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Tissue engineering of heart valves by recellularization of glutaraldehyde-fixed porcine valves using bone marrow-derived cells

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Abbreviations: BMC, bone marrow-derived cell; DAB, 3,3'-diamino-benzidine tetrahydrochloride; EB, ethidium bromide; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; FDA, fluorescein diacetate; GA, glutaraldehyde; H&E, hematoxylin and eosin; KDR, kinase-insert domain-containing receptor; M199, Medium 199; MF, myofibroblast; MHC, myosin heavy chain; MSC, mesenchymal stem cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; SEM, scanning electron microscopy; SM, smooth muscle; TCP, tissue culture plastics

Abstract

To increase the biocompatibility and durability of glutaraldehyde (GA)-fixed valves, a biological coating with viable endothelial cells (ECs) has been proposed. However, stable EC layers have not been formed successfully on GA-fixed valves due to their inability to repopulate. In this study, to improve cellular adhesion and proliferation, the GA-fixed prostheses were detoxified by treatment with citric acid to remove free aldehyde groups. Canine bone marrow mononuclear cells (MNCs) were differentiated into EC-like cells and myofibroblast-like cells *in vitro*. Detoxified prostheses were seeded and recellularized with differentiated bone marrow-derived cells (BMCs) for seven days. Untreated GA-fixed prostheses were used as controls. Cell attachment, proliferation, metabolic activity, and viability were investigated and cell-seeded leaflets were histologically analyzed. On detoxified GA-fixed prostheses, BMC seeding resulted in uninhibited cell proliferation after seven days. In contrast, on untreated GA-fixed prostheses, cell attachment was poor and no viable cells were observed. Positive staining for smooth muscle α-actin, CD31, and proliferating cell nuclear antigen was observed on the luminal side of the detoxified valve leaflets, indicating differentiation and proliferation of the seeded BMCs. These results demonstrate that the treatment of GA-fixed valves with citric acid established a surface more suitable for cellular attachment and proliferation. Engineering heart valves by seeding detoxified GA-fixed biological valve prostheses with BMCs may increase biocompatibility and durability of the prostheses. This method could be utilized as a new approach for the restoration of heart valve structure and function in the treatment of end-stage heart valve disease.

Keywords: bone marrow cells; endothelial cells; heart valve prosthesis; tissue engineering

Introduction

Replacement of heart valves with mechanical or biological prostheses is currently a common treatment for end-stage valvular diseases (Lupinetti *et al.,* 1997). These heart valve prostheses substantially reduce the mortality of patients, but the prostheses have severe limitations. Mechanical valve prostheses are associated with a high incidence of thromboembolism due to their poor blood compatibility, and require long-term use of anti-clotting medication (Lupinetti *et al.,* 1997). Biological tissue

valve prostheses, such as glutaraldehyde (GA)-fixed xenografts and cryopreserved homografts, suffer from limited durability and dysfunctions due to progressive tissue degeneration, and therefore may require successive surgery to replace the prostheses (Yacoub *et al.,* 1995). The major disadvantage of GA-fixed tissue valve prostheses is the degeneration of grafts, with subsequent calcification and tissue failure (Sodian *et al.,* 2000). This may result from an inability of host cells to repopulate the valve due to the GA-fixation process (Yacoub *et al.,* 1995).

Various strategies have been proposed to improve the performance of GA-fixed heart valve bioprostheses (Moritz *et al.,* 1990; Zilla *et al.,* 1997; Weissenstein *et al.,* 2000; Trantina-Yates *et al.,* 2001; Gulbins *et al.,* 2003). One method involves the use of a biological coating of viable endothelial cells (ECs) to increase the biocompatibility and durability of GA-fixed valves. By covering the surface of the valve prostheses with autologous ECs, valve degeneration and thromboembolic events may be reduced. However, stable EC layers have not been formed successfully on GA-fixed valves due to their inability to be repopulated (Fischlein *et al.,* 1992; Hoffmann *et al.,* 1992; Bengtsson *et al.,* 1993; Fischlein and Fasol, 1996). Methods that have previously been tested to improve cellular adhesion on GA-fixed prostheses include pretreatment of the prostheses with amino acid solutions (Fischlein *et al.,* 1994) or citric acid solutions (Gulbins *et al.,* 2003), and precoating the valves with fibronectin or growth factors (Fischlein *et al.,* 1994).

The purpose of this study was to develop a detoxifying treatment method for GA-fixed prostheses that would enable seeded bone marrow-derived cells (BMCs) to cover the luminal surface of the prostheses. Canine BMCs were used as a cell source instead of vascular-derived ECs to overcome the problems of limited availability of intact vasculature and the invasiveness during cell harvest, which results in morbidity at the donor sites (Kadner *et al.,* 2002). Seeding of BMCs on detoxified and untreated GA-fixed bioprosthetic tissues was investigated *in vitro*. GA-fixed porcine pulmonary valves were treated with a detoxifying agent to establish a more suitable surface for BMC attachment and proliferation by removing cytotoxic free aldehydes. The detoxified prostheses were seeded and recellularized *in vitro* with bone marrow-derived myofibroblast (MF)-like cells and EC-like cells for seven days. The attachment, proliferation, metabolic activity, and viability of the seeded cells were investigated and cell-seeded leaflets were histologically analyzed.

Materials and Methods

Detoxification of glutaraldehyde-fixed porcine valves

Commercially available GA-fixed porcine mitral valve prostheses (model = 342R, size = 33 mm, Hancock Heart Valve, Medtronic Inc., Houston, TX) were rinsed three times with sterile distilled water and transferred to Medium 199 (M199, Gibco BRL, Gaithersburg, MD). Valves were incubated for 24 h at 4^{o}C and then treated with 10% (w/v) citric acid solution (Sigma, St. Louis, MO) for 5 min to neutralize the cytotoxic effect of free aldehydes (Gulbins *et al.,* 2003). Residual reagents were removed by repeated washes in distilled water until neutral pH was achieved.

