Myofibroblasts and polyploid cells in the conjunctival surface after allogeneic hematopoietic stem cell transplantation

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For my grandparents

Arne and Signe

…and Oskar

I miss you

ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative treatment modality for hematological malignancies, such as leukemia and lymphoma. However, a major complication of the procedure is that the immune effector cells in the graft may become activated towards the host´s healthy tissues, resulting in a condition called Graft-versus-Host Disease (GvHD).

Ocular symptoms are among the most common manifestations of GvHD, afflicting at least 50% of all allo-HSCT recipients, typically through dry eye syndrome, “keratoconjunctivitis sicca”, with features of fibrosis.

This thesis describes a series of studies in which impression cytology was used to sample the ocular surface of allo-HSCT recipients and healthy individuals in order to identify and quantify two cell types – myofibroblasts (MFB) and polyploid cells (PP), both of which may play a role in the pathogenesis of ocular GvHD.

Myofibroblasts were identified through immunofluorescence staining for alpha-smooth muscle actin (αSMA). Moreover, in female allo-HSCT recipients with a male donor, cells of donor origin could be detected through sex chromosome-specific fluorescence-in situ-hybridization (FISH). FISH was also used to identify polyploid cells through their abnormally high chromosome content.

Our results indicate that myofibroblasts of donor origin are present, and increased with time, in the conjunctival surface after allo-HSCT. However, also recipient-derived myofibroblasts was a consistent finding, and were detectable many years after transplantation. These data indicate an ongoing concurrent myofibroblast differentiation from donor and recipient progenitors after allo-HSCT. The donor-derived *versus* total MFB ratio correlated significantly with ocular GvHD. Polyploid cell density increased soon after transplantation, peaked in the 3-12 month interval, and then disappeared.

Compared to post-transplant findings, normal conjunctiva displayed significantly fewer myofibroblasts, but there was a distinct seasonal variation in MFB density, exhibiting a minimum during December - February and a maximum in March - May. Polyploid cells, though occasionally found in the normal conjunctiva, were significantly fewer than after allo-HSCT.

In conclusion, our results demonstrate a higher than normal MFB density in the conjunctiva of patients after allo-HSCT. The donor-derived majority of myofibroblasts increased with time post-transplant, and was found to correlate with presence and severity of ocular GvHD. Myofibroblasts are cellular mediators of normal wound-healing, but also of fibrosis and connective-tissue disease. Our data suggests that myofibroblasts may play a role in the pathogenesis of chronic ocular Graft-versus-Host Disease. The role and significance of the high amounts of polyploid cells observed during the first year after allo-HSCT remains obscure.

POPULÄRVETENSKAPLIG SAMMANFATTNING

BAKGRUND

Hematologiska maligniteter, det vill säga cancersjukdomar i de blodbildande organen, är en av de vanligaste typerna av elakartad tumörsjukdom. Några exempel på sjukdomar i denna kategori är lymfom och leukemier. I Sverige insjuknar ungefär 3600 människor varje år i någon form av hematologisk malignitet.

Allogen hematopoietisk stamcellstransplantation (allo-HSCT) är en behandlingsform vid vilken patienten efter en stark cellgiftsbehandling erhåller nya friska *hematopoietiska* (blodbildande) *stamceller* från en donator. De nya stamcellerna återställer patientens benmärgsfunktion och de vita blodkroppar som bildas, särskilt *lymfocyter*, har dessutom kapacitet att utradera eventuella kvarvarande tumörceller. Allo-HSCT är en ofta botande behandling vid leukemier och lymfom.
Metodiken vid allo-HSCT har på senare år förfinats och gjorts tillgänglig för allt fler och äldre patienter. I Sverige genomgår numera 200-250 patienter allo-HSCT varje år, vanligen vid leukemi (75%) och lymfom.

Allo-HSCT är en komplicerad och långt ifrån ofarlig procedur. Det vanligaste problemet är den så kallade *”Graft-versus-Host”-sjukdomen* (GvHD), som beror på att att donatorns vita blodkroppar attackerar, och ibland förstör, mottagarens friska vävnader. Ungefär 70% av alla patienter som genomgått allo-HSCT drabbas av någon form av GvHD som ofta medför nedsatt livskvalitet och ibland allvarliga eller livshotande tillstånd.

Ögonsymptom är mycket vanligt efter allo-HSCT och medför skavkänsla, ljuskänslighet och torrhet, mera sällan sårbildning på hornhinnan och varaktig synnedsättning.

De mekanismer som ligger bakom ögon-GvHD är i stort sett okända. Dock verkar en sjuklig ökning av bindväven (*fibros*) i tårkörtlar och på ögonytan vara en viktig del av sjukdomsutvecklingen. Fibros är en faktor även vid GvHD i andra organ.

UTFÖRANDE

Vi har för provtagning av ögats yta använt *avtryckscytologi*, en skonsam och ofarlig metodik vid vilken ett sterilt cellulosafilter kortvarigt trycks mot ögonvitan. De celler som fastnar på filtret kan analyseras med FISH-metodik (*fluorescence in situ hybridization*) med avseende på innehåll av könskromosomer (XX hos kvinnor; XY hos män), och med avseende på förekomst av vissa proteiner (*immunofluorescence-analys; IF*). Dessa metoder kan appliceras på samma cellmaterial och om patienten är en kvinna som genomgått allo-HSCT från manlig donator kan man dels med IF identifiera celltyp, dels (med FISH) skilja ut celler som utgår från patient respektive donator. Cellfynden kan sedan ställas i relation till patienternas sjukhistoria och aktuella tillstånd, särskilt förekomst av GvHD. Genom att även undersöka ögonytan hos friska personer erhöll vi ett kontrollmaterial där ögonytans celler vid upprepad provtagning från olika lokalisationer, och vid olika yttre förhållanden kartlades.

RESULTAT

Arbete I (”*Donor-derived myofibroblasts in the ocular surface after allogeneic hematopoietic stem cell transplantation*”) beskriver fyndet av dessa celltyper i 8 kvinnliga patienter med manlig donator, med och utan ögonGvHD och med varierande förfluten tid efter transplantation. Vi fann:

- Celler med donatorursprung fanns i ögonytan hos samtliga patienter. Cellerna var s.k. *myofibroblaster*, en celltyp som har sårläkande uppgifter.
- *Polyploida celler*. Dessa var mycket stora celler med mångdubbelt fler kromosomer än normalt och var av patientursprung; de hade alltså inte utvecklats från donatorns celler.

Arbete II ("*Myofibroblasts in the normal conjunctival surface*") beskriver förekomsten av de ovan nämnda celltyperna i den friska ögonytan. Fynden relaterades till bakgrundsfaktorer som kontaktlinsbärande, ögonallergi och tidpunkt (säsong) för provtagning. Dessutom studerades även betydelsen av provtagningsteknik, lokalisation och intervallen mellan provtagningar. Vi fann:

- Myofibroblaster finns även i friska personers ögon, fastän i lägre antal än efter allo-HSCT. Vi observerade en årstidsvariation med ett större antal MFB under sommarhalvåret.
- Även polyploida celler finns i den friska ögonytan, men i lägre antal än efter allo-HSCT.

Arbete III *("Conjunctival polyploid cells and donor-derived myofibroblasts in ocular Graft-versus-Host Disease"*) beskriver en studie av en större patientgrupp, som alla genomgått allo-HSCT. Förekomsten av myofibroblaster och polyploida celler ställdes emot ett flertal kliniska faktorer, bl a ögonGvHD och tid sedan transplantation. Viktigaste fynd:

- Myofibroblaster med donatorursprung fanns hos nästan alla patienter, ibland så tidigt som en månad efter transplantationen. Hos en patient fann vi donator-MFB även i ett skede efter avstötning, dvs då enbart patientceller kunde påvisas i blod och benmärg.
- Det fanns en tidsberoende kontinuerlig ökning av MFB efter allo-HSCT. Polyploida celler fanns i kraftigt ökat antal under en period, 3-12 månader efter transplantationen.
- Det fanns ett samband mellan den relativa mängden donator-MFB och ögonGvHD.
- Myofibroblaster med patientursprung kunde påvisas även många år efter transplantation, trots att enbart donatorceller då fanns i blod och benmärg.

DISKUSSION OCH SAMMANFATTNING

Vårt huvudsyfte med dessa studier var att identifiera, karakterisera och ursprungsbestämma de celler som finns i ögats yta efter allo-HSCT, och att ställa fynden i relation till patienternas aktuella och tidigare sjukdomshistoria.

Vi har studerat två celltyper - myofibroblaster och polyploida celler - i ögats yta, dels hos friska personer (arbete II), dels hos patienter efter allo-HSCT (arbete I och III).

Myofibroblasten är en viktig cell vid sårläkning men kan också orsaka oönskad och farlig bindvävsökning vid olika sjukdomar. Våra resultat innebär att en ökning av myofibroblaster kan vara involverad vid ögon-GvHD. Denna tanke stöds av att merparten av ögats myofibroblaster efter allo-HSCT utvecklats från de (stam-)celler som erhållits från donatorn.

