Hypoxia Induces Near-Native Mechanical Properties in Engineered Heart Valve Tissue

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Background—Previous attempts in heart valve tissue engineering (TE) failed to produce autologous valve replacements with native-like mechanical behavior to allow for systemic pressure applications. Because hypoxia and insulin are known to promote protein synthesis by adaptive cellular responses, a physiologically relevant oxygen tension and insulin supplements were applied to the growing heart valve tissues to enhance their mechanical properties.

Methods and Results—Scaffolds of rapid-degrading polyglycolic acid meshes coated with poly-4-hydroxybutyrate were seeded with human saphenous vein myofibroblasts. The tissue-engineered constructs were cultured under normal oxygen tension (normoxia) or hypoxia (7% O₂) and incubated with or without insulin. Glycosaminoglycan production in the constructs approached that of native values under the influence of hypoxia and under the influence of insulin. Both insulin and hypoxia were associated with enhanced matrix production and improved mechanical properties; however, a synergistic effect was not observed. Although the amount of collagen and cross-links in the engineered tissues was still lower than that in native adult human aortic valves, constructs cultured under hypoxic conditions reached native human aortic valve levels of tissue strength and stiffness after 4 weeks of culturing.

Conclusions—These results indicate that oxygen tension may be a key parameter for the achievement of sufficient tissue quality and mechanical integrity in tissue-engineered heart valves. Engineered tissues of such strength, based on rapid-degrading polymers, have not been achieved to date. These findings bring the potential use of tissue-engineered heart valves for systemic applications a step closer and represent an important improvement in heart valve tissue engineering. (Circulation. 2009;119:290-297.)

Key Words: tissue engineering ▪ heart valves ▪ hypoxia ▪ insulin ▪ collagen ▪ tissue scaffold

Tissue-engineered (TE) heart valve replacements appear promising as autologous valvular substitutes that possess the ability to grow, adapt, and remodel, which is particularly relevant for pediatric and young adult patients. These attractive features distinguish TE valves from currently used valvular prostheses, which consist of foreign-body material and are nonviable. TE heart valves have been implanted successfully in the pulmonary position in sheep, showing a native-like function and tissue composition after 5 months.1,2 However, the valves did not show sufficient mechanical strength for systemic pressure applications.

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In native human aortic valves, the features responsible for maintaining the mechanical integrity and functionality of the tissue are the anisotropic mechanical and structural properties and the mature, well-developed collagen architecture present in the valve leaflets.3-5 The maturity of collagen is determined by the presence of collagen cross-links, which have been shown to directly influence the stiffness of human native aortic valve leaflets.6 So far, these critical features have not been sufficiently mimicked in engineered heart valves. Recently, a novel in vitro conditioning strategy was developed that mimics the diastolic phase of the cardiac cycle. This resulted in the fabrication of TE valves, based on a rapid-degrading polymer scaffold, that could withstand physiological systemic pressures during a 4-hour period in an in vitro setup.6 These dynamically conditioned valves were associated with considerable improvement in tissue quality and the development of anisotropic mechanical properties after 4 weeks of culturing in a bioreactor compared with a static control. Although these results are promising, native levels of tissue modulus and strength were not achieved in the engineered heart valves. Therefore, in addition to dynamic conditioning, alteration of the cellular niche by additional environmental and biochemical cues may further enhance tissue mechanical properties, and such was the aim of the present study.

Environmental factors of interest are the level of oxygen supplied during culturing and the addition of insulin. To date, growing valves were exposed to atmospheric air, which provided the engineered tissue with considerably more oxygen than under in vivo conditions.7 Various studies in cell cultures have demonstrated...
an upregulation of collagen on mRNA and protein expression level associated with hypoxia, ie, low oxygen tension. Similar to the described hypoxic responses of cells, insulin was also reported to enhance gene expression and protein synthesis of collagen in various cell types, gene expression of collagen cross-linking enzymes, and the synthesis of elastic fibers.

In the present study, the individual and combined contributions of hypoxia and insulin were evaluated for their potential to promote tissue development in a model system of TE heart valve equivalents based on human saphenous vein cells and a rapid-degrading polymer scaffold. The extracellular matrix composition and mechanical behavior of the engineered tissues were compared with those of native human aortic valves.

