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Molecular Understanding of Endothelial Cell and Blood Interactions with Bacterial Cellulose: Novel Opportunities for Artificial Blood Vessels

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1. Introduction

Cardiovascular disease (CVD) is the main cause of death or invalidism in high-income countries today. Moreover, worldwide demographic changes are aiding CVD's rapid progression towards the number one killer in middle- and low-income countries. The World Health Organisation estimates that if current trends are allowed to continue, about 20 million people will die from CVD by 2015. This group of disorders, which affect the heart and blood vessels, includes coronary heart disease, cerebrovascular disease and peripherial arterial disease, deep vein thrombosis and pulmonary embolism.

The main cause of these acute life-threatening conditions is atherosclerosis. Atherosclerotic plaques and restenosis can result in severe occlusions of peripheral and coronary arteries. Current treatments include drug therapy and bypass surgery, and depend on the severity of the disease. All treatments require molecular understanding of the processes that govern atherosclerosis. This is especially important when introducing artificial graft materials *in vivo*.

Generally, the first choice for vascular replacement graft material is the patient's own vessels, i.e., autologous vessels. If these are in shortage supply or do not exhibit sufficient quality due to, e.g., other diseases or previous surgery, artificial alternatives become necessary. Today, clinics use biomaterials such as expanded polytetrafluorethylene (ePTFE) and polyethylene terephtalate fibre (Dacron®) as prosthetic grafts for reconstructive vascular surgery. However, their performance is dismal in small diameter vessels (>6 mm) like coronary arteries and peripheral arteries below the knee, resulting in early thrombosis and intimal hyperplasia. Therefore, about 10% of patients with CVD are left untreated due to the lack of replacement material for small vessels.

Considering the large number of patients who need replacement vessels, the substantial demand for alternative small-caliber grafts is urgent, driving scientists to search for and develop new materials. Recently, this has even led to the use of completely biological vessels. However, the growth of such requires months, rendering them unsuitable for acute situations such as heart infarction, which demand a substitute vessel immediately.

2. What is the ideal vascular graft?

Several issues demand consideration when constructing a vascular graft: the mechanical properties of the graft must resemble those of a native blood vessel, and the graft must be biocompatible with its host. One important factor is compliance, i.e., how well the vessel withstands pressure from the bloodstream and whether it can maintain systemic pressure in the vascular system. Vascular grafts should also be "invisible" to the immune system and possess non-thrombogenic properties.

One interesting option is bacterial cellulose (BC), whose unique properties (strength, good integration into host tissue and flexibility that allows production in various shapes and sizes) make it an exciting candidate for vascular graft material. The most abundant biopolymer on earth, cellulose is insoluble in water and degradable by microbial enzymes. Several organisms such as plants, algae and bacteria can produce BC. Some members of the bacterial genus Acetobacter, especially *Gluconacetobacter xylinum*, synthesize and secrete cellulose extracellularly. The network structure of cellulose fibrils resembles that of collagen in the extracellular matrix (ECM).

This chapter describes how BC interacts with human endothelial cells (EC) and blood. Specifically, we will evaluate whether surface modifications could promote adhesion of EC and also whether BC's thrombogenic properties compare favorably with conventional graft materials. These properties are critical because materials intended as vascular grafts must satisfy many important features, including blood compatibility, cell interactions and mechanical properties.

3. Tissue engineering of blood vessels

Tissue engineering is a relatively new scientific discipline that combines cells, engineering and materials to improve or replace biological functions. Langer and Vacanti, two pioneers in the field, describe tissue engineering as an interdisciplinary area that applies the principles of engineering and life sciences to the development of biological substitutes that restore, maintain or improve tissue formation (Langer & Vacanti, 1993).

The basic concept of tissue engineering includes a physical support (3D-scaffold) composed of synthetic polymers or natural materials (collagen, elastin or fibrin). This support mimicks ECM and initially serves as a scaffold or template on which cells can organize and mature *in vitro* prior to implantation at the appropriate location.

Initial research in the mid-20th century focused on developing bioinert materials, eliciting a minimal host response characterized by passive blood transport and minimal interactions with blood and tissues. Although widely available, these industrial materials, including Teflon and silicone, were not developed specifically for medical applications. Later, the production of completely non-reactive substances became unrealistic.

Today, other biomaterials are being developed to stimulate reactions between proteins and cells at the molecular level in a highly precise and controllable manner. The key concept underpinning development of such biomaterials is that the scaffold should contain chemical or structural information that mimicks cell-cell communication and controls tissue formation, such as growth factors, the adhesion peptide Arg–Gly–Asp (RGD) and other molecules that mimic ECM components. RGD is the minimal sequence in basement membrane proteins such as fibronectin, fibrinogen and von Willebrand Factor, all required for cell adhesion (Pierschbacher & Ruoslahti, 1984).

A successful tissue engineered blood vessel must: be biocompatible, i.e., noninflammatory, nontoxic, nonimmunogenic and noncarcinogenic; infection-resistant and nonthrombogenic. It also must have appropriate mechanical properties, e.g., tensile strength, burst strength, good suture retention and compliance, and possess appropriate vasoactive physiological properties, including contraction or relaxation in response to neural or chemical stimuli and more.

The feasibility of constructing and using tissue engineered blood vessels was first demonstrated in landmark studies by L'Heureux (L'Heureux et al., 1998) and Niklason (Niklason et al., 1999). The vessels, which were produced using different in vitro techniques, had very good mechanical properties and functioned well in experimental animals. Although *in vitro* and experimental techniques have been developed since then, no clinical implantations have been made until now.

3.1 Biomaterials/biomaterial scaffolds

Williams defines biomaterial as any natural or man-made material that comprises the whole or part of a living structure or biomedical device that performs, augments, or replaces a natural function (Williams, 1999).

Many different materials have been investigated for biomaterial applications. They can be divided into natural materials, i.e., collagen (Weinberg & Bell, 1986; L'Heureux et al., 1998); fibrin (Cummings et al., 2004; Kumar & Krishnan, 2002); hyaluronic acid (Remuzzi et al., 2004; Turner et al., 2004); silk fibroin (Zhang et al., 2009) and BC (Backdahl et al., 2006; Klemm et al., 2001; Bodin et al., 2007; Fink et al., 2010)) and synthetic polymers, i.e., polyglycolic acid (PGA) (Niklason et al., 1999; McKee et al., 2003), polyethylene terephthalate (PET) (Sharefkin et al., 1983; Herring et al., 1984) and ePTFE (Zilla et al., 1987; Meinhart et al., 2005). The required properties for biomaterials vary with cell type, implantation site and strategy for tissue formation. Common demands for all biomaterials include biocompatibility, e.g., avoiding foreign body reactions, capsule formation and chronic inflammatory reactions. Additionally, materials intended to be in contact with blood require evaluation for thrombogenicity. Mechanical properties are important and depend on the target tissue. Since biomaterials used as vascular grafts must withstand blood pressure, they must be investigated for burst pressure, compliance, suture strength and fatigue before using them as implants.

