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Cardiovascular Tissue Engineering Based on Fibrin-Gel-Scaffolds

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1. Introduction

Cardiovascular disease is a major cause of death in the Western World. Novel drugs and innovative devices have enhanced the quality of life for patients with cardiovascular disease, but such treatments are not without limitations and complications. The major constraint with these current treatments is the inability for growth, repair and remodeling of the structure. The emergence of tissue engineering as an alternative therapy for cardiovascular disease has generated an intensity of research into the development of many components of the cardiovascular system, including heart valves, small-calibre vascular grafts and biological stent materials. The composition of the biomaterial used as a support for the developing cardiovascular structure is a key mediator of cell behaviour and function in the tissue, and the ideal scaffold biomaterial for development of a successful end-product continues to be a matter of debate. Fibrin, a major structural protein involved in wound healing, represents an ideal scaffold for the rapid synthesis of autologous tissue-engineered cardiovascular grafts, as its primary scaffold constituents (fibrinogen and thrombin) can be isolated directly from a blood sample of the patient requiring the graft. Fibrin gel scaffolds offer immediate high cell seeding efficiency and homogenous cell distribution by gelation entrapment, and have a degradation rate that can be controlled by protease inhibitors, e.g. tranexamic acid or aprotinin. Fibrin is also known to stimulate the secretion of reinforcing extracellular matrix (ECM) proteins by seeded cells. The potential to control the fibrin polymerisation process also offers the opportunity to produce complex 3-D structures, like heart valve prostheses and to embed porous, textile or metal (stent) structures. This book chapter reviews the properties of fibrin that make it an ideal scaffold candidate for applications in the area of cardiovascular tissue engineering, and documents the successful development of fibrin-based heart valves, vascular grafts and biostents for clinical application.

2. Scaffold materials

Scaffolds play a central role in cardiovascular tissue engineering. Essential requirements for the ideal cardiovascular scaffold are easy handling properties and the ability to mould

complex 3-D structures from the material, such as aortic roots or vessels with complex side branches. The scaffold material should neither be toxic, nor elicit any immunological side effects. The diffusion barrier of the scaffold material should have the lowest possible resistivity in order to guarantee an optimal nutrition supply in thicker tissues. Furthermore, both the mechanical and the chemical properties (e.g. the integration of growth factors) of the scaffold material should be modifiable. Controllable degradation of the material is also important in order to adapt the structural support of the scaffold with regard to the developing tissue.

A multitude of scaffolds are currently employed in the field of tissue engineering, e.g. synthetic polymers (polyurethanes, polyglyolic acid, polylactic acid, polyhydroxybutyrate, copolymers of lactic and glycolic acids, polyanhydrides, polyorthoesters) and natural polymers (chitosan, glycosaminoglycans, collagen), or biological scaffolds such as acellularised porcine aortic conduits (Bader et al., 1998; Chevallay & Herbage, 2000; Flanagan et al., 2006; Freed et al., 1994; Grande et al., 1997). Scaffold-related problems including cytotoxic degradation products, fixed degradation times, limited mechanical properties and the absence of growth modulation, etc. necessitate further extensive investigations in developing the ideal cardiovascular scaffold.

3. Fibrin as scaffold material?

Based on the assumption that successful tissue engineering should mimic the process of tissue regeneration, and that regeneration is closely related to haemostatis, fibrin (gel) seems to be an ideal candidate as a tissue engineering scaffold by virtue of its role as a "physiological scaffold" in tissue regeneration. Several influences of fibrin gel on tissue development have been described in the literature: it is known that fibrin gel is one of the major ligands for ß3 integrins, which leads to cell migration into a wound/tissue-engineered construct (Ikari et al., 2000; Nomura et al., 1999). Thrombin, fibrinogen, fibrin monomers and fibrinopepide B all increase DNA synthesis in smooth muscle cells (SMCs) and consequently the proliferation of the cell (Pakala et al., 2001).

3.1 Physiology of fibrin

Fibrin is the end-product of the coagulation cascade following the conversion of fibrinogen in the presence of thrombin and calcium (Figure 1). Fibrinogen is a soluble plasma glycoprotein, which is produced by the liver. Fibrinogen is an acute phase protein with a normal blood concentration of 1.4 – 3.5 g/l. The fibrinogen molecule has a length of 45 nm, a molecular weight of 340 KDa and consists of 2 subunits and 3 polypeptides chains - α, β and γ. During the polymerisation process, the fibrinopeptide A of the α-chain and the fibrinopeptide B of the ß-chain are cleaved by thrombin. The exposed N-terminal positions of the fibrinopeptides bind to the γ-chain of the fibrinogen and produce the so-called *proteofibrils*. In the subsequent step, the *lateral association* leads to apposition of the proteofibrils to form a 3-D fibrin network structure (Meyer, 2004). FXIIIa stabilises fibrin further by incorporation of the fibrinolysis receptors, alpha-2-antiplasmin and TAFI (thrombin activatable fibrinolysis inhibitor, procarboxypeptidase B), and binding to several adhesive proteins of various cells (Muszbek et al., 2008).

The polymerised fibrin gel matrix is a hydro-gel, which contains ~95-98% water. The water content can easily be exchanged against a buffer solution or a cell culture medium, allowing an optimal nutrition supply of the cells that are embedded after the gelation process.

