General introduction
Tissue engineering, a new approach for tissue repair

Many patients are in need for organ/tissue replacement or reconstructive surgery after disease or injury. Currently, patients in need for organ replacement or reconstructive surgery receive donor organs (allogenic) or transplants from healthy donor sites (autologous). Problems for using donor organs include shortage of organs and the possibility of immune rejection, even if immunosuppressive therapy is applied. Transplanting autologous healthy tissues from donor sites does not encounter immune response problems, but the availability of such tissues is limited. Currently, patients with burn injuries get skin transplants from autologous healthy donor sites. For jaw reconstruction, bone material from the patient’s iliac crest is used. Also patients in need for coronary bypass surgery often receive a vessel transplant from their saphenous vein.

Unfortunately, many health problems cannot be solved using tissue transplants, e.g. for replacement of meniscus, hip and intervertebral disks. Implants made of synthetic polymers, ceramics and metals give mechanical strength to the tissues and can, at least in part, repair the defect. Also transplants made of tissue derivatives, such as collagen or decellularized skin, are alternatives for tissue repair. However, to date none of the implants can provide the quality of the original tissues; they often fracture, induce immune responses and are difficult to anchor. A promising approach for organ replacement and reconstructive surgery is tissue engineering.

Tissue engineering, or regenerative medicine, aims at replacing or regenerating tissues that are lost due to diseases or injuries. Scaffolds or cells are implanted in the patient to guide tissue repair and to simulate autologous tissue formation and regeneration. Albeit the knowledge on tissue engineering is expanding, the poor vascularization of engineered tissues remains a principal constraint. Generally, the formation of new blood vessels (angiogenesis) is not fast enough to provide adequate oxygen, nutrient and waste product transport within the scaffold. The scope of this thesis is to influence scaffold vascularization by using fibrinogen variants as scaffolds in tissue engineering.

Tissue engineering

In tissue engineering, scaffolds or cells are implanted in the patient to mimic and guide in situ repair. The engineered scaffold should have tissue-like mechanical properties, display immunologic integrity and support the adhesion, migration and differentiation of cells. Ultimately, the scaffold should become degraded and replaced with cell-produced extracellular matrix. In the end, the morphology and functions of diseased/injured tissues should be completely restored.

The three principal approaches for tissue engineering are: (I) implantation of freshly isolated or cultured cells, directly or in combination with a scaffold. (II) The implantation of an in vitro engineered tissue, consisting of cells and a scaffold. (III) In situ tissue regeneration after implantation of a scaffold that stimulates tissue repair within in the body.

The central tissue engineering paradigm combines autologous cells, scaffolds and in vitro culture to restore, maintain or improve tissue function (Figure 1). Within the scaffolds, cells are necessary for tissue repair and extracellular matrix deposition. The extraction of cells from native tissue is generally achieved via biopsy and can result in low yields. Sufficient numbers of cells are either obtained or can be reached via in vitro expansion under appropriate culture conditions.
Another approach is using undifferentiated stem cells as starting material. These stem cells allow self-renewal, differentiate into specialized cells and govern high proliferative potentials. Stem cells may provide a massive supply of cells that have the potential to form any specialized cell in the body. Stem cells can be divided into two main groups: pluripotent embryonic and multipotent adult stem cells. Examples of adult stem cells are mesenchymal stem cells from bone marrow or from adipose tissue.

Important determinants for scaffolds are their strength, degradability, biocompatibility and influence on cell characteristics. The scaffold provides mechanical support and a suitable microenvironment for cells to effectively accomplish their functions. Scaffolds can be made of synthetic polymers, natural materials or combinations thereof (hybrid scaffolds). Synthetic materials can be reproducibly manufactured, easily manipulated and initially provide mechanical support. Most of the synthetic materials do not contain biologically functional molecules and are usually not or only slowly degraded. Hybrid scaffolds combine the natural and synthetic materials by the incorporation of functional molecules on a synthetic backbone. Whereas, natural materials facilitate cell-matrix interactions, integrate in the surrounding tissue and degrade upon new tissue generation.