A recent and popular approach involves electrospinning different materials to create nanofibre constructs. Both electrospun synthetic polymers and native ECM proteins have been used for cell seeding to construct vascular grafts (Hashi et al., 2007; Huang et al., 2001; Boland et al., 2004; Kenawy el et al., 2003).

3.2 Materials for vascular grafts

Jaboulay and Briau performed the first arterial transplantation in 1896, but imperfect anastomoses resulted in thrombosis (Jaboulay & Briau, 1896). Since then, more sophisticated techniques have been developed. The search for arterial vascular grafts began in 1952, when Voorhees discovered Vinyon N (nylon), the first fabric graft (Voorhees et al., 1952). A few years later, DeBakey discovered Dacron® in 1958 (Nose, 2008). Today, arterial and even especially venous autografts are used routinely in surgery, creating bypasses for patients with peripheral or coronary occlusive vascular diseases. However, autograft availability is limited, particularly for arteries.

Dacron® and ePTFE are still widely used as arterial replacements. Despite their success in replacing large diameter (>6 mm) high-flow vessels, these materials show thrombogenicity and compliance mismatch in low-flow or small-diameter vessels. Sophisticated techniques have been evaluated to enhance patency, including chemical modifications, coatings and seeding of the surface with different cells. In contrast to natural materials, synthetics often lack adhesion sites. Although passive materials can reproduce sufficient physiological mechanical strength, proper metabolic function and cellular signalling requires intact cellular machinery.

BC is an attractive material for biomaterial applications. Its structure resembles that of collagen, the component in arteries and veins that gives the blood vessel its strength. BC's manufacturing process allows versatility in shape and size, including tubes. Studies have shown successful growth of cardiac rat-derived myocytes and fibroblasts (Entcheva et al., 2004), rat-derived hepatocytes (Kino et al., 1998; Yang et al., 1994) and osteoprogenitor cells (Takata et al., 2001) from mice on cellulose-based materials. However, these matrices are based not on natural cellulose but rather on derivatives such as cellulose acetate and regenerative cellulose.

Although BC is biocompatible, it generally does not promote cell growth (Watanabe et al., 1993a). Thus, BC must be modified to support EC adherence. Modification of wet state BC is challenging because fibre structure and strength must be maintained. This is especially important for vascular grafts, which must withstand blood pressure.

4. Different approaches to engineered blood vessels

4.1 Collagen-based blood vessel model

Weinberg and colleagues developed a collagen-based blood vessel model (Weinberg et al., 1986). Improvement of the construct's mechanical properties is ongoing due to poor mechanical integrity.

4.2 Cell self-assembly model

The cell self-assembly model is made using intact layers of human vascular cells grown to overconfluence to form visible sheets of cells and ECM (L'Heureux et al., 1993, 1998). A sheet of smooth muscle cells (SMCs) is rolled around a mandrel to form the medial layer. Similarly, a sheet of fibroblasts is rolled over the SMC sheet media, forming an adventitial layer. Finally, ECs seeded onto the lumen of the matured vessel form a confluent monolayer. These constructs withstood more than 2000 mmHg pressure before bursting.

4.3 Cell-seeded polymeric scaffold–hybrid graft

This graft was developed by Niklason (Niklason et al., 1999). Vascular cells were seeded into a biodegradable scaffold (PGA) and cultured for 8 weeks under pulsatile radial stress (165 beats per minute and 5% radial strain). ECs seeded onto the lumen of the construct formed a confluent monolayer. Cultured under pulsatile conditions, the histological structure of the constructs resembled that of native arteries. Due to high collagen content, the constructs had a burst pressure greater than 2000 mmHg and they could contract. *In vivo* studies of tissue engineered blood vessels in Yucatan miniature pigs were promising.

With degradable material such as PGA, it is critical to ensure adequate strength of ECM (collagen, elastin) produced by the vascular cells. Degradation products may be toxic to the

cells. Non-degradable scaffolds offer durable support, but tissue acceptance is mandatory. Ideally, the scaffold should be compliant, similar to native vessels.

4.4 Acellularized construct

A rolled small intestinal submucosa (SIS) has been used as a small diameter vascular graft. A cell-free, 100-µm-thick collagen layer derived from small intestine, SIS is compliant, making it an interesting candidate for vascular implantation and requiring investigation (Roeder et al., 1999, Huynh et al., 1999).

4.5 Artificial artery generated in the peritoneal cavity

In the peritoneal cavity, artificial arteries are generated on silastic tubes. The arteries are lined by nonthrombogenic, mesothelial (endothelial-like) cells. The feasibility of this approach in humans is a matter of debate.

5. Bacterial synthesized cellulose as biomaterial

Cellulose, the most abundant biopolymer on earth, is insoluble in water and degradable by microbial enzymes. Several organisms, e.g., plants, algae and bacteria can produce cellulose, and the bacteria *Gluconacetobacter xylinum* can synthesize and secret cellulose extracellularly (Brown et al., 1976). BC is composed of linear nanosized fibrils of D-glucose molecules (Ross et al., 1991). The network structure of cellulose fibrils resembles that of collagen in the ECM of native connective tissue (Fig. 1) (Backdahl et al., 2006).

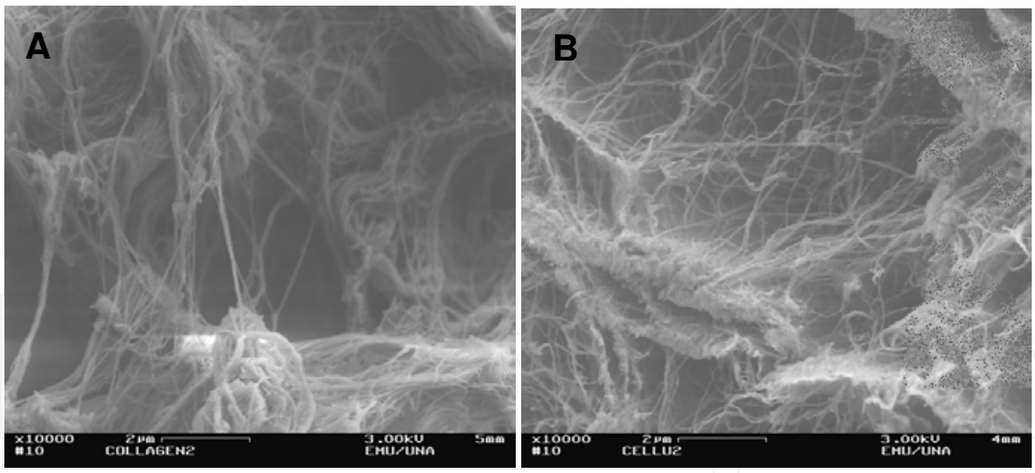

Fig. 1. SEM images of (A) collagen and (B) BC reprinted with permission from Backdahl et al., 2006. Copyright Elsevier 2006.

