

Tissue-Engineered Heart Valves

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Received September 1, 2009

Accepted October 30, 2009

Summary

Currently-used mechanical and biological heart valve prostheses have several disadvantages. Mechanical prostheses, based on carbon, metallic and polymeric components, require permanent anticoagulation treatment, and their usage often leads to adverse reactions, e.g. thromboembolic complications and endocarditis. Xenogenous and allogeneous biological prostheses are associated with immune reaction, thrombosis and degeneration, and thus they have a high rate of reoperation. Biological prostheses of autologous origin, such as pulmonary autografts, often burden the patient with a complicated surgery and the risk of reoperation. Therefore, efforts are being made to prepare bioartificial heart valves with an autologous biological component by methods of tissue engineering. They should be biocompatible, durable, endowed with appropriate mechanical properties and able to grow with a child. For this purpose, scaffolds composed of synthetic materials, such as poly(lactic acid), poly(caprolactone), poly(4-hydroxybutyrate), hydrogels or natural polymers, e.g. collagen, elastin, fibrin or hyaluronic acid, have been seeded with autologous differentiated, progenitor or stem cells. Promising results have been obtained with nanostructured scaffolds, and also with cultivation in special dynamic bioreactors prior to implantation of the bioartificial grafts into an animal organism.

Key words

Tissue engineering • Bioartificial heart valve • Nanofibrous scaffolds • Valve interstitial cells • Mesenchymal stem cells • Pulse flow bioreactor

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Introduction

Cardiovascular diseases represent a major worldwide health care issue. According to the World Health Organization, cardiovascular diseases killed 17.5 million people in 2005, which represents 30 % of mortalities. A considerable amount of these diseases is represented by heart valve failures. The number of patients requiring heart valve replacement is estimated to triple from approximately 290,000 in 2003 to over 850,000 by 2050 (Yacoub and Takkenberg 2005).

Heart valves are the aortic, pulmonary, mitral and tricuspid. In the surgical treatment of the aortic heart valve diseases, the currently preferred approach is heart valve replacement. In the case of mitral and tricuspid valves, conservative surgery of the patient's own native valve (i.e. valvuloplasty) is preferred in adulthood. Tricuspid and pulmonary valve operations are rare in adulthood, being performed in cases of significant regurgitation or endocarditis. On the other hand, clinical cases have been reported in which all four types of heart valves were replaced with prostheses, even simultaneously in one patient (Seeburger *et al.* 2009).

Currently used heart valve prostheses can be divided into two basic groups, namely mechanical and biological prostheses. Mechanical prostheses are mainly made of pyrolytic carbon combined with metallic and polymeric components. Although durable, these devices carry risks associated either with the valve itself, e.g.

thrombus formation, thromboembolic complications, endocarditis that occurs predominantly on the surface of foreign materials, or risks connected with permanent and long-lasting anticoagulation therapy, e.g. hemorrhage-bleeding complications occurring at 1 % patients per year (Aschermann *et al.* 2004). On the other hand, long-term survival of patients (for more than 20 years) with mechanical heart valves without anticoagulation therapy and without complications has been also described (Yildiz *et al.* 2006).

Xenogenous and allogeneous biological heart valve prostheses evoke an immune reaction of the patient's organism, thrombosis and undergo degeneration. The harvesting and implantation of autologous prostheses often requires complicated surgery with an increased risk of mortality. Therefore, an alternative way to improve the quality of heart valve prostheses is to construct bioartificial valves by tissue engineering methods.

The ideal valve prosthesis should be easy implantable, should develop a physiological hemodynamic performance without structural deterioration, should be non-thrombogenic, non-immunogenic and silent in its performance (Concha *et al.* 2004). Appropriate biomechanical properties of the tissue-engineered cardiac valve are extremely important, thus they are considered even to outweigh their biological properties. Otherwise, high pressure and stresses would result in valve failure, with potentially life-threatening consequences (Knight *et al.* 2008).

Mechanical valve prostheses

There are three major types of mechanical valves, namely caged-ball valves, tilting-disk valves and bileaflet valves, with many modifications on these designs.

The first artificial heart valve, implanted in 1952, was the caged ball, which utilized a metal cage to house a silicone elastomer ball (Hufnagel *et al.* 1958). During contraction of the heart, when the blood pressure in the heart chamber exceeded the value in the outside of the chamber, the ball was pushed against the cage and allowed the blood to flow. On completion of the heart's contraction, the pressure inside the chamber dropped, so the ball moved back against the base of the valve, forming a seal. A similar valve, commonly referred as the Starr-Edwards Silastic Ball Valve, was invented by Edwards and Starr in 1960 (Starr *et al.* 1985). It consisted of a silicone ball enclosed in a cage formed by metallic

wires originating from the valve housing. Caged ball valves have a high tendency to form blood clots, so the patient must have a high degree of anti-coagulation. In addition, the movement of the ball damages the blood cells. Therefore, although caged-ball valves achieved the first long-term success in prosthetic heart valve technology, the Edwards Lifesciences Company discontinued their production in 2007.

A more advanced type of artificial heart valves is represented by tilting-disk valves. The first clinically available tilting-disk valve was the Björk-Shiley valve, introduced in 1969 (Björk 1977). Tilting-disk valves had a single circular occluder, controlled by a metal strut. These valves were made of a metal ring covered by a fabric of expanded polytetrafluoroethylene (ePTFE). In this fabric, the suture threads were stitched in order to hold the valve in place. By means of two metal supports, the metal ring holds a disc which opens and closes as the heart pumps blood through the valve. The disc is usually made of a very hard carbon material (pyrolytic carbon), in order to allow the valve to function for years without wearing out. In some models of mechanical valves, the disc is divided into two parts, which open and close like a door.

The newest design of mechanical valves, introduced in 1979 by St. Jude Medical Company, is the bileaflet valve. It consists of two semicircular leaflets that rotate about struts attached to the valve housing. The entire valves, i.e. the two leaflets, housing and struts, are made of pyrolytic carbon and avoid the stainless steel (i.e. cobalt-chromium-molybdenum-nickel-alloy) that was used in earlier valve types. Only titanium is still a component of the housing body in some designs (Zilla *et al.* 2008). Although bileaflet valves are vulnerable to backflow, and thus they cannot be considered as ideal, they provided much more natural blood flow than the caged-ball or tilting-disk implants. These valves have a greater effective opening area, are better tolerated by the body, are the least thrombogenic of the artificial valves, and thus they require only mild anticoagulation therapy (Emery *et al.* 1979, Emery and Nicoloff 1979; for review see http://en.wikipedia.org/wiki/Heart_valve_prosthesis#Types_of_mechanical_heart_valves).