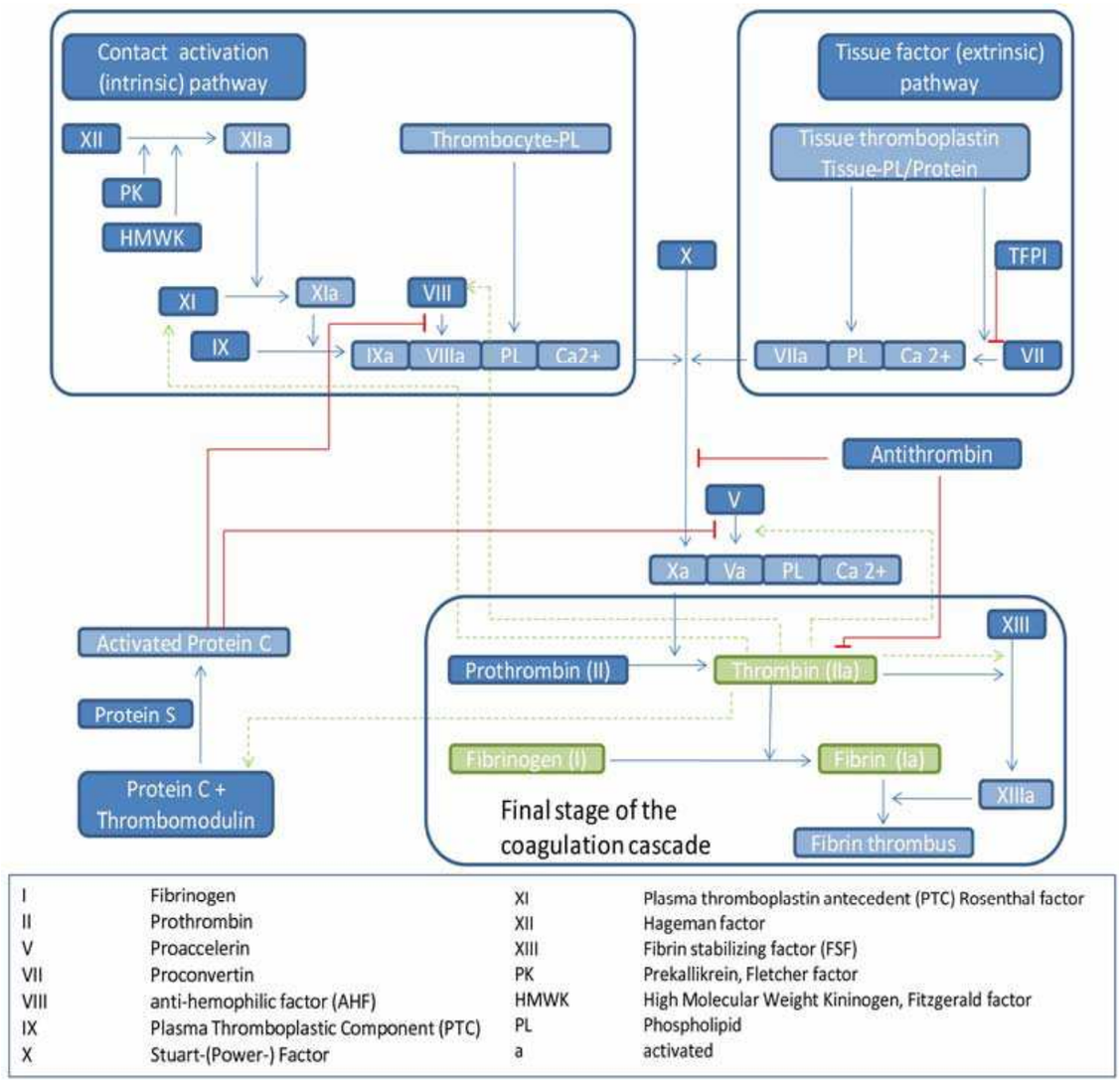

Fig. 1. Coagulation cascade: the conversion of fibrinogen into fibrin is triggered by thrombin and calcium

3.2 Production of autologous fibrin

The classical approach for production of autologous fibrin is the cryoprecipitation method: after the production of platelet-poor plasma (PPP), the plasma is frozen at -80°C and thawed overnight at +4°C. The precipitate formed contains ~60-70% of fibrinogen. After centrifugation, the supernatant is decanted and the precipitate is subsequently washed twice in rinsed water. After the precipitate is dissolved in water, overnight dialysis against calcium-free TRIS buffer solution is necessary to provide optimal conditions for the embedded cells.