Although BC is not a hydrogel in the true sense of the meaning, it is often referred to as such because of its high water content, insolubility in water and highly hydrophilic nature. Since BC consists of a highly entangled network of fibrils, it also provides strong mechanical properties that ensure the ability of tissue engineered blood vessels to withstand mechanical forces and prevent rupture. BC can be designed and shaped into three dimensional structures such as tubes or sheets (Backdahl et al., 2006). A major advantage of using BC rather than cellulose produced by any other organism is that BC is

completely free of biogenic compounds such as lignin, pectin and arabinan found in, e.g., plant cellulose. During the production process, it is also possible to modify several other properties including pore size, surface properties and layering of the material (Backdahl et al., 2008).

BC is used in various areas including food matrix (nata de coco), dietary fibres, acoustic or filter membranes and ultra-strength paper. In addition, BC has been suggested as a potential material for tissue engineering in several areas, e.g., as scaffold for tissue engineering of cartilage, blood vessels (BASYC®) and successful treatment of second and third degree burns, stomach ulcers and other situations that require a temporary skin substitute (Biofill®, Gengiflex®, XCell®) or to recover periodontal tissue (Gengiflex®) (Czaja et al., 2006; Svensson et al., 2005; Fontana et al., 1990).

5.1 Structure and morphology

Beginning with the water-soluble monosaccharide D-glucose, cellulose synthesis is produced extracellularly as pellicles at the air/liquid interface. Glucan chains of BC are extruded from several enzyme complexes and aggregated by van der Waals forces to form sub-fibrils, approximately 1.5 nm wide. BC sub-fibrils crystallise into microfibrils and then into bundles, which form a dense reticulated structure stabilized by hydrogen bonding. In culture medium, the bundles assemble into ribbons, forming a network of cellulose. This network of cellulose nanofibrils provides BC with high mechanical strength and a water retention capacity of about 99% (Iguchi et al., 2000).

The macroscopic morphology of BC varies with different culture conditions. In static conditions, BC accumulates on the surface of nutrient-rich culture medium, at the oxygen-rich air-liquid interface. Statically cultured BC has lower crystallinity than BC fermented during agitation.

Our molecular studies on EC-blood interactions with BC used BC synthesized by *Gluconacetobacter xylinum* (ATCC 1700178, American Type Culture Collection). Cellulose tubes were grown in corn steep liquid media at 30°C for 7 days. The cellulose was then purified by boiling, first in 0.1 M NaOH at 60°C for 4h and then in Millipore™ water. Finally, the cellulose was sterilised by autoclaving for 20 minutes. Due to the production process, BC consists of two distinctly different layers: one side has a compact network of fibrils with few if any pores, and the other side has a porous network structure. A density gradient arises between the sides.

5.2 Mechanical properties

The optimal scaffold is a biocompatible biomaterial that provides proper mechanical and physical properties, thus promoting cell adhesion and tissue formation. Prior to implantation into animals, BC tubes undergo extensive mechanical testing (burst strength, compliance and tensile strength). Films or sheets of BC show remarkable mechanical strength, due to high crystallinity, high planar orientation of the ribbons, ultrafine structure and a complex network (Iguchi et al., 2000). The mechanical properties of BC tubes are similar to those of pig carotid arteries (Backdahl et al., 2006). BC's compliance curve resembles that of a native artery more than any other synthetic material on the market, which is advantageous. Material density can be altered by varying the culture conditions or by post-culture modifications.

5.3 Biocompatibility

Integration of a material with the host tissue is essential for the success of tissue engineered blood vessels. According to Williams, the biocompatibility of a material is defined as "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1999). Therefore, an appropriate host response would involve a biomaterial that induces a very low inflammatory and foreign body response in the host tissue.

A study by Helenius et al. showed that BC is well integrated into the host tissue and does not induce inflammatory or foreign body responses (Helenius et al., 2006). They implanted BC pieces subcutaneously in rats and explanted them after 1, 4 and 12 weeks. Incorporation of the implant in the host tissue made it difficult to distinguish a clear interface between the implant and the host tissue (Helenius et al., 2006). These results are supported by another *in vivo* study, where BC tubes were implanted into the carotid arteries in pigs (Wippermann et al., 2009). Therefore, BC clearly has good biocompatibility and shows promising potential as scaffold material.

5.4 Surface modification

One challenge in the field of vascular grafts involves promoting EC attachment and spreading, since many biomaterials similar to BC exhibit limited support for cellular adhesion (Watanabe et al., 1993b). Over the years, many strategies have been developed to modify material surfaces.

To optimize cell-biomaterial interactions, manufacturers coat synthetic scaffolds with cell adhesive proteins such as collagen, fibronectin or laminin (Seeger & Klingman, 1988; Kaehler et al., 1989). However, varying protein composition results in a biofilm with passive protein adsorption, and that composition can modify over time (Vroman, 1987). Additionally, protein adsorption to BC is very low.

Much attention has focused on cell adhesion peptide RGD and its derivatives as possible alternative for stimulating reproducible and predictable cell adhesion (D'Souza et al., 1991; Hersel et al., 2003; Walluscheck et al., 1996; Gabriel et al., 2006). Most RGD modifications occur via covalent binding to the material (Massia & Hubbell, 1990). Although cellulose contains reactive hydroxyl groups that can be chemically modified, these very same hydroxyl groups participate in hydrogen bonding, which holds the cellulose fibre network together. Disruption of these bonds associates with loss of fibre ultrastructure (Sassi & Chanzy, 1995; Sassi et al., 2000). Dry films of BC have been modified with carboxymethyl and acetyl groups (Kim et al., 2002). However, surface modifications to wet state BC remain incompletely understood. Thus, modification of a BC hydrogel is especially challenging, since solvent exchange and cellulose modification typically destroy the hydrogel morphology.

Modification of a BC hydrogel is especially challenging because solvent exchange and cellulose modification typically destroy hydrogel morphology. Thus far, most modifications have been performed on dried BC. Consequently, a new method is needed to increase cell attachment without altering the structure of the BC network.

5.4.1 Xyloglucan

Xyloglucan (XG), the most abundant hemicellulose, is present in the primary wall of many plants. In contrast to cellulose, XG is water-soluble and interacts strongly with cellulose fibres (Hanus & Mazeau, 2006). We have taken advantage of these properties, which provide an elegant means of introducing cell adhesion peptide RGD with XG as a carrier molecule to BC.

BC and cotton linters, as reference material, were modified with XG and XG bearing a GRGDS pentapeptide (Bodin et al., 2007). Compared with organic solvents, modification in the water phase was clearly advantageous for preserving the morphology, as observed with SEM (Fig. 2). XG adsorption increased the wettability only to a minor extent, possibly explaining the decreased or undetectable adsorption of adhesive proteins shown by QCM-D. QCM-D studies further revealed that fibrinogen antibodies do not bind to BC, leading to the conclusion that cell enhancement would result from the presence of RGD epitopes, not from unspecific protein adsorption, e.g., fibronectin, from the cell culture medium. XG also enhances hepatocyte adhesion (Seo et al., 2004), and modification of BC with XG does not adversely affect ECs.

