Layer-by-Layer Incorporation of Growth Factors in Decellularized Aortic Heart Valve Leaflets

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Aortic heart valve disease is a growing health problem and a tissue-engineered aortic heart valve could be a promising therapy. In this paper, decellularized porcine aortic heart valve leaflets are used as scaffolds and loaded with growth factor and heparin via layer-by-layer electrostatic deposition (LbL technique) with the final purpose to stimulate and control cellular processes. Binding and subsequent release of heparin and basic fibroblast growth factor (bFGF) from aortic valve leaflets were assessed qualitatively by immunohistochemistry and quantitatively by radioactive labeling methods. It was observed that the amount of heparin and bFGF bound to aortic heart valve leaflets was directly proportional to the concentration of heparin and bFGF in the incubation medium. Release of heparin and bFGF from the decellularized heart valve leaflets at physiological conditions was sustained over 4 days while preserving the biological activity of the released growth factor.

1. Introduction

The incidence of heart valve disease is a growing public health problem.1 Potential treatment involves replacement of the valve by a bioprosthetic valve, a homograft, or a mechanical valve prosthesis. However, currently available prosthetic valves are all nonvital and lack the ability to grow, repair, and remodel in an in vivo environment in addition to several specific disadvantages.2,3 A living, tissue-engineered, aortic heart valve would overcome these limitations of current prostheses. For this purpose, several strategies could be followed. One approach to produce a tissue engineered aortic heart valve involves the preseeding of a scaffold with cells, followed by an in vitro stage of cell growth and tissue formation. Subsequently, the construct is implanted in vivo and tissue growth and remodelling is further allowed to take place.4,5 In this work decellularized porcine aortic heart valves are used as biological scaffolds. These heart valves have the advantage of enhancing cellular attachment and retaining many of the mechanical and structural properties such as tensile strength and the unique extracellular matrix composition of the aortic heart valve.6 During the decellularization of the porcine aortic heart valves, endothelial cells that cover the surface of the valve, interstitial fibroblasts, smooth muscle cells, and myofibroblasts and proteoglycans are removed. Finally, a matrix consisting of merely collagen and elastin remains.7–11

Upon reseeding of a decellularized matrix, migration, proliferation, and if necessary transdifferentiation have to take place to obtain a vital tissue. This is when growth factors come into play to stimulate and control cellular processes. Taking the short half-life of growth factors into account, it would be beneficial to develop a system that could load high amounts of growth factors combined with a controlled release of these growth factors. Several techniques aiming to incorporate bioactive components into tissue engineering matrices have been reported, including covalent as well as noncovalent coupling of growth factors to scaffolds.12,13 Because covalent coupling of growth factors can cause loss of biological activity of proteins, noncovalent binding of growth factors is preferred in this work.14

Basic fibroblast growth factor (bFGF) is a heparin-binding growth factor belonging to the FGF family which stimulates proliferation of fibroblasts.15 Heparin is a glycosaminoglycan with a well-known in vivo behavior and will be used in this work to replace the proteoglycans, which were removed during the decellularization process. Because proteoglycans act to resist compressive forces during valve opening and closure, they are important components of the heart valve.16 Moreover, glycosaminoglycans may function as a reservoir for growth factors and they are involved in adhesion, migration, proliferation, and differentiation of cells.17–19 Heparin has many advantages in combination with bFGF or other growth factors and many growth factors are known as heparin-binding growth factors. Binding of bFGF to heparin induces a conformational change in the bFGF molecule resulting in an increased resistance against thermal denaturation, enzymatic degradation, and a reduced inactivation at acidic pH.20–22 Yayon et al. suggested that binding of bFGF to heparin facilitates the binding of bFGF to high-affinity cell membrane receptors.23

In this paper, decellularized aortic heart valve leaflets are loaded with growth factors, using the layer-by-layer technique (LbL technique).24–27 This technique commonly involves the sequential adsorption of oppositely charged polymers (i.e., polyelectrolytes) on a charged surface.28,29 The driving force for the formation of LbL films comes from the alternate overcompensation of the surface charge after each deposition of an oppositely charged polyelectrolyte. bFGF was chosen as the positively charged polymer and heparin as the negatively charged polymer. Several studies reported on the use of synthetic matrices for incorporation and delivery of growth factors;

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however, the use of biological materials is rarely described.\textsuperscript{30–32} Here we aim to assess whether it is possible to perform the layer-by-layer technique on decellularized aortic heart valve leaflets, while preserving the biological activity of the released growth factor.