The cryoprecipitation method has two major disadvantages: (1) the efficiency of fibrinogen isolation is relatively low with only ~20-25% of the total fibrinogen content removed, and (2) the production process is time-consuming (~2 days). The low isolation efficiency is particularly problematic regarding the use of autologous fibrin gel scaffolds in paediatric

patients, as the volume of sampled blood needs to be kept to an absolute minimum. Therefore, the use of alternative precipitation methods with different chemicals has been evaluated: (1) ethanol (Kjaergard et al., 1992; Weis-Fogh, 1988), (2) ammonium sulphate alone, and (3) in combination with the cryoprecipitation method (Wolf, 1983), (4) albumin plus cryoprecipitation, and (5) polyethylene glycol (PEG) plus cryoprecipitation (Epstein et al., 1986). Heselhaus investigated each of these different precipitation methods with regard to their efficiency and their use in the development of fibrin scaffold materials for cardiovascular applications (Heselhaus, 2011):

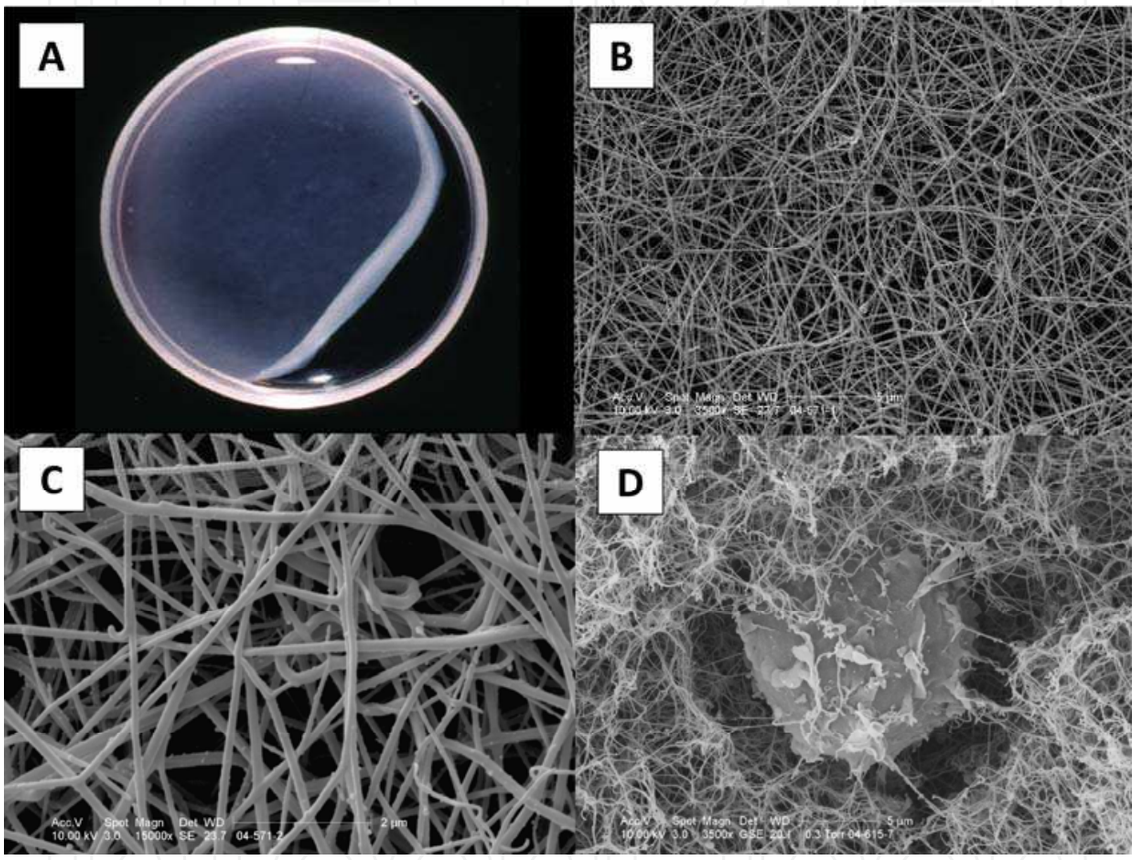

Fig. 2. [A] Fibrin gel after polymerisation in a 6-well plate. [B-D] Scanning electron microscopy (SEM) images demonstrating the nano-fibre network structure of the fibrin, which enables the gentle embedding of cells, with a vascular smooth muscle cell (SMC) shown in [D] immediately after the gelation of the fibrin within a web-like network surrounded by cell culture medium

Figure 3 demonstrates that all of the reported alternative methods are more efficient than the standard cryoprecipitation method. Here, the technique using ethanol as the precipitation reagent is observed as the most efficient method, with an isolation efficiency of ~80%, ~4-times higher than the efficiency of the standard method. The technique applying both albumin and cryoprecipitation indicates a false positive high result due to contamination of the precipitate with albumin (shown by a significant band in SDS gel electrophoreses) (Heselhaus, 2011).