Culture of BMCs

Bone marrow (30 ml from each dog) was aspirated from the humeri of anesthetized mongrel dogs (20-25 kg) and immediately mixed with heparin (100 unit heparin/ml bone marrow). The mixture was centrifuged on a Ficoll-Paque (Amersham Bioscience, Arlington Heights, IL) density gradient for 20 min at 1,500 rpm. Mononuclear cells were isolated from the buffy coat layer and washed three times in PBS (Sigma). The mononuclear cell fraction containing smooth muscle (SM) α-actin-positive cells (MF-like cells) was cultured in M199 (Gibco BRL) containing 10% (v/v) FBS (Gibco BRL) and 1% (v/v) penicillin and streptomycin (Gibco BRL). The mononuclear cell fraction containing CD31-positive cells (EC-like cells) was cultured in EGM-2 (Clonetics, San Diego, CA).

Characterization of BMCs

Cultured BMCs were stained immunochemically using antibodies against SM α-actin (Clone 1A4, 1:100 dilution) (Dako, Carpinteria, CA) and CD31 (Clone JC/70A, 1:20 dilution) (Dako). Cultured cells were fixed with a 4% (v/v) paraformaldehyde solution for 30 min and permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 10 min. After treatment with 3% (v/v) hydrogen peroxide (Sigma), cells were incubated at room temperature with primary antibodies for two hours and with biotinylated anti-mouse IgG secondary antibody (Vector Laboratory, Burlingame, CA) for 30 min. Signals were developed using a streptavidin biotin universal detection system (UltraTech HRP, Immunotech, Marseille, France) and a 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution (Vector Laboratory).

Semi-quantitative reverse transcription (RT)-PCR

Total RNA was isolated from cultured EC-like cells

and MF-like cells using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized with 5 μg of pure total RNA using SuperScript™ II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) (Choi *et al.*, 2004). cDNA was amplified with a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA) using the primers in Table 1. PCR was performed for 30 cycles of denaturing (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 45 s) with a final extension at 72°C for 7 min. PCR products were visualized by electrophoresis on 2% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide and analyzed with a gel documentation system (Gel Doc 1000, Bio-Rad Laboratories, Hercules, CA).

Cell seeding and *in vitro* maintenance

Valves were sequentially seeded with MF-like cells (SM α-actin-positive cells) followed by EC-like cells (CD31-positive cells). SM α-actin-positive cells were uniformly seeded onto the valve leaflets at a cell density of 8.0×10^6 cells per prosthesis. Two hours later, CD31-positive cells were uniformly seeded onto the valve leaflets at a cell density of 2.0×10^6 cells per prosthesis. Seeded valve prostheses were maintained *in vitro* in cell culture flasks (Bellco Glass Inc., Vineland, NJ) containing Medium 199 supplemented with 2% (v/v) FBS, human vascular endothelial growth factor (10 ng/ml; PeproTech, Rocky Hill, NJ), and human basic fibroblast growth factor (2 ng/ml; PeproTech) for one week (Figure 1). Samples were taken one, four and seven days after cell seeding.

Table 1. Oligonucleotide primers used for RT-PCR and predicted product sizes.

Targets	Sequences	Product sizes (bp)	References
eNOS	F: 5′-TCA ACC AGT ACT ACA GCT CC-3′ R: 5′-GTG GTT GCA GAT GTA GGT GA-3′	251	Kalra *et al.,* 2003
KDR	F: 5′-TTC CTG ACC TTG GAG CAT CT-3′ R: 5′-AGT CCA GCA TGG TCT GGT AC-3′	430	Murata *et al.,* 2000
SM α-actin	F: 5′-GCC AAC CGG GAG AAA ATG AC-3′ R: 5′-TCC TGT TTG CTG ATC CAC AT-3′	743	Ishida *et al.,* 2000
SM MHC	F: 5′-CAG GGC AAC ATG GAG GC-3′ R: 5′-GCC TCT TGA GCT GCT TGA CC-3′	409	Ishida *et al.,* 2000
Ribosome s17	F: 5′-GAA GGC GGC CCG GGT CAT CA-3′ R: 5′-GTA GGC TGA GTG ACC TG-3′	339	Ishida *et al.,* 2000

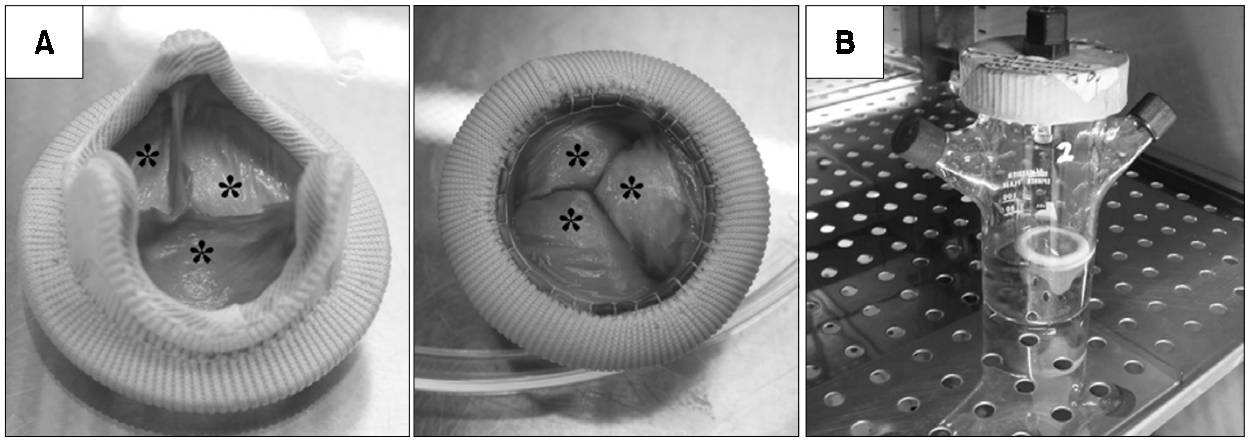

Figure 1. BMC seeding on detoxified GA-fixed heart valve prostheses. (A) Gross view of cell-seeded heart valve prostheses. Tri-leaflets (asterisks) are observed. (B) Cell-seeded prostheses were maintained *in vitro* in cell culture flasks for seven days.