Methods

Tissue Engineering of Heart Valve Constructs

Myofibroblasts, harvested from the human vena saphena magna and expanded by regular cell culture methods, were used as described previously. Characterization of the cells used for seeding was performed previously. Before seeding, all cells showed expression of vimentin. None showed expression of desmin, and a subpopulation showed expression of α-smooth muscle actin, which characterized them as a mixture of V- and VA-type myofibroblasts. The culture medium consisted of DMEM advanced (Gibco/Invitrogen Corp, Carlsbad, Calif) supplemented with 10% fetal bovine serum (Biochrom, Bremen, Germany), 1% Glutamax (Gibco), and 0.1% gentamicin (Biochrom). Cultures were maintained in a humidified incubator at 37°C and 5% CO₂, and the medium was replaced every 3 to 4 days. Before seeding, the (passages 6 to 8) were enzymatically detached with trypsin-EDTA (Lonza, Verviers, Belgium). Scaffold preparation was performed as described previously. Briefly, rectangular strips (5×35×1 mm) of rapid-degrading nonwoven polyglycolic acid meshes (PGA; Cellon, Bereldange, Luxembourg) were coated with a thin layer of poly-4-hydroxybutyrate (P4HB; TEPHA Inc, Cambridge, Mass). After a centrifuging step, the cells were resuspended in a fibrin gel, which served as a cell carrier, and were evenly distributed over the rectangular scaffold strips. To create static strain conditions, which have been shown to be beneficial for tissue development, the scaffolds were attached at the ends to the bottom of 6-well plates with polyeurethane-tetrahydrofuran glue (20% wt/vol). The medium for tissue culture was supplemented with additional gentamicin (0.3%) and L-ascorbic acid 2-phosphate (0.25 mg/mL). The latter was used to promote extracellular matrix production. The cell-seeded scaffolds will be further referred to as TE constructs.

Experimental Design

Four experimental groups were studied. The control group and the insulin group were both cultured under atmospheric air conditions (normoxia), whereas the insulin-plus-hypoxia group and the hypoxia groups were cultured under low oxygen tension, or hypoxia. TE constructs for the control group were cultured separately from the constructs for the insulin, insulin-plus-hypoxia, and hypoxia groups. All constructs were cultured according to the same protocol, with culture conditions kept strictly identical to those used in the other groups (ie, cell passage, cell seeding density, scaffold, and media supplements from identical batches). Because aortic valve leaflets are avascular tissue structures, oxygen transport to valvular cells occurs primarily through oxygen diffusion from the aortic blood. The oxygen concentration in arterial blood, which is 130 μmol/L, was therefore considered physiologically representative in the culture medium. The conversion of oxygen concentration (mmol/L) to partial oxygen pressure (mm Hg) in medium, which is very close to that of water, was calculated with Henry’s law using the coefficient of oxygen solubility in water (α), as follows:

\[ \text{CO}_2_{\text{medium}} = \alpha \cdot \text{PO}_2 \]

where \( \alpha = 1.39 \cdot 10^{-3} \text{ mmol} \cdot \text{L}^{-1} \cdot (\text{mm Hg})^{-1} \).

Using the formula, the oxygen tension (mm Hg) in culture medium could be calculated from the oxygen concentration (130 μmol/L) and was 93 mm Hg. Previously, Anderson et al investigated the diffusion of oxygen from gas to culture media at various partial pressures. From these data, the oxygen pressure in air required to obtain an oxygen tension of 93 mm Hg in the medium was estimated at 53 to 57 mm Hg, which corresponds to 7% to 7.5% O₂. Therefore, a gas mixture consisting of 7% O₂, 5% CO₂, and 88% N₂ was supplied to the hypoxia groups. Despite the claim that this oxygen concentration may be more representative of physiological conditions, in the present study, 7% O₂ levels are termed hypoxic and 20% O₂ normoxic, for consistency with conventional terminology in cell culture studies.

The control group was cultured with normal tissue culture medium; the culture medium for the insulin groups was supplemented with insulin (2.5 μg/mL). The insulin concentration was determined from the literature and confirmed by metabolic activity tests of a range of concentrations (data not shown). The hypoxia groups were placed in a closed bell jar (Figure 1), which was flushed with the hypoxic gas mixture daily. In all groups, constructs were sacrificed for analysis at 2 and 4 weeks (n=5 per group at each time point). During culturing, the constructs were placed on a shaking table to promote gas exchange and diffusion.

Mechanical Behavior

After 2 and 4 weeks of culturing, the mechanical properties of the TE constructs were measured with a uniaxial tensile tester equipped with a 20-N load cell (Kammrath & Weiss, Dortmund, Germany). The dimensions of the rectangular constructs were measured with an optical imaging profiler (SensoFar PLμ 2300, SensoFar-Tech, Barcelona, Spain), and each sample was tested at a speed of the initial length (l₀) per minute. The ultimate tensile stress, indicative of tissue strength, was assessed from the stress-strain curves. The slope of the linear part of the curve represented Young’s modulus of elasticity (indicative of tissue stiffness). These parameters were compared with averaged tensile data in the circumferential direction of native adult human aortic valve leaflets from previous work.