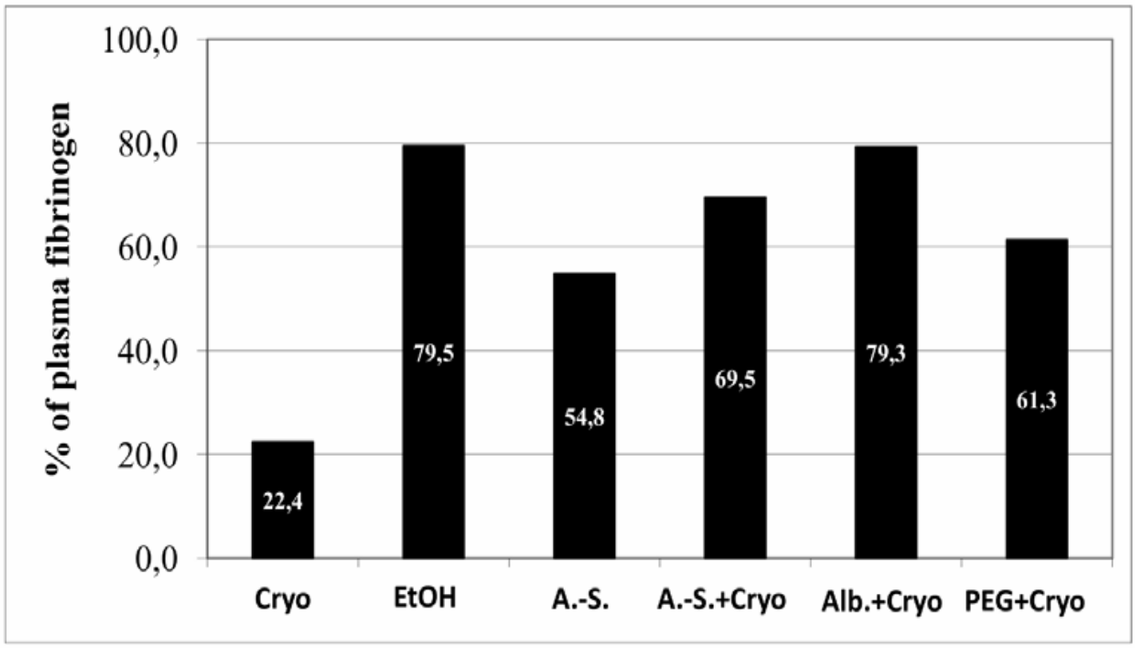

Fig. 3. Comparison of different precipitation methods for the isolation of fibrinogen (Cryo – cryoprecipitation; EtOH – ethanol; A.-S. – ammonium sulphate; Alb.–albumin; PEG – polyethylene glycol)

3.3 Control of scaffold degradation (aprotinin vs. tranexamic acid)

In nature, fibrin production and fibrinolysis are finely balanced. Figure 4 demonstrates the process of fibrinolysis induced by plasmin and the role of different regulatory factors, which stimulate and inhibit the fibrinolysis, respectively.

In the context of *in vitro* cultivation, both of these processes are restricted by the limited concentration of substrates. This offers the opportunity to adapt the degradation of the fibrin scaffold material to the individual need during the tissue maturation process. Until recently, the most commonly used substrate to control the degradation of fibrin gel was aprotinin with a concentration of 130 K.I.U. per mL medium (Ye et al., 2000). Aprotinin is a polypeptide serine protease inhibitor, which stops fibrinolysis by inhibiting kallikrein, plasmin and platelet-activation factors. Aprotinin was used as anti-fibrinolyticum for many years in the clinic, but negative side-effects in the post-operative recovery phase of cardiothoracic patients led to the suspension of aprotinin in the worldwide market.

Therefore, tranexamic acid (trans-4-aminomethely-cyclohexane-1-carboxylic acid; *t*AMCA) was evaluated as alternative drug to control fibrin gel degradation *in vitro* and as far as possible *in vivo*. Tranexamic acid is clinically approved and competitively inhibits the conversion of plasminogen into plasmin via reversibly binding to the lysine-binding site on plasminogen.

Cholewinski et al. (2009) demonstrated that tranexamic acid at a concentration of 160 mg per mL medium has a comparable inhibition effect on fibrinolysis in comparison with aprotinin. Furthermore, no negative side-effects with regard to proliferation, apoptosis, necrosis or the burst strength of the produced fibrin gel scaffolds could be demonstrated (Cholewinski et al., 2009).

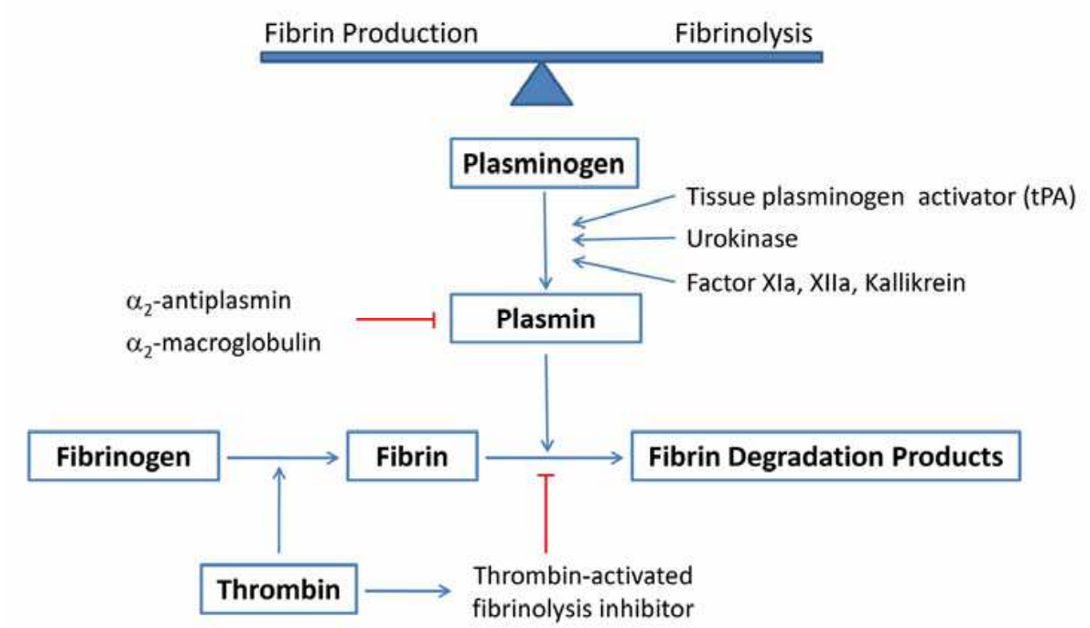

Fig. 4. Balance between fibrin production and fibrinolyis (→positive and --| negative effect)