Scanning electron microscopy

Scanning electron microscopy (SEM) of the valve leaflets was performed before and after cell seeding. Specimens were fixed, dehydrated and dried. Dried specimens were mounted on aluminum supports and coated with platinum using a Sputter Coater (Cressington Scientific Instruments Inc., Cranberry, PA). SEM (JEOL, Tokyo, Japan) was performed at 5 kV.

DNA quantification

To measure cell growth on the prostheses, the number of cells was determined by quantitative DNA assays performed in triplicate. DNA was isolated

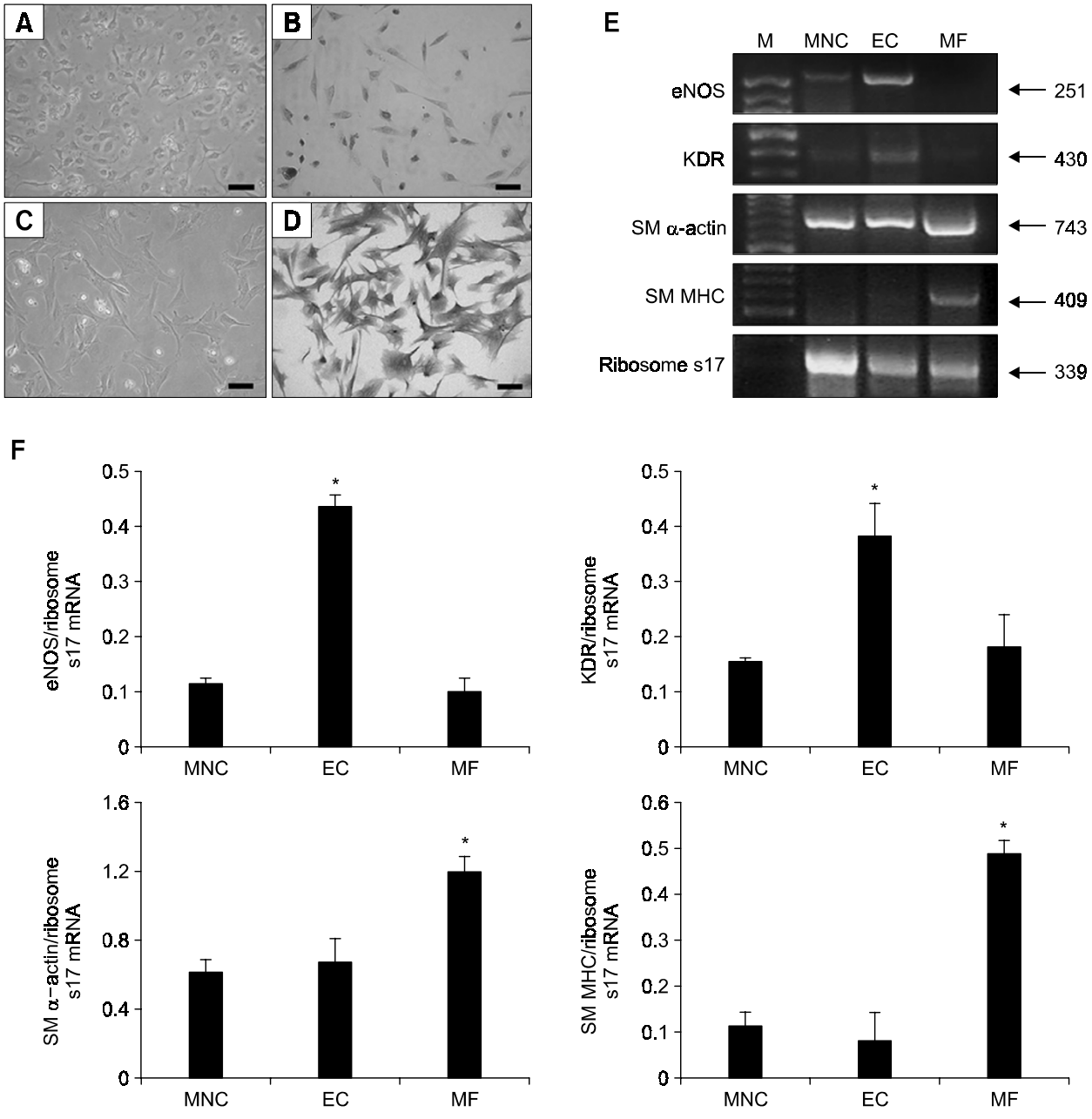

Figure 2. Characterization of cultured canine BMCs. BMCs cultured in EC culture condition showed the cobblestone morphology typical of ECs (A) and stained positively for CD31, a specific marker of ECs (B). BMCs cultured in MF cell culture condition had morphology similar to that of MFs (C) and stained positively for SM α-actin, a specific marker of MF (D). (E) The mRNA expression of markers of ECs (eNOS and KDR) and markers of MFs (SM α-actin and SM MHC) in BMCs cultured in EC-like and MF-like cell culture conditions. (F) Expression of each gene mRNA was normalized with house keeping Ribosomal s17 mRNA. The scale bars indicate 10 μm. *$P < 0.05$ compared with the other groups.