2. Materials and Methods

2.1. Electrophoretic Mobility. Polystyrene beads (10 \( \mu \text{m} \), sulfonated; Microparticles GmbH) were coated with poly(ethyleneimine) (PEI; 22 kDa; Sigma–Aldrich), heparin (Diosynth Biotechnology), and basic fibroblast growth factor (bFGF; PeproTech) in combination with bovine serum albumin (BSA; Sigma–Aldrich) or Tween 20 (Sigma–Aldrich). The electrophoretic mobility of the coated polystyrene beads was measured using a Malvern Zetasizer 2000 (Malvern Instruments).

Adsortion of the polymers on the polystyrene beads was performed by the incubation of the beads during 30 min in the appropriate macromolecule solution followed by two successive washing steps with water, PEI, heparin, and bFGF, respectively, used in a concentration of 1 mg/mL, 1 mg/mL, and 1.5 mg/\( \mu \text{L} \).

2.2. Cell Culture. Human dermal foreskin fibroblasts were obtained from ATCC (CRL-2522). Cells were cultured in Eagle’s minimum essential medium (EMEM; ATCC), supplemented with streptomycin/penicillin, and 20% heat inactivated fetal bovine serum (FBS; Gibco) and grown at 37 °C in a humidified atmosphere containing 5% CO\(_2\). Experiments were performed at population doubling 30.

2.3. Matrix Preparation. Decellularization of the porcine aortic heart valves was performed using a patented detergent-enzymatic treatment.\textsuperscript{33,34} First, isolated porcine aortic heart valves were alternatively subjected to a mild alkaline hypotonic (pH 8) and hypertonic buffered solution (pH 8), supplemented with phenylmethylsulfonyl fluoride (PMSF), streptomycin/penicillin solution, and butylated hydroxyanisole (BHA). Performing these steps resulted in the rupture of the native cells and partially extracted cytoplasmic elements and soluble extracellular matrix. Second, to digest nucleic acids and cellular membranes, the valves were treated enzymatically (DNase I, RNase A, RNase inhibitor, and phospholipases A2, C, and D). Finally, the aortic heart valves were washed in a magnesium and calcium-free chelating solution. All products were purchased from Sigma–Aldrich.

2.4. Collagen Determination of Aortic Heart Valve Leaflets. The total collagen content of six lyophilized decellularized aortic valve leaflets was determined according to the protocol of The International Standards Organisation; ISO/DIS 3496.2. The lyophilized decellularized aortic valve leaflets were hydrolyzed using 6 M HCl at 110 °C during 24 h to release hydroxyproline. Hydroxyproline was oxidized with dimethylaminobenzaldehyde in perchloric acid and with chloramin-T which resulted in the formation of pyrrole-2-carboxylic acid and pyrrole. Pyrrole was determined using a color reaction with Ehrlich’s reagent (p-dimethylaminobenzoaldehyde) in perchloric acid and isopropanol to give dipyrryl-phenylmethene salt and ms-tetra-(4-dimethyl-aminophenyl)-porphin. The latter was determined spectro photometrically at 558 nm. To calculate the percentage of collagen, a standard curve of hydroxyproline solutions subjected to the color reaction was used. A conversion factor of 8 was used.\textsuperscript{8}

2.5. Scanning Electron Microscopy. Decellularized aortic heart valve leaflets were dried on a silicone wafer followed by sputtering with gold. SEM images were recorded with a Quanta 200 FEG FEI scanning electron microscope operated at an acceleration voltage of 5 kV.

