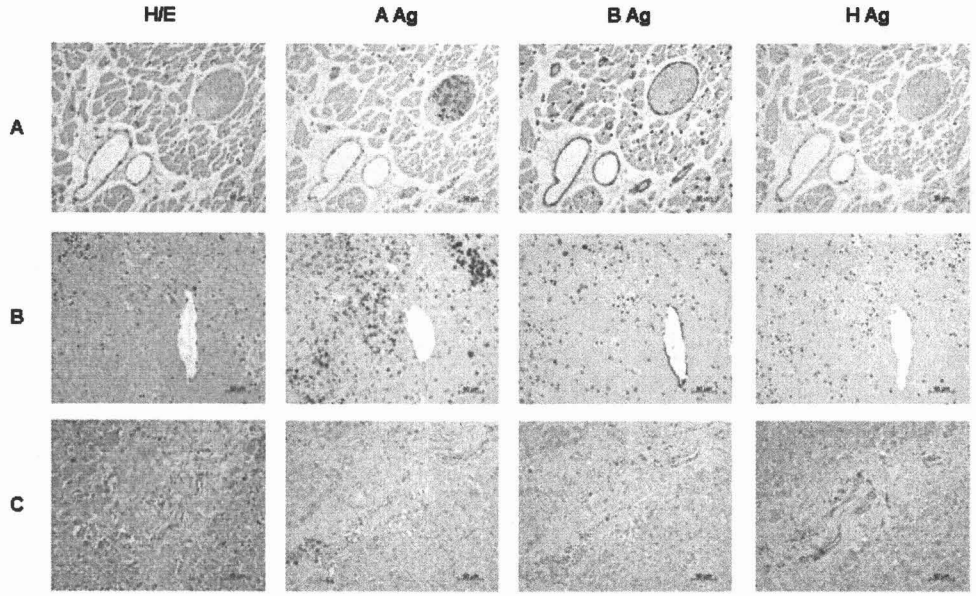

Figure 4. ABO Ag staining in autopsy-derived tissue. Representative pictures from ABO Ag-stained heart, liver and solid bone marrow sections. (A) heart and (B) bone marrow from a 61-year-old patient, who died of grade IV acute GvHD 145 days after bidirectional ABO-incompatible HSCT (A in B). (C) liver from a 60-year-old patient, who died of CMV-ileitis 102 days after major ABO-incompatible HSCT (A in O). Haematopoietic cells are positively stained for donor-specific A antigen, whereas recipient-specific B antigen (A and B) and H antigen (C) persisted in all endothelial cells. H/E, morphology staining with haematoxilin/eosin.

Donor-type endothelial cell chimerism is a rare event in tumour neoangiogenesis after HSCT

Tumour samples from 4 patients were analysed for endothelial cell chimerism. One patient had an extramedullary relapse of a multiple myeloma after ABO-incompatible HSCT (patient #49, B in O). In this case ABO immunohistochemistry on the tumour biopsy 181 days after HSCT exclusively showed recipient-type H antigen expression on endothelial cells, none of the cells expressed donor-type B antigen. Furthermore, 3 patients developed secondary carcinomas 12 to 21 years after ABO-identical, gender-mismatched HSCT and were analysed for endothelial cell chimerism by combined immunohistochemistry/FISH. One patient with invasive ductal carcinoma of the breast had no signs of donor-derived endothelial cells (patient #52). In contrast, low numbers of donor-derived endothelial cells were detected in

tumour vessels in two other patients, 1.2% of the total amount of vWF-positive endothelial cells in a mucoepidermoid carcinoma of the parotid gland (patient #50, figure 2B) and 2.5% in a papillary thyroid carcinoma (patient #51), respectively. In conclusion, the large majority of endothelial cells in the investigated tumours had their source in the endogeneous progenitor cell pool and did not arise from the transplanted haematopoietic stem cells.

5.3.5. Discussion

This study provides clear evidence that replacement of endothelial cells by bone marrow-derived donor stem cells does not represent a major repair mechanism for blood vessels after allogeneic HSCT. Overall, donor-derived cells did not engraft into recipient endothelium after HSCT, therefore endothelial cell chimerism cannot be a general explanation for tolerance after ABO-incompatible HSCT [Stussi et al., 2006b].