Den polyploida cellen karakteriseras av ett kromosominnehåll mångdubbelt högre än det normala, och har mestadels studerats inom ramen för tumörtillväxt, men vissa studier sätter den i samband med vävnaders svar på immunologisk stress. Efter allo-HSCT ökar antalet polyploida celler kraftigt under det första året, det vill säga under den tid som kronisk GvHD, ett slags immunologisk stress, debuterar. Det är inte otänkbart att fyndet av polyploida celler kan få betydelse för förståelsen av kronisk ögon-GvHD.

Sammanfattningsvis beskrivs i denna avhandling, för första gången, förekomsten av myofibroblaster och polyploida celler både hos friska personer och hos patienter efter allogen stamcellstransplantation. Vi har observerat en kraftig ökning av båda celltyper efter alloHSCT. Den markanta relativa ökningen av myofibroblaster med donatorursprung vid samtidig GvHD är ett fynd som skulle kunna bana väg för ny behandling vid detta tillstånd.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Donor-derived myofibroblasts in the ocular surface after allogeneic haematopoietic stem cell transplantation.**
Hallberg D, Wernstedt P, Hanson C, Wettergren Y, Stenberg K, Brune M, Stenevi U.

Acta Ophthalmol. Scand. 2006; **84**: 774-780.

II. **Myofibroblasts in the normal conjunctival surface.**
Aguilar X, Hallberg D, Sundelin K, Hanson C, Stenberg K, Brune M, Stenevi U.

Acta Ophthalmol. 2010; 88: 407-412.

III. **Conjunctival polyploid cells and donor-derived myofibroblasts in ocular Graft-versus-Host Disease.**
Hallberg D, Stenberg K, Hanson C, Stenevi U, Brune M.

Manuscript

LIST OF ABBREVIATIONS

aGvHD	Acute Graft-versus-Host Disease
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
APC	Antigen-presenting cell
Auto-HSCT	Autologous hematopoietic stem cell transplantation
BM	Bone marrow
cGvHD	Chronic Graft-versus-Host Disease
DAPI	4,6-diamidino-2-phenylindole
ECM	Extracellular matrix
FICT	Full intensity conditioning transplantation
FISH	Fluoresence-in situ-hybridization
G-CSF	Granulocyte colony-stimulating factor
GvHD	Graft-versus-Host Disease
GvL	Graft-versus-Leukemia Effect
HLA	Human leucocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IC	Impression cytology
IF	Immunofluoresence
IFNγ	Interferon gamma
IL	Interleukin
MFB^{TOT}	Total myofibroblast density
MFB^{XX}	Recipient-derived myofibroblasts
MFB^{XY}	Donor-derived myofibroblasts
MOMP	Monocyte-like mesenchymal progenitor
MtF	Male-to-female
NP40	Nonyl phenoxypolyethoxylethanol
PBS	Phosphate-buffered saline
PBSC	Peripheral blood stem cell
PP	Polyploid cell density
RICT	Reduced intensity conditioning transplantation
TBI	Total body irradiation
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
αSMA	Alpha-smooth muscle actin

“The jester laughs ´cause the world is full of fools”

- Thorin, *A Wolf in Wolf´s Clothing*

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INTRODUCTION

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic and autologous hematopoietic stem cell transplantation (HSCT) is a treatment modality for myeloid and lymphatic malignancies and also for selected solid tumors. Both procedures include the collection and infusion of hematopoietic stem cells (HSCs) either from a donor (allo-HSCT) or from the patient her-/him-self (auto-HSCT). However, auto-HSCT and allo-HSCT constitutes different treatment principles which are detailed below. Notably, the main topic of this thesis, the Graft-versus-Host Disease, is not seen after auto-HSCT.

Autologous HSCT

Autologous HSCT entails the collection and cryogenic storage of the patient´s own hematopoietic stem cells. It is commonly used in the treatment of newly diagnosed myeloma and relapsed lymphoma patients. Using autologous HSC as a rescue after a myeloablative chemo-/radiotherapy, hematopoietic and immunological recovery is typically quick with a relatively low risk of complications. Thus, the sole principle of auto-HSCT is to deliver a high, sometimes curative, cytotoxic treatment without jeopardizing the patient´s life or long-time well-being. However, the low transplant-related mortality after auto-HSCT comes at the price of a substantial risk of disease relapse.

Allogeneic HSCT

Hematopoietic stem cell transplantation (HSCT) from an HLA-identical sibling or a matched unrelated donor is a potentially curative treatment for patients with myeloid and lymphatic malignancies. The procedure may be introduced as a primary treatment approach in chronic, slowly progressing disease, such as myelodysplastic syndromes, or as a relapse-preventive measure for acute leukemia or lymphoma patients in chemotherapy-induced remission (Baron et al, 2004; Porter et al, 2001). Apart from its use as a treatment for hematological malignancies, allo-HSCT is a valuable tool for non-malignant diseases including congenital or acquired immune deficiencies (Burroughs et al, 2010), severe aplastic anemia (Young et al, 2010) and hemaglobinopathies (Galanelloi et al, 2010).

Allo-HSCT involves an immunological treatment component in addition to the chemo-radiotherapy. Donor effector cells may attack and eradicate malignant cells present after the cytotoxic therapy, a mechanism denominated *graft-versus-leukemia effect* which presents a markedly reduced relapse-

risk compared with auto-HSCT. However, this benefit comes at the cost of slower immunological recovery, and also a higher risk of transplant-related morbidity and mortality due to infections and immunological complications. *Graft-versus-host disease* (GvHD) is the most common side early and late complication after allo-HSCT and is decribed in detail below. Against the background of its resulting side effects, indications for allo-HSC must be carefully considered, and the procedure applied only when the risk of malignant relapse outweighs the risk of transplant-related complications.

Procedure overview

In Sweden, approximately 250 allo-HSCT procedures are performed each year, at six centers. Approximately 75% of those allografts are indicated by leukemias, while lymphomas and non-malignant indications account for 15% and 10%, respectively.

Allo-HSCT is a complex, labor-intense and expensive endeavor, demanding extensive coordination between clinic, caregivers, laboratory medicine and external resources (such as donor registries). Donor and patient must be screened and the harvested cells transported and analyzed before infusion to the patient. Combined, allo-HSCT presents a considerable challenge on not only the clinical and technological resources but also with respect to logistical support and coordination structure.

Donor selection

The primary requirement for a successful allo-HSCT is the identification of a suitable donor. Donor choice is based on the demands for (i) a rapid and sustained successful engraftment; (ii) an operative and effective GvL effect and (iii) the need to minimize post transplant complications, particularly acute and chronic GvHD. Traditionally, the donor was a sibling matched for class 1 and class II Human Leukocyte Antigens (HLA) molecules encoded for by genes in the Major Histocompatibility Complex (MHC) on chromosome 6 (Petersdorff et al, 2007). However, if an HLA-identical and medically fit sibling donor is not available, an unrelated registry donor can be identified in 50-75% of cases. Unrelated adult donors now accounts for more than half of allo-HSCT performed. (Mullighan et al, 2006; Nowak et al, 2008)

Stem cell collection

Up until the late 90´s, the predominant source of hematopoietic stem cells was the donor´s actual bone marrow tissue, aspirated from the iliac crest. In more recent years, peripheral blood stem cell

(PBSC) mobilization and collection has to a large extent replaced bone marrow aspiration as the source of hematopoietic stem cells (Jansen et al, 2002; Bacigalupo et al, 2000). For PBSC collection, the donor is pretreated with granulocyte colony stimulating factor (G-CSF), which causes increased stem cell activation and the release of hematopoietic progenitor cells into the peripheral blood. The mononuclear fraction containing the PBSCs is collected through continuous-flow centrifugal apheresis. The process presents few risks of complication and causes a minimum of discomfort for the donor. For the patients, the shift from BM to PBSC as the source of stem cell has led to a more rapid engraftment of donor cells, but also to an increased risk for chronic Graft-versus-Host Disease (Mohty et al, 2002).

In later years, with the establishment of cord blood banks and registries, in which hematopoietic stem cells collected from umbilical cord blood are cryogenically stored, a third stem cell source has arrived, and indeed increasingly used (Sanz et al, 2002; Lee et al, 2010)

Pre-transplant conditioning

Prior to stem cell infusion, the recipient must be pretreated – *conditioned* – with chemotherapy and/or total body irradiation (TBI). The reason for pre-transplant conditioning is twofold: (i) the recipient´s immune defense must be suppressed to enable successful engraftment of the transplanted HSCs, and (ii) the malignant clone must be forced into recession in order to give time for donor immune effector cells to establish the graft-versus-leukemia effect (see below). The various conditioning courses may be divided into two main groups: *full* and *reduced* intensity regimens.

Full intensity conditioning transplantation (FICT) is, as the name implies, the more aggressive of the two alternatives encompassing a very toxic, pre-transplant myeoablative regimen. The aims are to irrevocably suppress the recipient´s immune functions and to completely eradicate the malignant clone. The primary downside of this strategy is the risk of acute extra-medullary organ damage and bacterial and fungal infections acquired in the cytopenic phase. The acute risks of the regimen act as a limiter as to which patient should be considered a candidate for a FIC transplant.