2.6. Aortic Valve Tissue Staining with Hematoxylin and Eosin. Decellularized aortic valve leaflets were dehydrated in ethanol and embedded in paraffin. Sections of 5 \( \mu \text{m} \) were cut, stained with hematoxylin and eosin, and examined by light microscopy.

2.7. Immunohistochemistry of Heparin and bFGF Binding. 2.7.1. Rhodamine Labeling of Heparin. Heparin was labeled with rhodamine isothiocyanate (RTIC; Sigma–Aldrich) by slowly adding 12 mg RTIC in 60 mL of borate buffer (0.1 M; pH 8.5) to a vigorously stirring solution of 300 mg heparin in 60 mL of borate buffer. After an overnight reaction, the solution was dialyzed for 7 days against distilled water with regular renewal of the water. Finally, RTIC-heparin was obtained as a purple powder after lyophilization. Coupling of heparin to rhodamine isothiocyanate occurred through amide bond formation between primary amines of heparin and isothiocyanate groups of RTIC.

2.7.2. Visualization of Heparin Binding. Decellularized aortic heart valve leaflets were incubated in a 1 mg/mL RTIC-heparin in PBS solution (Gibco). Two washing steps by soaking the aortic valve leaflets in excess PBS were performed to remove nonadsorbed RTIC-heparin, and subsequently, the tissues were dehydrated in ethanol and embedded in paraffin. Paraffin sections (5 \( \mu \text{m} \)) were cut with a microtome and visualized with a Nikon EZ-C1 confocal microscope.

2.7.3. Visualization of bFGF Binding. RTIC-bFGF-loaded aortic heart valve leaflets were incubated in aqueous medium containing 1 mg/\( \mu \text{L} \) bFGF and 0.1% Tween 20 in an acetate buffer (50 mM; pH 5). Two washing steps by soaking the aortic valve leaflets in excess acetate buffer were performed to remove nonadsorbed bFGF, and subsequently, the tissue was embedded in Tissue Tek (Sakura). Cryo sections (10 \( \mu \text{m} \)) were cut with a cryo-microtome, washed in PBS containing 1% BSA, and incubated overnight at 4 °C with a biotinylated bFGF antibody (1/100 dilution of a 1 mg/mL stock in PBS containing 1% BSA; PeproTech). After washing with PBS containing 1% BSA, fluorescent staining was performed with Cy5-labeled streptavidin (1/25 dilution of a 1 mg/mL stock solution; Zymed Laboratories) followed by washing with PBS. Microscope images were recorded on a Nikon EZ-C1 confocal microscope.

2.8. \textsuperscript{125}I and \textsuperscript{123}I Labeling of Heparin. Heparin was labeled with \textsuperscript{125}I according to the Bolton Hunter method. Briefly, 100 mg heparin was incubated during 3 h with a solution of water-soluble Bolton Hunter (5 mg/mL; Pierce) at pH 9 (borate buffer 0.1 M) in an ice bath, followed by an overnight dialysis of the reaction mixture against PBS (pH 7.4) to remove unbound Bolton Hunter reagent. Binding of the Bolton Hunter by amide binding to heparin was possible due to the presence of few primary amine groups in heparin.\textsuperscript{35} The heparin–Bolton Hunter was incubated with \textsuperscript{125}I in a vial (coated with 70 \( \mu \text{g} \) iodogen) in PBS during 20 min at room temperature. After the incubation period, residual \textsuperscript{125}I was separated from the \textsuperscript{125}I-heparin by size exclusion chromatography on a PD-10 column. The concentration of the \textsuperscript{125}I-heparin solution was determined via size exclusion chromatography (PL aquagel–OH 30 8 \( \mu \text{m} \), 300 \times 7.5 mm; Polymer laboratories, Church Stretton, U.K.) coupled with a spectrophotometer at a wavelength of 210 nm. The mobile phase was PBS (pH 7.4), the flow rate 1 mL/min. The obtained \textsuperscript{125}I-heparin solution was further diluted in PBS. Labeling of heparin with \textsuperscript{125}I was performed via a similar method.