5.4.1.1 Increased cell spreading and adhesion on XGD-modified BC

XG-RGD-modification increased cell adhesion by 20%, and also increased the metabolism of seeded ECs as compared with unmodified BC. In contrast, the proliferation rate was less affected, presumably due to biological variation between cell donors. Our results (Fink et al., 2011a) concur with studies on RGD-grafted regenerated cellulose, which showed that an adhesion peptide enhances adhesion by approximately 20% (Bartouilh de Taillac et al., 2004). Another study showed that cellulose binding proteins bound to different adhesion peptides improve adhesion and spreading of human microvascular cells to cellulose (Andrade et al., 2010). In our study, the absence of serum negatively influenced cell adhesion on unmodified BC but did not act similarly in modified BC, further indicating that increased adhesion is peptide specific.

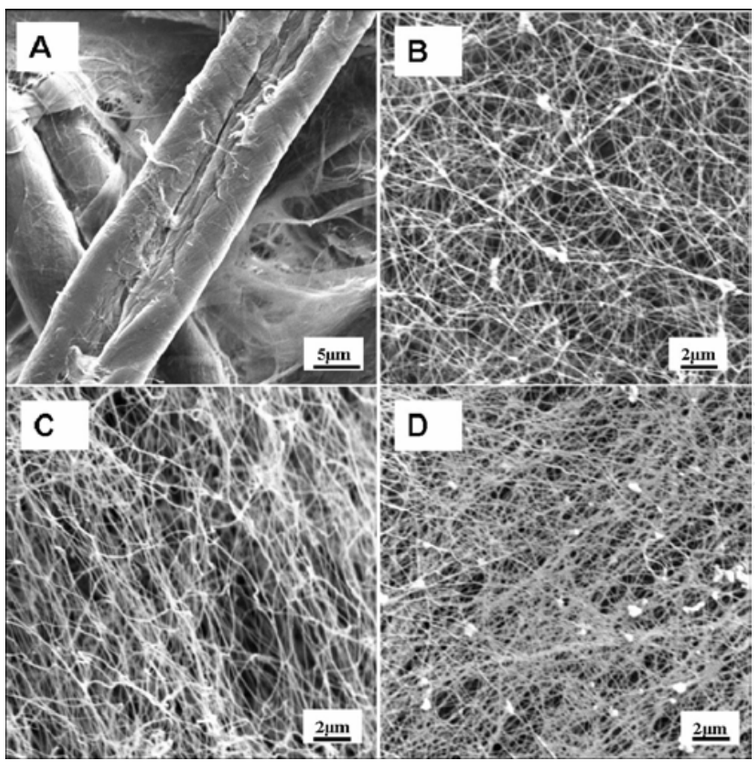

Fig. 2. SEM micrograph of BC morphology of (A) cotton linter, (B) unmodified BC, (C) XG-RGD modified BC and (D) acetone treated BC. Reprinted with permission from Bodin et al., 2007. Copyright American Chemical Society 2007.

Initial cell attachment is crucial to subsequent behaviour such as spreading, proliferation and cell differentiation on substrates. The extent of cell spreading is an important parameter for the biocompatibility of materials. EC adhesion to the ECM proteins is normally followed by cell spreading, a process in which cells reorganize the f-actin cytoskeleton, resulting in flattening and spreading of the cell. These polymerised actin filaments attach the cells to the substrate via focal adhesions. Cells grown on RGD-modified BC spread out, displaying a well-organized actin cytoskeleton with prominent f-actin fibres. They also grow in clusters, which we believe is a step towards achieving a confluent monolayer.

6. Endothelial cells as cellular source for graft lining

The endothelium is composed of a monolayer of squamous epithelial cells that line the inside of blood vessels in a confluent layer, with a total area of 350-1000 m^2 and a weight of 0.5-1.5 kg (Pries et al., 2000; Jaffe, 1987). The morphology of these cells is flat, resembling a cobblestone pattern. This morphology is essential to maintaining good blood flow without turbulence. ECs function not only as a physiological barrier, separating the blood from surrounding tissues, but also as a dynamic layer of cells that displays antithrombotic properties in its resting state. This is achieved by physically preventing elements in the blood from contacting prothrombotic elements in the subendothelium and by active synthesis of various mediators. Endothelial functions help maintain blood vessel function.

The endothelium upholds delicate balances in the vasculature, i.e., vasoconstriction/vasodilatation, anticoagulant/procoagulant properties, blood cell adherence/nonadherence and growth promotion/inhibition. It regulates vascular tone, maintains hemostasis, controls vascular structure and mediates inflammatory and immunological responses.

The endothelium responds to inflammatory conditions by regulating its own permeability and releasing a variety of substances. It mediates inflammation with pro-inflammatory mediators including cytokines such as the interleukins (IL) (e.g., IL-1β, IL-6, IL-8), platelet-activating factor (PAF) and also by expressing endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule 1 (ICAM-1), inflammatory mediators that control the interaction between EC and circulating blood cells and leukocytes, leading to extravasation of leukocytes.

During an inflammatory response, adhesion molecule P-selectin is expressed on ECs after exposure to leukotrine B4 or histamine, which are produced by mast cells. Tumor necrosis factor alpha (TNF-α) and lipopolysaccharides (LPS) induce P-selectin expression and the synthesis of E-selectin, another selectin that appears a few hours after the inflammatory process begins. Because the interactions between these selectins and their corresponding glycoprotein ligands (sialyl-Lewis^x moiety) on leukocytes are relatively weak and reversible, leukocytes are unable to attach firmly to the endothelium. Instead, they "roll" along the surface of the vessel wall. The interactions are enhanced as other integrins are induced on the endothelium.

Leukocyte integrins LFA-1 and Mac-1 normally adhere only weakly to leukocytes. On the other hand, IL-8 and other chemokines bound to the endothelial surface trigger a conformational change in LFA-1 and Mac-1 on the rolling leukocytes, increasing adhesiveness and consequently firmly anchoring the leukocytes to the endothelium. Rolling is arrested and the leukocytes squeeze between the ECs into the subendothelial tissue, a process known as diapedesis.

6.1 Angiogenesis and vessel remodeling

ECs regulate vessel structure by producing both growth promoting and growth inhibiting factors. SMC growth is stimulated by platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor alpha (TGF-α), endothelin and angiotensin II. Growth is inhibited by nitric oxide (NO), prostacylin (PGI_2), some FGFs, insulin-like growth factor 1 (IGF-1) and thrombospondin.

Angiogenesis is regulated by a variety of growth factors. Hypoxia and inflammatory cytokines such as FGF increase vascular endothelial growth factor-A (VEGF-A) levels through autocrine and paracrine mechanisms. VEGF-A, an endothelial-specific growth factor that consists of a heparin-binding homodimer, is a major regulator of EC function and angiogenesis. VEGF-A activates several EC functions, e.g., proliferation, migration and NO-release, processes that participate importantly in new blood vessel formation. VEGF-A also increases vessel wall permeability. Both VEGF and FGF induce EC production of proteases such as matrix metalloproteinases (MMPs) and plasminogen activator (PA). At least twenty MMPs participate in angiogenesis (Kroll & Waltenberger, 2000; Lamalice et al., 2007).