Tissue Composition of TE Constructs

After tensile testing, biochemical assays were performed on the TE constructs to evaluate the extracellular matrix composition. For DNA and glycosaminoglycan (GAG) analyses, lyophilized tissue samples were digested in papain solution (phosphate buffer, L-cysteine, EDTA, and papain) at 60°C for 16 hours. DNA content was determined with the Hoechst dye method and a standard curve from calf thymus DNA. GAG content was determined with a modification of the assay described by Farnacle et al and a standard curve from chondroitin sulfate from shark cartilage.
cartilage. For collagen analysis, the digested samples were hydrolyzed in 6 mol/L HCl (Merck, Darmstadt, Germany). Hydroxyproline residues were measured on the acid hydrolysates by reverse-phase high-performance liquid chromatography after derivatization with 9-fluorenlymethyl chloroformate (FMOC; Fluka, Buchs, Switzerland). The same hydrolysates were used to measure the number of mature collagen hydroxylysyl pyridinoline cross-links by high-performance liquid chromatography, as described previously. The number of hydroxylysyl pyridinoline cross-links was expressed per collagen triple helix. Hydroxyproline and GAG content were normalized to dry weight and to the amount of DNA to obtain a measure for the amount of matrix components produced per cell. The tissue properties of the engineered constructs were compared with native tissue values obtained from human adult aortic valves previously used for mechanical testing.

**Histology**

To evaluate the development of tissue formation over time in the TE constructs, histology was performed on the engineered tissues and on adult human aortic valve tissue, to serve as a reference. The constructs were fixed with 3.7% formaldehyde (Merck) and embedded in paraffin. Tissue sections of 10-μm thickness were cut and stained with Safranin-O/Fast Green to visualize sulfated GAGs and collagens. To study cellular phenotypes in the engineered tissues, immunohistochemical stainings with antibodies against vimentin, desmin, and α-smooth muscle actin were performed on tissue sections from all groups. Cell nuclei were stained with DAPI.

**Statistical Analysis**

Descriptive statistics (mean±SD) were derived for collagen content, cross-links, and mechanical properties (modulus and ultimate tensile stress). Statistical differences between the groups were determined for both time points by univariate ANOVA, followed by Bonferroni post hoc tests. Correlations between collagen and cross-link amounts and mechanical properties were investigated among all groups with Pearson’s correlation test. Differences with \( P \leq 0.05 \) were considered significant. Statistical analysis was performed with SPSS 13.0 software (SPSS Inc, Chicago, Ill).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

### Results

**Effect of Insulin and Hypoxia on Tissue Composition**

An overview of all tissue properties is presented in the Table. The addition of insulin in the medium under normoxic conditions resulted in significantly greater GAG and collagen production (Figure 2) and more collagen cross-linking (Figure 3) in the constructs after 2 weeks of culturing compared with the control group. This difference in collagen production and cross-linking disappeared after 4 weeks; however, the weight percentage of collagen remained higher. Culturing under hypoxic conditions induced an increase in collagen per DNA, as well as cross-link production, after 2 and 4 weeks compared with the control group. In the combined hypoxia and insulin group, more collagen and more cross-links were observed after 2 and 4 weeks, and GAG production was increased after 4 weeks compared with the control group. Both hypoxic groups showed less collagen and GAGs (per DNA and per dry weight) than the insulin group after 2 weeks. This difference was absent after 4 weeks. The number of cells in all treated groups was higher than in the control group after 4 weeks of culturing.
Effect of Insulin and Hypoxia on Mechanical Behavior

The tissue stiffness (modulus) and strength (ultimate tensile stress) for all groups are shown in Figures 4 and 5, respectively. The insulin group showed an initial increase in tissue stiffness after 2 weeks of culturing. Remarkably, after 4 weeks, this no longer differed from the control group. The hypoxia group showed no difference in stiffness or strength after 2 weeks compared with the normoxic control group; however, after 4 weeks, higher values for tissue stiffness and strength were found. The addition of insulin under hypoxic conditions resulted in stiffer tissue after just 2 weeks compared with the control and hypoxia groups. After 4 weeks, tissue stiffness and strength were distinctly greater than in the control group, but they were not different from the hypoxia group. After 4 weeks of culturing, a positive linear correlation was identified between collagen cross-links and tissue stiffness and between collagen amount and tissue stiffness. The mechanical data furthermore showed that the effect of hypoxia was most pronounced in the last 2 weeks of culturing, during which an increase in stiffness and strength was observed in the hypoxia groups.