\textsuperscript{125}I-heparin was used to investigate the binding of the heparin to decellularized aortic heart valve leaflets, while \textsuperscript{123}I-heparin was used to assess the release of heparin from decellularized aortic heart valve leaflets due to its longer half-life.

2.9. \textsuperscript{123}I and \textsuperscript{125}I Labeling of bFGF. bFGF was labeled with \textsuperscript{125}I by the incubation of 50 \( \mu \text{g} \) bFGF with \textsuperscript{125}I in a vial coated with 70 \( \mu \text{g} \) iodogen in PBS containing 0.1% Tween 20 during 20 min at room temperature. After the incubation period, residual \textsuperscript{123}I was separated from \textsuperscript{123}I-bFGF on a PD-10 column by size exclusion chromatography using acetate buffer (pH 5; 50 mM) containing 0.1% Tween 20 as eluent. The concentration of the radioactive bFGF was calculated via the percentage of nonspecific adsorption of bFGF on the PD-10 column and the original amount of bFGF, and the concentrated \textsuperscript{123}I-bFGF solution was further diluted in acetate buffer (pH 5; 50 mM) containing 0.1% Tween 20. Labeling of bFGF with \textsuperscript{125}I was performed via a similar method.

Similar to the iodine labeled heparin, \textsuperscript{123}I-bFGF was used to investigate the binding of the growth factor to decellularized aortic heart valve leaflets, while \textsuperscript{125}I-bFGF was used to assess the release of bFGF from decellularized aortic heart valve leaflets.

2.10. Binding and Subsequent Release of Heparin from Decellularized Aortic Heart Valve Leaflets. Decellularized aortic heart valve leaflets were incubated during 1 h under shaking conditions (400
Polystyrene beads were incubated during 1 h under shaking conditions (400 min−1) with 3 mL of heparin solution, followed by two successive washing steps during 10 min in PBS. After deterioration of the radioactivity of 125I-heparin, the leaflets were lyophilized followed by the assessment of their dry mass.

Release of heparin from the loaded heart valve leaflets was performed in physiological medium (PBS). The adsorbed and released iodine labeled heparin was measured in a Packard cobra automated γ-counter. All experiments were performed in triplicate.

2.11. Binding and Subsequent Release of bFGF from Decellularized Aortic Heart Valve Leaflets. Decellularized aortic heart valve leaflets were incubated during 1 h under shaking conditions (400 min−1) with consecutively 3 mL of heparin solution (1 mg/mL) and 3 mL of bFGF solution, each followed by two successive washing steps during 10 min in PBS after heparin binding and in acetate buffer containing 0.1% Tween 20 after bFGF binding. After deterioration of the radioactivity of 125I-bFGF, the leaflets were lyophilized followed by the assessment of their dry mass.

Release of bFGF from the loaded heart leaflets was performed in physiological medium (PBS containing 0.1% Tween 20). The adsorbed and released iodine labeled bFGF was measured in a Packard cobra automated γ-counter. All experiments were performed in triplicate.

2.12. Preservation of the Biological Activity of the Released bFGF. To investigate the preservation of the biological activity, decellularized aortic heart valve leaflets were loaded with heparin and heparin/bFGF, as described in sections 2.10 and 2.11 followed by their release in EMEM containing 0.5% FBS. As controls, solutions of heparin and heparin/bFGF of equal concentration as the release medium were used. Human dermal fibroblasts were seeded in a 96-well plate at a density of 2500 cells per well and incubated with the test medium. After 1, 3, and 5 days, the cells were quantified using a p-nitrophenyl phosphate (pNPP; Sigma-Aldrich) cell viability assay. Cells were incubated with a pNPP solution under acidic conditions during 2 h at 37 °C and 5% CO2. During this incubation period, p-nitrophenyl phosphate was hydrolyzed by acid phosphatase resulting in the release of p-nitrophenol and phosphate. The reaction was stopped with NaOH, resulting in the formation of a yellow color, which was spectrophotometrically measured at 405 nm. All experiments were performed in triplicate.