Proteases digest the basement membrane, allowing ECs to invade surrounding tissue, where they proliferate and migrate to form a sprout. The sprout elongates and the ECs differentiate to form a lumen. ECs in the newly formed vessel produce PDGF-BB, which attracts mural cells (pericytes to capillaries/SMC to larger arteries and veins) and stabilises the vessel. Expressed on ECs, heparin sulphate proteoglycans and their glycosaminoglycans (GAG) side-chains play an important role in angiogenesis because they bind circulating growth factors like VEGF (Kroll & Waltenberger, 2000).

6.2 Regulation of vascular tone

ECs regulate vessel tone and, consequently, local blood flow by managing the communication between the blood and the underlying SMCs, and by releasing substances that influence SMCs to relax or contract. In addition, ECs synthesise both vasodilating and vasoconstricting agents.

Vasodilatation is mediated through PGI_2 and (NO/endothelium-derived relaxing factor (EDRF) and endothelium-derived hyperpolarizing factor (EDHF), where NO plays a central role. Vasoconstricting agents released by ECs include endothelin, angiotensin II and thromboxane A_2 (TXA_2).

Shear stress, bradykinin, thrombin, serotonin and various drugs stimulate ECs to release prostacyklin, thus stimulating adenylate cyclase, which increases cyclic adenosine monophosphate (cAMP) in SMCs. NO is synthesized from L-arginine by NO synthase and diffuses to SMCs, where it activates guanylate cyclase to produce cyclic guanosine monophosphate (cGMP). This leads to decreased intracellular calcium and muscle relaxation. Sensing mechanical changes in the environment, f-actin mediates mechanical induction of NO, leading to signal transduction into the cell.

The eNOS gene contains a shear stress regulatory element (SSRE) that increases or decreases eNOS activity (Balligand et al., 2009). Acetylcholine stimulation of M1 muscarinic receptors releases endothelium-derived hyperpolarizing factor (EDHF), changing membrane potential (Pagliaro et al., 2000). Endothelin consists of three isoforms, ET-1, ET-2 and ET-3. ECs produce endothelin-1, the most potent mediator of vasoconstriction. Two endothelin receptors are found in the vasculature: ET_A on SMCs and ET_B on ECs. Binding of endothelin-

1 to the ET_A-receptor results in signal transduction and smooth muscle relaxation. On the other hand, activation of ET_B on ECs stimulates NO and PGI_2 production. In contrast, angotensin II is a much weaker vasoconstrictor. Renin cleaves angiotensinogen to angiotensin I, which is then converted to angiotensin by endothelial angiotensin-converting enzyme (ACE) (Nordt & Bode, 2000).

7. Blood compatibility of biomaterials is a challenge

A nonthrombogenic surface is the key to a successful vascular graft. Non-thrombogenicity can be achieved by various surface modifications. Since ECs could provide a nonthrombogenic surface, intense investigation has focussed on endothelialisation in this context. In our studies, we used ECs passage 4 from non-diseased human saphenous veins, by-products of coronary bypass surgery.

The thrombogenicity of a biomaterial is an essential factor for any material that will be in contact with blood. Although the thrombogenic property of cellulose has been extensively researched as it has been used for haemodialysis membranes (Fushimi et al., 1998; Mao et al., 2004), the thrombogenicity of BC remains undetermined because it is a relatively new material for vessel grafts. Therefore, one of our studies focussed on delineating the blood compatibility of BC in comparison with ePTFE and PET vascular grafts, which are both used clinically as graft material (Fink et al., 2011b).

The endothelium's most important function in relation to biomaterials is hemostatic control. Under normal physiological conditions, ECs express thrombo-resistant molecules, but they must be able to switch to a procoagulant state upon injury to initiate coagulation and clot formation. Since blood is transported under high pressure, minimization of blood loss requires a rapid response. Some molecules are continuously secreted by ECs while others are only produced upon stimulation. Molecules can be expressed on the surface or secreted into the blood stream. The different endothelial anti- and procoagulant factors are discussed below in their biological context.

7.1 Primary hemostasis – Platelet adhesion, activation and aggregation

Prostacylin I_2 (PGI_2), nitric oxide (NO) and adenosine diphosphatase (ADPase) suppress platelet activation, aggregation and platelet-wall-interaction. Both NO and PGI_2 are secreted and act in a paracrine manner, whereas ADPase is expressed on the EC surface. Platelet inhibition by PGI_2 is mediated through a guanosine nucleotide binding receptor. This receptor-mediated signal transduction increases cAMP levels and inhibits platelet activation and the release of proaggregatory compounds such as TXA_2 (Moncada, 1982). PGI_2 production is stimulated by diverse agonists such as thrombin, histamine and bradykinin, and synthesised via arachidonic acid (AA) and prostaglandin (PGG_2) (Wu, 1995).

ECs produce EDRFs, which are responsible for acetylcholine-induced vasorelaxation. The most important EDRF is NO, which synthesises nitric oxide synthase (NOS) by converting L-arginine. NO is a small molecule, so it diffuses easily. When NO enters platelets, it inhibits their adhesion and activation via guanylyl cyclise (Radomski et al., 1987c). PGI_2 and NO have synergistic effects on inhibition of platelet adhesion, activation and aggregation and also reverse platelet aggregation (Radomski et al., 1987a; b)

7.2 Secondary hemostasis – Coagulation

The endothelium physically separates coagulation factor VIIa from tissue factor (TF) and prevents platelet exposure to collagen and von Willebrandt factor (vWF) (Fig. 3).

Thrombomodulin (TM) is expressed on the surface of ECs. Thrombin binds to TM, thereby undergoing a conformational change that results in enhanced affinity for protein C. Thrombin is the only enzyme capable of activating protein C. Activated protein C cleaves and inactivates clotting factors Va and VIIIa (Esmon, 1993). The thrombin-TM complex, effectively removes thrombin from the blood and internalises it, leading to its degradation. The TM molecule can also bind FXa, thus inhibiting prothrombin activation (Thompson & Salem, 1986). Protein S, also synthesised by ECs, binds to the endothelial surface and protein Ca to form a complex, thus enhancing FVa and FVIIIa inhibition (Fig. 3).

The endothelium expresses heparin sulphate proteoglycans with anticoagulant activity on its surface. Heparin is a cofactor for antithrombin III, a plasma protein present that can inhibit thrombin, IXa, FXa and XIIa. The complex binding of thrombin to antithrombin III occurs slowly. This process is accelerated by the interaction with heparin, which has many binding sites for antithrombin, and serves to localise and increase its activity more than a thousand-fold. The β-isoform of antithrombin is more highly effective than the α-isoform. Moreover, the β-isoformis effectively inhibits thrombin-induced SMC proliferation (Swedenborg, 1998). Synthesised in the liver and also by ECs, tissue factor pathway inhibitor (TFPI) forms a complex with Xa and inactivates the VIIa-tissue factor complex by binding to it.