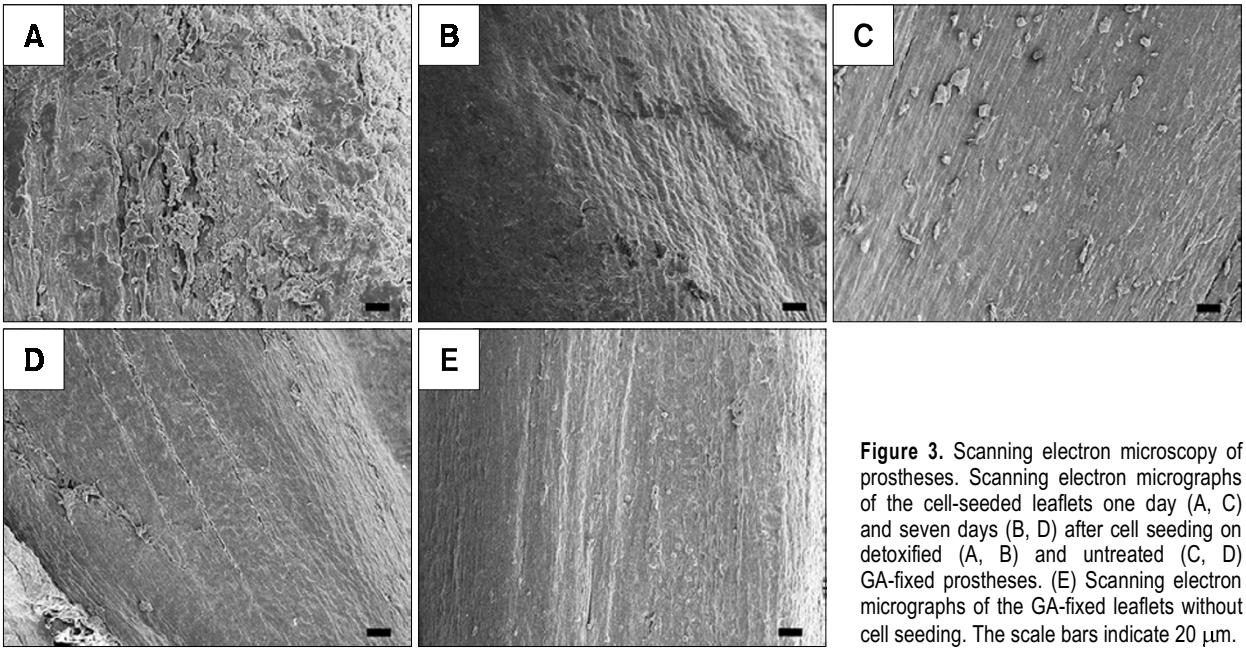

Figure 3. Scanning electron microscopy of prostheses. Scanning electron micrographs of the cell-seeded leaflets one day (A, C) and seven days (B, D) after cell seeding on detoxified (A, B) and untreated (C, D) GA-fixed prostheses. (E) Scanning electron micrographs of the GA-fixed leaflets without cell seeding. The scale bars indicate 20 μm.

using a Wizard Genomic DNA Purification kit (Promega, Madison, WI) according to the manufacturer's protocol. DNA content was measured with an ultraviolet absorbance spectrophotometer (UV-160A spectrophotometer, Shimadzu, Kyoto, Japan) at 260 nm. Cell numbers were calculated using a DNA standard curve for identical cells.

MTT assay

Mitochondrial metabolic activity of cells cultured on tissue culture plastics (TCP) and on GA-fixed prostheses was measured in triplicate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) assay (Liu *et al.*, 2005). MTT stock solution (1 mg/ml; Sigma) was added to cultured cells and incubated at 37°C for 4 h. Following removal of the medium, formazan precipitates were solubilized by the addition of 1 ml 0.1N HCl solution containing 10% (w/v) sodium dodecyl sulfate and 45% (w/v) dimethylformamide. Absorbance at 540 nm was measured using a microplate reader (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland).

Cell viability assay

The viability of cells seeded on the GA-fixed prostheses was measured by double staining using fluorescein diacetate (FDA, green fluorescence; Sigma) and ethidium bromide (EB, red fluorescence; Sigma) (Ryu *et al.,* 2004). The staining solution was prepared by combining 10 ml of FDA solution (5 mg/ml in acetone) and 5 ml of EB solution (10 μg/ml in PBS). Samples were incubated in FDA/EB solution for 5 min at 37°C, washed twice in PBS, and observed under confocal microscopy (Fluoview BX50, Olympus, Tokyo, Japan).

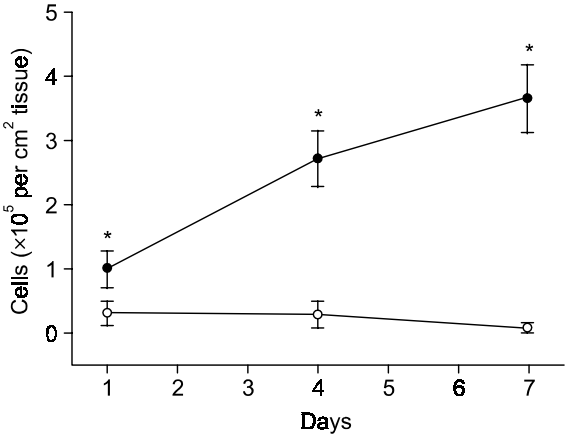

Figure 4. BMC proliferation on detoxified (solid circles) and untreated (open circles) GA-fixed prostheses. BMCs cultured on untreated prostheses showed decreased proliferation, while BMCs cultured on detoxified prostheses showed uninhibited proliferation. *$P < 0.05$ compared with untreated group.