2.13. Statistical Analysis. Descriptive statistics are expressed as mean ± standard deviation. Data regarding preservation of the biological activity of the released bFGF were analyzed via 3-way ANOVA. p-Values < 0.05 are considered significant.

3. Results and Discussion

3.1. Electrophoretic Mobility. Growth factors are often provided in combination with bovine serum albumin (BSA) as cryoprotectant during lyophilization and to avoid nonspecific adsorption to the surface of the recipient upon reconstitution in aqueous medium. Taking into account the pKa values of 9.59 for bFGF and 4.97 for BSA it is reasonable to assume that at physiological conditions (i.e., pH 7.4; 150 mM NaCl) electrostatic complexes will be formed in solution between bFGF and BSA. This will hamper the use of electrostatic forces to incorporate bFGF as such in an electrostatically assembled multilayer film (further denoted as LbL film) onto a solid surface. Therefore, we initially investigated the parameters involved in LbL build-up using polystyrene beads as template and electrophoretic mobility measurements as detection technique. To avoid nonspecific adsorption of bFGF, Tween 20 was used as an alternative for BSA. Tween 20 is a nonionic blockcopolymer surfactant and in presence of bFGF, electrostatic complexation in solution between Tween 20 and bFGF will be avoided, allowing the growth factor to participate in the formation of a LbL film.

Electrophoretic mobility measurements showed the polystyrene beads to exhibit a γ-potential of −50 ± 1.89 mV. To mimic the surface charge of an aortic heart valve leaflet, exhibiting a positive surface charge due to the presence of collagen, the polystyrene beads were coated with a layer of polyethyleneimine (PEI), which is commonly used in LbL assemblies as adhesion promoter in a first layer, resulting in a positive γ-potential of 18 ± 2.58 mV. Upon deposition of a heparin layer charge reversal took place and a γ-potential of −48 ± 3.37 mV was measured. In a next step, the beads were incubated with a solution of 1.5 µg/mL bFGF combined with respectively 0.1% BSA or 0.1% Tween 20, resulting in a γ-potential of −33.2 ± 5.76 mV, respectively, −12.6 ± 3.07 mV, indicating that, compared to the presence of BSA, the presence of Tween 20 allowed the γ-potential to increase more pronounced upon deposition of bFGF (Figure 1). As a control (data not shown), PEI/heparin coated beads were incubated with a 0.1% BSA and a 0.1% Tween 20 solution, resulting in respectively a γ-potential of −31.8 ± 4.83 mV and −31.9 ± 3.91 mV. Another set of controls (data not shown) included the incubation of the PEI and heparin-coated beads with a 1.5 µg/mL solution of bFGF in a polypropylene vial precoated with BSA or Tween 20, which resulted in respectively a γ-potential of −26.7 ± 3.82 mV and −15.6 ± 3.2 mV.

In all cases, adsorption of bFGF resulted in an increase of the γ-potential due to the positive charge of the growth factor at physiological conditions. The highest increase of the γ-potential was obtained when the beads were incubated with a 1.5 µg/mL solution of bFGF containing Tween 20 or when the beads were incubated with a 1.5 µg/mL solution of bFGF in a vial which was precoated with Tween 20. These results indicate an ideal formation of a LbL film using bFGF in combination with Tween 20. All the experiments in this paper, including the formation of LbL film on the heart valve leaflets, were performed using bFGF in combination with 0.1% Tween 20.