3.4 Fibrin as autologous growth factor delivery system (PDGF)

The autologous source of fibrin, whole blood, contains a number of important growth factors. One major growth factor is the platelet-derived growth factor (PDGF), a cytokine produced in megacaryocytes and stored in the α-granules (Cianciolo et al., 1999; Ross et al., 1986). PDGF has been described as one particular factor that positively influences proliferation, migration and ECM secretion of fibroblasts and SMCs (Claesson-Welsh, 1996). PDGF is released in conjunction with the platelet release reaction (Witte et al., 1978). Platelet activation induces the release of a variety of low-molecular weight growth factors and more than 60 of these growth factors are directly involved in tissue repair mechanisms such as chemotaxis, cell proliferation, angiogenesis, ECM deposition and remodelling (Borzini & Mazzucco, 2007). Platelets are easily isolated from blood, and can be concentrated in a low volume of plasma known as platelet-rich plasma (PRP). Thus, PRP is a storage vehicle for platelet growth factors. In addition to the abundant mixture of growth factors, PRP also contains proteins known to act as cell adhesion molecules and matrix for bone, connective tissue and epithelial migration, namely fibrin, fibronectin and vitronectin (Marx, 2004).

PRP can be used as a fibrin-based scaffold material by inducing gel polymerisation. The use of PRP as an autologous scaffold material and growth factor delivery vehicle has attracted the attention of researchers in the field of tissue engineering as a new possibility to optimise the composition of the "ideal" autologous scaffold.

To further optimise fibrin gel scaffolds, the use of PRP as a basis for autologous gel scaffolds instead of platelet-poor plasma (PPP) or pure fibrinogen solution has been evaluated. It was postulated that the use of PRP in contrast to PPP or fibrinogen solution as a basis for human autologous fibrin gels leads to an increased release of autologous PDGF-AB, which may have a consequent positive effect on tissue development. Therefore, a protocol for plasma preparation and subsequent plasma gel production was developed and the release kinetics of PDGF-AB from autologous plasma gels were investigated. Wirz and colleagues (2011)

analysed (i) the concentration of PDGF-AB in the PRP vs. PPP during gel preparation, (ii) the influence of plasma gels and particularly PDGF-AB (autologous and recombinant) on growth behaviour and cell proliferation, and (iii) the secretion of ECM by human umbilical cord artery smooth muscle cells (HUASMCs) in both plasma-based and pure fibrin gels. The study demonstrated that the use of PPP leads to almost complete loss of PDGF, whereas the use of PRP in combination with a concentration step (by a factor of two) retains almost serum levels of PDGF. The subsequent steps in the protocol allow the removal of all platelets without losing the PDGF in the concentrate (Wirz et al., 2011).

But is the absence of platelets in the fibrin-based scaffolds good or not? To answer this question, Wirz et al. compared PDGF release from such fibrin-based scaffolds with and without incorporated platelets. Whereas the release of PDGF of platelet-rich and platelet-free fibrin gels were similar during the first 3 days, the platelet-rich gels showed a significantly higher level of PDGF in the supernatant after the first medium exchange, indicating that the platelets with the α-granules continue to work as a 'natural' reservoir of the growth factor PDGF (Wirz et al., 2011).

Nevertheless, against all expectations, the significantly higher level of PDGF-AB in fibrin gels was shown to have neither a significant influence on cell proliferation nor on the ECM synthesis of vascular SMCs. Although the vascular SMCs are the basic cell source for cardiovascular tissue engineering, these cells do not express the receptor for PDGF-A and lose the receptor for PDGF-B at a very early stage of *in vitro* primary cell culture (P0 to P4) (Wirz et al., 2011). Therefore, PDGF-rich fibrin gels may ultimately have a positive effect on fibroblasts and mesenchymal stem cells, for example, but not on primary vascular SMCs.