Since the late 90´s, reduced intensity conditioning transplant (RICT) has sailed up as a gentler alternative to FICT, and hence applicable to a wider range of patients, partially circumventing the organ toxicity and early risk associated with FIC transplants (Storb et al, 2001; Deeg, 2010). The therapeutic objective of the reduced condition is not immediate myeloablation, but to achieve an immunosuppression providing the transplanted cells with an engraftment window. In addition, most RIC regimens are moderately toxic to the recipient´s bone marrow, and cause a short but marked cytopenia associated with risk of infection. Thus, although based on the same basic principle as the

myeloablative pre-transplant regimen, the reduced conditioning regimens, with their moderate anti-leukemic effect, to a larger extent relies on the graft-versus-leukemia (GvL) effect.

The major advantage of the RICT approach is that it can be used on a wider patient spectrum, most importantly those sub-optimalized for FICT through age or comorbidities. However, the incomplete myeloablation is associated with an increased rate of relapse, and the incidence of chronic GvHD is similar to what is reported from transplantations preceded by myeloablative conditioning. The recipient will also be immunologically compromised until complete hematopoietic reconstitution has been achieved, a process which may take several months or years (Mohty et al, 2007).

Graft-versus-Leukemia Effect

A significant benefit of allo-HSCT is the graft-versus-leukemia (GvL) effect (Imamure et al, 2009). As the transplanted cells have successfully engrafted, and hematopoietic function restored, the reconstituted immune function will engage and eradicate residual leukemic cells. Donor T-cells activate against anything not recognized as “self”, including malignant cells. This ongoing sentinel activity is the basis for GvL, which accounts for the reduced frequency of relapse after allo-HSCT compared to auto-HSCT. However, this protection from leukemic recurrence comes at the price of certain transplant-related complications, chiefly among them graft-versus-host disease, For a review see Riddell et al, 2003.

GRAFT-VERSUS-HOST DISEASE

Graft-versus-Host Disease (GvHD), the "evil twin" of GvL, is the most common complication after allo-HSCT, affecting up to 80% of graft recipients (Ferrara et al, 2009). GvHD is commonly defined as the activation of donor-derived immunocompetent cells against healthy tissues of the recipient, and is, apart from relapse, the most frequent cause of transplant-related mortality (Shlomchik et al, 2007; Edinger et al, 2009). Risk factors for GvHD include HLA mismatch, advanced donor or recipient age, the combination of female donor and male recipient, aggressive conditioning regimens and the use of peripheral blood as stem cell source. GvHD comes in two separate forms: acute and chronic GvHD, which are quite distinct entities in terms of symptom presentation, localization, histopathology and pathophysiology.

Acute GvHD

Typically clinical presentations of acute GvHD (aGvHD) occur in skin, liver and gastrointestinal tract. The onset is often sudden and characterized by skin rash, jaundice and diarrhoea. Acute GvHD was previously defined as GvHD symptoms and signs occurring before 100 days post-transplant. However, with the advent of reduced intensity conditioning transplants, the onset of typical aGvHD symptoms may occur later. The pathophysiology of aGvHD is well known, the underlying process being a damage of epithelial cells in the target tissues i.e. the basal membrane cells of the skin, intestinal crypts and bile ducts (Ball et al, 2008; Goker et al, 2001). Around 80% of aGvHD patients respond to treatment with high-dose corticosteroids, whereas mortality is high in non-responding patients.

Chronic GvHD

Chronic GvHD (cGvHD) typically presents later than 100 days after transplantation, sometimes following a period of acute GvHD, sometimes being a de-novo phenomenon. The clinical manifestations of cGvHD are heterogeneous, exhibiting a wide range of symptoms from lichen-type features in mucous membrane of mouth, eyes and vagina, to serosal inflammation and fibrosis of pleurae and skin (Cutler et al, 2006; Mohty et al, 2010). Chronic GvHD is common, affecting between 40 and 70% of all allo-HSCT recipients (Jagasia et al, 2007; Pérez-Simón et al, 2008). The common denominator in post-inflammatory, end-stage cGvHD is fibrosis reminiscent of those found in autoimmune disorders, including sclerodermoid skin lesions, oral or vaginal lichen planus, pulmonary fibrosis, liver cirrhosis and dry eye syndrome (Vogelsang et al, 2004; Shulman et al 2006; Arora et al, 2009). Typical features of skin cGvHD include sclerotic features, which may also include

dyspigmentation, obliteration of sweat glands, partial hair loss and nail dystrophy (Ratanathanathorn et al, 2001; Mohty et al, 2002; Häusermann et al, 2008)

The mucosal membranes are favored targets of cGvHD. Oral cGvHD may present as lichen planus-like plaques, ulcers or mucosal dystrophy (Imanugli et al, 2008). In female patients, gynecological symptoms are common, including mucosal inflammation, tenderness, dyspareuni, vaginal stenosis and syneciae of the labia minor (Spinelli et al, 2003).

More serious – or even life threatening – manifestations of cGvHD may involve the lungs (bronchiolitis obliterans), liver (bile duct cirrhosis) or gastrointestinal tract (Vargas-Díez et al, 2005). The treatments consist of non-specific immunosuppression including local or systemic corticosteroids and calcineurin inhibitors (Mohty & Apperley, 2010).

Immunological mechanisms of Graft-versus-Host Disease

Acute GvHD is fairly well studied, and has been shown to be primarily mediated through interaction between the recipient´s own remaining antigen-presenting cells (APC:s) with donor-derived helper (CD4+) and cytotoxic (CD8+) T-cells (Jacobsohn et al, 2007; Sun et al, 2007). The reconstituted donor-derived T-cell population is introduced into an already inflammatory environment, a "cytokine storm" due to tissue injury caused by pre-transplant conditioning. Lacking anergic de-selection *vis-á-vis* recipient antigens, these T-cells become activated, initiates clonal expansion and attacks recipient tissues. This may be effected through either direct cytotoxic interaction (cell-cell interaction-dependent apoptosis or localized release of cytotoxic granules; CD8+ T-cells), or indirectly, through CD4+ helper T-cell release of acute-phase proinflammatory cytokines such as interleukin 2 (IL-2) and gamma-interferon (IFNγ) (Reddy et al, 2003; Pacsezny et al, 2010).

Chronic GvHD is poorly defined from an immunological perspective. A growing body of evidence indicates that an autoimmune process is at work in cGvHD (Toubai et al, 2008). It has been suggested that cGvHD is effected through a continuation of recipient-specific antigen presentation, the proinflammatory "relay torch" passed on to donor-derived APC:s (Shlomchik et al, 2007; Tyndall et al, 2008). This model describes CD8+ (cytotoxic) T-cell activation through cross-presentation of host antigens on MHC class I. This suggestion is compelling in view of the similarity between clinical cGvHD symptoms and symptoms featured in certain protean autoimmune disorders (such as Sjögren´s syndrome).

Chronic GvHD is characterized by a late-phase inflammation profile, dominated by production of interleukins 1, 6, 10 and 13; an unspecific cytokine profile found in many chronic inflammatory and

profibrotic disorders. Increased levels of IFNγ, tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) have also both been seen to associate with cGvHD (Liem et al, 1999; Holler et al, 2002; Martin et al, 2008; Skert et al, 2009; Poloni et al, 2011). From a histopathological perspective, the inflammatory micro-milieu is dominated by a diffuse, low-level cell infiltration, predominantly macrophages and T-cells, bundles of alpha-smooth muscle actin-expressing fibroblasts (myofibroblasts) and a marked loss of tissue architecture and homeostasis (Sloane, 1990; Shulman et al 2006).

Recent studies have reported B-cell and autoantibody involvement in cGvHD, a process in which recipient-specific antigens are recognized by donor-derived B-cells, resulting in production of recipient-targeting antibodies (Patriarcha et al, 2006; Daileker et al, 2007; Shimabukuro-Vornhagen et al; 2009; Hofmann et al, 2009). Reportedly, therapy with anti-CD20 (anti-B-cell) monoclonal antibody (rituximab) has shown to alleviate symptoms of cGvHD (Kharfan-Dabaja et al, 2010).

THE OCULAR SURFACE IN GRAFT-VERSUS-HOST DISEASE

Structure and function of the ocular surface

The ocular surface consists of two types of epithelial layer: the corneal and the conjunctival epithelia. The corneal epithelium, a non-keratinized stratified squamous epithelium, is fully transparent, allowing for transmission of light into the interior of the eye.

Along the limbal border, the corneal epithelium interfaces with the conjunctival epithelium, which is opaque and of stratified columnar or cuboidal type. The conjunctiva is continuous from the limbus, over the sclera (bulbar conjunctiva) and lines the inside of the eyelids (tarsal conjunctiva), participating in the first-line defence against ocular infection.

The corneal and conjunctival epithelia exhibit differential cytokeratin profiles. The corneal epithelium express cytokeratin 3 (CK3), which is absent in conjunctival epithelium. Conversely, the conjunctiva expresses cytokeratin 19 (CK19) while the corneal epithelium does not (Ryder et al, 1990; Elder et al, 1997). Hence, the two epithelial types may be separated through immunohistochemical staining. This technique is in clinical application for the diagnosis of corneal stem cell deficiency (Singh et al, 2005), in which the corneal epithelium fails to sustain itself and is invaded by conjunctival epithelium, resulting in a loss of transparency.