3.2. Characterization of the Aortic Heart Valve Leaflets. 3.2.1. Collagen Content of the Aortic Heart Valve Leaflets. Collagen fibers provide most of the mechanical and tensile strength of heart valves. The collagen content of decellularized
3.3.1. Qualitative Analysis by Immunohistochemistry. The LbL film on the decellularized aortic heart valve leaflets consisted of heparin (Figure 3), which is negatively charged, and bFGF, which is positively charged at physiological conditions (i.e. pH 7.4). Heparin was red fluorescent labeled with rhodamine and incubated with aortic heart valve leaflets at a concentration of 1 mg/mL heparin. Subsequently, the heart valve leaflets were rinsed with PBS to remove nonadsorbed heparin followed by incubation of the heart valve leaflets in a 1 µg/mL bFGF solution. After the incubation period and washing steps, microtome sections were made of the leaflet followed by immunohistochemical staining with bFGF antibody. Figure 4 shows confocal microscopy images of stained heart valve leaflets. The rhodamine-labeled (red fluorescent) heparin was visible as small stripes presumably along the collagen fibers. Although the binding of heparin to the tissue in the cryosections was homogeneously distributed throughout the heart valve leaflet, we saw in the paraffin sections that binding of heparin was higher to the surface of the tissue compared to the binding deeper in the tissue. The antibody staining against bFGF was assigned the blue color, as both rhodamine and Cy5 are considered red fluorophores, however, with a sufficient wavelength gap to record them separately. The bFGF was apparently also present in a stripe-like pattern, similar to that of heparin. Control experiments with heart valve leaflets without heparin coating yielded much lower signal of bFGF (data not shown). For this reason binding of bFGF to leaflets without heparin coating was not studied in the following experiments. Taken together, these images indicate that both heparin and bFGF are well adsorbed to the decellularized heart valve leaflets.

Binding of heparin and bFGF to the decellularized aortic heart valve leaflet can occur through electrostatic interaction, although other binding mechanisms may be involved as well. Decellularized aortic heart valve leaflets contain collagen and elastin as major components. Heparin can interact through electrostatic interaction with collagen type I through the positively charged groups. It is also described in literature that collagen contains high-affinity receptor for heparin explaining the high affinity between heparin and collagen type I.39–41 Interaction between heparin and bFGF can be explained by ionic interaction between both 2-O-sulfate groups and the N-sulfate group of heparin molecules and certain lysine and arginine residues in bFGF.30

3.3.2. Quantitative Analysis by Radioactive Labeling. Decellularized aortic heart valve leaflets were incubated with radioactive labeled heparin (125I-heparin) within a concentration range from 0 to 5.073 mg/mL. Another set of experiments included incubation of decellularized aortic heart valve leaflets with heparin (1 mg/mL) followed by incubation with radioactive labeled bFGF (125I-bFGF) within a concentration range from 0 to 1.764 µg/mL. A concentration of 1 mg/mL of heparin was used as it is a commonly used concentration to construct LbL films. bFGF was used within the µg/mL range because the effective concentration of this growth factor is within the ng/mL range. Previously, Somers et al. used the wet weight of the decellularized aortic heart valve leaflets to express the amount of bound heparin and bFGF to the tissue.32 Because the wet weight of the leaflets is characterized by a high variability (data not shown), using this data to express the amount of bound polymer to the decellularized aortic heart valve leaflets is merely an approximation. In this work, we used the mass of the lyophilized aortic heart valve leaflets to express the amount of bound heparin and bFGF to the tissue. Figure 5 demonstrates that the amount of bound heparin (Figure 5A) and bFGF (Figure 5B) is directly proportional to the concentration of the medium in which the heart valve leaflets were incubated. However, one has to keep in mind that these curves are probably the first part of a curve leading to a plateau indicating saturation of the binding of heparin and bFGF to the decellularized aortic heart valve leaflets.42

3.3.3. Release of Heparin and bFGF from the Decellularized Aortic Heart Valve Leaflets. Decellularized aortic heart valve leaflets were loaded with 125I-heparin in a 1 and 4 mg/mL solution and with 125I-bFGF in a 0.316 and 1.265 µg/mL.
solution. Release of heparin and bFGF, evaluated at physiological conditions via $^{125}$I labeling of the polymers due to the longer half-life of $^{125}$I compared to $^{123}$I, was mainly driven by competition with salt ions. For both components, the release profile is characterized by an initial burst release, followed by a sustained release over 4 days. The initial burst release can be explained by the release of material which was loosely bound near the surface of the leaflets. The sustained release can be explained by the release of polymer, which was located more deeply into the structure of the leaflets. After an incubation period at physiological conditions of 4 days 70% of the originally bound heparin and 80% of the originally bound bFGF has been released (Figure 6).