Histological and immunohistochemical analyses

For histological analyses, specimens were fixed in 10% (v/v) buffered formaldehyde and dehydrated with an ascending series of graded ethanol. Spe-

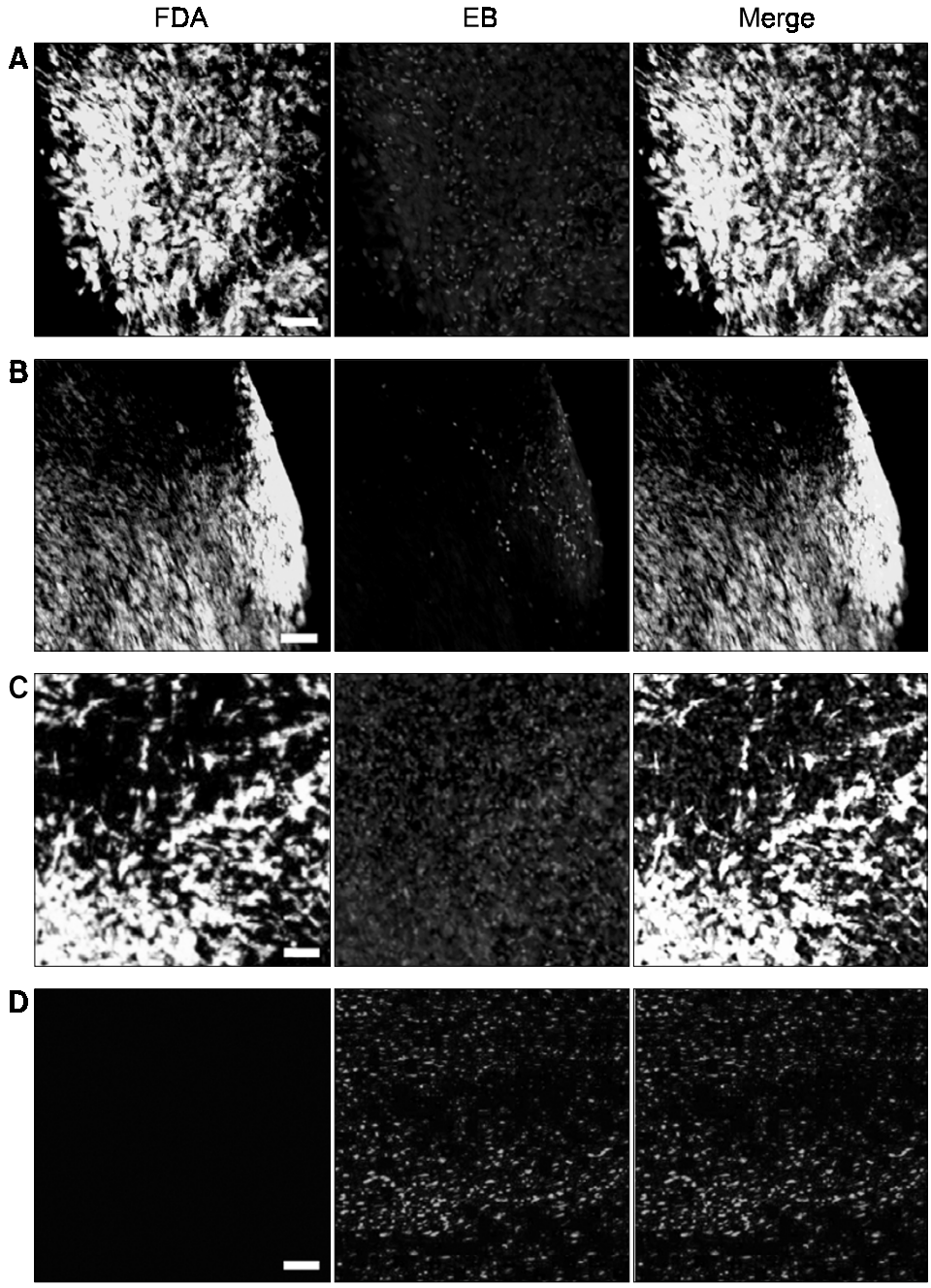

Figure 5. Confocal microscopy of cell-seeded leaflets of detoxified (A, B) and untreated (C, D) GA-fixed prostheses stained with FDA and EB one day (A, C) and seven days (B, D) after cell seeding. Figure in the far left column indicate viable cells (green signals and figures in the middle column indicate dead cells (red signals), respectively. The scale bars indicate 20 μm. All photographs were taken at the same magnification.

cimens were embedded in paraffin, sectioned at 4 μm, and processed for hematoxylin and eosin (H&E) staining. For immunohistochemical analyses, 4 μm-thick sections were stained using antibodies against CD31, SM α-actin, and PCNA (Clone PC10, 1:200 dilution; Dako). Sections were deparaffinized and incubated at room temperature with primary antibodies for two hours and biotinylated anti-mouse IgG secondary antibodies for 30 min. Staining signals were developed using the streptavidin biotin universal detection system and DAB substrate solution.

Results

Differentiation and characterization of BMCs

Cultured canine BMCs were able to differentiate into EC-like cells and MF-like cells *in vitro*. BMCs grown in the EC culture condition showed the characteristic cobblestone morphology of ECs (Figure 2A) and

