Enhancing mechanical properties of tissue-engineered constructs via lysyl oxidase crosslinking activity

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Abstract: A number of strategies have been investigated to enhance the mechanical stability of engineered tissues. In this study, we utilized lysyl oxidase (LO) to enzymatically crosslink extracellular matrix (ECM) proteins, particularly collagen and elastin, to enhance the mechanical integrity of the ECM and thereby impart mechanical strength to the engineered tissue. Vascular smooth muscle cells (VSMCs) were liposomally transfected with the LO gene. Both Northern and Western analyses confirmed increased LO expression. Increased LO activity was demonstrated using a fluorescent enzyme substrate assay and by observation of the presence of increased levels of desmosine, a product of LO crosslinking, in the ECM. The mechanical effects of altered crosslink densities within tissue-engineered constructs were demonstrated in a VSMC-populated collagen gel model. When smooth muscle cells transfected with lysyl oxidase were seeded in collagen gels, the tensile strength and elastic modulus in these constructs increased by approximately two-fold compared to constructs seeded with mock-transfected VSMCs. Also, desmosine levels in the LO-populated collagen gels were higher than they were in mock-seeded gels, as demonstrated via immunohistochemical staining. Compositional analysis of the ECM deposited by the transformed cells showed similar collagen and elastin levels, and cell proliferation rates were similar as well, thus attributing increased mechanical properties to ECM crosslinking. J Biomed Mater Res 66A: 513–521, 2003

Key words: tissue engineering; vascular grafts; lysyl oxidase; gene therapy; ECM; biomechanics

INTRODUCTION

Tissue engineering has been successful in mimicking the histologic and biochemical appearance of various natural tissues as well as mimicking many tissue functions. Scaffold materials are intended to encourage the growth, migration, and organization of tissue cells, providing support while the tissue is forming. In time, scaffolds should degrade to make room for tissue to form. Ideally the scaffolds will be replaced with host cells and new ECM, which in turn should provide functional and mechanical properties similar to native tissue.

Unfortunately, mechanical properties remain a challenge in many applications, including vessels,[1] nerves,[2] skin,[3] cartilage,[4] and bone.[5] Thus the practical applications of tissue engineering have been limited. Increasing extracellular matrix production though the introduction of stimulatory factors, such as transforming growth factor-β[6] (TGF-β), or subjecting engineered scaffolds to cyclic mechanical strain conditions[7] have been shown to enhance tissue integrity. Glycation, the nonenzymatic crosslinking of ECM proteins that is brought about by reducing sugars, previously has been proposed as a strategy for enhancing the stiffness and strength of tissue equivalents.[8] Since the ECM plays an important role in a tissue's mechanical integrity, crosslinking of the ECM may be an effective means of improving the mechanical properties of constructs for tissue-engineering applications.

ECM crosslinking can result from the enzymatic activity of lysyl oxidase (LO), tissue transglutaminase,[9] or nonenzymatic glycation.[8] LO is a copper-dependent amine oxidase responsible for the formation of lysine-derived crosslinks in connective tissue, particularly in collagen and elastin.[10] Desmosine, a product of LO-mediated crosslinking of elastin, commonly is used as a biochemical marker of ECM crosslinking.[11] The LO-catalyzed crosslinks are present in various connective tissues within the body, including bone, cartilage, skin, and lung, and are be-

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lieved to be a major source of mechanical strength in tissues.

Additionally, the LO-mediated enzymatic reaction renders crosslinked fibers less susceptible to proteolytic degradation.[1] An example of the effect of altered extracellular matrix crosslinking on mechanical properties can be seen in the thoracic aortas of spontaneously hypertensive rats (SH). Such aortas are significantly less compliant than are those of normotensive animals[13] because of the elevated activity of lysyl oxidase in the SH smooth muscle cells. Also, tissue equivalents treated with β-aminopropionitrile (BAPN), a LO inhibitor, display significantly reduced mechanical properties.[8]

In the present study, we investigated a novel strategy for enhancing the mechanical performance of potential tissue-engineered scaffolds. We hypothesized that LO would form crosslinks within newly deposited extracellular matrix of engineered tissue, resulting in a structure with enhanced mechanical properties. Vascular smooth muscle cells genetically engineered to produce LO were incorporated into collagen gel scaffolds and subjected to dynamic tensile testing after *in vitro* culture. This novel strategy successfully demonstrated enhancement of the mechanical properties of the tissue-engineered construct.