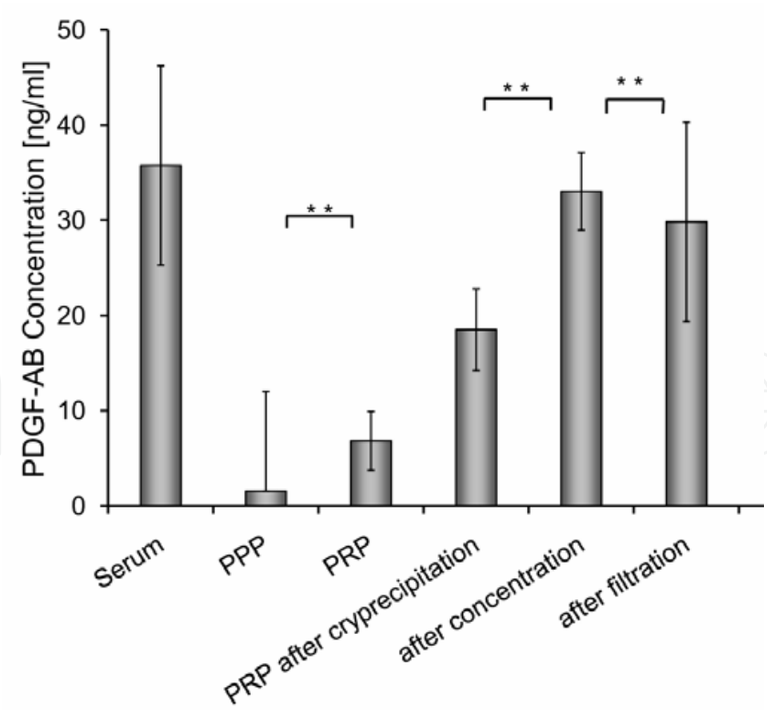

Fig. 5. PDGF-AB release of platelet poor plasma (PPP), platelet rich plasma (PRP) before and after cryoprecipitation, after concentration of the plasma volume (by a factor of two), and after sterile filtration process (0.22µm filter)

4. Examples of fibrin-based cardiovascular tissue engineering

4.1 Heart valve

The principles of fibrin-based cardiovascular tissue engineering can be demonstrated using the example of a completely autologous heart valve prosthesis as shown in Figure 6 (Jockenhoevel et al., 2001b). For paediatric application, the umbilical cord is the optimal cell source. The umbilical cord contains the myofibroblasts/vascular SMCs, which are responsible for the structural and mechanical stability of the neo-tissue. Furthermore, the endothelial cells of the umbilical cord vein are an ideal cell source for the luminal coating of cardiovascular structures. The endothelial cells are mainly responsible for the haemocompatibility of the tissue-engineered graft. The third component of the umbilical cord that is applicable to tissue engineering is the blood, from which the compounds for autologous fibrin-gel synthesis can be extracted.

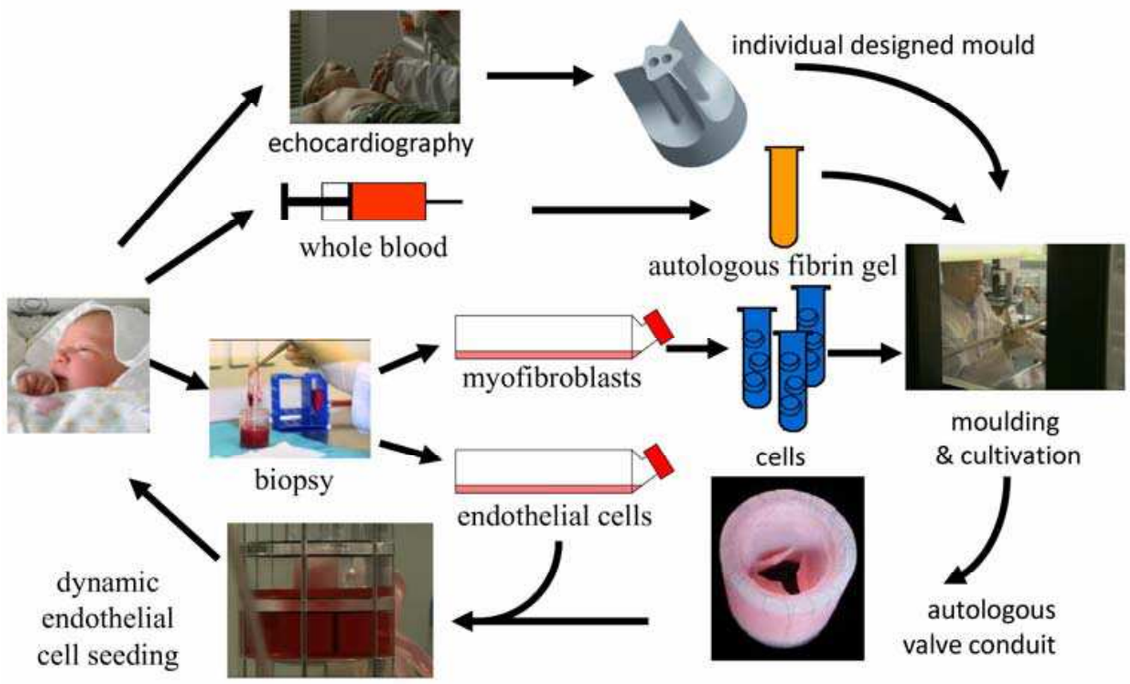

Fig. 6. Principles of fibrin-based tissue engineering using the example of a completely autologous heart valve prosthesis

Based on clinical imaging methods such as echocardiography, it is possible to manufacture a mould that is customised for the individual. This allows the surgical correction of complex congenital heart defects with a shape-optimised implant.