The ocular surface is further covered by the tear film, which consists of three layers. The tears, the aqueous phase, are produced by the lacrimal glands situated in the upper temporal part of the orbit. Mucin, a proteoglycane produced by goblet cells in the conjunctival surface, enables hydrophilic retention of the tear film on the ocular surface. Outermost is a layer of lipids, produced by the Meibomian glands, which inhibits evaporation of the aqueous phase.

Ocular Graft-versus-Host Disease

Ocular manifestations of GvHD – the main focus of this thesis – are among the most common sequlae after allo-HSCT, is clinically evident in approximately 50% of all recipients, and may occur in both the acute and chronic variants of GvHD. Symptoms range from slight ocular dryness, irritation and photophobia, through inflammatory states (such as conjunctivitis, chemosis and blefaritis) up to and including severe corneal and conjunctival ulceration, corneal melting and permanent loss of visual acuity (Kerry et al, 1999; Ogawa et al, 1999; Tabbara et al, 2009; Riemens et al, 2010; Westeneng et al, 2010).

In the chronic form of ocular GvHD, a fibrotic thickening of the upper tarsal plates is often noted, which may progress into degradation of eyelid function and contraction of the tarsal plate. In the most severe cases fusion of the tarsal and bulbar conjunctivae can be observed. Other common findings include conjunctival epithelial roughening, dystrophy or defects as well as cyst formation on the tarsal conjunctiva and ciliae loss (Anderson et al, 2004; Filipovich et al, 2005; Kim et al, 2006; Teär-Fahnehjelm et al, 2008).

Histopathologically, fibrotic obstruction of the lacrimal glands and tear ducts are routinely observed and tear substitution is used by approximately two-thirds of all allo-HSCT recipients (Ogawa et al, 2003). In summary, after HSCT ocular GvHD may inhibit many daily activities, such as outdoor sports, vehicle driving and computer usage and may have negative impact on quality of life.

FIBROSIS AND FIBROBLASTS

Fibrosis is characterized by a pathological over-deposition of extracellular matrix components, primarily collagen, due to an abnormal healing response. It may be initiated by a variety of factors, such as autoimmune or allergic disease, infection, radiation or chemical injury and other forms of persistent inflammation. No matter what the initiating factor is, the end result is the same; loss of tissue homeostasis, architecture and function. An obliteration of capillaries is often seen. Disruption of the microcirculation may result in tissue ischemia, which then in turn accelerates the fibrotic process.

In the fibrotic lesion, fibroblasts are the most prolific cell type, depositing extracellular matrix and, according to recent findings, propagating the chronic inflammation through antigen presentation and cytokine production.

Fibroblasts

The fibroblast is a mesenchymal-type, ubiquitous cell, found in virtually all tissues. Although its main role seems to be the turnover and maintenance of stroma and supporting tissues, there has recently developed an interest in the fibroblast as a participant in the immune response, in some cases even implicating fibroblasts as culprits and main effector cells in autoimmune fibrotic diseases (Smith et al, 2005).

One of the major physiological functions of the activated fibroblast is deposition of collagens, elastins, proteoglycans and other extracellular matrix (ECM) components during late-stage inflammation and wound healing, along with its ability to effect tissue contraction through expression of alpha-smooth muscle actin (αSMA).

The αSMA-expressing fibroblast (termed *myofibroblast; MFB*) has a crucial role in wound closure to minimize the pathogen exposure area and accelerate the regenerative response. In normal wound healing, myofibroblasts appear in massive numbers once the primary inflammation is resolved, effect wound healing and scar formation and then disappear through apoptosis (Desmouliére, 1995; Gabbiani, 2003). However, a persistent myofibroblast presence is commonly seen in terminal fibrosis and protean disease conditions, for example keloid scarring and Dupuytren´s contracture (Hinz et al, 2007).

The traditional, rather simplistic view, of fibroblasts as caretaker cells are now changing into a more complex picture, as it has been shown that fibroblasts, like leukocytes, exhibit phenotypic and

functional diversity and are capable of modulatory interactions with the immune system (Smith et al, 2005).

Origin(s) of the fibroblast

Traditionally, the presence of fibroblasts in granulation tissue during wound healing and fibrotic disorders are seen as originating within the quiescent population of stromal mesenchymal cells, responding to late-stage inflammatory cytokines and activation factors released from disturbed extracellular matrix (ECM) (Smith et al, 1997). While this is doubtless true, and may constitute the most common pathway of fibroblast derivation, at least two other differentiation routes of fibroblasts have been identified.

A second source of fibroblasts is epithelial-to-mesenchymal transition (Taylor et al, 2010), a somewhat disputed phenomenon in which epithelial-type cells triggered by tissue stress or injury may assume fibroblast morphology and characteristics. In some instances, endothelial-to-mesenchymal transition has also been reported (Zeisberg et al, 2007).

Recently, a third source of fibroblasts have been reported – the monocyte-like mesenchymal progenitor (MOMP; Seta et al, 2007) - or "fibrocyte" (Quan et al, 2004), which is described as a bone marrow-derived cell present in the peripheral circulation. The circulating progenitor may extravasate at the site of injury or inflammation and adopt mesenchymal properties such as production of collagen I and III, expression of mesenchymal markers (fibronectin and others), and the ability to myodifferentiate (i.e. express αSMA) under the influence of TGFβ. Moreover, the MOMP/fibrocyte has been shown to express various hematopoietic markers such as CD45 (a pan-leukocyte marker), CD14 (monocyte marker) and CD34 (hematopoietic progenitor marker), indicating a hematopoietic-lineage origin (Kuwana et al, 2003).

Fibroblasts in ocular and systemic cGvHD

Several studies have demonstrated that fibroblast-like cells of bone marrow origin participate in the pathogenesis of fibrosis. For a review see Lin et al, 2008. Fibrosis is a hallmark of chronic GvHD and several studies have indicated that fibroblasts of donor origin can be found in the fibrotic lesions of ocular cGvHD (Ogawa et al, 2005). Shirai et al (2009) reported that in patients with chronic myeloid leukemia (CML), fibroblasts exhibiting the CML-specific chromosomal marker, the Philadelphia chromosome, were detected in skin and connective tissues.

In a series of studies Ogawa and co-workers (1999, 2001, 2005), showed that (i) bone-marrow-derived (CD34+) fibroblasts participate in the fibrotic obstruction of the lacrimal gland; (ii) these

fibroblasts were partially of donor origin, and (iii) the presence of donor-derived fibroblasts were associated with chronic GvHD involving the lacrimal gland.

Plasticity of the hematopoietic stem cell?

Fibroblasts aside, the presence of other non-hematopoietic, donor-origin cell types have been demonstrated in allo-HSCT recipients. The cell types involved are mainly of the epithelial or mesenchymal lineages, suggesting that some form of trans-, or crosslinear differentiation mechanism may be active after allo-HSCT. Eberwein et al, 2009, reported the presence of donor-derived conjunctival epithelium in gender-mismatched allo-HSCT recipients.

Other studies have described a donor-derived subset population in alveolar, nasal and gastrointestinal epithelium and among hepatocytes, myocytes as well as vascular and endometrial endothelium after allo-HSCT (for review, see Rovó et al, 2008). The current general consensus favors the transdifferentiation model, although alternate theories for the appearance of non-hematopoietic cell types, including fagocytosis-mediated horizontal gene transfer, have been proposed (Waterhouse et al, 2011).

AIMS

Paper I: To identify and characterize donor cells in ocular surface impression cytologies from recipients of gender mismatched (male-to-female) allogeneic hematopoietic stem cell transplantation.

Paper II: To quantify myofibroblasts and polyploid cells in the ocular surface of healthy volunteers and to determine their seasonal and anatomical variations.

Paper III: To quantify myofibroblasts and polyploid cells in a larger group of allo-HSCT recipients and investigate the associations between these cell types and various clinical factors, including presence of ocular and systemic GvHD, time elapsed since transplantation and conditioning regimen.

METHODS

Patient selection

In paper I, the patients enrolled were gender-mismatched (male-to-female; n=9) allo-HSCT recipients with a clinical history of ocular GvHD, but without currently active symptoms. In the study described in paper III, forty-six allo-HSCT recipients were enrolled, to a total of 70 samples. Male-to-female recipients were actively sought (MtF group; 25 patients; 37 samples), to make possible the identification of donor cells. Remaining patients were enrolled at regular visits or after referral due to ocular symptoms after allo-HSCT (21 patients; 33 samples). The aim was to obtain imprints from patients with a wide spectrum of systemic and ocular GvHD, and at varying time points after transplantation. Clinical background data were obtained from medical records.

For the study described in paper II, 35 healthy individuals (25 women, 10 men; “screening group”) were enrolled. Twelve of these (10 women, 2 men; “1-year group”) were followed by sampling 2-5 times per month. The 1-year group was selected according to availability.