MATERIALS AND METHODS

Cell culture procedures

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aortas of adult Sprague-Dawley rats, as previously described.[14,15] VSMCs (passage 3–4) were maintained in low glucose (1g/L) of Dulbecco's modified Eagle's medium (DMEM, Sigma) that contained 10% fetal bovine serum (FBS, Whittaker), 2 m*M* of L-glutamine, 1 unit/mL of penicillin, and 1 μg/mL of streptomycin (GPS, Sigma). Cell number was determined by Coulter counting (Beckman Coulter, Multisizer™ 3). For certain experimental sets, the media was supplemented with 150 μg/mL of β-aminopropionitrile (BAPN, Sigma) for inhibition of lysyl oxidase activity.

Transformation of smooth muscle cells

Rat lysyl oxidase cDNA previously was cloned and generously provided by Dr. Herbert Kagan.[16] The 1680-bp transgene was cloned into a commercially available mammalian expression vector, pcDNA3.0 (Invitrogen). VSMCs were seeded at 75,000 cells/well into 6-well plates. Twelve h following seeding, 3 μL of Fugene 6 cationic liposome formulation (Roche Biochemical) were added to 100 μL of serum-free DMEM and allowed to incubate at room temperature for 5 min. Three μg of plasmid DNA then were added to the mixture and allowed to incubate at room temperature for 15 min. The Fugene 6/DNA complex then was added to each well. Cells were incubated with Fugene 6/DNA complex for 9 h, and fresh medium then was added. As a control, mock wells received the plasmid DNA vector with no lysyl oxidase cDNA.

RNA isolation and hybridization

Total RNA was isolated from cultured VSMCs with Trizol™ reagent (Invitrogen) 4 days post-transfection. The RNA was fractionated on a formaldehyde–agarose gel, transferred to a nitrocellulose filter, and immobilized on the filters by UV irradiation. The filters were hybridized with a randomly primed ^{32}P-labeled LO cDNA probe corresponding to a 1.6-kb fragment. The filters were washed twice in 0.325*M* of NaCl, 0.03*M* of sodium citrate, 0.1% sodium dodecyl sulfate (SDS), pH 7.0, buffer at 42°C, and then twice with 32.5 m*M* of NaCl, 3.6 m*M* of sodium citrate, and 0.1% SDS, pH 7.0, at 60°C. The filters were examined by autoradiography with Kodak XAR film at −80°C for 24 h. Lysyl oxidase mRNA expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western analysis

LO- and mock-transfected SMCs were seeded at 5000 cells/cm^2 in 24-well plates and cultured to confluency. The medium was removed and the cells were washed three times in PBS. Blue lysis buffer [0.16*M* of tris-HCl, pH 6.8; 20% (v/v) glycerol; 4% SDS (w/v); 0.005% bromophenol blue; and 0.02% β-mercaptophenol] was used to lyse the cells and solubilize cellular proteins. The BCA protein assay kit (Pierce) was used to determine protein concentration within the samples.

Thirty μg of total protein were separated on a 10% SDS-PAGE gel and blotted to a Hybond-ECL nitrocellulose membrane (Amersham-Pharmacia Biotech). A protein ladder was used to confirm the correct band size of LO at 32 kDa. The membrane was stained with amido black (Sigma) to visualize the protein ladder. The membrane was treated with 5% milk reagent in tris-buffered saline Tween (TBST) overnight at 4°C and incubated with rabbit anti-lysyl oxidase for 3 h at room temperature. A 20mer peptide corresponding to the N-terminal 20 amino acids of LO was used to raise this antibody in rabbits (Research Genetics, Inc.). The serum then was purified using a HiTrap affinity column (Amersham Pharmacia) and used at a 1:250 dilution. After five washes (5 min each) with TBST, peroxidase-labeled goat anti-rabbit IgG (BioRad) was used at a 1:5000 dilution and incubated for 1 h at room temperature. The membrane was washed again in TBST and treated with enhanced chemiluminescence reagent (Amersham) for detection.