Comparison of Engineered Tissue With Native Human Aortic Valves

The stress-strain curves of the engineered tissue strips in the present study and of native human aortic valve leaflets

Figure 2. Evolution of matrix properties over time. GAG/dw indicates sulfated GAGs (µg) per dry tissue weight; Hyp/dw, hydroxyproline (µg) per dry weight; and Ins, insulin. After 2 weeks, the insulin-treated tissues contained the highest amounts of hydroxyproline and GAGs of all groups. After 4 weeks of culturing, the GAG, hydroxyproline, and cross-link concentrations in the hypoxic groups had increased considerably relative to both normoxic groups. *Difference vs control group; †difference vs insulin group. Single or double symbols indicate $P<0.05$ or $P<0.01$, respectively.

Figure 3. Evolution of cross-link concentration over time. HP/TH indicates the number of collagen cross-links expressed per collagen molecule; Ins, insulin. Higher collagen cross-link concentrations were found in the hypoxic groups than in the control group after 2 and 4 weeks. The number of cross-links in the insulin groups was much higher after 2 weeks of culturing, after which it appeared to stabilize. *$P<0.05$ and **$P<0.01$ vs control group, respectively.

Figure 4. Tissue stiffness (E) in the engineered constructs after 2 and 4 weeks of culturing. After 2 weeks, both insulin-treated groups, under normal and hypoxic conditions, showed a significant increase in tissue stiffness compared with the control group. After 4 weeks, both groups cultured under hypoxic conditions showed a distinct increase in tissue stiffness compared with the untreated control, in contrast to the insulin group. The bold line indicates the average stiffness of human native aortic valve leaflets in the circumferential direction. Statistical comparisons were performed only between groups from the same culturing period. *Difference vs control group; †difference vs insulin group; ‡difference vs hypoxia group. Single or double symbols indicate $P<0.05$ or $P<0.01$, respectively. Ins indicates insulin.

Figure 5. Ultimate tensile strength (UTS) in the engineered constructs after 2 and 4 weeks of culturing. After 2 weeks, the UTS did not differ among the groups. A dramatic increase in tissue strength was observed in the hypoxic groups after 4 weeks compared with the normoxic control. The bold line indicates the average UTS of human native aortic valve leaflets in the circumferential direction. Statistical comparisons were performed only between groups from the same culturing period. *Difference vs control group; †difference vs insulin group; ‡difference vs hypoxia group. Single or double symbols indicate $P<0.05$ or $P<0.01$, respectively.
Figure 6. Stress-strain curves of engineered tissue strips after 4 weeks of culturing. The control group (a) and the insulin group (b) were both cultured under normoxic conditions. The hypoxic groups, cultured with (c) and without (d) the addition of insulin, showed improved tissue stiffness and strength. The bold, dashed line overlaid in each graph indicates the stress-strain curve of human aortic valve leaflets in the circumferential direction (data obtained from Balguid et al).
Discussion

To ensure durable functioning of TE valves, we believe that the complex anisotropic mechanical behavior of native aortic valves should be realized (ie, they should be much stiffer and stronger in the circumferential than in the radial direction of the valve leaflet), in association with a highly organized collagen matrix. Although mechanical anisotropic behavior was shown to be achieved in TE valves by mechanical conditioning during culturing, the tissue stiffness and strength needed improvement.\(^6\) Both insulin and hypoxia have previously shown stimulatory effects on engineered tissue equivalent in terms of matrix production,\(^8–10,12\) and this may be a key factor in attaining native properties in TE heart valves. The aim of the present study was to enhance tissue development during culturing by the addition of insulin to the culture medium and by reducing the oxygen level to physiologically relevant values.

Under normoxic conditions, the addition of insulin enhanced the tissue matrix and mechanical properties, particularly during the first 2 weeks of culturing. The effects of hypoxia on these parameters, however, were much more pronounced during the last 2 weeks of culturing. The addition of insulin did not result in any further optimization of tissue properties during this period, which suggests that hypoxia alone can trigger this accelerated increase in tissue development in a later stage. Although the exact mechanism behind the cellular response to insulin is not exactly clear, insulin was shown to stimulate proliferation and collagen production of collagen-synthesizing cells.\(^24\) Furthermore, an insulin-induced increase in mRNA expression of collagen I and lysyl oxidase (a cross-linking enzyme) and an inhibiting effect on
collagen mRNA were described previously in fibroblast studies. In the present experiments, the addition of insulin indeed resulted in higher collagen production per cell and a greater absolute amount of collagen, specifically in the first 2 weeks of culturing, with increased proliferation after 4 weeks compared with the control group. Insulin was further reported to enhance the synthesis of elastic fibers. In the present study, mature elastic networks could not be detected in any of the groups, but because it is an essential matrix component for the mechanical behavior of heart valves, obtaining elastin in TE constructs will be an important aspect of future studies.