Grading of ocular GvHD

All sampling and clinical grading was performed by two ophthalmologists, both familiar with ocular GvHD and imprint cytology. Ocular status at sampling was graded according to a four-level scale as outlined below (adapted from Filipovich et al, 2006 and Riemens et al, 2010). The grading was done through review of medical records, without knowledge of laboratory study results.

Stage I: Grittiness, foreign body sensation, redness, mucous or mucopurulent secretion, tearing, photophobia with or without pain. Objective findings were discrete and unspecific hyperemia with smooth conjunctival uptake of fluorescein. The conjunctival manifestations were seen over the tarsal plate in everted upper eye lids. The corneal staining with fluorescein, i.e. small, sporadic points in the epithelium, became overt one to two minutes after installment of fluorescein. The findings correspond to those of conjunctivitis. Some patients used lubricant eye drops one to three times per day, while some did not use any at all.

Stage II: Conjunctivitis more advanced with chemosis and inflamed eyelids (i.e. blepharitis). Sharp vision was not always present. Follicular inflammation was seen in the conjunctiva of the lower eyelids, along with small cyst formations in the superior tarsal conjunctiva. After application of fluorescein, areas of epithelial loss and degeneration in the lower part of the corneal epithelium were seen in the corneal microscope. Lubricant eye drops were used at least three times per day.

Stage III: Grittiness, photophobia, ocular pain, redness and threadlike mucous secretions during daytime were commonly seen, often affecting visual acuity. Swollen eyelids, ciliae loss, symblepharon and fibrosis of the superior tarsal plates were often noted, along with shortened tear-film break-up time (<10 seconds). Corneal erosions and ulcers were sometimes apparent. Lubricant eye drops were used 8-16 times per day, along with intermittent use of topical corticosteroids.

Stage IV: As in stage III, but with corneal involvement more pronounced and more severe degrees of eyelid dysfunction. Corneal ulceration and melting were routinely observed, along with fibrosis of the tarsal plates.

Characterization of systemic GvHD

During the course of chronic GvHD, most patients in addition to local treatment typically need systemic corticoid steroid treatment, often for a prolonged period of time. Rapid tapering of steroids may trigger a flare-up of GvHD, and to prevent this a moderate prednisolone dose is used for several months. Clinically, it is difficult to differentiate between, on one hand an ongoing inflammatory GvHD process causing e.g. oral dryness, and on the other hand a post-inflammatory fibrosis - due to a recent episode of GvHD - which may be associated with the similar symptoms and signs.

Corticoid steroid treatment will often be maintained in both these situations. Therefore, for the purpose of this study (Paper III), ongoing prednisolon treatment was considered to be a marker of systemic GvHD. This definition is a pragmatic tool for the identification of patients who, according to the attending physician, have - or recently had – systemic GvHD.

Impression cytology

Impression cytology (IC) is a minimally invasive technique for sampling the superficial cell layer of the ocular surface. First described in 1977, it is now a standard tool in the diagnosis and evaluation of ocular surface pathologies (Calonge et al, 2004; Singh et al, 2005).

The sampling entails application of a sterile, non-abrasive membrane to the ocular surface. For this purpose, we have utilized Millipore well inserts (Ø: 12mm) with an acetate cellulose or polarized PTFE membrane (Fig. 1) pre-mounted on a plastic holder enabling easy handling without forceps or other manipulation tools. An additional benefit for using well inserts is that they are also individually packaged and sterilized.

Figure 1: Millipore well insert used for impression cytology.

The sampling, following administration of a topical anesthetic, was performed through gently touching the membrane to the corneal or conjunctival surface for one or two seconds. The sample was then allowed to air dry before it was re-sealed in its original container and stored at -18˚C awaiting analysis.

The patient may experience mild irritation or foreign body sensation for a few hours after sampling. Patients were supplied with artificial tear substitute after sampling and a recommendation to apply these once every 2 hours for the next 24-hour period.

Fluorescence-in situ-hybridization (FISH)

FISH is a method developed for cytogenetic detection of specific DNA sequences and chromosome identification. It is in wide-spread laboratory use, mainly for evaluation of chromosomal abnormalities in cancer and fetal genetic diagnostics.

When used to identify X and Y chromosomes, FISH is a convenient tool for the identification of donor cells in a sex-mismatched donor/recipient pair. In short, the method is based on the association (hybridization) of a fluorochrome-tagged oligonucleotide, or probe, complementary for a DNA sequence specific for the X-, and Y chromosomes, tagging each with a different fluorochrome. Several studies have demonstrated the efficacy of FISH-analysis on IC samples (Egarth et al, 2005; Eberwein et al, 2009; Catanese et al, 2011).

In preparation for FISH analysis, IC samples were thawed and fixed in ethanol and acetic acid (3:1) for 15 minutes, and then allowed to air dry. The membrane was removed from the plastic holder with a sterile scalpel and placed on a glass slide. The membrane was flattened by pressure from a cover slip

and 5 µl ready-to-use XY probe mix (CEP X^{Orange}/Y^{Green}; Vysis Inc.) was applied, whereupon a cover slip was placed on top and sealed in place with rubber cement.

Samples were allowed to denature for 5 minutes (85°C) on a heat block, and placed in a dark moisture chamber (39°C) overnight. The membrane was then removed from the slide and washed in 2x saline sodium citrate (SSC) + 50% formamide (pH: 7,6) for 20 minutes, 2xSSC (pH: 7,0) for 15 minutes and, finally, 2xSSC + 0,05% nonyl phenoxypolyethoxylethanol (NP40; pH: 7,0) for 10 minutes. The washing steps were performed in a sterile 12-well cell culture plate at 43°C in an incubator.

Immunofluorescence (IF)

IF is a well established immunohistochemical technique useful in the identification and visualization of cellular antigens in a vareity of settings, both for clinical diagnostics and for research purposes. The method is based on the interaction of immunoglobulins (antibodies) with their specific epitope. The antigen-antibody complex is then visualized by association with a fluorochrome, either conjugated directly to the antibody (direct IF) or with a secondary antibody specific for the primary (indirect IF).

With the correct fixation and preprocessing, the IF method is capable of visualization of most types of cellular components, including membrane-bound, cytoplasmic and nuclear antigens, on a vareity of sample types – for example paraffin-embedded biopsies, cryosectioned material or cytological impression samples. Moreover, with the appropriate choice of fluorochrome and attention paid to pretreatment options, it is possible to perform IF and FISH analyses at the same time, in the same sample.

Following FISH, membranes were washed in phosphate-buffered saline (1xPBS; pH: 7,0) for 2 minutes before being transferred into blocking solution - 5% normal horse serum (Vector) in 1xPBS (papers II and III) or 5% (w/v) non-fat dry milk in 1xPBS (paper I) - and allowed to incubate for 30 minutes. All washing and incubations steps were performed on a sterile 12-well cell culture plate, using a new well for each washing and incubation step. Each washing step utilized 1 ml buffer, and were performed at 37°C. Blocking, primary and secondary antibody incubations utilized a quantity of 300µl working solution per incubation and were performed at room temperature in the dark.

After blocking, the membrane was incubated with primary antibody [aSMA (clone C5E) anti-human mouse IgG (Abcam); diluted 1:500 in 1xPBS] and allowed to incubate overnight. The samples were then washed thrice in 1xPBS for five minutes each and incubated with secondary antibody (TRITC-conjugated anti-mouse horse IgG (Vector) at dilution 1:300 in 1xPBS) for 1 hour.

The samples were again washed in 3x5 minutes 1xPBS plus 1x5 minutes in dH_2O, before mounting with Vectashield (Vector) mounting medium for fluorescence, containing DAPI nuclear counterstain.

Cell quantification

The mounted samples are evaluated under a UV-microscope at 400x magnification, using a calibrated frame grid. On each membrane, 10 frames were analyzed, excluding frames lacking cells or containing more than one layer of cells.

In each frame, the total number of nuclei (DAPI positive), the number of aSMA-positive cells (Texas Red fluorescence) and the number of polyploid cells were counted, and the genotype for each determined. The ten scores for each cell type from each frame were pooled, and the mean percentage of each cell type was calculated.

RESULTS

Impression cytology - safety and feasibility

Papers I-III: Impression cytologies (IC) could be performed all patients without sequelae. Slight irritation and redness were occasionally described by some patients, but these manifestations were short-lived, usually subsiding within 2-4 hours. There were no instances of post-sampling bleeding, infection or aggravation of ocular problems. Although the sampling procedure itself was sometimes experienced by the patient as uncomfortable, the general conclusion is that IC is a safe, practical and ethically sound method for cytological sampling of the ocular surface.

Conjunctival IC samples yielded a monolayer of cells covering 25-100% of the membrane, equal to approximately 5000-20000 cells. Although minor areas showed multicellular layers, the cells were typically well separated, allowing for a clear view of each individual cell. The nuclei (as visualized by DAPI counterstain) were discrete and retained structural coherency throughout the staining and analysis procedure.

Donor-derived cells are present in the ocular surface after allo-HSCT

Paper I: Nine female allo-HSCT recipients (median age 40 (31-61) years; median time since transplantation 20 (10-72) months) with male donors were included, to a total of 17 samples.