The results of endothelial cell chimerism in animal models are controversial [Bailey et al., 2004; Ziegelhoeffer et al., 2004] and reports in humans remain scarce. So far, endothelial cell chimerism has not been analysed after ABO-incompatible HSCT, but it has been described after gender-mismatched HSCT. Two earlier studies, which did not find donor-type endothelial cells in the stromal constituents of bone marrow up to 3 years after HSCT [Athanasou et al., 1990; Simmons et al., 1987] were contrasted by several reports showing endothelial cell chimerism in skin, gut, heart and bone marrow after gender-mismatched HSCT [Murata et al., 2007; Thiele et al., 2004a; Jiang et al., 2004; Thiele et al., 2004b; Kvasnicka et al., 2003; Suratt et al., 2003]. All these studies used XY in-situ hybridisation for the detection of donor-derived cells. Reported numbers of donor-type bone marrow-derived endothelial cells after HSCT varied from 2% in non-GvHD-affected skin and in gastrointestinal tract up to 40% in the lung. However, it has to be mentioned, that the latter study analysed only 2 patients and histopathological diagnoses of the 2 lung biopsy specimens included diffuse alveolar damage in one patient and bronchiolitis obliterans and GvHD in the other patient. These pathologies may have promoted an enhanced engraftment of bone marrow-derived cells. In any case, the detection of endothelial cell chimerism depends crucially on the methods applied for chimerism analysis.

X and Y chromosome analysis by in-situ hybridisation is generally performed in combination with immunohistochemical staining of a single endothelial cell marker such as CD31, CD34 or vWF. In our experience, several immunohistochemical endothelial and haematopoietic cell markers, a careful morphological analysis and XY in-situ hybridisation are necessary to

conclusively determine endothelial cell chimerism. For instance, CD34 is not only expressed on all endothelial cells but also on haematopoietic precursor cells [Krause et al., 1996] and without leukocyte-specific CD45 staining, perivascular cells dislocated into the luminal part of the vessels and extravasating or adhering leukocytes, in particular monocytes could be misinterpreted as endothelial cells. In addition, cell morphology is often severely impaired and the integrity of the blood vessels disrupted due to the pretreatment (heat, enzyme digestion) of the tissue required for XY in-situ hybridisation. Furthermore, fetal stem cells transferred into maternal blood during pregnancy may engraft in the bone marrow and persist at low levels in organs or, in the case of tissue injury, home to the damaged organ and differentiate as part of the maternal repair response [Bianchi et al., 1996; Koopmans et al., 2005; O'Donoghue et al., 2004]. For these reasons determining endothelial cell chimerism in females after gender-mismatched transplantation by the method of XY in-situ hybridisation may not be sufficient to prove the presence of donor-type endothelial cells. Therefore, in the present study, we combined 3 different methods, i.e. immunohistochemical staining, XY in-situ hybridisation and STR analysis. The results obtained by STR consistently detected DNA with recipient-specific pattern in endothelial cells and DNA with a donor-specific pattern in blood cells, third party DNA was never detected. Moreover, no cells with more than diploid sex chromosomes were detected in any tissue specimen analysed by XY in-situ hybridisation, making a possible cell fusion unlikely [Wang et al., 2003].

ABO blood group staining may also have several technical and biological limitations. ABO Ag have variable expression levels in different tissues and vessels within the same patient, but there is also a considerable interpatient variability. This supports the antigenic heterogeneity of vascular endothelial cells amongst different organs in the human body [Page et al., 1992]. In our study, samples were obtained from normal skin (prospective study) and from skin suspected to have GvHD (retrospective study), therefore the observed interpatient variability may be due to a loss of vessels and to denudation of the vessel wall depending on the grade of GvHD [Biedermann et al., 2002].