The production of the basic heart valve structure is realised using an injection moulding technique: at the outset, the first of two essential components is produced, and consists of myofibroblasts suspended in a buffer solution that is supplemented with calcium and thrombin. The second component consists of the autologous fibrinogen solution. With a 2-piston-dual syringe system, both components are injected into the customised mould, which consists of a negative and positive stamp. The gelation process begins immediately and homogenously entraps the myofibroblasts in the fibrin gel. After the gel polymerisation is

complete, the newly moulded heart valve conduit is decast from the mould and transferred into a bioreactor system. A suitable nutrition supply and biomechanical stimulation are essential for the maturation of these tissue-engineered structures, as they are too fragile at the outset for direct implantation (Flanagan et al., 2007).
After preconditioning in the bioreactor system, the mechanically stable heart valve prosthesis has been developed. The lining of the luminal surface with autologous endothelial cells is the final production step and is important for complete immunological integrity and physiological haemostasis. At the end of the production chain, a completely autologous heart valve implant is realised, which has the ability to withstand the mechanical properties in the low pressure/pulmonary circulation, as demonstrated previously (Flanagan et al., 2009).

4.2 Vascular graft

Autologous vascular grafts based on a fibrin scaffold have also been developed for applications in a number of settings, including coronary artery and peripheral artery bypass procedures, arteriovenous access grafts for haemodialysis patients, or in the paediatric setting for congenital pulmonary artery reconstruction. Fibrin alone does not possess sufficient mechanical properties for direct implantation into the high pressure setting of small-calibre arteries (< 6 mm); therefore, the fibrin-based grafts are supported by a bioabsorbable, macroporous mesh (poly(L/D)lactide 96/4; PLA) with a controlled degradation profile, which acts as a temporary supporting system upon implantation until the implanted cells have remodelled the fibrin matrix into an autologous tissue (Tschoeke et al., 2009).

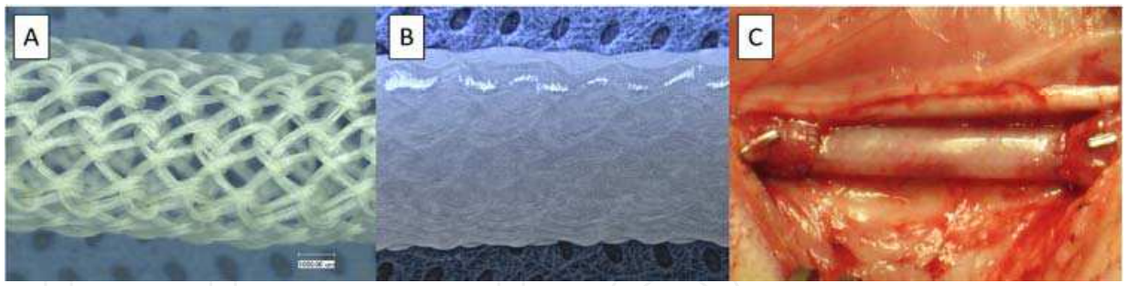

Fig. 7. Bioresorbable, macroporous mesh with a pore size of ~ 1mm before [A] and after [B] the embedding into the fibrin/cell matrix. [C] Fibrin-based vascular graft after implantation in the arterial circulation (ovine carotid model)

The synthesis of the fibrin-based vascular graft is based on similar principles to those used in developing the autologous fibrin-based heart valve (i.e. injection moulding technique). Once the vascular grafts are constructed in a tubular mould, however, the mould acts as the bioreactor system and is connected to a flow support that imparts pulsatile luminal flow to the graft. We have shown for the first time that fibrin-based vascular grafts attain a supraphysiological burst strength sufficient for arterial implantation after just 21 days of mechanical conditioning in such a bioreactor system (Tschoeke et al., 2008). We followed up this report with a preclinical study in a large animal model, which presented data on the first series of fibrin-based grafts to be implanted in the arterial circulation (ovine carotid model) (Koch et al., 2010). In this model, the grafts showed no evidence for thrombus

formation, aneurysm, calcification or infection, and remained patent for at least 6 months *in vivo*. The grafts maintained a functional endothelial lining *in vivo*, and the fibrin-based scaffold was completely replaced by autologous connective tissue elements (e.g. collagen, elastin) after 6 months of implantation (Koch et al., 2010).

4.3 BioStent

During recent years, percutaneous stent angioplasty has become well established in the treatment of peripheral and coronary atherosclerosis. Nevertheless, the patency rates in small-calibre vessels, particularly those of the femoral and femoropopliteal region, are not satisfactory (Cejna et al., 2001; Grenacher et al., 2004; Schillinger et al., 2006).The incessant problem of *instent restenosis* is a result of: (1) the proliferation stimulus resulting from the pressure trauma of stent implantation (>10 bar in non-self-expanding stents), which leads to (2) an ingrowth of myofibroblasts through the gap of the stent structure, and (3) an increased production of ECM proteins; (4) finally, acute thrombotic occlusion can occur in the stented segment of the vessel. Occlusion rates of more than 20% after 6 months could be overcome with a viable stent prosthesis that includes a confluent, functionally active endothelial cell layer from the outset.