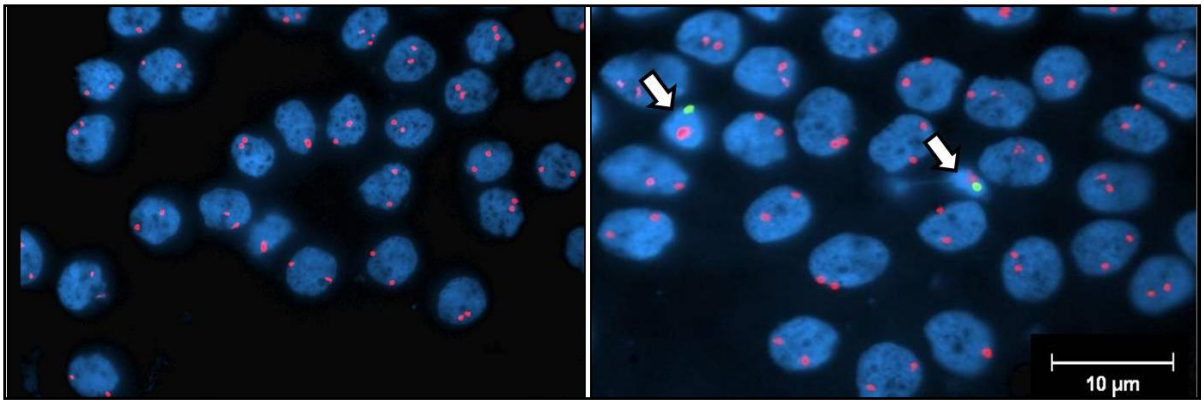

Figure 2: Left: XX-positive FISH signals in a conjunctival imprint from a non-transplanted woman. Right: XY-positive cells (donor-derived; arrows) in a female allo-HSCT recipient with a male donor.

Impression cytology proved efficient in sampling the conjunctival - but not the corneal – epithelium. XY-FISH analysis showed a mixed donor/recipient chimerism (Fig. 2), dominated by recipient-type cells (XX genotype; 86%), but with a distinct complement of donor-type cells (XY genotype; 9%). The recipient-type cells found were of conjunctival epithelial (CK19+; 70%) and goblet cell (PAS+; 10%) type. No donor-derived CK19+ cells were found.

Mixed chimerism of myofibroblasts after allo-HSCT

Paper I: A majority (2/3) of the donor cell compartment could, through IF staining for the alpha-smooth muscle actin (αSMA), be confirmed as myofibroblasts (Fig. 3). These cells also exhibited a weak CD45 expression. There was also a smaller compartment (3%) of recipient-derived αSMA+ myofibroblasts.

Paper III: In a larger, longitudinal study (37 samples) of gender mismatched (male-to-female) allo-HSCT recipients (n=25; MtF group), mixed myofibroblast chimerism could be seen in 28 (76%) samples. The mixed chimerism was retained even in the longest follow-up (95 months post-transplantation). In one patient, donor-derived myofibroblasts could be detected as early as three weeks post-transplant.

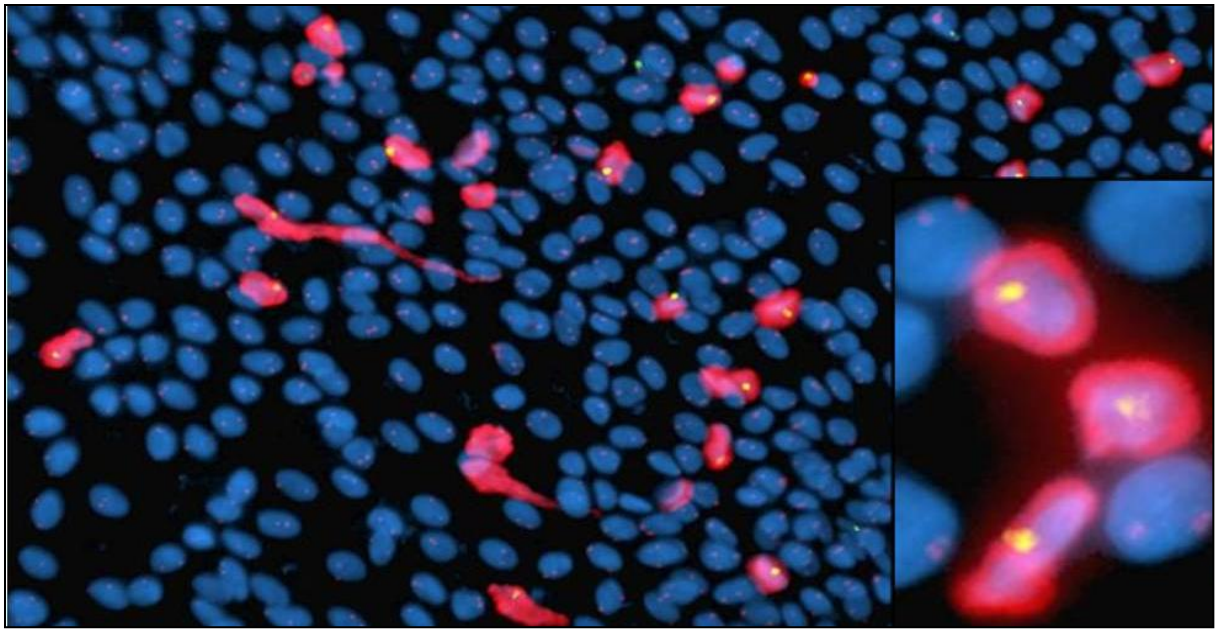

Figure 3: αSMA+ cells (red) of donor origin in the ocular surface after gender-mismatched allo-HSCT.Y-chromosome FISH signal appears as a yellow dot.

Donor-derived (but not recipient-derived) myofibroblasts increase with time after transplantation

Paper III: Forty-six allo-HSCT recipients (32 women, 14 men; median age 49 (19-67) years), all relapse-free, were enrolled at a median time from transplant to first sampling of 14 (1-122) months. Thirteen patients were sampled more than once – at different time points – to a total of 70 samples. Ten patients were also sampled before transplantation. The median total myofibroblast density post-transplant (regardless of origin; MFB^{TOT}) was significantly higher compared to pre-transplant samples (7.0 (0.6-78)% vs. 4.1 (0.6-11)%; p=0.01).

The material was arranged into four time categories: Early (0-3 months; n=17), Intermediate 1 (>3-12 months; n=18), Intermediate 2 (>12-36 months; n=16) and Late (>36 months; n=19). MFB^{TOT} could be seen to increase over time (Fig. 4a) in a statistically significant correlation (p=0.0001).

In the MtF group (25 patients, 37 samples), donor-type myofibroblast (MFB^{XY}) content was significantly increased over time post transplantation (Fig 4c; p=0.01), while recipient-derived myofibroblasts (MFB^{XX}) did not vary over time (Fig. 4d).

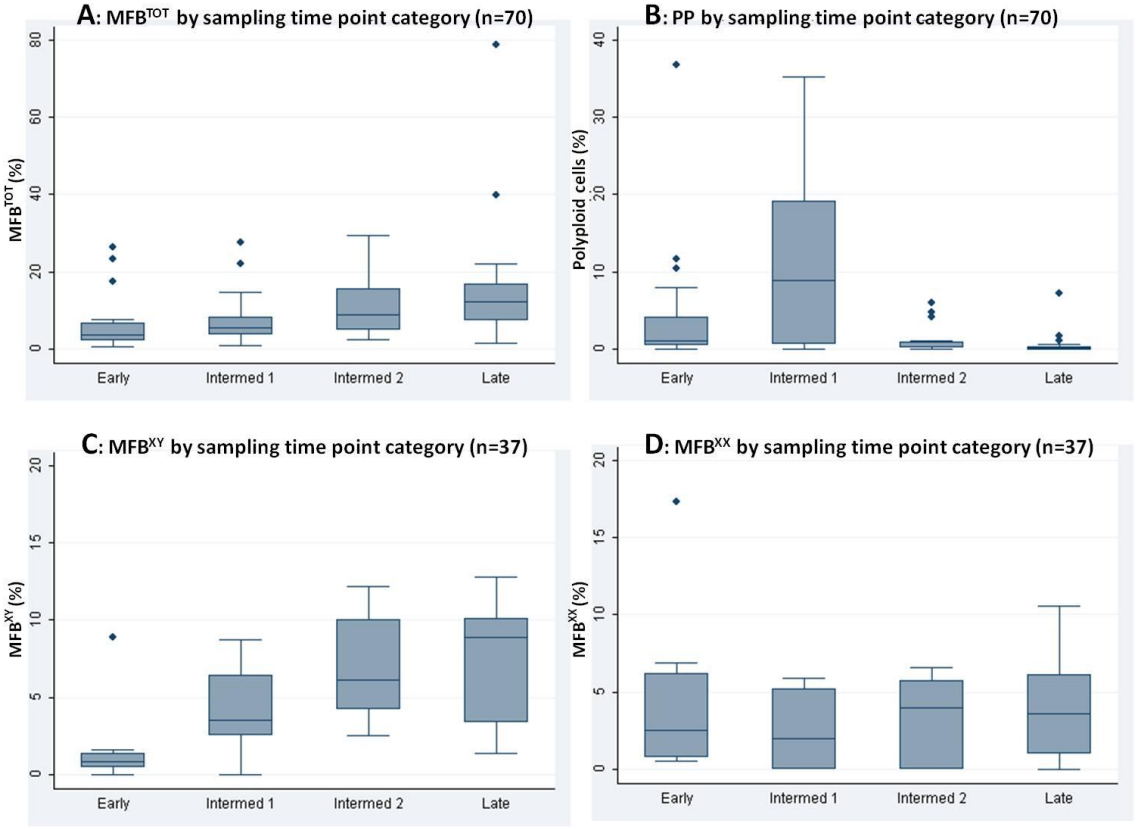

Figure 4: Time-associated kinetics of myofibroblasts and polyploid cells. A: Association between total myofibroblast density (MFB^{TOT}) and time categories in all samples. B: Association between polyploid cell density (PP) and time categories in all samples. C: Association between donor myofibroblast density (MFB^{XY}) and time categories in the MtF group. D: Association between recipient myofibroblast density (MFB^{XX}) and time categories in the MtF group.