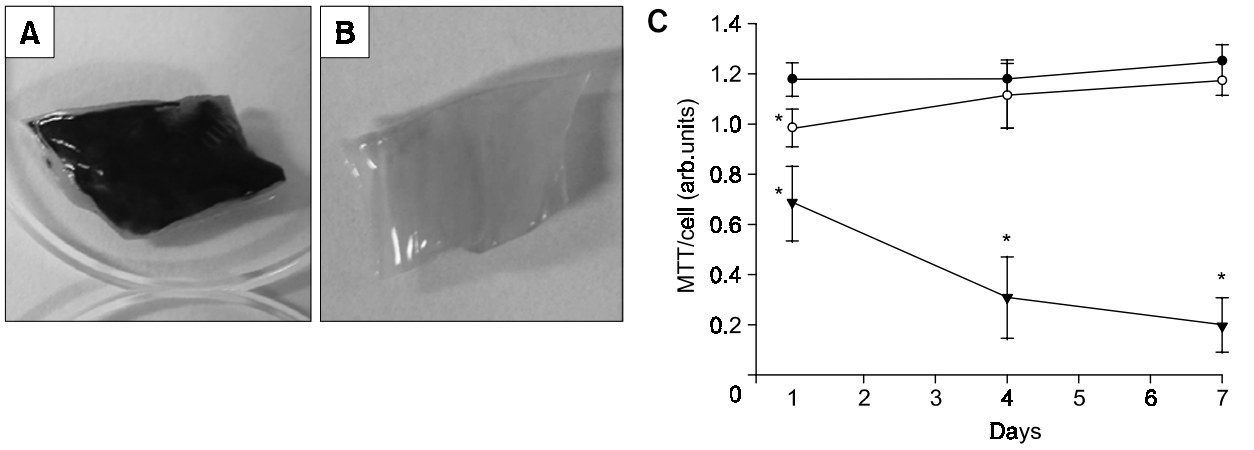

Figure 6. MTT staining of BMCs seeded on detoxified (A) and untreated (B) GA-fixed prostheses seven days after cell seeding. Formation of formazan precipitates was observed on detoxified prostheses (A), but not on untreated prostheses (B). (C) Metabolic activity of BMCs cultured on TCP (solid circles), detoxified (open circles), and untreated (solid inverted triangles) GA-fixed prostheses. *$P < 0.05$ compared with TCP.

stained positively for CD31, a marker of ECs (Figure 2B). BMCs grown in the MF culture condition had morphology similar to MFs (Figure 2C) and expressed SM α-actin, a marker of MFs (Figure 2D). The mRNA markers of ECs, endothelial nitric oxide synthase (eNOS) and kinase-insert domain-containing receptor (KDR), were expressed at a higher extent in the BMCs cultured in the EC-like cell culture condition than freshly isolated bone marrow MNCs and cells cultured in the MF-like cell culture condition. The mRNA markers of MFs, SM α-actin and SM myosin heavy chain (MHC), were expressed at a higher extent in BMCs cultured in the MF-like cell culture condition than freshly isolated bone marrow MNCs and cells cultured in the EC-like cell culture condition (Figure 2E and F).

Seeding on GA-fixed prostheses

GA-fixed valve prostheses, with or without detoxifying treatment, were seeded with EC-like cells and MF-like cells differentiated from bone marrow MNCs, and maintained *in vitro* for one week. Pretreatment of GA-fixed prostheses with 10% (w/v) citric acid improved the efficiency of BMC seeding. SEM analysis showed that BMC seeding on detoxified GA-fixed valves resulted in even cell attachment one day after seeding (Figure 3A) and formation of a confluent layer of cells over the surface of the valve leaflets after one week (Figure 3B). In contrast, BMC seeding on untreated GA-fixed valves resulted in scattered BMCs on the surface one day after seeding (Figure 3C) and disappearance of these cells after seven days (Figure 3D).

Cell proliferation on GA-fixed prostheses

Detoxified GA-fixed prostheses allowed adhesion and proliferation of seeded BMCs over a culture period of seven days. The initial cell seeding density of 3.0×10^5 cells/cm^2 resulted in 1.1×10^5 cells/cm^2 remaining attached to the detoxified GA-fixed valve after one day in culture, an adhesion percentage of 36.7% (Figure 4). For the untreated GA-fixed valve, the cell adhesion efficiency was 17.2%. Seeded BMCs also grew more rapidly in the detoxified GA-fixed valve than in the untreated GA-fixed valve. After seven days in culture, the average cell density of the detoxified GA-fixed valve was 3.7×10^5 cells/cm^2, while that of the untreated GA-fixed valve was 0.1×10^5 cells/cm^2, corresponding to a 236% increase and 90.9% decrease in cell density compared to the initial cell adhesion densities for the detoxified and untreated GA-fixed valves, respectively (Figure 4).

Cell viability on GA-fixed prostheses

Viability of BMCs seeded on GA-fixed prostheses was examined by fluorescent microscopy with differential staining for viable and nonviable cells: FDA stains the cytoplasm of viable cells green and EB stains the nuclei of nonviable cells orange-red. Confluent viable cells were present on the surface of detoxified GA-fixed prostheses one day after seeding (Figure 5A). Most of the cells showed FDA-positive staining (green fluorescence) seven days after cell seeding, indicating that they remained viable during the *in vitro* culture periods (Figure 5B). In contrast, cell viability decreased over time on untreated GA-fixed prostheses. One day after seeding on untreated valves, more than half of the cells