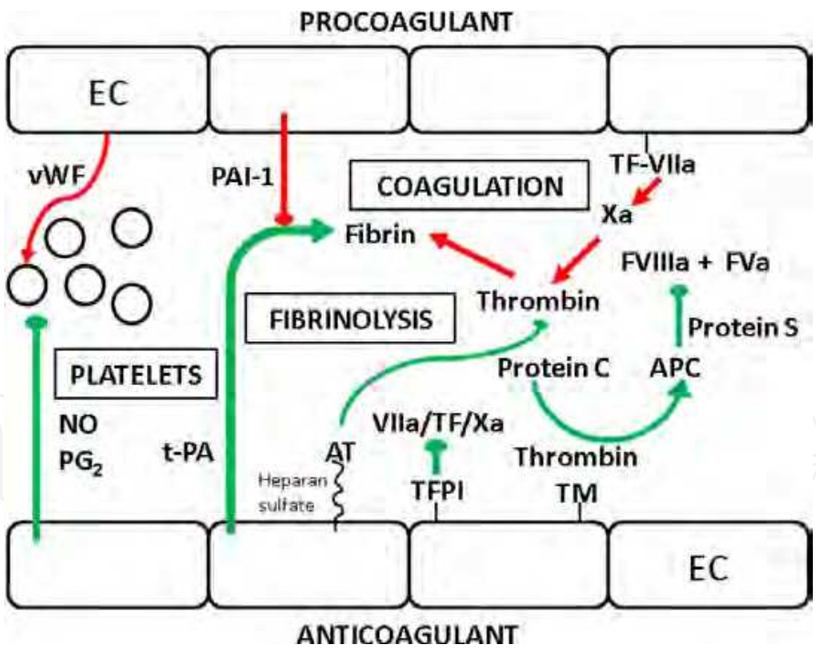

Fig. 3. Schematic illustration of the regulation of coagulation by ECs.

7.2.1 Procoagulation factor

The endothelium also participates importantly in the initiation of coagulation, which arrests bleeding. It expresses a variety of procoagulant factors, including vWF, coagulation factors V and VII, TF and high molecular weight kininogen (HMWK).

ECs synthesise vWF, a platelet adhesion molecule that secretes following stimulation by thrombin, and stores it in vesicles (Weibel-Palade bodies). vWF possesses binding sites for coagulation factor VIII, collagen (exposed after injury) and platelets (GPIb- XI-V), and acts as a bridging molecule in platelet aggregation and activation (Ruggeri, 1994). Importantly, the absence of vWF leads to severe bleeding disorders.
ECs also secrete TF, which is found mainly in the subendothelium at sites not normally exposed to the bloodstream. The basal production of TF is low in comparison with the underlying SMCs and fibroblasts, but it can increase 10- to 40-fold upon stimulation. In addition, ECs have binding sites for factor VII, IX, IXa, X and Xa. Binding to factor IXa inhibits EC decay in the presence of factors VIII and X, which provide an additional feedback mechanism for cell-bound procoagulant activity (Jaffe, 1987; Vane et al., 1990).

7.2.2 Fibrinolysis

The endothelium also helps regulated fibrinolysis (Fig. 3). The degradation of fibrin requires plasmin. Plasminogen binds to the cell surface and facilitates plasmin conversion by two PAs, tissue type plasminogen activator (tPA) and urokinase (uPA) (van Hinsbergh). Physiologically, the most important PA in vascular fibrinolysis is tPA. Indeed, tPA enhances the conversion of plasminogen 100-fold when it binds to fibrin. The release of tPA is either constitutively or pathway-mediated. Thrombin, FVa, bradykinin, PAF and shear stress all induce the synthesis and release of tPA from ECs (Emeis, 1992; Giles et al., 1990; Brown et al., 1999). When tPA binds to the EC surface, it is protected from degradation by the two PA inhibitors (PAI), PAI-1 and PAI-2, which are also released by ECs. PAI-1 requires vitronectin, present in ECM, to maintain its activity; it is the main inhibitor to tPA. Recombinant t-PA (rt-PA) is the most frequently used substance for inducing thrombolysis by pharmacological means (Noble et al., 1995; Bennett et al., 1991).

7.2.3 Biomaterial-induced coagulation

Evaluation of coagulation induced by biomaterials is mostly studied in terms of platelet adhesion, partial thromboplastin time (PTT), protein adsorption by QCM-D or ellipsometry (Liu et al., 2009; Mao et al., 2004;van Oeveren et al., 2002; Keuren et al., 2003). The QCM-D method is surface-sensitive, but the distance from the surface to where measurement is possible is limited. Currently, it is not possible to attach BC to quartz crystals. Therefore, cellulose other than BC must be used. However, this material could be used for QCM-D measurements as a model surface, complementing other studies. Because ellipsometry is an optical method, it is not possible to use native BC for this assay either. Automated calibrated thrombin generation is very sensitive and has become a widespread method for quantitative analysis of coagulation kinetics in blood plasma (van Oeveren et al., 2002; Gerotziafas et al., 2005; Hemker et al., 2003). Thrombin generation is also considered the most sensitive method to assay thrombogenicity.
Since BC is used in a wet state, finding appropriate analysis techniques has been challenging. To our knowledge, ours is the first study to investigate the thrombogenic properties of BC compared with other graft materials (Fink et al., 2011b). We also developed a modified automated calibrated thrombin generation assay (Fink et al., 2010). This makes it possible to follow thrombin generation, in the presence of a material, in real time rather than using an endpoint assay. Our assay has led to new insights into the kinetics of thrombin generation induced by a material surface, which otherwise would have been missed.

Most methods that study the coagulation process measure coagulation in the bulk without regards to where it began or the kinetics describing the propagation from the initiation point. Our method (Fink et al., 2011b) makes it possible to visualize the exact initiation point of coagulation and determine how coagulation propagates (Kantlehner et al., 2000). The captured images are used to calculate the coagulation time of the plasma at the surface (surface coagulation time) and into the bulk (propagation). Such factors are highly relevant to the study of material interactions with blood.