MFB^{XY}/MFB^{TOT} ratio increases with time and correlate with ocular GvHD

Paper III: In the MtF group, the MFB^{XY}/MFB^{TOT} ratio was calculated for each sample and could be seen to correlate significantly with time post transplantation (p=0.011). The material was then sorted according to severity (grading) of ocular GvHD (Grade 0: n=18; grade 1: n=10; grade 2: n=5; grade 3: n=4). MFB^{XY}/MFB^{TOT} correlated significantly (p=0.034) with ocular GvHD.

Myofibroblasts are present in the normal conjunctiva

Paper II: Thirty-five healthy individuals (25 women, 10 men; median age 45 (18-79) years; "screening group") were recruited into a screening study and sampled 1-10 times over a 5-month period (January-May, 2008) to a total of 83 samples. Myofibroblasts were detected in 94% of all subjects, at an average of 3.4% (SD: ±0.46%) at the first sampling. The myofibroblast density in normal conjunctivae was lower compared to samples taken after allo-HSCT, but not compared to pre-transplantation samples.

Anatomical variation of myofibroblast density in the normal conjunctiva

Paper II: Nine individuals (5 women, 4 men; median age 50 (27-60) years) were recruited from the screening group to determine the myofibroblast density variations in different sampling locales. A total of 36 samples were obtained, one each from the superior, inferior, nasal and temporal bulbar conjunctiva on each subject.

The greatest density of myofibroblasts could be found on the superior and inferior conjunctiva (22 (13-46)% and 9.7 (6.5-15)%, respectively. The nasal and temporal conjunctiva exhibited a myofibroblast density of 1.7 (0.6-2,9)% and 1.9 (0.8-5.4)%, respectively. Typically, a decreasing density could be seen in proximity to the limbus. Repeated samples of the same area did not result in higher myofibroblast counts.

Seasonal variation of myofibroblast density in the normal conjunctival surface

Paper II: Twelve volunteers (10 women, 2 men; median age 50 (40-65) years; "one-year group") were recruited from the screening group and sampled 2-5 times per month for one year, to a total of 484 samples. The results showed a significant cyclical variation in myofibroblast density during the course of the year, with a minimum (1.2 ± 0.5%) in the December-February interval and a maximum (4.1 ± 1.5%) in the March-May interval, suggesting a possible association with environmental factors, such as pollen count, UV-exposure or pollution.

Presence of polyploid cells after allo-HSCT

Paper I: *En passant*, the observation was made that the conjunctival surface of allo-HSCT recipients contained, in proximity to the limbal border, abnormally large cells. These cells were exclusively of recipient origin, and exhibited an abnormally high number of X-chromosomes (2-18n), as visualized through FISH analysis.

The giant cells were polyploid rather than polynuclear - i.e. the chromosomes were contained within one, large nucleus (Fig.5) - and comprised, on average, 2% of the total of number of cells. Polyploid cells were found in all 9 patients.

Paper III: Polyploid cells were found in 55/70 post-transplantation samples, to a median density of 0.6 (0-37)% and with a median chromosomal content of 8 (6-24)n. Cells with less than 5n were disregarded as possibly mitotic. In imprints obtained before transplantation (n=10), polyploid cells (≥5n) were found in 7/10 samples, to a median of 0.3 (0-1,7)%. No donor-derived polyploid cells were detected.

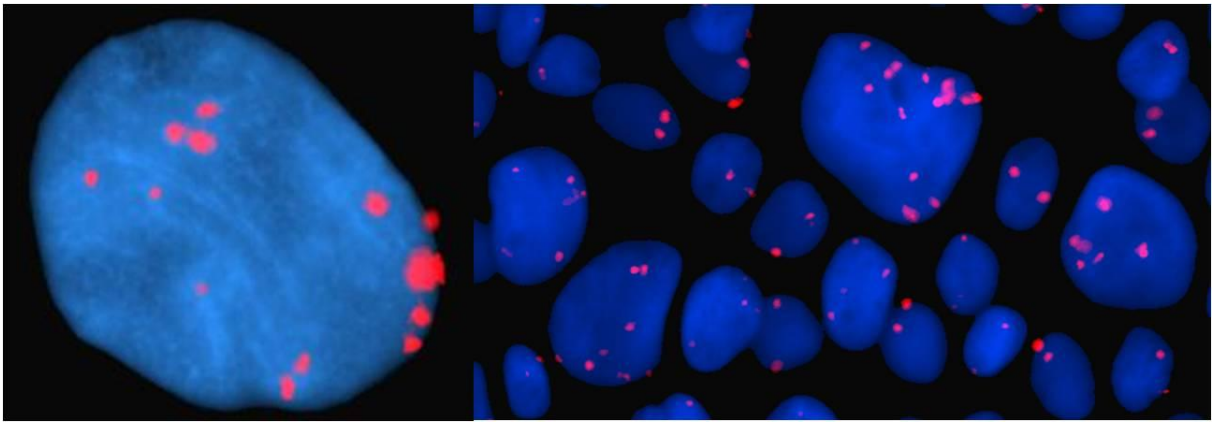

Figure 5: Polyploid cells of varying ploidy (4-14n) in the conjunctival surface after allo-HSCT

Polyploid cell density correlates with time after transplantation but not GvHD

Paper III: Polyploid cells differed considerably between time categories (Fig 4b). In the "Early" and "Intermediate 1" categories, the median densities of polyploid cells (PP) were 1 (0-37)% and 8.9 (0-35)%, respectively, while in the "Intermediate 2" and "Late" categories, a significantly lower PP density was found; 0.3 (0-6)% and 0 (0-7.2)%, respectively. The association between PP and time post-transplantation was statistically significant (p=0.001). No correlation between PP and ocular or systemic GvHD were observed.

Polyploid cells are present in low numbers in the normal conjunctival surface

Paper II: In the "screening group" (35 individuals), polyploid cells were present in 48/83 samples. If at all present, the polyploid cells were in low numbers (0.06 (0.01-0.33)%).

Case report

Paper III: A 58 year-old woman, with a 5-year history of chronic lymphocytic leukemia and who received a matched sibling, gender mismatched PBSC allograft in 2006, was followed with repeated imprints from 2 weeks to 52 months post-transplant. Her post-transplant course, detailed in Paper III, was complicated with episodes of rejection, relapse, re-transplantation and donor lymphocyte infusions. In this patient, donor-derived ocular myofibroblasts could be seen as early as one month post-transplant. At 9 months, with a fully rejected stem cell graft and 100% T-cell and myeloid recipient chimerism, her conjunctival myofibroblasts nevertheless were of donor origin. Conversely, at 52 months, at this point with complete donor T-cell chimerism in the peripheral blood and bone marrow, and with >97% donor chimerism in the bone marrow $CD34^+$ fraction, the ocular myofibroblasts still exhibited mixed chimerism.

DISCUSSION

Transplant-related mortality has decreased in later years, but chronic GvHD remains a major complication after allo-HSCT. Many allo-HSCT recipients find their quality of life impaired due to cGvHD symptoms which causes disability, fatigue, reduced work capacity and divers organ dysfunctions. Extensive cGvHD, apart from relapse, is the major clinical post-transplant problem, also associated with increased mortality due to late infections, organ damage and side effects of immunosuppressive medication.

Ocular cGvHD, typically in the form of dry eye syndrome, affects a majority of allografted patients. The ocular symptoms are normally rather mild, presenting as conjunctivitis, grittiness or foreign body sensation. However, ocular cGvHD may, in some cases, progress into involvement of the cornea, causing erosion and ulceration, which in turn may cause permanent loss of visual acuity.

The pathogenesis of ocular cGvHD has been poorly elucidated, although fibrotic changes of the ocular surface and surrounding tissues are typically observed. Fibrosis and obstruction of the lacrimal and meibomian glands are common findings. Theoretically, concomitant dysfunction of the lacrimal and meibomian glands could cause a synergistic combination effect of (i) reduced tear production and (ii) impaired ability to inhibit tear-film evaporation – resulting in dry eye syndrome. Dysfunction of the conjunctival epithelium, with resultant loss of mucin-producing goblet cells - and reduced capacity for tear-film retention - may also be a contributory factor in the pathogenesis of dry eye syndrome in ocular cGvHD.