Biological valve prostheses

The adverse reactions that accompany implanted mechanical prostheses stimulated the development of biological prostheses. Stented glutaraldehyde-fixed

valves, usually of animal origin, were developed in the 1960s; however, biological prostheses became more reliable and commercially available in the 1970s (Senthilnathan *et al.* 1999). At present, the following types of biological prostheses are available: porcine xenograft valves and bovine pericardial valves (i.e. implants derived from other species than human), allograft valves (also called homografts, and derived from human donors other than the patient) and autografts (i.e. derived from autologous patient's tissue) (Vesely 2005).

Both xenografts and allografts can evoke fibrotic tissue production, leading to valve dysfunction with incidence of 2-4 % patients/year, and the incidence of obstructive thrombosis is in the range of 0.2-1.8 % per year (Popelová *et al.* 2007). Re-surgery of these types of biological valve prostheses 15 years after their implantation into the body is indicated at 65 % of patients under age of 60. Both increasing age of the allograft donor and younger recipient age has been associated with allograft failure and a need for allograft valve reoperation due to accelerated valve degeneration (Yap and Yii 2004). Five-year survival of the allograft in right ventricular outflow tract reconstruction was 25 % for recipients less than 1 year of age, 61 % for those between 1 and 10 years, and 81 % for those older than 10 years (Forbess *et al.* 2001). Age-dependent degeneration can be caused by many reasons, such as immunological response or progressive overgrowth of the implanted allograft by patient's fibrotic tissue. In addition, young patients exhibit increased hemodynamic demands on the allograft, as they are more active and have higher metabolic needs. In addition, valve procurement, duration of the allograft ischemia, time between valve harvest and valve implantation, and also the surgical method for implantation influence the long-term durability of the implant allograft (Yap and Yii 2004). Dehiscence of the prosthesis is also a serious complication that affects both bioprostheses and mechanical prostheses.

As for pericardial and other valves prepared from non-valvular tissues, Boenhoeffer *et al.* (2000) reported the first successful percutaneous valve replacement in a human patient, created from bovine jugular vein sutured into a platinum stent and placed in the pulmonary valve position. Two years later, another successful human percutaneous implantation of an aortic valve was performed. This valve was made of bovine pericardium sutured on a 23 mm balloon-expandable stainless steel stent, developed by Edwards Lifescience PVT (Irvine, CA) (Cribier *et al.* 2002).

Pulmonary autografts have been shown to be resistant to long-term degeneration and were confirmed to be able to grow in children (Concha *et al.* 2004). They can be used to replace the aortic root and all infected tissue in aortic valve endocarditis. Another indication for using an autograft is older age of the patients, where the graft can last longer than the patient (Senthilnathan *et al.* 1999). On the other hand, a pulmonary autograft implanted into the aortal position, i.e. the Ross operation, is a complex and demanding surgery with slightly increased mortality, where 20 % of patients need re-operation 10 years after surgery (Popelová *et al.* 2007). This type of surgery involves replacing the aortic valve by the autologous pulmonary valve, and the pulmonary valve is then replaced by a pulmonary homograft.

A novel approach to the development of autologous heart valves is offered by methods of tissue engineering. It involves constructing valves using autologous cells and extracellular matrix (ECM) molecules, or a bioactive artificial material simulating the natural ECM. This procedure allows the histological structure of the natural valve to be fully respected. Deep knowledge of this structure, and also of the characteristic features of the cell types present in heart valves, is necessary for successful heart valve tissue engineering.

Heart valve structure

Microscopically, the heart valve (Fig. 1) is composed of three layers (Fig. 2), namely ventricularis, closest to the inflow surface and rich in radially aligned elastin fibers, fibrosa, closest to the outflow layer, containing predominantly circumferentially aligned, macroscopically crimped, densely packed collagen, and spongiosa, located in the central part and rich in glycosaminoglycans (GAGs) and loosely packed collagen (Mendelson and Schoen 2006, Knight *et al.* 2008). Elastin restores the contracted configuration of the cusp during systole. Collagen fibers are inelastic and incapable of supporting large strains. Crimped collagen cords radiate primarily from the commissures. They confer strength and are responsible for withstanding diastolic stress; they maintain coaptation during diastole, when the cusp has the maximum area. During valve closure, the crimping expands in the radial direction, and increases in size with minimal stress. GAGs absorb shocks during the valve cycle and accommodate the shear of the cuspal layers. This structure enables the leaflets to be extremely soft and pliable when unloaded, and

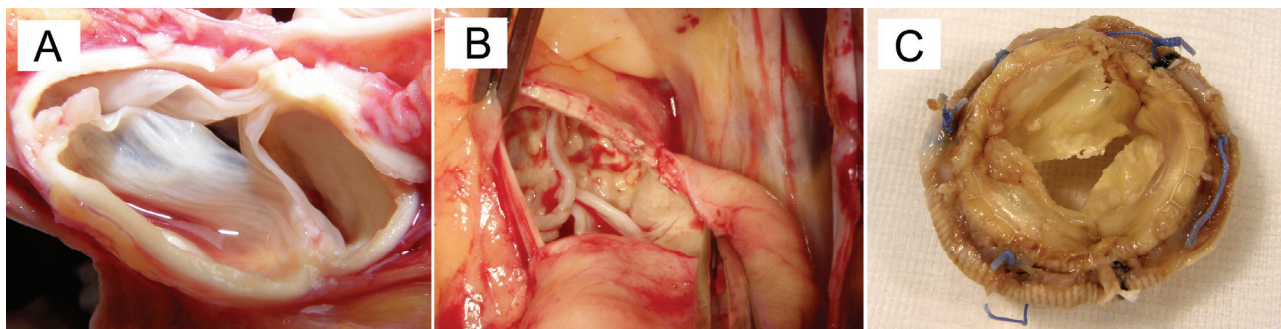


Fig. 1. A three-cusp aortic heart valve without pathological changes from an explanted heart with dilated cardiomyopathy (**A**), a perioperative picture of a three-cusp aortic valve with degenerative changes (**B**), and a biological prosthesis changed by endocarditis with a cusp perforation and vegetation (**C**).