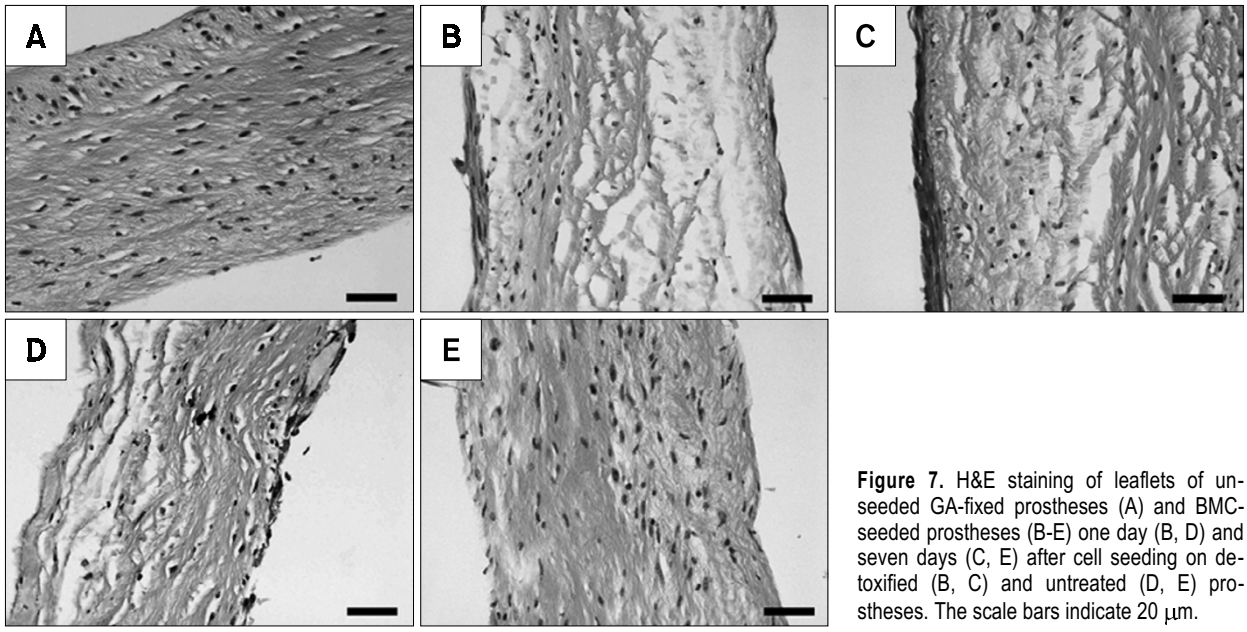

Figure 7. H&E staining of leaflets of unseeded GA-fixed prostheses (A) and BMC-seeded prostheses (B-E) one day (B, D) and seven days (C, E) after cell seeding on detoxified (B, C) and untreated (D, E) prostheses. The scale bars indicate 20 μm.

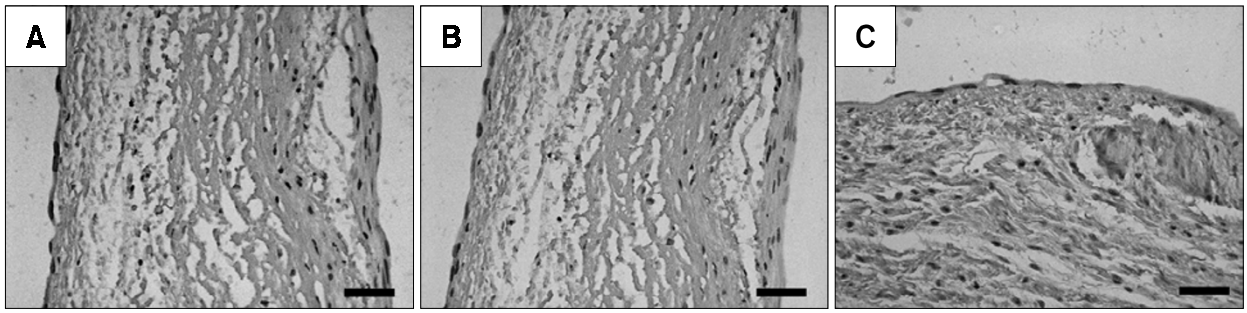

Figure 8. Immunohistochemical analysis of BMC-seeded detoxified prostheses seven days after cell seeding: (A) PCNA; (B) SM α-actin; (C) CD31. The scale bars indicate 20 μm.

stained with EB (red) and only a few cells showed FDA-positive staining, indicating few viable cells on the surface (Figure 5C). After seven days no viable cells were found on the untreated GA-fixed prostheses and all of the cells showed EB-positive staining (Figure 5D).

MTT assay

The MTT cytotoxicity assay showed the effect of the detoxifying treatment on the relative survival of BMCs on the GA-fixed valves, as measured by metabolic activity. BMCs cultured on detoxified prostheses showed a similar metabolic activity to those cultured on TCP, while BMCs cultured on untreated prostheses showed significantly reduced metabolic activity (Figure 6). These results verify the cytocompatibility of the detoxified GA-fixed prostheses. Cell survival and metabolic activity remain high on the detoxified GA-fixed prostheses for up to seven days after seeding.

Histological and immunohistochemical analyses

H&E staining of the valves one day after cell seeding showed that a high density of cells was present on the leaflets of the detoxified prostheses (Figure 7B), while only scattered BMCs were present on the leaflets of untreated prostheses (Figure 7D). Seven days after seeding, a stable and confluent cell layer was achieved on the detoxified prostheses (Figure 7C), but no cells were observed on the surface of the leaflets of the untreated prostheses (Figure 7E).

The cells lining the leaflet surfaces of detoxified GA-fixed valves stained positively for PCNA, a marker of proliferating cells, at seven days after *in vitro* culture (Figure 8A). PCNA positive cells were only observed on the leaflet surfaces and not in the

interstitial region. The cells on the leaflet surfaces also stained positively for SM α-actin, a MF marker, (Figure 8B) and for CD31, an EC marker (Figure 8C). However, positive staining for PCNA, CD31, and SM α-actin was not observed on the surface of untreated GA-fixed prostheses at seven days after seeding.

Discussion

Successful engineering of heart valve tissue requires a safe and reliable scaffold. Although GA-fixed valve prostheses have the disadvantage of cytotoxicity due to the fixation procedure, we used these prostheses as scaffolds for heart valve tissue engineering for the following reasons. The manufacturing process of these prostheses has been standardized; cross-linking by GA-fixation reduces immunogenicity; and the fixation process facilitates good mechanical properties and surgical handling (Gulbins *et al.,* 2003). Therefore, these prostheses have several potential advantages as tissue engineering scaffolds which could be applied for clinical use in the future.