Figure 1. The central paradigm of tissue engineering. Autologous (stem) cells are taken from the patient (1) and expanded during in vitro culture (2). The cells are seeded in a porous scaffold, together with growth factors (3). The engineered scaffold is further developed during in vitro and/or bioreactor culture (4). Finally, the regenerated tissue is implanted into the site of damage to integrate with the natural tissue (5). The image is originated from the PhD thesis of Julian H. George: Electrospinning and Tissue Engineering, published in 2009.
Tissue engineering is a promising approach for repair after several injuries and diseases, albeit many aspects still need further improvement. Until now, the developments of avascular (cartilage) and thin tissues (skin, bladder) have been successful in this field of research. However, for most tissues the applications are still in early stages of development. One of the main problems in tissue engineering is the vascularization of the scaffold. The limited blood supply results in the deprivation of oxygen and nutrients of cells within the scaffold. Without vascularization, the maximum diffusion rate of oxygen (~100 to 200 µm) determines the size of the scaffold. Since no vascular system is established in the scaffold at the moment of implantation, the tissue engineered scaffold will soon display an oxygen gradient that declines towards the interior of the scaffold. Within the scaffold the lack of oxygen (hypoxia) limits cellular respiration and growth and thus creates a potentially lethal environment. Local reduction of cell viability has indeed been attributed to the declining oxygen gradient, and mesenchymal stem cells were shown to die when exposed to anoxia for 5 days. Hypoxia in the scaffold results in significantly lower cell densities in the center and uneven extracellular matrix deposition. In addition, metabolically active cells in the peripheral regions of the scaffold hinder oxygen penetration towards the center of the scaffold. Oxygen is important in controlling angiogenesis, glucose metabolism, cell differentiation and cell survival.

Various cell types, among which endothelial cells, macrophages, fibroblasts and keratinocytes, are able to sense low oxygen levels and secrete growth factors or enhance their

Figure 2. Angiogenesis in a fibrinous matrix. Angiogenic factors activate endothelial cells (1). The vascular permeability is increased and a fibrinous matrix is formed (2). The basement membrane of endothelial cells is degraded. The fibrinous matrix is locally degraded and endothelial cells invade, migrate and proliferate to form an angiogenic sprout (3). Finally, the new capillary is stabilized by smooth muscle cells and pericytes (4). This image was originally published by M.A. Engelse et al. *Semin Thromb Hemost.* 2004;30;1:71-82.
proteolytic activity to promote vascularization of tissues\textsuperscript{15,22,23}. From tumor angiogenesis, we learned that hypoxia inducible factors (HIF) are important oxygen sensors in cells and their accumulation is important in regulating angiogenesis\textsuperscript{21}. In hypoxic conditions HIF forms a DNA-binding complex that activates the expression of many genes that are involved in cell survival, metabolism and angiogenesis. HIF regulated genes include important angiogenic factors, such as vascular endothelial growth factor A (VEGF-A), stromal cell-derived factor-1 and angiopoietin-2\textsuperscript{24,25}. By increasing the knowledge on angiogenesis and the cellular response in the scaffold, the problem of poor scaffold vascularization can be conquered.

**Angiogenesis**

Angiogenesis is the formation of new vessels from pre-existing vessels; an important process during neovascularization of tissues. Angiogenesis plays a major role in embryonic development, whereas in adults it is limited to the female reproductive system, pathological conditions and tissue repair. Repair associated angiogenesis is usually accompanied by the presence of inflammatory cells, vascular leakage and fibrin deposition\textsuperscript{26,27}. Fibrinogen leaks from the vasculature and forms a temporary fibrinous matrix, which not only acts as a sealing matrix, but also facilitates tissue repair and angiogenesis. The inner layer of blood vessels is covered with a quiescent lining of endothelial cells which respond to angiogenesis activating factors. Known stimuli for angiogenesis are hypoxia, VEGF-A, placenta growth factor, fibroblast growth factor, platelet-derived growth factor, interleukin-8 and angiopoietin-1 and -2\textsuperscript{28,29}. Upon activation of angiogenesis, endothelial cells from adjacent vessels locally degrade fibrin, mainly by matrix metalloproteinases and/or plasmin activities\textsuperscript{30-32}. The endothelial tip-cells form the leading edge of a new angiogenic sprout. These cells locally degrade the matrix, migrate towards the angiogenic stimulus and branch\textsuperscript{33}. The adjacent endothelial cells, called stalk-cells, follow the sprouting tip-cells, create the vascular lumen and proliferate (Figure 2). The tip- and stalk-cell phenotype of endothelial cells is regulated via delta like ligand - Notch signaling\textsuperscript{34,33}. Recently, it was shown that tip- and stalk cells are not completely different types of endothelial cells, and they can switch position during capillary formation\textsuperscript{35}.