In the present study, low-level endothelial cell chimerism was observed in 2 patients with minor ABO-incompatible HSCT (O in A), yet this finding could not be confirmed by XY in-situ hybridisation. Alternative explanations are invading leukocytes or altered ABO Ag expression caused by changes in A and B glycosyltransferases due to leukaemia [Brody and Beizer, L. H., 1965]. The amount of CD45-positive leukocytes counted in the 2 skin biopsies was not higher than in other samples without endothelial cell chimerism. However, H antigen expression was also seen in skin biopsies of blood group type A obtained from 2 patients

before HSCT. In addition, aberrant ABO antigen expression was seen in 3 patients after ABO-identical HSCT. Patient #9 (A in A) showed A and H antigen, patient #30 (A in A) showed A and B antigen and patient #43 (O in O) showed H and B antigen. This could be explained by genetic mutation of the ABO-gene and/or aberrant ABO Ag expression, a phenomenon, that has earlier been described in rejection sites after hepatic transplantation or in gastric and colonic tumours of blood group O individuals [Bloom et al., 1994; David et al., 1993].

Murata et al. elegantly showed cells of donor-specific male XY karyotype concurrently determined as CD31-positive and CD45-negative endothelial cells in the GvHD-affected dermis of 13 gender-mismatched transplanted patients [Murata et al., 2007]. Strikingly, the maximal percentage of chimeric endothelial cells was 9.5% during the effector phase of acute GvHD, but after this phase, the proportion of chimeric cells went down to 2%. In the present study, endothelial cell chimerism was detected at similar levels in two patients (0.9% and 3.3%), both patients had a limited chronic GvHD at the time of biopsy and a history of mild acute GvHD (grade I and II respectively). Since there was no intimal lymphocytic infiltrate or vascular proliferation, i.e. signs of an acute phase of vascular inflammation, a correlation with the grade of GvHD could not be concluded in our study. Interestingly, GvHD does not result in widespread endothelial cell destruction, like it is seen in hyperacute rejection in solid-organ transplantation, yet the exact mechanism of immunological escape after HSCT remains unexplained [Biedermann, 2008].

Analysis of tumour tissue arising after HSCT revealed a relevant amount, i.e. 1.2% and 2.5, of donor-derived endothelial cells incorporating into the growing vascular bed of the recipient. In line with this result, Peters et al. also found that secondary tumours after HSCT can induce mobilisation of bone marrow-derived stem cells to areas of neovascularisation [Peters et al., 2005]. This study highlighted substantial differences between human tumours and many mouse models by showing only 4.9% of the analysed human tumour endothelial cells being of bone marrow origin [Peters et al., 2005; Lyden et al., 2001]. Recent observations in mouse tumour and ischaemia models clarify this issue by showing transplanted donor-derived progenitor cells temporarily incorporated in the endothelium only in the early phase of neoangiogenesis, but these cells disappeared subsequently [Nolan et al., 2007].

The mechanism how bone marrow-derived stem cells may be involved in postnatal angiogenesis and vessel repair is still in debate. It has been shown in a mouse model that neighbouring endothelial cells are responsible for vascular remodelling rather than bone marrow-derived cell incorporation [Kinnaird et al., 2004]. Hereby, bone marrow-derived

stromal cells may play a pivotal role by producing arteriogenic cytokines leading to paracrine stimulation and proliferation of local endothelial cells [Kinnaird et al., 2004]. These data indicate that paracrine signalling is an important mediator of cell therapy and underpins that in the adult organism bone marrow-derived stem cells can act as "cytokine factories" promoting vascular remodelling.

In conclusion, the absence of endothelial cell chimerism after HSCT has several implications. First, it supports the concept of a relative resistance of endothelial cells to GvHD. Second, it excludes endothelial cell chimerism being a mechanism to explain tolerance after ABO-incompatible HSCT. Third, it indicates that, although the existence and potential of bone marrow-derived endothelial progenitor cells have been shown beyond any doubt, transplanted haematopoietic stem cells do not hold a major role in physiological turnover of endothelial cells, repair of vascular injury and tumour neoangiogenesis after allogeneic HSCT.

5.3.6. Acknowledgements

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6. General Discussion

ABO blood group-mismatched haematopoietic stem cell transplantation provides an excellent model to study the mechanistic backgrounds of accommodation, tolerance and the biology of bone marrow-derived endothelial progenitor cells. One major objective of this thesis was to investigate the possibility of vessel repair by donor-derived endothelial cells after allogeneic haematopoietic stem cell transplantation in GvHD-affected and non-affected tissues. By this, a potential mechanism for immunological tolerance after antigen-mismatched transplantation was analysed.