The addition of insulin induced a substantial increase in GAG production. The gel-like GAG molecules may adopt the function of fibrin in this system and help retain matrix proteins, including other GAGs in the tissue matrix. In the present study, no histological analysis of the hyaluronic acid content or of differences between the classes of sulfated GAGs was performed, because that was beyond the scope of the study. However, from the literature, it is clear that this may be of interest, because it has been demonstrated that both the pattern of disaccharide sulfation and the production of hyaluronic acid vary in response to mechanical loading conditions. Because each class of GAGs regulates specific biological functions, there are certainly implications for tissue engineering applications. Low oxygen concentrations may also influence the pattern of disaccharide sulfation and the production of hyaluronic acid in a manner similar to that of mechanical conditioning; however, this would require a separate study.

Interestingly, the hypoxic insulin group did not show an increase in GAG production. This discrepancy may be explained by the impact of scaffold degradation on cell metabolism. Under normal culture conditions, the scaffold degrades within 2 to 4 weeks, showing a drastic decline in mechanical integrity after 7 days of culturing (data not shown). The hypoxic environment may have induced a moderately higher acidity in the culture medium. This could accelerate scaffold degradation and further decrease the pH in the initial culture period. An acidic environment has been reported to interfere with tissue development via suppression of cell metabolism and a negative effect on GAG and collagen biosynthesis. Despite the argument that hypoxia enhanced collagen cross-linking in as little as 2 weeks, one might speculate that hypoxic conditions should be postponed until a significant part of the scaffold has degraded, to avoid the effects of acidity.

The present study demonstrated that by culturing under low oxygen tension, engineered heart valve tissue attained the mechanical characteristics of native adult aortic valves. These results were achieved in a model system of rectangular tissue strips, which allowed for simultaneous testing of various conditioning parameters (biochemical and mechanical) in a large number of samples under strictly controlled culture conditions. Obviously, caution is required in the extrapolation of these data to heart valve geometry. However, tissues of such strength have not been achieved previously, and hence, these findings are a major step forward in heart valve tissue engineering.

The tissue stiffness and strength of treated groups were comparable to native values. Native valve leaflets appeared slightly more flexible than the hypoxic engineered tissues in the lower strain range (0% to 10%). Dynamic conditioning in a strain-based bioreactor was demonstrated previously to result in anisotropic mechanical behavior. Because this system imposes constant movement on the engineered valve, it may additionally result in improved flexibility in the lower strain range.

Lesser concentrations of matrix proteins were measured in the engineered tissues than in human native valves. Yet, the mechanical properties of the engineered tissues were similar to those of adult native tissue. This discrepancy may be explained by the difference in matrix structure between the engineered and native tissues. Native aortic valves consist of a 3-layered tissue matrix, of which 1 layer, the fibrosa, bears the largest part of the load during the cardiac cycle. In the engineered tissues, the collagen distribution is relatively homogeneous, which allows a relatively homogeneous stress distribution over the tissue.

In the present study, engineered tissues were compared with adult human aortic valves as a benchmark. For future clinical applications, it is questionable whether TE valves need to resemble the 3-layered native valve structure before implantation, because prenatally, aortic valves show a homogeneous matrix distribution, mainly consisting of proteoglycans. Not until after birth does a 3-layered structure in the valve gradually develop, together with collagen fiber alignment and maturation. In addition, remodeling was observed in TE heart valves that were implanted in the pulmonary position in sheep. Twenty weeks after implantation, the initially homogeneous collagenous engineered tissue developed into a native-resembling 3-layered tissue structure. Although care must be taken in the extrapolation of these results to patient application, these in vivo results provide promise for the functionality of TE heart valves.

The results presented here strongly indicate that oxygen tension is a key parameter that promotes the development of human venous myofibroblasts into heart valve tissue equivalents. The development of a hypoxia heart valve bioreactor system, to combine hypoxia with mechanical conditioning, is a topic of ongoing research. This would further allow for evaluation of the effect of hypoxia on functional behavior, tissue composition, collagen orientation, and mechanical behavior in a valvular geometry.

Conclusions
The results presented here demonstrate for the first time that mechanical properties comparable to those of native aortic valve tissue can be achieved under hypoxic culturing conditions during a 4-week culturing period. These findings bring the potential use of engineered heart valves for systemic applications a step closer and can be considered an important improvement in heart valve tissue engineering.

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Disclosures

None.

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