The vascular system is a highly branched and hierarchically organized network that regulates homeostasis through the transport of gas, liquids, nutrients, waste products, signaling molecules and cells. Establishment of the vascular network is important for oxygen and nutrient delivery to cells within tissue engineered scaffolds. Two approaches can stimulate vascular network formation in the scaffold (I) pre-vascularization of scaffolds prior to implantation and (II) promotion of scaffold angiogenesis by the host \textit{in situ}\textsuperscript{12}. In both approaches the formation of the vascular network in the scaffold is stimulated, resulting in proper tissue homeostasis. The establishment of a vascular network in the scaffold can lead to relevant cell-based tissue engineered constructs.

Angiogenesis not only relies on stimulation and migration of endothelial cells; the extracellular matrix also plays an important role herein. In healing wounds and tumor stroma, fibrin was shown to act as a perfect matrix for angiogenesis. Tumors increase the vascular permeability, leading to platelet activation and fibrin deposition, and thereby generate a pro-
angiogenic environment. The hemostatic plug that is formed after wounding has fibrin as a major matrix component and provides a temporary structure, wherein cells can migrate during tissue repair. Immediately after wounding, inflammatory cells e.g. neutrophils and monocytes, are recruited towards the injured area and granulation tissue is formed. The granulation tissue is mainly composed of fibrin, fibronectin and hyaluronic acid and contains macrophages, endothelial cells and fibroblasts. The formation of granulation tissue coincides with the formation of new capillaries, a process that is stimulated by macrophage- and platelet-derived growth factors. Fibroblasts in the granulation tissue gradually replace the fibrinous matrix by a collagenous matrix. Finally, this results in the ‘mature’ extracellular matrix of healed tissue that contains various matrix proteins, such as collagen, vitronectin, fibronectin, fibrin and laminin.

The interaction between endothelial cells and the extracellular matrix regulates cell migration, tube formation and vessel stabilization. Integrins on endothelial cells provide the physical interaction with the extracellular matrix and play a role in vascular remodeling. The integrins αVβ3, αVβ5 and α5β1 on endothelial cells can bind to RGD (Arg-Gly-Asp) sites of the fibrinogen molecule. Indeed, the formation of a fibrinous matrix accelerates the process of angiogenesis, while collagen type I matrices can impair tube formation in vitro. Within tissue engineering, fibrin was shown to accelerate wound healing, and fibrin injection increases the blood flow in the infarcted myocardium. Besides its role in facilitating angiogenesis, fibrin stimulates extracellular matrix protein synthesis and matrix remodeling. The fibrin matrix provides specific cell adhesion sites and serves as a reservoir for storage and release of growth factors. In this thesis, the in situ vascularization is influenced by variants of the natural scaffold for angiogenesis; fibrin. Fibrinogen can be autologously obtained, purified from donor plasma or recombinantly produced.

**Fibrinogen**

Fibrinogen is a 340 kDa hexameric glycoprotein, composed of pairs of three gene products, namely Aα2-, Bβ2- and γ2-chains that are present on chromosome 4q22-32. Fibrinogen is synthesized in the liver and circulates in blood at approximately 2 to 4 g/L. Disulfide bonds connect the N-terminal ends of the three chains, resulting in a half-fibrinogen molecule. Dimerization of two half-molecules in an anti-parallel configuration by additional disulfide bonds results in the intact fibrinogen molecule. Fibrinogen has three distinct domains; two terminal D-domains that are linked to the central E-domain by triple-stranded coiled coil regions (Figure 3). Electron microscopy and protein crystallization revealed the trinodular structure spanning a total length of 45 nm. Fibrin is a natural polymer that is formed by the enzymatic polymerization of fibrinogen (Figure 4). During the last step of the coagulation cascade, thrombin (factor IIa) cleaves off the fibrinopeptides A and B from the N-terminal ends of the fibrinogen Aα- and Bβ-chains. Fibrin monomers interact with each other via ‘knob-hole’ binding and form protofibrils. Hereby, soluble fibrinogen molecules are converted to insoluble fibrin polymers and that form the network of fibrin fibers. Transglutaminase (factor XIIIa) cross-links adjacent α-α and γ-γ chains and thereby stabilizes the fibrin network.
**Figure 3. The crystal structure of human fibrinogen.** The $\alpha_2$-B$_2$- and $\gamma_2$-chains are given in red, green and blue, respectively. The central E-domain, coiled coil regions and distal D-domains are depicted, including the A- and B-knob locations. The carboxy-termini of the $\alpha$-chains fold back towards the center of the molecule, but are not given in this crystal structure. This image was originally published by J.M. Kollman et al. *Biochemistry*. 2009;48;18:3877-3886.