In a first step, it was found, that ABH histo-blood group antigens are expressed in the bone marrow on normal erythropoietic cells as early as the basophilic erythroblastic maturation stage and on all bone marrow endothelial cells. Pure red cell aplasia (PRCA) after major ABO-incompatible haematopoietic stem cell transplantation is caused by anti-A/B isoagglutinins produced by recipient-type plasma cells. The eradication of ABH histo-blood group antigen expressing erythropoietic bone marrow cells in such patients indicates a direct rejection mechanism mediated by anti-donor isoagglutinins. According to this mechanism, we observed a clear association between the recovery of reticulocytes and the decrease of anti-donor isoagglutinins in patients recovering from PRCA.

In addition, as shown by repetitive anti-A/B isoagglutinin titre measurements, anti-recipient isoagglutinins are not produced following minor ABO-incompatible haematopoietic stem cell transplantation, and anti-donor isoagglutinins decrease and stay low in the post-transplant period following successful major ABO-incompatible haematopoietic stem cell transplantation. The hypothesis that anergy, regulation or deletion of B cells may play a key role in tolerance after minor ABO-incompatible haematopoietic stem cell transplantation appears very likely because anti-A/B isoagglutinin titres decline in the post-transplant course in the absence of IgG, IgM, or complement deposition on recipient endothelial cells as shown by direct immunofluorescence in skin biopsies (own unpublished observations). The exact mechanism of this surprising lack of anti-recipient isoagglutinins after minor ABO-incompatible haematopoietic stem cell transplantation was not elucidated during this thesis. Therefore, it can only be speculated on the pivotal role of B cell tolerance following ABO-incompatible haematopoietic stem cell transplantation. ELISPOT assays adjusted from a previously published protocol [Fan et al., 2004] for the analysis of B cells after

haematopoietic stem cell transplantation were never specific enough in our hands to detect A or B blood group antigen-specific B cells. We used commercially available synthetic blood group A or B trisaccharides conjugated to bovine serum albumine (BSA) to coat the plates. Non-specific binding sites were blocked with 10% fetal calf serum (FCS), 1% BSA, 3% BSA, 1% casein or 1% milk, nonetheless antibody-producing cells bound to the plates with a strong cross-reactivity (see Introduction, Figure 6). In addition, we tested different antigen concentrations, different cell concentrations and cells from different ABO blood group types, compared cultured peripheral blood mononuclear cells, peripheral mononuclear cells from the bone marrow and splenocytes and altered washing steps. Due to dissatisfactory results despite considerable efforts we finally had to give up the attempts to establish the A/B blood group antigen-specific ELISPOT assay. One possible reason for the observed cross-reactivity and for the failing of our analyses could be the structural similarity of neo-glycoproteins (A-BSA and B-BSA, Dextra Laboratories, Reading UK). Thus we suggest the use of newly constructed A/B trisaccharides for the coating of the ELISPOT plates. Alternatively the old immunological technique of plaque forming assays (PFA) using red blood cells and complement to detect anti-A/B producing B cells and plasma cells might be revisited to investigate the mechanisms leading to B cell tolerance after minor ABO-incompatible haematopoietic stem cell transplantation. However, to address and resolve this question remains a worthwhile future experimental goal.

In general, lymphocytes respond to antigen on cell surfaces presented by MHC molecules. If the antigen presenting cells are overwhelmed with excess antigen, which is the case after antigen-mismatched haematopoietic stem cell transplantation, lymphocytes bind to free antigen which prevents them from getting the second signals they need to become activated. This condition is known as high-zone tolerance [Swinton et al., 1994]. It is overcome as the excess antigen is removed by phagocytes and serum proteases. Eventually, the antigen concentration is reduced to a stimulatory level. However, continuous exposure to high concentrations of circulating antigens would continuously induce high zone tolerance and by this exhaustion and deletion in a normal mature immune system. Whether in this manner B cell deletion might be responsible for the induction of tolerance after minor ABO-incompatible haematopoietic stem cell transplantation remains to be elucidated. It seems reasonable to speculate that persistent systemic exposure of susceptible cells to A/B histo-blood group antigens plays a pivotal role in the pathway to tolerance.