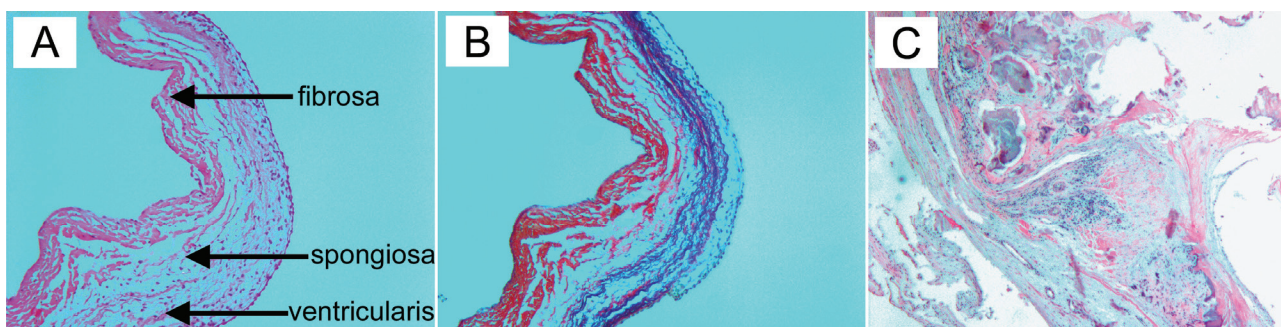


Fig. 2. Hematoxylin-eosin staining (**A**), and Sirius Red (nuclei and elastic fibres - black, collagen fibres - red) staining (**B**) of a three-cusp aortic heart valve without degenerative changes, and hematoxylin-eosin staining of an aortic valve with degenerative changes, calcifications, and inflammatory infiltration (**C**). Microscope Leica 020-519.011DMLB 100S, blue filter, obj $\times 100$ (**A**, **B**), and $\times 50$ (**C**).

practically inextensible when back pressure is applied. In addition, they are anisotropic, having much higher tissue compliance in the radial direction than in the circumferential direction (Mendelson and Schoen 2006, Knight *et al.* 2008).

Cell types present in heart valves

Several cell types are present in heart valves, i.e. endothelial cells (EC), valve interstitial cells (VIC), fibroblasts, smooth muscle cells (SMC) and nerve cells.

EC create a non-thrombogenic surface layer and control immune and inflammatory reactions. Valve EC differ from vascular EC particularly in their reaction to mechanical stress, as they align perpendicularly to the blood flow, whereas EC from the aorta align in parallel to the flow (Butcher *et al.* 2004) (Fig. 2). In addition, EC are an important modulator of the VIC function (Schoen 2008).

VIC, the most numerous cell type in the valve, display morphological and functional characteristics of fibroblasts, smooth muscle cells and myofibroblasts. Five distinct phenotypes of VIC have been recognized, i.e. embryonic progenitor endothelial/mesenchymal VIC,

quiescent VIC, activated VIC, adult progenitor VIC and osteoblastic VIC (Schoen 2008). VICs synthesize ECM proteins, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) that mediate matrix remodeling (Taylor *et al.* 2003). VIC have an activated myofibroblast-like phenotype during valve development and maturation, various diseases (e.g. myxomatous mitral valve), adaptation of an early pulmonary to aortic autograft (i.e. Ross operation) or during VIC remodeling (tissue-engineered valves *in vitro* and *in vivo*) (Figs 2 and 3) (Aikawa *et al.* 2006, Schurch *et al.* 1998). In their quiescent state, VIC have a fibroblast phenotype, characterized by the presence of vimentin and very low levels of α -smooth muscle actin, MMP-13 and non-muscle myosin heavy chain (SMemb). Taken together, VICs maintain the stability and integrity of the valves and are involved in synthesis and remodeling of the valve ECM (Fayet *et al.* 2007).

SMC in the valves have been observed in their less and more mature forms, i.e. in synthetic and contractile phenotypes, respectively. Terminally differentiated contractile SMC, characterized by the presence and mutual interaction of smooth muscle α -actin and smoothelin, formed small bundles in the

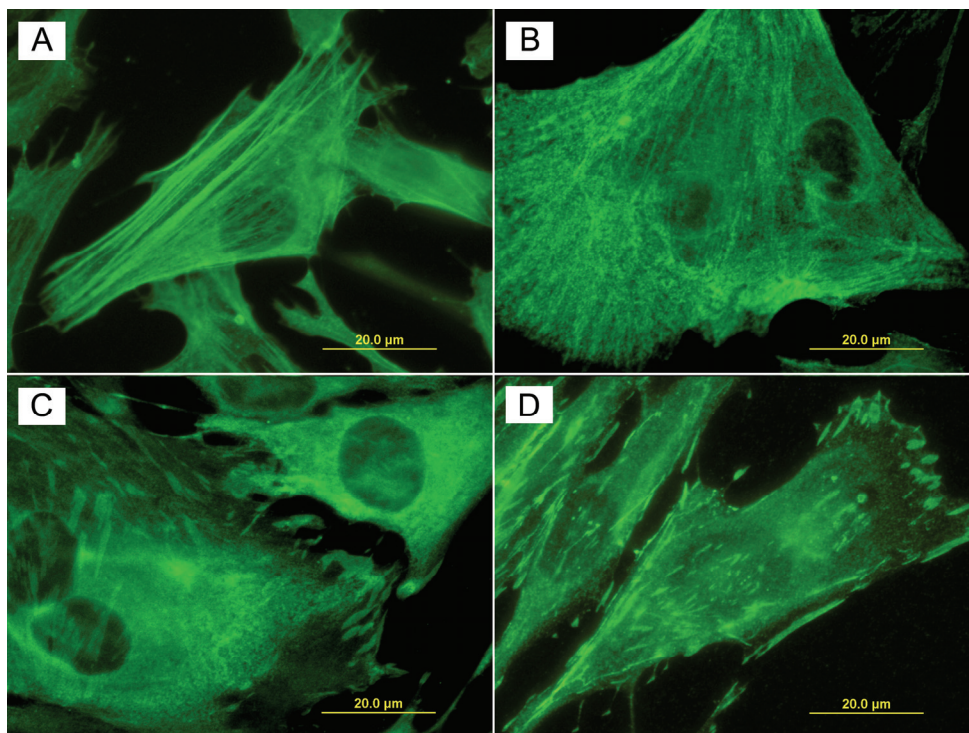


Fig. 3. Immunofluorescence staining of alpha-actin (A), myosin (B), vinculin (C), and talin (D) in valve interstitial cells from a pig aortic heart valve on day 3 after seeding. Olympus XI 51 microscope, DP 70 digital camera, obj. $\times 100$, oil immersion, scale = 20 μm .

ventricularis, where they were oriented circumferentially. In addition, these cells were also distributed as individual cells throughout all valvular layers (Cimini *et al.* 2003).

The role of neurons in the heart valves is still not entirely clear. The valve leaflets contract in response to a variety of neurotransmitters, e.g. 5-hydroxytryptamin (Chester *et al.* 2000). Nerve branches are often associated with small vessels and are found predominantly in the basal third of the valve cusps (Weind *et al.* 2000).