**Figure 4. Fibrinogen polymerization.** Soluble fibrinogen is converted into fibrin monomers after thrombin-catalyzed removal of fibrinopeptides A and B. Soluble monomers bind each other via ‘knob-hole’ binding and form insoluble fibrin protofibrils. Finally, polymers that also branch are formed and result in an insoluble fibrin matrix. This image was originally published by H.C.F. Côté et al. *Blood*. 1998;92;7:2195-2212.
Fibrinogen is synthesized as high molecular weight (HMW) with intact Aα-, Bβ- and γ-chains (A). SEM images were published by E.L. Kaijzel et al. *J Thromb Haemost.* 2006;4;9:1975-1981. Partial degradation of one Aα-chain results in low molecular weight (LMW) fibrinogen (B), whereas partial degradation of both Aα-chains results in low molecular weight' (LMW′) fibrinogen (C). Alternative splicing of the γ-chain(s) can result in γ'-fibrinogen (D), SEM images were published by K.R. Siebenlist et al. *Blood.* 2005;106;8:2730-2736. Alternative splicing of the Aα-chains can result in αE-fibrinogen (E), SEM images were published by M.W. Mosesson et al. *Biophys Chem.* 2004;112;2-3:209-214.
Fibrinogen heterogeneity

Fibrinogen in human plasma shows high degrees of heterogeneity caused by alternative mRNA processing, posttranslational modifications and proteolytic degradation\(^6\). Splice variants that are present in human plasma are the Aα-Extended splice variant (1 to 3%) and the γ’ splice variant (5 to 8%) (Figure 5). The splice variant Aα-Extended (αE) fibrinogen has 236 additional amino acids on the C-terminal ends of the Aα-chains and a molecular mass of 420 kDa\(^6\). The αE-fibrin matrix has thinner fibers with more branch-points, when it is compared to the structure of ‘native’ fibrin\(^6\). γ’-fibrinogen is the result of alternative splicing of the γ-chain, and is also called anti-thrombin I. Here, 4 amino acids are replaced by 20 amino acids on the C-terminal end of the γ-chains\(^6\). This splice variant shows increased thrombin and factor XIII subunit B binding and reduced platelet aggregation activity. In the majority of studies, the presence of γ’-fibrinogen results in a more dense matrix with thinner fibers\(^6\).

Most of the fibrinogen heterogeneity in plasma is due to partial proteolytic degradation of the Aα-chains, resulting in the fibrinogen molecular weight variants. High molecular weight (HMW) fibrinogen represents ‘native’ fibrinogen with a molecular mass of 340 kDa (70% of total plasma fibrinogen). In low molecular weight (LMW) fibrinogen one of the Aα-chains is partially degraded resulting in a molecule with a mass of 305 kDa (24% of total plasma fibrinogen). Partial degradation of both Aα-chains results in low molecular weight’ (LMW’) fibrinogen (6% of total plasma fibrinogen) with a molecular mass of 270 kDa\(^6\). Although LMW- and LMW’-fibrinogen are deprived from HMW-fibrinogen, the specific enzyme(s) responsible for this conversion have not yet been identified\(^6\). Three major C-terminal residues of the Aα-chains have been described by Nakashima et al.; Asn-269, Gly-296 and Pro-309\(^7\). A number of functional domains are present in the αC-domains and the loss of these domains in LMW- and LMW’-fibrin may affect the matrix characteristics and cell-matrix interactions. The C-terminal region of the Aα-chain contains a RGD-site (Aα572-574) for binding to endothelial cell integrins, also interaction sites for factor XIII and plasminogen are present in the C-terminal region of the Aα-chain\(^6\). The αC-domains enhance lateral aggregation and fiber thickness\(^7\).