3.4. Preservation of the Biological Activity of the Released bFGF. An important issue regarding binding and release of bFGF from decellularized aortic heart valve leaflets is the preservation of the biological activity of the released growth factor. As bFGF stimulates proliferation of human dermal fibroblasts, we performed a comparative cell proliferation study between on one hand freshly prepared heparin, heparin/bFGF solutions, and on the other hand heparin, heparin/bFGF, released from the aortic heart valve leaflets. Aortic heart valve leaflets loaded with respectively heparin (1 mg/mL) or heparin (1 mg/mL)/bFGF (1 µg/mL) were incubated for 4 days in EMEM containing 0.5% FBS. From the binding data (Figure 5) one can calculate the amount of adsorbed heparin (i.e., 5.3 µg/mg tissue) and bFGF (25.5 ng/mg tissue). As the release after 4 days at physiological conditions of heparin and bFGF is, respectively, 70 and 80% (Figure 6), the concentration of heparin and bFGF in the release medium can be calculated considering the mass of the leaflets. These solutions were diluted in cell medium to obtain a bFGF concentration of 10 ng/mL, which should according to literature be optimal for cell proliferation.

Cell proliferation was assessed by culturing human dermal fibroblasts in the presence of the diluted release medium, followed by analysis of the cell viability with a common pNPP assay after, respectively, 1, 3, and 5 days. Figure 7 shows the comparative results between heparin and heparin/bFGF released from aortic heart valve leaflets and freshly prepared heparin and heparin/bFGF solutions of equal concentrations as calculated for the released heparin and heparin/bFGF. The cell viability
was expressed as a percentage relative to cells cultured in heparin- and bFGF-free medium as control. These series of experiments demonstrated that heparin did not influence cell proliferation, as within the heparin group no statistical significant difference between days 1, 3, and 5 was observed. In contrast, bFGF did stimulate cell proliferation as relative cell numbers increased as a function of time. Within the group of heparin/bFGF, statistical significant differences between days 1 and 3, days 1 and 5, and days 3 and 5 were observed. These findings suggested that the activity of the released bFGF was preserved after being bound to an aortic heart valve leaflet and subsequently being released.

4. Conclusions

In this work, we performed the layer-by-layer technique on decellularized aortic heart valve leaflets as biological matrices. Binding of heparin and bFGF to aortic valve leaflets was shown followed by release of bFGF and heparin while preserving their biological activity. We also demonstrated that, to incorporate growth factors within a multilayer film, it is preferable to combine the growth factor with Tween 20 instead of BSA to avoid nonspecific adsorption of bFGF.

Human dermal mesenchymal fibroblasts are an attractive source of progenitor cells for myofibroblasts, making them an interesting cell type for the repopulation of the decellularized aortic heart valve leaflets. In future research we will focus on the interaction between heparin/growth factor-loaded heart valve leaflets with human dermal mesenchymal fibroblasts. So far we were not yet able to demonstrate repopulation of heparin/bFGF-loaded heart valve leaflets. This is likely due to the limited pore size of the leaflets that does not allow spontaneous inward migration of human dermal mesenchymal fibroblasts. On the other hand, improvement could be obtained when using an optimal growth factor cocktail, for example, incorporated within a polyelectrolyte multilayer film, comprising of growth factors such as bFGF, transforming growth factor beta1 (TGF-β1), and epidermal growth factor (EGF), as reported by Narine et al., to stimulate proliferation, transdifferentiation, and migration of in vitro cultured human dermal mesenchymal fibroblasts. These investigations are currently ongoing.

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References and Notes