Tissue-engineered heart valves

Currently, the following approaches are used for developing tissue-engineered valves (Vesely 2005):

Decellularized xenogenic tissues for cell seeding or direct implantation

It has been assumed that the antigenicity of xenogenic tissues, such as bovine or porcine pericardium, porcine small intestinal submucosa, and porcine or ovine heart valves is caused by cell debris (Vesely and Boughner 1989, White *et al.* 2005). In addition, porcine aortic valves need to be cross-linked by glutaraldehyde before implantation, which can cause cytotoxic and other adverse reactions. Glutaraldehyde-fixed pericardium was resistant to collagenase degradation and cell infiltration, but significantly prone to increased calcification (Tedder *et al.* 2009). Homografts (i.e. allografts), which do not require glutaraldehyde cross-linking, can last 15-20 years

and have better mechanical properties than acellular glutaraldehyde-fixed xenografts. This favorable behavior likely results from the presence of interconnected sheets of collagen, layers and tubes of elastin, highly non-linear mechanics, anisotropy and viscoelasticity of native valve tissue (Vesely and Boughner 1989).

The decellularization technique has a different impact on tissue preservation and valve efficiency. Aggressive decellularization by enzymes (DNAase, RNAse), detergents, spit freezing and radiation, used in SynerGraft technology, resulted in catastrophic failure of decellularized xenogenic heart valves (Simon *et al.* 2003). On the other hand, deoxycholic acid completely decellularized heart valves, while structural proteins were retained and appeared to be intact (Booth *et al.* 2002). Decellularization of carotid arteries by distilled water, 0.025 % trypsin, 1 % Triton X-100 and ammonium hydroxide caused various structural changes of their ECM, such as less compact and more extended adventitial collagen, and increased spacing between fibrils. These alterations led to changes in mechanical properties, which involved a higher tensile modulus, lower extensibility, and decreased residual stress of the decellularized arteries, suggesting significant fiber network disruption (Williams *et al.* 2009).

Implantation of decellularized allograft and xenograft valves induces tissue regeneration *in vivo* with efficient repopulation of the matrix by interstitial cells, but graft endothelialization is not sufficient. Complete re-

population of detergent-decellularized ovine pulmonary valves with endothelial cells was achieved *in vitro* in a dynamic bioreactor (Lichtenberg *et al.* 2006). In the bioreactor, EC were adapted to a relatively high flow of the medium by a progressive increase of flow rate from 0.1 l/min to 2.0 l/min. If differentiated EC for seeding on the valve are less available, mesenchymal stem cells (MSC) can relatively easily be isolated from the bone marrow (e.g. bioptically from the iliac crest) and phenotypically converted into EC *in vitro* (Gojo *et al.* 2003, Marion and Mao 2006). MSC also provide a source for fibroblasts/myofibroblasts and smooth muscle cells. When MSC were seeded onto a decellularized porcine pulmonary valve pre-coated with fetal bovine serum and fibronectin, they colonized the scaffold, and endothelium lining, and the presence of fibroblasts and myofibroblasts was proved in the valve graft (Iop *et al.* 2009).

Another approach in engineering heart valves is the use of modified decellularized bovine pericardium (ABP) as a scaffold for cell colonization. ABP has advantages of good mechanical properties, being composed of native ECMs including collagen, elastin and various GAGs (Lai *et al.* 2006). The properties of this material were further improved by conjugation with RGD-containing polypeptides, which act as ligands for cell adhesion receptors. As a result, 10 days after seeding, human MSC attached much better, proliferated faster and ingrew deeper into the modified ABP scaffolds (Dong *et al.* 2009).

Constructs containing polymerized extracellular matrix and entrapped cells

Similarly as in the approach reported above, this technology also utilizes purely biological materials for valve tissue engineering. However, the cell carriers are made of more defined materials, represented by isolated ECM molecules, such as collagen, elastin, hyaluronic acid, fibronectin and fibrin.

Type I collagen is an important component of the vessel wall, and a major matrix protein of the bone and other connective tissues. Collagen as a biomaterial has a number of useful properties. It is biodegradable, biocompatible, non-antigenic, haemostatic, easily modifiable, acts synergetically with other bioactive components, is biologically plastic due to its high tensile strength and minimal expressibility, and is compatible with synthetic polymers (Lee *et al.* 2001). Excellent cell adhesion on collagen is mediated by cell membrane integrins, such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Tulla *et al.* 2001). Pure

collagen, due to its fast degradation, often needs cross-linking or needs to be used in a blend with other polymers. Heart valve-shaped collagen scaffolds, prepared from decellularized porcine pericardium treated with penta-galloyl glucose, allowed time-dependent cell infiltration and collagen synthesis *in vivo*, while no calcium deposits were found (Tedder *et al.* 2008). However, fully cross-linked collagen reduces the cell infiltration and remodeling capacity, and thus might prevent tissue regeneration. On the other hand, non-cross-linked scaffolds may degrade too quickly and their functionality is threatened. A tubular vascular hybrid collagenous scaffold colonized with canine jugular smooth muscle cells resisted rupture pressure up to 110 mm Hg (Baguneid *et al.* 2006). Collagen scaffolds composed of 1-5 % collagen prepared by rapid prototyping allowed adhesion, proliferation of VIC *in vitro* (Taylor *et al.* 2006). This technique is advantageously used to prepare scaffolds of predetermined size and three-dimensional shape.

Elastin has been proposed for engineering of vascular grafts, heart valves, skin substitutes, or elastic cartilage (Daamen *et al.* 2007). In heart valves, there are several elastin architectures, i.e. sheet-like orientation in the ventricularis, sponge-like orientation in spongiosa and tubular to circumferential in the fibrosa (Scott and Vesely 1996, Daamen *et al.* 2007). Elastin peptides have been shown to reduce proliferation and migration of vascular smooth muscle cells *in vitro* and *in vivo* (Ito *et al.* 1997, Karnik *et al.* 2003). One of the problems connected with elastin as a biomaterial is its tendency to calcification, which occurs in cardiovascular prosthetic implants, e.g. bioprosthetic heart valves and aortic homografts. Elastin and also collagen may serve as nucleation sites for mineralization, independent of cellular components of the prostheses. Calcification can be reduced by treatment with aluminium chloride, ethanol/EDTA, the presence of glycosaminoglycans, or basic fibroblast growth factor (for review see Daamen *et al.* 2007). Elastin can be used in various forms, including an insoluble form present in autografts, allografts, xenografts, or in a soluble form. Scaffolds prepared from insoluble collagen and solubilized elastin induced angiogenesis, and increased elastic fiber synthesis without inducing calcification (Daamen *et al.* 2007). In other experiments, elastic lamina and collagen matrix scaffolds, prepared from rat aorta, were implanted into the host rat aorta. Reduced unwanted reactions, namely leukocyte adhesion, bromodeoxyuridine (BrdU) incorporation (i.e. a marker

of DNA synthesis) and neointimal formation, were found on matrices composed of elastic lamina compared to collagen matrix (Liu *et al.* 2004).