Fibrosis is a hallmark of chronic ocular GvHD, and the myofibroblast might be considered the hallmark of fibrosis. The myofibroblast is a cell with a pivotal role in wound healing and inflammation, but has also been implicated as a culprit in fibrosis and connective-tissue disorders.

The myofibroblast density was found to be significantly lower in healthy controls than allografted patients. However, also in the normal ocular surface, the presence of myofibroblasts was a consistent observation, suggestive of an ongoing state of tissue repair or remodeling in the conjunctiva, speculatively as a result of exposure to various environmental factors, such as chemical pollution, UV radiation or pollen, or due to the normal wear and tear of eyelid motion. In Paper II, we found that myofibroblast density in healthy conjunctivae exhibited marked seasonal variation, closely matched by the ambient pollen concentration. However, the causality between these two factors remains unsubstantiated.

In the allo-HSCT recipient, the combination of a male donor and a female recipient provides a useful set-up for the identification of differentiated cells derived from the infused donor graft. The identification of donor and recipient genotypes by FISH, chimerism analysis, is applied not only in research, but also in clinical surveillance of engraftment kinetics and detection of minimal residual disease after sex mismatched allo-HSCT. In Paper I and III, the method was used to identify and quantify donor cells in the conjunctivae of allografted patients.

In male-to-female allo-HSCT patients, donor-derived myofibroblasts could be seen as early as three weeks post transplantation, i.e. at the same time as donor-derived leukocytes become detectable in the peripheral blood. This indicates that a progenitor capable of fibroblastic differentiation is transferred to the patient via the graft. This progenitor may or may not be identical with the hematopoietic stem cell.

The observations made in the single-patient case accounted for in paper III, illustrates several of our key findings. In this patient, conjunctival myofibroblasts of donor origin were detectable already at one month post-transplant. Later in the clinical course, during an episode of complete rejection of the donor hematopoietic system, a majority of conjunctival myofibroblasts were still donor-derived. This observation is intriguing, but a cautious interpretation would be that the hematopoietic stem cells are not the only progenitor of myofibroblasts.

The total myofibroblast density (irrespective of origin; MFB^{TOT}) increased with time after transplantation in the whole patient cohort (46 patients; 70 samples). In the male-to-female group (25 patients; 37 samples) we found that the increase of myofibroblasts with time was due to an absolute increase of donor-derived myofibroblasts (MFB^{XY}). Another observation was the statistically significant correlation between the MFB^{XY}/MFB^{TOT} ratio and the presence and severity of ocular GvHD. This ratio is a constructed variable, representing the relative relationship between donor-, and recipient-derived myofibroblasts regardless of quantitative value. Its association with GvHD suggests that it is the chimeric distribution within the myofibroblast population - rather than the total number of myofibroblasts - that has an impact on (or simply reflects…) ocular cGvHD.

The hematopoiesis in a fully engrafted, not relapsed, allo-HSCT recipient is exclusively of donor origin. However, a curious observation in the male-to-female group was the continuous finding of recipient myofibroblasts retained even after the longest follow-ups (95 months post-transplant). This result is indicative of a myofibroblast differentiation pathway independent of the hematopoietic stem cell. The finding supports the prevailing notion that, indeed, myofibroblasts have several routes of differentiation. Again our case report is illustrative; at more than four years post-transplant, with

ongoing extensive chronic GvHD, and with hematopoietic cells 100% donor-derived, her ocular myofibroblasts still displayed a mixed chimerism.

The most obvious candidate for the origin of the recipient-derived myofibroblast is the patient´s own pool of stromal fibroblasts. These cells reside – in a quiescent state – in the connective tissues of the ocular surface, and may undergo clonal expansion in response to reparative stimuli. Epithelial-, or endothelial-to-mesenchymal transition is another possible mechanism through which a recipient-type myofibroblast population may be retained.

With respect to the origin of the donor-derived myofibroblasts, several candidate pathways of differentiation are suggested by contemporary studies.

Mesenchymal stem cells (MSC), is one possible source of donor-derived myofibroblasts. The MSC can be found in peripheral blood and represents a stem cell population reportedly distinct from HSC, specifically lacking hematopoietic markers such as CD34 and CD45. However, several observations are inconsistent with this hypothesis. Firstly, most of our patients have received G-CSF mobilized PBSC products, and MSCs, albeit abundant in bone marrow, has not been found in G-CSF mobilized blood (Wexler et al, 2003). Secondly, donor-derived MSCs have not been seen to be capable of long time engraftment after allo-HSCT (Rieger et al, 2005; Stute et al, 2002).

Many studies have demonstrated the existence of a circulating fibroblast progenitor – the fibrocyte or monocyte-like mesenchymal progenitor – which may be recruited into the tissues during wound healing and inflammatory conditions. The fibrocyte is a unique cell type expressing both hematopoietic lineage markers (CD45, CD34, CD14) and mesenchymal characteristics such as collagen production and the ability to myodifferentiate in response to TGFβ stimuli (Mori et al, 2005). It is possible that the allogeneic stem cell graft, both BM and PBSC, contain a sub-population of fibrocytes, which would explain the presence of donor-derived myofibroblasts in allo-HSCT recipients. Fibrocytes have been shown to extravasate at the site of an ongoing inflammation, undergo differentiation and help bolster tissue regeneration (Abe et al, 2001). Whether or not the fibrocytes derive from HSC or constitute a stem cell population in their own right remains to be clarified.

The origin of the polyploid cell, and its significance in health and after allo-HSCT, remains elusive, although recent studies indicate that non-malignant polyploidization may occur during high oxidative stress conditions, in replicative senescence and in response to specific inflammatory stimuli (Hanselmann et al, 2000; Storchova et al, 2004). Evidence has been presented that transient polyploidization may appear in normal granulation-phase wound healing and that these cells – their

larger gene-expression base lending them increased resistance to an adverse environment – arise through selective pressure in the injured tissue (Oberringer et al, 1999; Lee et al, 2011).

In the normal conjunctiva, polyploid cells are observed sporadically, generally in low numbers. Most of these are tetraploid, which may represent cells undergoing mitosis. However, cells of higher ploidy grade (>5n) are exceedingly rare.

In the allo-HSCT recipient, a high density of polyploid cells is a routine observation during the first year post-transplantation. In comparison to pre-transplantation samples and healthy controls, these cells exhibit higher numbers, larger size and a shift towards higher ploidy (>5n). Polyploidization correlates closely with time post-transplantation, reaching a maximum in the 3-12 month interval, followed by a decrease to pre-transplantation levels. It is interesting to note that this peak in polyploid cells occurs during the same time frame as a high incidence of GvHD manifestation. However, no association between polyploid cells and ocular or systemic GvHD could be detected.

Thus, the role and significance of polyploid cells after allo-HSCT remains obscure. Speculatively, the presence of conjunctival polyploidization during the first year after allo-HSCT may represent an adaptation or survival mechanism in response to a changing immunological milieu. Other possibilities include polyploid cells as a late effect of chemotherapy administered before transplantation, or the product of a viral infection.

Conclusions

This thesis describes the time-related and disease-dependent kinetics of two cell types – myofibroblasts and polyploid cells – in the conjunctival surface in health and after allo-HSCT.

Myofibroblasts are a consistent observation in the normal conjunctiva, exhibiting a distinct seasonal variation with low numbers in the winter and high numbers during the summer. Polyploid cells are found rarely and in low numbers.

In allo-HSCT recipients, our results indicate (i) the presence of a donor graft progenitor cell capable of rapid and sustained differentiation to ocular myofibroblasts; (ii) long-term retention of recipient ocular myofibroblasts differentiated from a non-hematopoietic progenitor; (iii) an association between the donor/recipient myofibroblast ratio and GvHD.

However, it remains to be demonstrated whether the influx of donor myofibroblasts is a bystander effect of another inflammatory process or, in fact, represents a primary mechanism of the fibrotic process often seen in ocular cGvHD.

Polyploid cell density increase dramatically after allo-HSCT, peak in the 3-12 month interval and then disappear. Whether or not these cells are involved with the pathogenesis of GvHD remains to be elucidated.

Perspectives

Chronic GvHD remains one of the major hurdles after allo-HSCT, and its pathogenesis is still only partially elucidated. As allo-HSCT becomes increasingly common – and early survival rates improve – cGvHD becomes a more and more urgent clinical problem.

Speculatively, intervention of the fibrotic process often seen in cGvHD may help control the disease at an early stage and prevent symptoms from developing.

Although the practical clinical relevance of the findings presented in this thesis remains uncertain, it is hoped that this contribution to the knowledge base may provide insight into the growing understanding of the mechanisms behind chronic GvHD.

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"Are you suggesting coconuts migrate?"

-Monty Python & the Holy Grail

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“God don't like people not doing much work.
People who aren't busy all the time might start to think”

-Terry Pratchett, *Small Gods*