Scanning electron microscopy of HMW-fibrin shows an open structure with thick fibers. On the contrary, LMW-fibrin is composed of a dense structure with thinner fibers (Figure 5)\(^8\). In vitro angiogenesis studies showed increased ingrowth of endothelial cells and formation of tube-like structures in HMW-fibrin, when compared to unfractionated-fibrin. Whereas, LMW-fibrin severely reduced the tube-like structure formation by endothelial cells in vitro. These results were confirmed in vivo, the reduction to 50% HMW-fibrin by addition of LMW-fibrin, resulted in decreased angiogenesis and perfusion of the matrix\(^8\). These data give evidence that rate of vascularization in tissue engineered scaffolds can be influenced by using different fibrinogen variants.
Aim of the study

Establishment of a vascular network in tissue engineered scaffolds is a problem and currently limits the size and development of scaffolds. In wound healing and tumor growth, fibrinogen forms a temporary fibrin matrix that facilitates cell adhesion, migration, proliferation and angiogenesis. Also in tissue engineering fibrin was shown as appropriate matrix for scaffold vascularization. By using specific (naturally occurring) fibrinogen variants, the rate of vascularization can be influenced. These variants can be either isolated from plasma or recombinantly produced. Stem cells are often the cellular component of tissue engineered scaffolds; their response to fibrinogen variants is important for scaffold development. Understanding of the effects of naturally occurring fibrinogen variants on cellular characteristics is required for the development of tissue engineering applications.

The main aim of this thesis is to evaluate the effects of naturally occurring fibrin(ogen) variants as scaffolds in tissue engineering, with specific emphasis on angiogenesis and stem cell behavior, in order to gain more insight in refinement of scaffold vascularization. Objectives of this study are: (I) to unravel the mechanism of the endothelial cell response on the fibrin molecular weight variants. (II) To investigate the stem cell response on the fibrin molecular weight variants. (III) To explore the possibilities for other naturally occurring fibrinogen variants on angiogenesis and wound healing. (IV) To investigate whether recombinant and plasma-derived fibrinogen variants have similar effects on angiogenesis.
Outline of the thesis

The focus of this thesis is on the use of fibrinogen variants to restore, maintain or improve tissue function of injured or diseased tissues, using a tissue engineering approach. First, a survey of the different aspects of fibrin matrices for tissue engineering is given in Chapter 2.

Angiogenesis was shown to be altered in matrices composed of molecular weight variants of fibrinogen$^{68}$. Unraveling the mechanisms behind these differences is important for the development of 3-dimensional HMW- and LMW-fibrin scaffolds for tissue engineering. Functional characteristics and gene expression of endothelial cells on HMW- and LMW-fibrin are described in Chapter 3.

Another application of fibrin in tissue engineering is coating of a synthetic vascular graft. In Chapter 4A, the barrier integrity and formation of a quiescent endothelial monolayer on HMW- and LMW-fibrinogen coatings are described. This is followed by a discussion of the observations of Sahni et al. on the addition of intact fibrinogen that induces an increase in endothelial permeability via fibrinogen domain $\beta_{15-42}$ binding to VE-cadherin (Chapter 4B).

Stem cells are important for tissue engineering, since they have high proliferative capacity and differentiate into multiple cell types. Chapter 5 describes adipose tissue-derived mesenchymal stem cells during expansion and differentiation in normal and low oxygen environments, in combination with the fibrinogen molecular weight variants.

The $\gamma'$-fibrinogen variant was shown to possess unique structural characteristics and its levels in plasma associated with venous and arterial thrombosis. In Chapter 6 the effects of fibrinogen $\gamma$-variants on angiogenesis and in vivo wound healing are described.

Recombinant fibrinogen has advantages in homogeneity, quality and consistency, when compared to commercially available plasma-derived fibrinogen. In Chapter 7 the comparison of recombinant HMW-fibrinogen with plasma-derived HMW-fibrinogen is made.

Finally, in Chapter 8 the results are discussed in the context of previous and recent developments in the field of tissue engineering and angiogenesis. In addition, a brief overview of the conclusions and future directions are provided.
Reference List