Several groups have studied seeding of human ECs on biological valve prostheses. However, seeding on GA-fixed valves has yielded poor results, mainly due to continual release of toxic GA and remaining free aldehyde groups (Fischlein *et al.,* 1992; Hoffmann *et al.,* 1992; Bengtsson *et al.,* 1993; Fischlein and Fasol, 1996). In the present study, the initial seeding of BMCs on extensively rinsed untreated prostheses was successful, but the cells rapidly lost adhesion and did not survive seven days. The cytotoxicity of GA-fixed valves was significantly reduced by pre-incubation in cell culture medium and citric acid solution. During the GA-fixation process, GA covalently cross-links collagen fibers through the amino groups of the proteins, resulting in reduced immunogenicity and improved mechanical properties relative to fresh porcine aortic valves (Gulbins *et al.,* 2003). After fixation, unbound GA can be washed out by rinsing with cell culture medium, but GA covalently bounded with collagen fibers can not simply be washed out, and the free aldehyde groups of this remaining GA causes cytotoxicity. The cross-linking of collagen fibers by GA-fixation also results in a hydrophobic surface, thus reducing the capability of cells to adhere, since cells attaching through pseudopodia that use non-covalent bonds require a hydrophilic surface (Gulbins *et al.,* 2003). This hydrophobicity, combined with the toxicity of free aldehyde groups, results in poor adhesion and cell viability.

Our studies show that pretreatment with citric acid increased both cell adhesion and viability following seeding with BMCs. Citric acid reacts with free aldehyde groups, thus reducing cytotoxicity (Gulbins *et al.,* 2003). In addition, citric acid is a strong organic acid that binds to amino groups of the collagen fibers, increasing the hydrophilicity of the surface and enhancing cellular attachment (Gulbins *et al.,* 2003). Therefore, the detoxification treatment changed the surface properties of the prostheses to improve cellular adhesion.

Establishing a reliable cell source is also vital for the successful tissue engineering of heart valves. Various cell types have been used, including dermal fibroblasts (Shinoka *et al.,* 1997), vascular-derived cells (Hoerstrup *et al.,* 2000; Sodian *et al.,* 2000), and mesenchymal stem cells from bone marrow (Hoerstrup *et al.,* 2002; Kadner *et al.,* 2002; Perry *et al.,* 2003a; Perry and Roth, 2003b). However, dermal fibroblasts resulted in contracted and immobile leaflets (Shinoka *et al.,* 1997). Vascular-derived cells are the most frequently used and have produced positive results in previous attempts to fabricate tissue engineered heart valves (Hoerstrup *et al.,* 2000). However, this requires the sacrifice of intact tissues and additional invasive surgery to harvest cells. In addition, the characteristics of vascular-derived cells are different from those of valvular cells, which may affect the function of tissue-engineered heart valves (Flanagan and Pandit, 2003).

BMCs are an attractive source of cells for the development of tissue-engineered heart valves that may overcome the limitations of other cell sources. Bone marrow aspiration is less invasive, and cell isolation is associated with much lower morbidity at the donor sites, than blood vessel biopsy (Kadner *et al.,* 2002). Using patients' own BMCs, autologous valvular grafts could be constructed that may avoid immune rejection. Recently, a clinical case report demonstrated the feasibility of tissue engineering of human blood vessels with autologous bone marrow MNCs (Matsumura *et al.,* 2003). In addition, cell therapy using autologous bone marrow MNCs or bone marrow AC133-positive cells has been clinically tested in humans for neovascularization in limb ischemia (Tateishi-Yuyama *et al.,* 2002) or infarcted myocardium (Stamm *et al.,* 2003). These results demonstrate the applicability of BMCs as an autologous cell source in the clinical treatment of cardiovascular diseases. We have previously reported the tissue engineering of small-diameter blood vessels and vascular smooth muscles using BMCs (Cho *et al.,* 2004; 2005a; b), and several studies have used the bone marrow as a cell source for the tissue engineering of heart valves (Hoerstrup *et al.,* 2002; Kadner *et al.,* 2002; Perry *et al.,* 2003a). In the present study, the potential of BMCs to differentiate

into valvular cells has been shown, and the applicability of BMCs for the construction of tissue-engineered heart valves was verified. We have identified culture conditions that allow for the proliferation and differentiation of the multiple cell types necessary for valvular tissue regeneration. Genetic and immunochemical characterization demonstrated that cultured BMCs could differentiate into EC-like cells and MF-like cells by exposure to the appropriate culture conditions.

In summary, this study demonstrated the tissue engineering of biological valvular prostheses *in vitro* using detoxified GA-fixed prostheses and BMCs. The heart valves engineered with valvular cells induced from BMCs showed tissue regeneration with a CD31-positive layer. Before such recellularized valves can be used in clinical procedures, *in vivo* experiments are necessary to prove whether the seeded BMC layer improves biocompatibility and durability of the GA-fixed valve prostheses. Because physical stress on the valvular structures plays an important role in bioprosthetic degeneration, a large animal model such as an ovine model should be used to test the valves under physiological conditions. In addition, the method of cell seeding on prostheses needs to be further improved. Although the detoxified prostheses demonstrate improved biocompatibility, the initial cell adhesion ratio is low. This may be due to the immediate detachment of BMCs from the leaflets after cell seeding. It is possible that cell seeding and *in vitro* maintenance in a static culture condition is inefficient, and that dynamic cell seeding (Kim *et al.,* 1998) and preconditioning of the seeded prostheses in a pulsatile bioreactor might improve cell adhesion ratio and tissue regeneration. Further characterization of valvular cells differentiated from bone marrow MNCs is also necessary, and additional studies related to the growth, remodeling, durability, calcification and function of tissue-engineered heart valves with respect to long-term implantation are required.

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