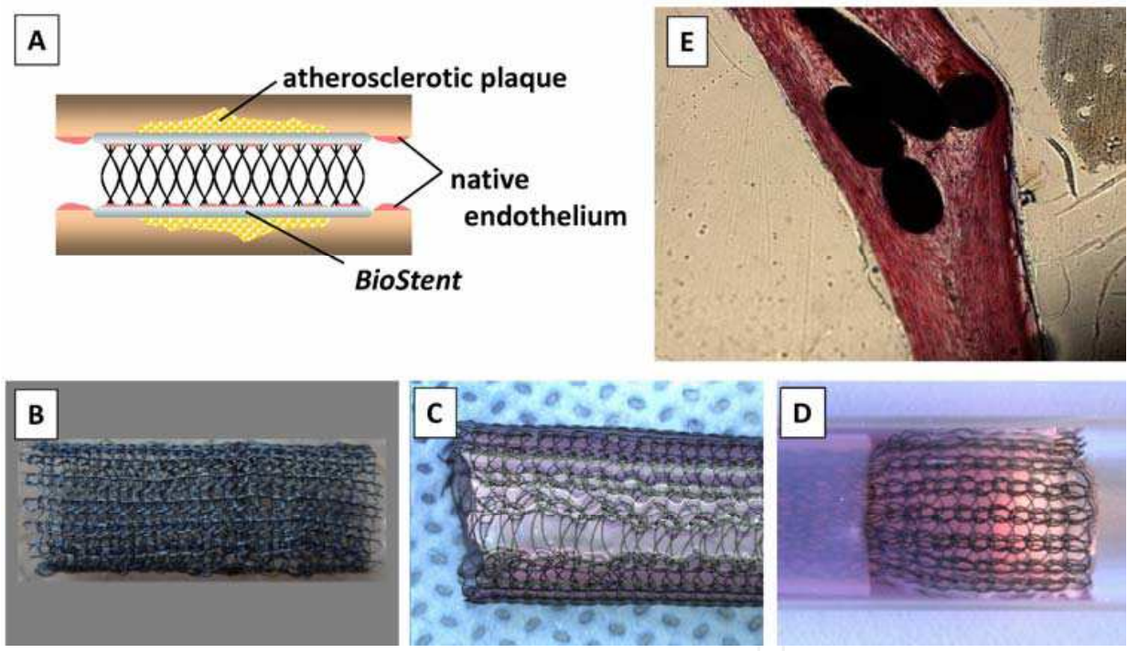

Fig. 8. Concept of the BioStent [A]: the knitted, self-expanding Nitinol-Stent [B] is embedded into a fibrin-cell matrix [C] and can be deployed without tissue destruction [D]. H&E staining demonstrates a complete coating of the Nitinol stent structure with viable tissue [E]

The *BioStent* concept merges the principles of self-expanding stent technology with those of vascular tissue engineering: the moulding process of vascular grafts based on a fibrin gel scaffold allows the complete integration of a self-expanding stent structure within the tissue-engineered vessel. This process leads to a complete exclusion of the atherosclerotic section of the vessel from the blood stream on the one hand, and coating of the neo-lumen with a functional endothelial cell layer on the other hand (Figure 8).

The *BioStent* concept is a platform technology, i.e. by applying different cell types, a number of alternative therapies can be addressed, including (1) endobronchial stenting using respiratory epithelium, (2) oesophageal stenting using mucosa cells, or (3) urological stenting using urothelial cells, etc.

5. Summary

In our opinion, fibrin gel combines a number of important properties of an ideal scaffold for cardiovascular tissue engineering:

- Fibrin gel is a naturally-occurring scaffold that can be isolated as an autologous substrate from blood of the patient in question (Heselhaus, 2011);
- Starting with a cell suspension in fibrinogen solution, fibrin gel scaffolds offer immediate high cell seeding efficiency and homogenous cell distribution by gelation entrapment, with a minimal loss of cells during the seeding procedure; furthermore, there is no time-consuming cell ingrowth from the scaffold surface to the deeper parts of the scaffold (Jockenhoevel et al., 2001a);
- Polymerisation as well as degradation of the fibrin gel is controllable and can be adapted to tissue development through the use of the protease inhibitors, such as aprotinin and tranexamic acid (Cholewinski et al., 2009);
- Local, covalent immobilisation of different growth factors is possible, while PRP gels can be developed to enhance the content and delivery of growth factors (Wirz et al., 2011);
- Production of complex 3-D structures such as heart valve conduits or vascular grafts with complex side branches is possible through the use of an injection moulding technique (Flanagan et al., 2007; Jockenhoevel et al., 2001b);
- Textile-reinforced fibrin-based grafts can be implanted in the arterial circulation and function for at least 6 months *in vivo* (Koch et al., 2010);
- Fibrin-based tissue engineering can be merged with self-expanding stents to create a platform technology for cardiovascular, and other, diseases.

These properties highlight the significant potential for creation of functional, autologous implantable cardiovascular prostheses in future using tissues derived from the patient.

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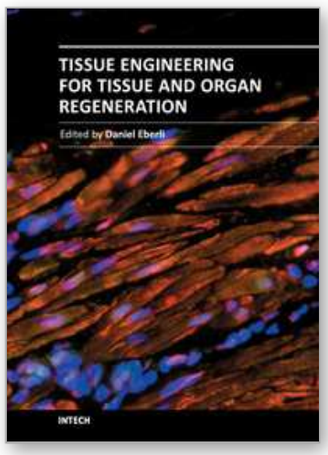

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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