Hyaluronic acid (HA) is a major component of cardiac jelly during heart morphogenesis (Masters *et al.* 2005). Hyaluronic acid and chondroitin sulphate compose about 90 % of the total GAGs in human heart valves (Ramamurthi and Vesely 2005). It is non-immunogenic, non-thrombogenic, viscoelastic, and can be cross-linked to form a hydrogel (Masters *et al.* 2005). During wound healing, hyaluronic acid supports cell invasion into the provisional matrix (Clark 2001). Photopolymerization of HA did not reduce the viability of previously entrapped VIC (Masters *et al.* 2005). Degradation products of methacrylated HA have been observed to induce proliferation of EC, VIC, and also angiogenesis and ECM production, which can in turn improve the mechanical and biological properties of the gel. In addition, low molecular weight HA stimulated elastin production (Masters *et al.* 2005). Similarly, a composite material made from hyaluronan and elastin supported synthesis of elastin by neonatal smooth muscle cells compared to standard tissue culture polystyrene. On the composite gels, the cells proliferated and produced a higher ratio of insoluble elastin (Ramamurthi and Vesely 2005). Interaction between VICs and valvular endothelial cells, and their relationship to the formation of intercellular matrix have also been studied. Increased interstitial cell matrix synthesis and more confluent endothelial cell monolayers were observed after supplementation of collagen hydrogel with chondroitin sulfate (Flanagan *et al.* 2006). It seems that the interaction of VICs and valvular endothelial cells is essential for valve remodeling (Ruel and Lachance 2009).

Human fibronectin is a dimer composed of two nonidentical polypeptide chains which are disulfide-bonded at their carboxyl termini (Faralli *et al.* 2009). The fibronectin structure, based on homologous repeating units termed I, II and III, gives a strong elasticity to the fibronectin fibrils. Fibronectin can stretch about four times its original length. During its stretching, novel cryptic sites are exposed, e.g. those located in the III1 repeat, which further promotes the fibronectin fibril assembly and stimulates cell growth and contractility (Hocking and Kowalski 2002). Fibronectin supports and guides cell adhesion, migration and alignment *via* integrin adhesion receptors on cells, namely integrins $\alpha_3\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_4\beta_1$, $\alpha_4\beta_7$ (Zhou *et al.* 1999). The cell adhesion mediated by $\alpha_5\beta_1$ integrins is optimal

on the native fibronectin or its pre-stretched states, where the distance between the RGD-loop on the FN-III₁₀ module and a fibronectin synergy site, which is located on the Fn-III₉ module, is 32 Å. Stretching increased the RGD-synergy site distance to 55 Å, which impaired cell spreading (Krammer *et al.* 2002). Integrin $\alpha_v\beta_3$ does not recognize the synergy site and thus it is less sensitive; this means that $\alpha_v\beta_3$ -mediated cell adhesion is affected only if relatively high stretches are applied. Fibronectin was also observed to promote collagen contraction by fibroblasts. It was accompanied by a pronounced abundance of both phosphorylated and total paxillin, i.e. an integrin-associated protein present in focal adhesions. Other ECM proteins or glycosaminoglycans did not increase the collagen contraction (Liu *et al.* 2006). Fibronectin serves as a depot for soluble factors, and binds other components of ECM, e.g. thrombospondin-1, which modulate cell cellular responses to ECM (Midwood *et al.* 2004). Other molecules mediating biological activities of fibronectin are syndecans and heparin sulphate proteoglycans, which interact with the heparin-binding domain of fibronectin via the heparin sulphate chain (Faralli *et al.* 2009). Fibronectin can be used as an effective coating of artificial materials in order to support the adhesion and spreading of cells (Wittmer *et al.* 2007). Fibronectin coating of polytetrafluoroethylene (PTFE) prostheses enhanced their endothelialization regardless of whether they were pre-seeded with EC *in vitro* prior to their implantation into dogs (Seeger and Klingman 1988). Co-adsorbed albumin improved poor cell spreading on fibronectin pre-adsorbed on hydrophobic surfaces (Horbett 1993). A possible explanation is that the co-adsorption of albumin and fibronectin modified the geometrical conformation of fibronectin to be accessible for the cell adhesion receptors. This phenomenon, referred to as “albumin rescuing”, caused a higher availability of fibronectin-cell binding domains on the surfaces of artificial materials, such as tissue culture polystyrene, microbiological polystyrene, and Teflon AF[®], if low concentrations of albumin were used (Koenig *et al.* 2003).

Fibrin is a provisional matrix protein participating in wound healing. Thus, it represents a biocompatible and biodegradable material interacting with tissue without inducing inflammation, foreign body reaction, tissue necrosis or extensive fibrosis (Bense and Woodhouse 1999; Jackson 2001). Fibrin can be advantageously used as an autologous material derived from the patient's blood. Encapsulation of the cells inside

the fibrin gel has been applied for molding a valve conduit containing myofibroblast cells (Ye *et al.* 2000, Jockenhoevel *et al.* 2001, Mol *et al.* 2005, Grassl *et al.* 2002). *In vitro* experiments confirmed that fibrin promotes matrix synthesis through the release of platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF- β) (Jockenhoevel *et al.* 2001). As demonstrated by our earlier experiments, fibrin layers of various thicknesses can be prepared, ranging from ultrathin two-dimensional layers to a bulk three-dimensional fibrin (Filová *et al.* 2009a, Riedel *et al.* 2009). These layers promoted the adhesion, spreading and phenotypic maturation of endothelial cells, thus they could be used for endothelialization of vascular and valve replacements. Disadvantages of fibrin include its poor mechanical properties, relatively quick degradation, and shrinkage due to structural changes and contraction of the newly synthesized collagen bundles (Jockenhoevel *et al.* 2001). In addition, increased intimal thickening has been reported in ePTFE grafts coated with fibrin and implanted into pigs (Walpoth *et al.* 2007). Mechanical properties of fibrin gels can be tailored by introducing it into composite materials. Three-dimensional matrices prepared from type I collagen, fibrin or a mixture of collagen and fibrin in a 1:1 ratio with embedded rat aortic smooth muscle cells exhibited a different stress-strain profile (Cummings *et al.* 2004). Pure collagen had the highest linear modulus and pure fibrin the lowest, while collagen-fibrin mixtures underwent the highest compaction. The gel compaction was further amplified, if cyclic mechanical strain was applied to the construct. At the same time, the proliferation activity of smooth muscle cells (which is generally considered as undesirable in vascular constructs) was reduced (Cummings *et al.* 2004). Dynamic conditioning of molded fibrin-based heart valve seeded with a mixture of smooth muscle cells and fibroblasts from ovine carotid artery in a bioreactor increased the cell attachment, expression of α -smooth muscle actin (i.e. a marker of phenotypic maturation towards the contractile phenotype), enhanced deposition of types I and III collagens, fibronectin, laminin and chondroitin sulphate (Flanagan *et al.* 2007). ECM deposition can further improve the remodeling, mechanical properties and function of a graft. Fibrin is degraded by proteolytic action of plasmin and MMPs, and is gradually replaced by collagen and other ECM components produced by cells (Bense and Woodhouse 1999, Clark 2001). Fibrin degradation and remodeling can be slowed down by adding aprotinin, an inhibitor of

plasmin, or ϵ -aminocaproic acid, an inhibitor of fibrinolysis, into the cultivation medium (Grassl *et al.* 2003), or by chemical or mechanical fixation of the gel (Jockenhoevel *et al.* 2001).