A second possibly important factor for the development of tolerance is the maturity of the immune system at the time of transplantation. In previous studies, immature B cells have been shown to be more sensitive to tolerance induction than mature B cells [Cambier JC et al., 1976]. Engagement of the B cell receptor (BCR) leads to apoptosis of immature B cells. B cell tolerance by deletion occurs in the naïve and immature human neonate in reaction to non-self A/B antigens following ABO-incompatible heart transplantation [West et al., 2001a]. Similar outcomes have been reported for liver transplantation performed in early infancy [Ohdan et al., 2007]. However, the developmental window of susceptibility to tolerance induction to graft A/B antigens is narrow and remains to be determined in detail. The newly engrafted immune system of haematopoietic stem cell recipients has many characteristics of an immature immune system, e.g. recipients of allogeneic haematopoietic stem cell transplantation need to be vaccinated again for childhood diseases, even though the donor had a normal immune reaction against these pathogens. In conclusion, induction of tolerance after haematopoietic stem cell transplantation might develop in a similar way as the immature human immune system in early infancy.

Another main objective of this thesis was the tracking of transplanted haematopoietic endothelial progenitor cells and the analysis of the origin of vascular endothelial cells in skin biopsies of patients after allogeneic haematopoietic stem cell transplantation. Using ABO- and gender-mismatch for discrimination of donor- and recipient-type cells, heart and bone marrow blood vessels demonstrated no evidence of donor-derived cells incorporated in vascular endothelium in a patient 11 months after haematopoietic stem cell transplantation [Mueller et al., 2006]. This apparent lack of endothelial cell chimerism following haematopoietic stem cell transplantation was then confirmed in a large study involving 52 patients with and without GvHD. A clear strenght of this study including a large number of patients was the combinatorial use of three different methods to distinguish between donor and recipient-type cells, i.e. immunohistochemistry for ABH histo-blood group antigens, in-situ hybridisation for X and Y sex chromosomes and molecular analysis of highly polymorphic short tandem repeats (STR). Each of these methods alone would in our opinion not have been sufficient to conclusively analyse donor-type endothelial cell chimerism. Endothelial cell replacement by bone marrow-derived donor cells does not seem to occur routinely after allogeneic haematopoietic stem cell transplantation and therefore endothelial cell chimerism cannot be accounted as a general mechanism leading to tolerance after ABO-incompatible haematopoietic stem cell transplantation. In addition, donor bone marrow-

derived endothelial cells do not seem to contribute to *in vivo* blood vessel formation after haematopoietic stem cell transplantation, neither in physiological cell turn-over nor in tumour neo-angiogenesis.

In a pilot project we extracted CD133-positive haematopoietic stem cells from a bone marrow aspirate of a healthy donor and cultured these cells in an endothelial growth promoting medium (PromoCell) [Ehrbar M at al., 2005]. Circulating endothelial progenitor cells (EPC) are defined by at the least three markers CD133, CD34 and VEGFR2. We found, that 1.3% of the total nucleated cells in the bone marrow aspirate were CD133-positive. At around day 16 of the cell cultivation cells lost the marker CD133, in turn all cells became positive for CD31, indicating that these cells were differentiating into endothelial cells. To date, it is not clear whether EPC act differently in pathological situations. To investigate this question, it would be interesting to compare the numbers of circulating EPC and their origin with respect to donor or recipient type before and after allogeneic haematopoietic stem cell transplantation, both in patients with and without GvHD. In conclusion, we did not find any evidence for endothelial cell chimerism and EPC are most likely not recruited in higher numbers after routinely performed haematopoietic stem cell transplantation.

Taken together, these results improve our understanding of haematopoietic stem cell plasticity after allogeneic haematopoietic stem cell transplantation. The immunological mechanism that induces tolerance against ABH histo-blood group antigens remains to be elucidated, however, endothelial cell chimerism was firmly excluded from the list of potential explanations. Whether the intensive immunosuppression given to recipients of haematopoietic stem cells could conceivably mask the contribution of ABH histo-blood group antigens to graft rejection or a profound mechanism leading to tolerance is underlying still needs to be clarified in future studies. We assume, if tolerance in ABO-incompatible haematopoietic stem cell transplantation is not explained by changes on the endothelial cell (recipient) side, the underlying mechanism leading to durable tolerance must lie on the side of the donor-derived immune cells.