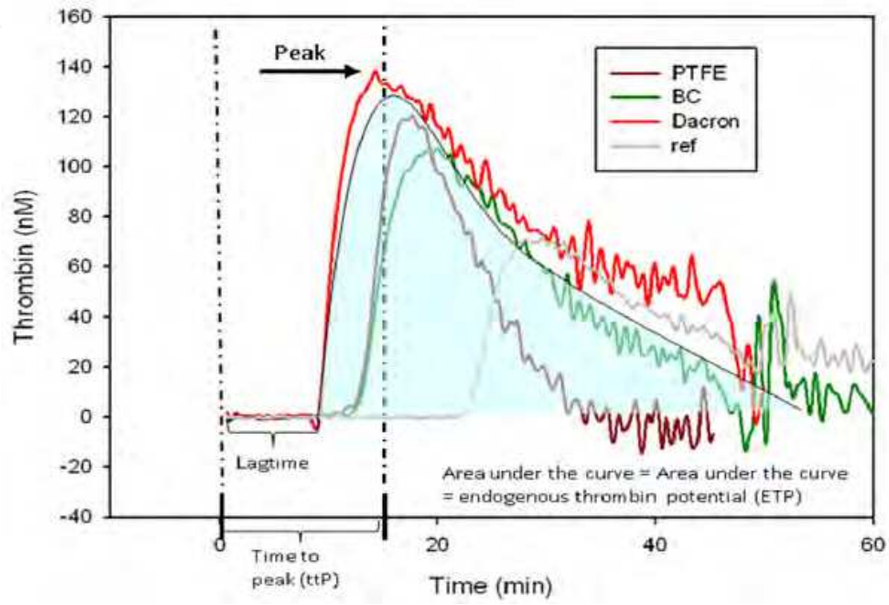

Fig. 4. Thrombogram generated from thrombin generation assay displaying lagtime, time to peak (ttPeak), peak and endogenous thrombin potential (ETP).

We measured the levels of thrombin and factor XIIa using calibrated automated thrombography, which displays the concentration of thrombin and factor XIIa, respectively, in clotting plasma with or without platelets (platelet-rich plasma/platelet-free plasma, PRP/PFP). The splitting of a fluorogenic substrate is monitored for either thrombin or factor XIIa and compared with a known thrombin or factor XIIa activity, respectively, in a parallel non-clotting sample. To evaluate thrombin and factor XIIa generation exclusively induced by the biomaterial surfaces, we fixed material samples with heparinised O-rings and analysed them by calculating the average rate of fluorescence increase over a period of 60 min.

7.2.3.1 Biomaterial induced coagulation of biological cellulose

We compared biomaterial-induced coagulation of BC with clinically used graft materials, i.e., ePTFE and PET. In addition, we visualised coagulation propagation at the material surfaces and into the plasma bulk.

Thrombin generation experiments revealed dramatic differences between the tested materials (Fink et al., 2010). Both ePTFE and BC generate longer lagtimes and time to Peak (ttPeak) values than PET (Fig. 4). Furthermore, BC generates the lowest 'Peak', indicating a

slower coagulation process at the surface. These results are also supported by the measurements of factor XIIa generation and analysis of surface coagulation times, where BC had the lowest FIIa generation and slowest propagation of coagulation into the bulk (Fig. 5). Compared with PET, thrombin generation in the whole blood Chandler-Loop system depicted the same response, yielding decreased accumulation of thrombin-antithrombin III complex (TAT) on both BC and ePTFE. Since the measurements are performed after one hour and not continuously, the difference in coagulation speed cannot be observed. On the other hand, this assay is performed in whole blood during flow conditions that more closely resemble an *in vivo* situation compared to measurements in platelet-free plasma during static conditions. It is interesting and promising that these two systems show similar results.

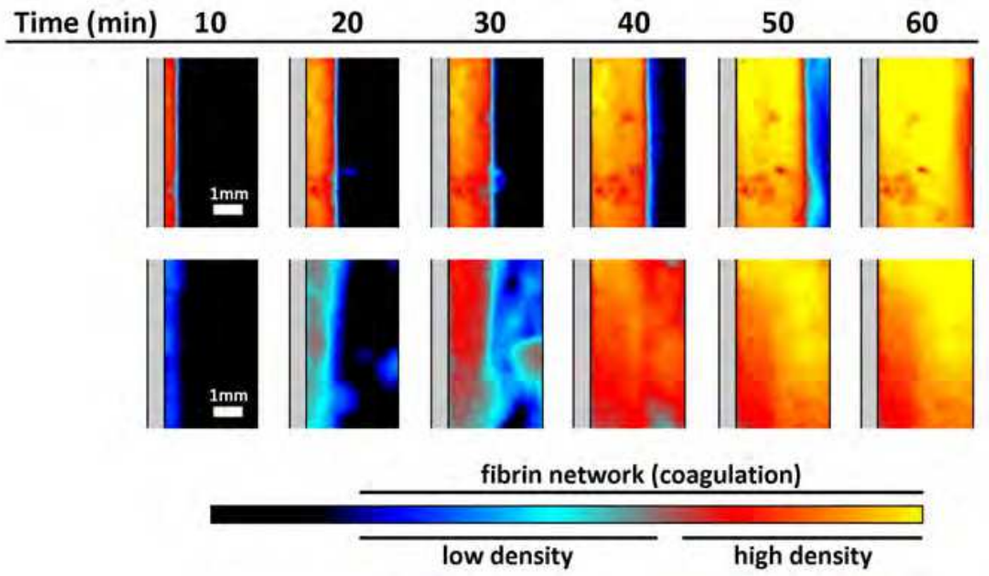

Fig. 5. Representative time-lapse images from a comparative experiment in the imaging of coagulation setup. Graft material samples are attached along the left wall in the images. The colour represents the density of the formed fibrin network (Courtesy of Lars Faxälv, PhD).

Hypothetically, the slower coagulation process on BC (Fig. 5) could be an advantage when blood contacts biomaterial applications, because it would provide time for the blood flow to divert and dilute activated coagulation products. The whole blood model also shows that 4 mm BC tubes perform well regarding anti-thrombogenic properties and perform better compared with ePTFE than 6 mm tubes. Measurements of thrombin generation correlate very well with the XIIa generation assay and visualisation of propagation. Together these methods potentially could provide fast screening methods for evaluating the thrombogenicity of biomaterials.

The amount of TAT generated depends on blood velocity (e.g., shear rate) in the loop system. Higher velocities associate with increased TAT generation. Shear rates are higher in the 4 mm loop system compared with the 6 mm system, and the narrower material also exhibits greater coagulation activation. Interestingly, however, platelet consumption does not increase, suggesting platelet activation. The amount of TAT generated on ePTFE increased 18-fold on 4 mm tubes as compared with 6 mm tubes, but we detected only a 3-fold increase for BC tubes. In comparison with the other tested materials, the platelet

consumption of BC is remarkably low, especially compared with heparinised polyvinyl chloride (PVC), which is known to have low thrombogenic properties (Johnell et al., 2005). In addition, cellulose showed no visible sign of clotting following one hour of incubation with whole blood containing only small amounts of soluble heparin.

7.3 Complement system

The immune complement system (CS) is part of the innate immune system. Its main task is to protect the body from pathogenic agents like bacteria, viruses and fungi. On contact with a foreign surface, e.g., a bacterial surface, the CS activates in a cascade that either destroys the bacterial surface or releases bioactive degradation products, or both, causing inflammatory reactions in the surrounding tissue.