Degradable synthetic scaffolds pre-seeded with cells

Biological molecules used in the previous approach are often of xenogeneic origin (except fibrin, which can be derived in considerable quantities from the patient's own blood), and this can cause adverse reactions in the patient's organism. Therefore, in recent tissue engineering, great attention is being paid to synthetic cell carriers. Synthetic scaffolds used in heart valve tissue engineering have mostly been made of bioresorbable polymers, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(caprolactone) (PCL), polyhydroxyalkanoates, and copolymers or blends of these polymers, e.g. a blend of PGA or PLA with poly(4-hydroxybutyrate) (P4HB) (Hoerstrup *et al.* 2002, Sodian *et al.* 2000). For example, very good results were obtained with PGA/PLLA nonwoven meshes seeded with mesenchymal stem cells (MSC). After 4-week cultivation in roller bottles, the cell-material constructs were implanted into sheep for 24 weeks. Although 2 sheep died, in the other animals MSC differentiated into cells that were positively stained for α -smooth muscle actin, von Willebrand factor (i.e. a marker of endothelial cells) and vimentin (a marker of fibroblasts), and created a histological structure similar to a native valve (Sutherland *et al.* 2005).

In comparison with nature-derived materials, synthetic polymers are of more defined and controllable properties, and can be more easily reproduced. However, incompletely degraded polymers can evoke inflammation; the space formerly occupied by polymer and its interstices are replaced by fibrotic tissue, and the perfusion of deep parts of the scaffold is limited (Mendelson and Schoen 2006). Other promising polymers for the construction of bioartificial valves are soft and elastic hydrogels. Their mechanical properties correspond better to the properties of soft tissues than those of hard hydrophobic polymers such as PGA, PLA or PCL. Macroporous hydrogels prepared from recently synthesized degradable polymethacrylates induced only a minimal sterile inflammatory reaction in the surrounding tissue, probably only due to the surgery, and no foreign body reaction when implanted in the spinal cords of rats (Přádný *et al.* 2006).

Advanced technologies, e.g. rapid prototyping

and nanospider technology, have been used recently for creating the nanoarchitecture of bioartificial constructs (Shih *et al.* 2006). The reason for this approach is that the natural tissues are built on hierarchically-organized macro-, micro- and nanoscale levels. Nanofibers seem to provide cells with a similar environment to the native ECM. A variety of polymers, such as poly(lactic-co-glycolic acid) (PLGA), poly-L-lactide (PLLA), PCL, poly(ethylene oxide terephthalate)-poly(butylene terephthalate) (PEOT-PBT), collagen or chitosan, have been successfully electrospun. The diameter of the electrospun nanofibers ranged from ten to one thousand nanometers, and created a two- or three-dimensional structure with high surface-to-area ratios (Shih *et al.* 2006). Nanofibers made of type I collagen had multiple effects on MSC behavior, e.g. they increased the cell viability, induced a more polygonal and flattened cell morphology with numerous filipodia-like protrusions, and enhanced cell migration. Another positive feature was that the MSC displayed a lower activity of alkaline phosphatase, i.e. an enzyme involved in calcification, compared to the cells on standard tissue culture polystyrene (Shih *et al.* 2006). A technique enabling on-site layer-by-layer 3D tissue generation has already been developed, with the possibility to entrap different cell types simultaneously in one procedure (Yang *et al.* 2009). Fibroblasts seeded into a ten-layer collagen/PCL cell-fiber construct were found to be evenly distributed throughout the scaffolds, they proliferated, and deposited ECM during 14 days of culture (Yang *et al.* 2009).

In our experiments focused on the use of nanotechnology in tissue engineering of bioartificial valves, nanofibrous scaffolds, made of PCL, PLA and gelatine by electrospinning in a nanospider machine, provided good supports for the adhesion, growth and phenotypic maturation of VIC derived from a pig aortic valve. These cells formed well-developed vinculin-containing focal adhesion plaques and a rich α -actin cytoskeleton. These effects were more apparent in scaffolds with nanofibers aligned in parallel than on randomly-oriented nanofibers (Filová *et al.* 2009b).

Response of valve cells to dynamic loading *in vivo*

The aortic heart valve has a specific shape and undergoes a specific load during the cardiac cycle. Systole and diastole lead to different changes in shear stress and pressure load that bend the valve and subject

the valve to tensile and compressive forces. Different loads act upon the ventricularis and fibrosa, i.e. the inflow and outflow side of the leaflet. This is caused by pulsatile blood flow and by the mechanics of valve opening and closure. The blood flow on the inflow (i.e. ventricularis) side is strongly unidirectional and pulsatile, whereas the flow on the arterial (i.e. fibrosa) side is much slower and more oscillatory (Kilner *et al.* 2000). The pressure gradients across the normal aortic valve in the systole, when the valve is open, are practically zero. In the diastole under physiological conditions, when the valve is closed, there is a pressure gradient between the left ventricle and the ascending aorta of about 80 mmHg. The shear stresses acting on the aortic valve leaflet surfaces range from 30-1500 dynes/cm² (Weston *et al.* 1999). Both valvular endothelial cells (VEC) and valvular interstitial cells (VIC) are sensitive to mechanical forces (Butcher *et al.* 2008). VIC isolated from the pulmonary valve were less stiff than those from the aortic valve, and these differences correlated with α -smooth muscle cell actin content and the amount of collagen biosynthesis (Merryman *et al.* 2006). VICs are capable of remodeling the ECM, which is dependent on the mechanical loading. After the Ross operation, i.e. placing the pulmonary valve in aortic position, the change towards a higher pressure resulted in increased synthesis of collagen and glycosaminoglycans by pulmonary VIC. VEC *in vivo* align circumferentially on the leaflet surface, which is perpendicular to the direction of fluid flow, in contrast to arterial endothelial cells, which align in parallel (Deck 1986). It was further demonstrated that this alignment difference was regulated by changes in integrin clustering (followed by immunofluorescence of β_1 integrins), focal adhesion plaque formation (followed by immunofluorescence of vinculin), integrin-mediated signaling (through focal adhesion kinase) and cytoskeletal (i.e. f-actin) arrangement (Butcher *et al.* 2004). Moreover, DNA microarrays have shown statistically significant differences (approximately 10 %) in the genetic profile of porcine vascular (aortic) endothelial cells and valvular endothelial cells (Butcher *et al.* 2005). Steady shear stress was protective against oxidation damage, inflammatory reaction and chondro/osteogenic differentiation in both endothelial cell types (Butcher *et al.* 2005). Simmons *et al.* (2005) for the first time identified distinct endothelial phenotypes on opposite sides of the aortic valve. Over 584 transcripts were found to be differentially expressed *in situ* by the endothelium on the fibrosa versus ventricularis side of the

valve. The fibrosa side of the valve is more sensitive to lesion formation; however, this vulnerability is balanced by an enhanced antioxidative gene expression profile, which means that the fibrosa of the normal valve may be protective against inflammation and lesion initiation. Side-specific differences in valvular endothelial gene expression may be due to the differences in hemodynamic profile on each side of the valve (Butcher *et al.* 2008). The valvular endothelium is probably derived from specific regions of endocardial endothelium during embryonic development (Eisenberg and Markwald 1995).