In the light of the growing need for new strategies to overcome the organ shortage, this research may contribute to the development of new techniques and may have implications for the clinical applicability of new approaches in the field of transplantation medicine such as the transplantation across the ABO blood group barrier.

Furthermore, this study added some more aspects in terms of the scientific controversy about cellular plasticity after allogeneic haematopoietic stem cells transplantation. High hopes were created to replace any kind of tissue by bone marrow-derived stem cells, but only poor evidence for such a plasticity was found and cell fusion evolved as the explanation for the misinterpretation of the potential ability of haematopoietic stem cells to cross beyond lineage barriers. Here, it was clearly shown that the frequency of transplanted haematopoietic stem cells that differentiated into non-haematopoietic cells is too low to be of therapeutic relevance. However, haematopoietic bone marrow-derived stem and progenitor cells might play a role in therapeutic approaches for tissue repair by providing themselves signals to the damaged organ inducing self repair or if they receive additional contributing signals to provide organ repair beyond haematopoiesis.

8. Appendix

8.1. References

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8.2. List of Abbreviations

AA	Aplastic anaemia
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloblastic leukaemia
BMT	Bone marrow transplantation
CISH	Chromogen in-situ hybridisation
CLL	Chronic lymphoblastic leukaemia
CML	Chronic myeloblastic leukaemia
EC	Endothelial cell
ELISA	Enzyme-linked imunosorbent assay
ELISPOT	Enzyme-linked immunospot technique
EPC	Endothelial progenitor cell
FISH	Fluorescence in-situ hybridisation
G-CSF	Granulocyte-colony stimulating factor
GvHD	Graft-versus-host disease
HHT	Hereditary haemorrhagic teleangiectasia
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplantation
HvGD	Host-versus-graft disease
mAb	Monoclonal antibody
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MM	Multiple myeloma
MPS	Myeloproliferative syndrome
NAb	Natural antibody
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PECAM	Platelet/endothelial cell adhesion molecule
PRCA	Pure red cell aplasia
STR	Short tandem repeat
vWF	von Willebrand factor

8.3. Curriculum Vitae

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Date of birth: 13th April 1976

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Education and Work Experience:

• February 2004 - present	PhD-thesis at the Laboratory for Transplantation Immunology, University Hospital Zürich under supervision of Prof. Dr. A. Oxenius, Prof. Dr. H. Hengartner and Prof. Dr. J.D. Seebach
• 10th September 2003	M.Sc Diploma in Biomedical Health Sciences, Radboud University Nijmegen, The Netherlands
• February – July 2003	3rd part Masters Thesis in the group of Prof. Dr. J.D. Seebach, Laboratory for Transplantation Immunology, University Hospital Zürich
• September 2002 – January 2003	2nd part Masters Thesis in the group of Prof. Dr. R.G. Richards, Interface Biology, AO Research Institute, Davos Platz
• September 2000- August 2002	Study of **"Biomedical Health Sciences"** at Radboud University Nijmegen, The Netherlands *Major topic:* "Pathobiology" *Minor topics:* "Neurology" and "Pharmacology"
• March 1999 – March 2000	Research fellow (later recognised as 1st part of my Masters Thesis in Neurology) in the group of Prof. Dr. M.E. Schwab, Dept. of Neuromorphology, Brain Research Institute, Zürich
• October 1996 – February 1999	Study of **"Human medicine"** at University of Zürich
• January 1996	Matura Typus B (with Latin)
• 1989 – 1996	Gymnasium Freudenberg in Zürich

Languages: German, English, Dutch, French, Italian

Awards/ Grants:

- Best Trainee Oral Presentation Award at the Basic Science Symposium, Halifax, Nova Scotia, Kanda, September 5-8, 2007
- Stipend from the Barth-Fonds/.011 from ETH Zürich for travel expenses to the Basic Science Symposium 2007 Halifax, Nova Scotia, Canada, September 5-8, 2007
- TTS Travel Award from The Transplantation Society for the Basic Science Symposium 2007, Halifax, Nova Scotia, Canada, September 5-8, 2007
- Research grant from the Forschungskommission und Nachwuchsförderungskommission der Universität Zürich for 24 months, Start: 1st of November 2005
- Travel Grant Award from the Hartmann-Müller Foundation and the Transplantation Society for the 8th International Xenotransplantation Congress and the 2nd International Symposium on ABO incompatibility in Transplantation, Göteborg, Sweden, September 10-14, 2005
- Best Student Oral Presentation at European Cells and Materials IV (ECMIV) held at Davos congress centre June 30th-July 2nd 2003. Title: Immunohistological identification of the osteoclast formation regulator osteoprotegerin ligand (OPGL/RANKL/TRANCE) in human bone tissue.
- ESB student award in the category best oral presentation at 18th European Conference on Biomaterials held in Stuttgart October 1-4, 2003. Immunohistological identification of the receptor activator of NF-κB ligand (RANKL) in human, ovine and bovine bone tissue.

Publications:

- Puga Yung G, Valli P.V., Starke A., **Mueller R.J.**, Fehr T., Cesar-Özpamir M., Schanz U., Weber M., Wüthrich R.P., Seebach J.D., Stussi G. Measurement of ABO antibodies in ABO-incompatible living-donor kidney transplantation. *Transplantation* 84: S20-S23, 2007
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- **Mueller R.J.**, Zeuner A., Ruscio G., Puga Yung G., Stussi G. and Seebach J.D. ABH histo-blood group antigen expression during erythropoietic stem cell differentiation.

Abstracts:

- **Mueller R.J.**, Stussi G., Nikolic M., Meyer-Monard S., Odermatt B., Biedermann B., Halter J., Schanz U., Seebach J.D. Bone marrow-derived stem cells do not replace recipient endothelium after allogeneic hematopoetic stem cell transplantation. Basic Science Symposium of The Transplantation Society in collaboration with The Canadian Society of Transplantation, September 5-8, 2007, Halifax, Nova Scotia, Canada

- **Mueller R.J.**, Stussi G., Nikolic M., Meyer-Monard S., Schanz U., Seebach J.D. Short tandem repeat analysis to determine endothelial cell chimaerism after haematopoietic stem cell transplantation. Annual Congress of the Swiss Society of Allergology and Immunology, April 19-20, 2007, Basel, Switzerland *Swiss Med Wkly* 2007, 137 (Suppl.157):S27

- **Mueller R.J.**, Stussi G, Ruscio G, Zeuner A, Halter J, Schanz U, Seebach JD. Expression of ABH histo-blood group antigens during haematopoietic cell differentiation. Annual Congress of the Swiss Society of Allergology and Immunology, Zürich, Switzerland, March 30-31, 2006; *Swiss Med Wkly* 2006, 136 (Suppl.149):S52

- **Mueller R.J.**, Stussi G, Halter J., Schanz U., Odermatt B., Biedermann B.C., Seebach J.D. No evidence for endothelial cell chimerism after ABO-incompatible allogeneic hematopoietic stem cell transplantation. 8th International Xenotransplantation Congress and 2nd International Symposium on ABO Incompatibility in Transplantation, September 10-14, 2005, Göteborg, Sweden

- **Mueller R.J.**, Stussi G, Halter J, Schanz U, Odermatt B, Biedermann B, Seebach JD. No evidence for endothelial cell chimerism after ABO-incompatible haematopoietic stem cell transplantation. Annual Congress of the Swiss Society of Allergology and Immunology, Bern, Switzerland, March 3-4, 2005, *Swiss Med Wkly* 2005, 135 (Suppl.144):S24

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- **Mueller R.J.**, Stüssi G., Biedermann B.C., Seebach J.D. ABH histo-blood group antigen expression on endothelial cells after haematopoietic stem cell transplantation. Cellular Interactions in the Immune System, Annual Congress of the Swiss Society of Allergology and Immunology, Geneva, Switzerland, April 17-18, 2004, *Swiss Med Wkly* 2004, 134 (Suppl.140):S41

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