Consisting of more than 30 different cell-bound and soluble proteins that circulate as inactive zymogens under nonpathological conditions, the immune CS is present in blood and serum. Its most important factor is complement factor 3 (C3). Cleavage of C3 by C3 convertase creates C3a and C3b, causing a cascade of further cleavage and activation events. Three different pathways lead to the creation of C3 convertase: the classical pathway, the alternative pathway and the mannose-binding lectin pathway. The classical complement pathway typically requires antibodies for activation, whereas the alternative and lectin pathways can be activated by C3 hydrolysis or antigens without the presence of antibody.

7.3.1 Classical pathway

Classical convertase is initiated when antibodies bind to a surface such as a bacterium. When factor C1 binds to an antibody, it cleaves is cleaved and binds to additional factors forming the classical convertase (C4b2a) (Kinoshita, 1991).

7.3.2 Alternative pathway

The alternative pathway is triggered by either spontaneous C3 hydrolysis, which forms C3a and C3b, or covalent binding of C3b from the classical and lectin pathways to a surface. The C3b molecule is capable of covalently binding to a pathogenic membrane surface in its vicinity. If there is no pathogen in the blood, the C3a and C3b protein fragments will deactivate when they rejoin with each other. Upon binding with a cellular membrane, C3b is binds to factor Ba and P, forming the alternative pathway C3-convertase (C3bBbP). A characteristic feature of the alternative pathway is a feedback mechanism that leads to accelerated C3 activation. Such mechanisms are not present in the classical pathway (Medicus et al., 1976; Rother et al., 1998).

7.3.3 Mannose-binding lectin pathway

A variant of the classical pathway, the Mannose-binding lectin pathway does not require antibodies. Activation of this pathway occurs when mannose-binding lectin (MBL) binds to mannose residues on the pathogen surface. Subsequently, the MBL complex can split C4 and C2, generating C3-convertase, as in the classical pathway (Petersen et al., 2001). This pathway will not be discussed further in this chapter.

The convertases from both the classical and alternative pathways cleave C5 into C5a and C5b. The C5b molecule associates with C6, C7, C8, and C9, forming the C5b-9 membrane attack complex (MAC), which is inserted into the cell membrane and initiates cell lysis. The C5b-9, also called the terminal complement complex (TCC), may exist as a soluble active

form denoted sC5b-9. This soluble form can be measured to assess complement activation . The C5a and C3a fragments are anaphylatoxins that participate in the recruitment of inflammatory cells and trigger mast cell degranulation. Therefore, these anaphylatoxins participate in many forms of acute and chronic inflammation including sepsis (Guo et al., 2004; Ward, 2008).

7.3.4 Complement activation

Extracorporal treatments such as haemodialysis and cardiopulmonary bypass activate the CS. Contact between blood and biomaterials may generate degradation fragments of complement C3a and C5a and soluble C5b-9. These fragments result in chemotaxis of leukocytes, cytokine release and generation of prostaglandins, resulting in a life-threatening condition termed "whole body" inflammation. Biomaterial induced CS is activated by both the classical and alternative pathways (Nilsson et al., 2007).

The Chandler-Loop system was also used to assess CS activation. The complement activation parameters (C3a and C5b-9) were much higher for BC compared with the other materials, for both 4 and 6 mm tubes. Cellulose is known to induce complement activation in hemodialysis membranes (Frank et al., 2001). The mechanisms underlying these results for BC are still unclear and require further investigation. Bacterial fragments could still be present in the material. However, endotoxin values are well within the limit for cardiovascular devices. It is also possible that exposed hydroxyl groups induce complement activation through the alternative pathway (Arima et al., 2009; Toda et al., 2008). The physiological significance *in vivo* of this complement activation remains undetermined. Interestingly, platelet activation is low even when complement activation is high. According to the literature, platelet consumption and complement activation are closely related (Fushimi et al., 1998; Hamad et al., 2008; Peerschke et al., 2006; Gyongyossy-Issa et al., 1994).

8. What are the future therapeutic possibilities?

This chapter has presented possible approaches to modifying BC that enhance EC growth *in vitro*. The XG method, an easy one-step procedure carried out in water, is an elegant technique for modifying BC to promote EC. Its advantage is the preservation of the fibre structure, thus maintaining its strength. The modification of BC with the XG technique is far from limited to the RGD peptide. Different peptide sequences or other active groups and growth factors could be attached to the XG molecule. Platelets could potentially adhere to exposed RGD peptides. Therefore, other peptides, more specific to ECs, or different combinations of peptides should be explored.

Measurements of thrombin generation correlated well with the XIIa generation assay and visualisation of the propagation of coagulation. Together, these methods could offer potential fast screening methods for evaluating the thrombogenicity of biomaterials and future surface modifications.

BC could be used for vascular grafts in two different approaches: (i) implantation as a tube without cells or (ii) seeding prior to implantation. The ideal BC modification would provide an initial nonthrombogenic surface and promote long-term endothelialisation. Future modifications of BC could include heparinisation or combinations of surface modifications, e.g., different peptides or coatings. Heparin is a potent antithrombotic

agent that functions by binding antithrombin. In recent years, heparinised ePTFE grafts (Propaten®) have been developed (Losel-Sadee & Alefelder, 2009). Although encouraging outcomes for below-knee bypass are reported, the compliance mismatch of ePTFE grafts still remains. However, this is an exciting modification and preliminary studies on heparinised BC tubes show considerably lower amounts of thrombin on the hep-BC surface.

Our studies show that modification of BC with the adhesion-promoting peptide RGD results in increased EC adhesion, metabolism and spreading. Furthermore, BC induces slower coagulation than clinically available materials such as Gore-Tex® and Dacron® and induces the least contact activation as evaluated by Factor XIIa generation. In addition, BC consumes low quantities of platelets and generates low thrombin values as compared with Dacron® and Gore-Tex®.

9. Conclusion

Our work demonstrates that it is possible to introduce an adhesion peptide to BC that enhances EC adhesion without altering the fibre network or mechanical properties. The anti-thrombogenic properties of BC, especially 4 mm tubes, are promising as compared with conventional graft materials. Therefore, BC emerges as a promising, novel vascular graft material for small-caliber grafts. Molecular studies confirm that BC exhibits low thrombogenicity and extensive EC adhesion, which are beneficial when introducing artificial materials *in vivo* during by-pass surgery. Together with the modification methods presented in this chapter, BC has the potential to become a material for artificial vessels, thus underlining the importance of increasing molecular understanding of EC and BC interactions to create novel opportunities for artificial blood vessels.

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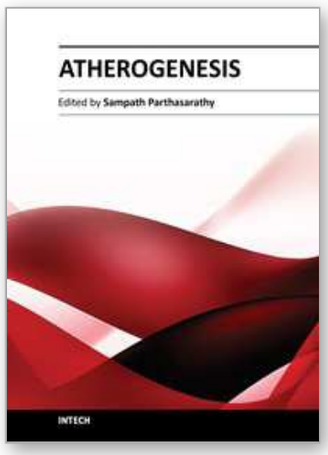

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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