Dynamic cultivation systems for valve tissue engineering

For successful tissue engineering, including heart valve tissue engineering, it is necessary to simulate the physiological mechanical loading of the tissues *in vivo* by dynamic cultivation *in vitro*. Currently, two types of dynamic systems are used for *in vitro* tests: (1) simple systems, intended for unidirectional stretching or bending of samples that are not preformed in the shape of the valve and (2) sophisticated systems, with more physiological dynamic loading of the valve-shaped samples or bioengineered valves together with the entire aortic root. These systems use either steady or pulsatile flow produced by rotary or membrane pumps. The aim of the dynamic culture is to produce a physiological flow curve through the valve, to apply pressure and shear stress, and to bend the valves. An advanced bioreactor, i.e. the flow loop bioreactor, ensures a complex hemodynamic environment (Butcher *et al.* 2008). Proper mechanical stimulation represents the primary strategy for producing a functional tissue *in vitro* (Syedain *et al.* 2008). Especially for the construction of bioartificial heart valves, mechanical loading of the tissue is absolutely necessary. This loading stimulates growth and maturation of VIC and the formation of ECM by these cells. For differentiated VIC, this behavior is usual, but for MSC cells, mechanical conditioning is required for their differentiation towards VIC phenotype. In tissue engineering of the valves, pulsatile flow of the culture medium through the valve, or pulsatile back-pressure on the valve has been applied (Mol *et al.* 2005). Mechanical stimulation had fundamental effects on the differentiation of sheep bone marrow-derived stem cells on PGA/PLLA scaffolds. Increased tissue formation and expression of markers of endothelial cell phenotype has been observed

only in the group with both cyclic and laminar flow (Engelmayr *et al.* 2006). It is known that cells adapt their behavior to constant environmental stimuli; that is why the cells initially respond but then return to baseline level after sustained stimulation (Li *et al.* 2005, Syedain *et al.* 2008). A fibrin-based construct with porcine VIC exposed to constant 15 % cyclic distension increased collagen content, ultimate tensile strength and tensile modulus after 3 weeks. Constructs subjected to cyclic distension with strain, which was incremented from 5 % to 15 % over the 3 weeks of cyclic distension, improved their mechanical properties (ultimate tensile strength, tensile modulus) as well as their collagen production compared to the constructs exposed to a constant strain amplitude (Syedain *et al.* 2008). Similarly, VIC and MSC that were stretched on collagen-coated flexible substrates increased their collagen synthesis at 14 % stretch (Ku *et al.* 2006). *In vivo*, VIC are exposed to large strain values during each cardiac valve cycle, mounting by 24 % in the radial direction and by 11 % in the circumferential direction (Lo and Vesely 1995). In another study, PGA/PLLA 50:50 nonwoven fibrous scaffolds-polyacrylamide gel composites were seeded with smooth muscle cells and exposed to unidirectional cyclic three-point flexure at physiological frequency and amplitude in a bioreactor for three weeks. This stimulation resulted in increased concentration and improved structural-mechanical properties of the collagen in the scaffolds (Engelmayr and Sacks 2008).

Cultivation of cells in a dynamic bioreactor enabling pulsatile flow of the culture medium had beneficial effects also on vascular cells in our experiments. Rat aortic smooth muscle cells and bovine pulmonary artery endothelial cells of the line CPAE, co-cultured on Aramid (i.e. aromatic polyamide) nanofiber meshes in a dynamic bioreactor, formed a continuous bilayer. Both cell types in the bilayer were well-stained for their differentiation markers, i.e. α -actin for smooth muscle cells and von Willebrand factor for endothelial cells. On the contrary, in the static culture system, only small islets of both cell types were observed, and the staining intensity for both differentiation markers was much weaker (Fig. 4) (Straka *et al.* 2009).

Dynamic cultivation is also a part of a novel approach to valve tissue engineering, known as “in-body tissue architecture technology”. In experiments conducted by Nakayama *et al.* (2009), this technique started with the insertion of a silicone tube into the dorsal subcutaneous space of a rabbit. After encapsulation with connective

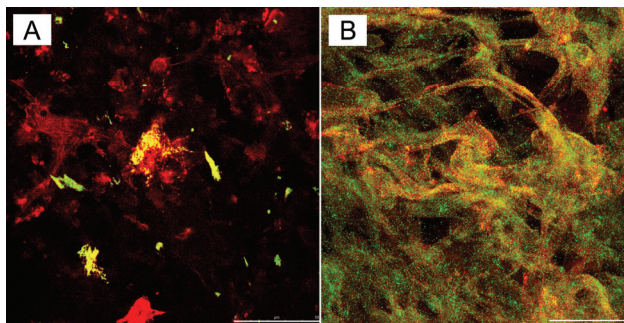


Fig. 4. Immunofluorescence staining of α -actin in smooth muscle cells (AlexaFluor 488, green) and von Willebrand factor (AlexaFluor 633, red) in endothelial cells under static (**A**) and dynamic culture conditions (**B**) on Aramid nanofibre mesh after 17 days in culture. Leica SPE confocal microscope, obj. $\times 40$, zoom $\times 1.5$, oil immersion, scale = 50 μm .

tissue, the rod was harvested and a polyurethane belt in the shape of three successive semi-ovals was rolled tightly around the connective tissue. The assembly was then re-inserted into the same rabbit to achieve further encapsulation. Finally, the autologous conduit-valve-shaped construct with trileaflet valves was obtained by removing both the implanted rod and the polyurethane belt (Nakayama *et al.* 2009). A similar technology can also be used for creating vascular grafts or vascular stents covered with autologous tissue membranes (Huang *et al.* 2009). An important consideration is that full maturation of the newly formed tissue constructs and marked improvement of their mechanical properties can be achieved in a dynamic bioreactor (Huang *et al.* 2009).

Future directions and questions to be resolved

New materials for cardiovascular tissue engineering, decellularization techniques and dynamic bioreactors matching the condition in a healthy organism are being developed. The nanostructured materials mentioned above are promising, as they modulate both protein adsorption and cellular response in a more physiological manner. Some xenotransplants prepared by special advanced techniques are also promising, e.g. the usage of xenotransplants from $\alpha 1,3$ -galactosyltransferase gene-knockout pig with prolonged survival (Ezzelarab *et al.* 2005). Novel heart valve replacements also contain DNA aptamers on their surface, which serve as capture molecules for circulating endothelial progenitor cells in the bloodstream. This approach would enable to skip the pre-endothelialization of the graft *in vitro*, which is often long-lasting and accompanied by a risk of infection

(Schleicher *et al.* 2009).

Advanced bioreactors are able to provide cells with a complex hemodynamic environment that can establish and maintain desirable cell phenotype (Butcher *et al.* 2008). However, it is still not clear what dynamic loading is appropriate to influence cell phenotype changes, stimulate and maintain cell differentiation and induce ECM remodeling without damage to the cell-material construct. Our experience with vascular and heart valve tissue engineering in pulse and perfusion bioreactors suggests that the initial loading of the cell-material constructs pre-formed under static cell culture conditions must be relatively low (i.e. much lower than those in arterial position in the living organism) in order to avoid damage to the cell and material. Then the loading should gradually be increased, according to the maturation capability of the tissue (Chlupáč *et al.* 2006, 2008, Straka *et al.* 2009). Similarly, in experiments carried out by other authors with cardiomyocytes, perfusion with culture medium below 1.5 ml/min supported the contractile properties of cardiomyocytes (Brown *et al.* 2008). However, excessive shear stress above 2.4 dyn/cm² triggered p38 activation in neonatal cardiomyocytes and initiated their apoptosis (Dvir *et al.* 2007). Also in experiments *in vivo*, bioengineered prostheses are usually implanted into a pulmonary position, because the right side of the heart produces lower stress on the valve. In addition, any blood clots formed on the implant can be filtered in the lungs, and will not reach the general circulation (Korossis *et al.* 2000, Sutherland *et al.* 2005).

Concerning the implantation technique, there are requirements for the development of mini-invasive procedures, catheterization techniques, suitable stents, fine instruments for valve implantation, and advanced imaging systems, e.g. 3D echocardiography, computer tomography (CT). These procedures can in turn extend the spectrum of percutaneous interventions, which are currently reserved for patients having a high risk associated with surgical treatment.

Despite the indisputable progress that has been made in the construction of heart valve prostheses, many questions still remain to be fully answered, such as whether we can completely avoid xenogeneic and allogeneic materials. An attempt to avoid xenogeneic proteins, and simultaneously to preserve the biological properties of the material, involved the use of enzymatically cross-linked collagen-mimetic dendrimer with a triple-helical structure (Khew *et al.* 2008). Another

promising possibility is the use of recombinant proteins and peptides, such as analogues of collagen, elastin or silk fibroin (Bini *et al.* 2006, Gelain *et al.* 2007, Kyle *et al.* 2009).

Further important questions are which cell types or co-cultures should be preferably used – whether differentiated, progenitor or stem cells, and whether the development of bioengineered heart valve can be tracked in respect of expression of genes involved in cell differentiation or calcification. As mentioned above, autologous differentiated cells are often available in small quantities, they proliferate relatively slowly and may have a limited life span, and therefore progenitor and stem cells are used. Among these cells, there is a tendency to avoid MSC in heart valve and vascular tissue engineering due to a high tendency to calcification of this cell type, which is derived from bone marrow. Alternative sources of progenitor cells are human umbilical cord cells (Hoerstrup *et al.* 2002), endothelial progenitor cells from peripheral blood (Zisch 2004), adipose-derived stem cells (Bunnell *et al.* 2008), skeletal muscle satellite cells (Danišovič *et al.* 2008) or amniotic fluid-derived cells (Schmidt *et al.* 2008).

Another issue is whether it is necessary to use valve-shaped grafts, or whether they can be tailored from a flat tissue sheet formed before implantation, which would be much simpler. If the second option is chosen, maturation of the construct in an appropriate dynamic bioreactor prior to implantation is highly recommended, due to the complicated architecture of the heart valve, as well as the high diversity and specialization of the heart cell types as mentioned above.

Other questions that need further intensive investigation are which scaffold properties (e.g. material, pore size or interfiber distance, stiffness, elasticity or degradation rate) are crucial for cell colonization and differentiation. As is known from bone tissue engineering, the appropriate pore size for bone tissue ingrowth inside the scaffolds ranges in hundreds of micrometers, optimally from ~400 to 600 μm . For fibrovascular tissue, this size has been reported to be much smaller, i.e. below 100 μm (for a review, see Pamula *et al.* 2008, 2009). On the other hand, in decellularized bovine pericardium (ABP), a pore diameter of ~25 μm , accompanied by about 60 % pore interconnectivity and porosity, was unsuitable for cell ingrowth, and better results were obtained with synthetic scaffolds with values of 250 μm and 90 %, respectively (Wei *et al.* 2005, Dong *et al.* 2009). In the latter study

(Dong *et al.* 2009), the attractiveness of ABP for cell colonization was considerably improved by treating ABP with acetic acid, which increased the scaffold pore size and porosity.

Conclusions

Currently used mechanical and biological heart valve prostheses have serious disadvantages. Therefore, efforts are being made to prepare bioartificial heart valves with an autologous biological component using tissue engineering methods. However, approaches for the development of the tissue-engineered heart valves still have several limitations and there are unresolved questions. Promising approaches seem to be the use of modified decellularized xenogenic or allogenic scaffolds (particularly those modified by RDG-containing polypeptides) or the use of xenotransplants from $\alpha 1,3$ -galactosyltransferase gene-knockout pig with prolonged survival. The development of materials containing polymerized extracellular matrix and entrapped cells enables an analysis of molecular mechanisms of cell-to-extracellular matrix interactions, cell reaction to mechanical stimulation, as well as cell-cell interaction in co-cultures of different cells types. Some advanced technologies hold out high hopes, e.g. rapid prototyping and nanospider technology for creating a nanoarchitecture to promote the colonization of scaffolds with cells. Analyses of the adhesion, proliferation, migration and differentiation potential of several cell types, including stem and progenitor cells, in cultures on degradable synthetic scaffolds with different chemical compositions, porosity or elasticity and under various dynamic conditioning, are also important tasks for further research in this area. In terms of implantation techniques, further development of appropriate tools can be anticipated, as well as stents for percutaneous valve implantation.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by the Centre for Cardiovascular Research (grant No. 1M6798582302 provided by the Ministry of Education, Youth and Sports of the Czech Republic) and by the Academy of Sciences of the Czech Republic (grant No. 1QS500110564). Mr. Robin Healey (Czech Technical University, Prague)

is gratefully acknowledged for his language revision of the manuscript. We also thank Mrs. Jana Voborníková for her excellent technical assistance in preparing the manuscript.

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