Fluorometric peroxidase assay

H_2O_2 is a product of LO deamination reaction, and its presence provides a measurement of the activity of this

enzyme.[17] In this assay, homovanillic acid (HVA, Sigma) is converted to a fluorescent product in the presence of H_2O_2 (excitation: 325 nm, emission: 425 nm). Cells were cultured to confluence in a 6-well plate and subsequently incubated in serum-free DMEM overnight. Five hundred-μL samples (n = 5 wells) of conditioned media were used for analysis. Blanks and standards contained the same volume of DMEM as the experimental samples to normalize for autofluorescence.

All reactions were performed in a 2-mL final reaction volume of PBS (pH 7.8) containing 50 ng/mL of $CuSO_4$ (Sigma), 5 μL of horseradish peroxidase (HP, Sigma; 10 mg/mL in 0.1*M* of K-phosphate buffer, pH 6.0), and 20 μL of HVA (50 mg/mL, Sigma). As a negative control, 100 μL of BAPN (50 mg/mL), a lysyl oxidase inhibitor, were added to the reaction mixture. One hundred μL of LO substrate (1,5-diaminopentane, 100 mg/mL, Aldrich) were added. All reactions then were incubated while shaking at 37°C for 1.5 h. Samples were placed on ice to stop the reaction, and the entire 2-mL volume was used for the fluorometric measurement.

Desmosine quantification

Desmosine content was determined via radioimmunoassay, as previously described.[18] In brief, cells (n = 6 wells) were scraped in 6*N* of HCl, placed in a 1.5-mL microfuge, and hydrolyzed for 24 h at 104°C. The hydrolysate was cooled, and the solution was evaporated in a savant evaporator. The residue was redissolved in water and subsequently analyzed for desmosine content by a radioimmunoassay.[18]

Desmosine immunostaining

All incubation steps were performed at room temperature. The cells (n = 3 wells per group) were washed with phosphate-buffered saline (PBS) and then lysed in 25 m*M* of NH_4OH for 1 h. The wells were rinsed with PBS, and the remaining matrix was fixed with 10% formalin for 10 min and then treated with 0.3% H_2O_2 for 10 min. Next the wells were incubated in blocking serum (from the anti-rabbit VECTASTAIN Elite ABC System kit, Vector Laboratories) for 2 h, followed by addition of anti-desmosine antibody (Elastin Products) at 1:250 dilution in blocking serum overnight.

The wells were washed with PBS and incubated with a biotinylated anti-IgG antibody (anti-rabbit VECTASTAIN Elite ABC System kit, Vector Laboratories) for 40 min. Diaminobenzidine tetrahydrochloride (Peroxidase Substrate Kit, Vector Laboratories) was used to detect secondary antibody, yielding gray/black staining.

Wells stained in the absence of primary antibody were used as negative controls. Samples were analyzed by light microscopy (Zeiss Axiovert 135). Images of immunostained wells were captured though a CCD camera coupled to a Macintosh G3 computer with Scion Image software. Images were converted to gray scale, and histogram analysis was performed. The average gray-scale intensity was obtained from images and used to compare the staining intensity between samples. Background intensity, derived from negative controls, was subtracted from each image.

Analysis of extracellular matrix proteins

Matrix composition was determined as previously described.[8] VSMCs were cultured in the presence of 1μCi/mL of ^{3}H-glycine (Sigma) for 8 days. The cells (n = 3 wells per group) were removed nonenzymatically by incubation in 25 m*M* of NH_4OH for 1 h at room temperature, and the remaining ECM was washed three times with 70% ethanol. The wells were dried under a laminar flow hood for 30 min. Enzyme solutions were prepared in 25 m*M* of tris HCl (pH 8.0) with 5 m*M* of $CaCl_2$, and enzymes were used sequentially in the order of trypsin (20 μg/mL, Sigma), elastase (58 U/mL, ICN Corp.), and collagenase (76 U/mL, Sigma).

After sequential enzymatic digestion, the culture vessels were treated with 1*N* of NaOH for an additional 2 h to confirm that all ECM material had been digested. The total matrix produced was normalized to a final cell number. The relative amounts of glycoproteins (trypsin-sensitive), elastin (elastase-sensitive), and collagen (collagenase-sensitive) were quantified as percentages of the total matrix radioactivity released.

Preparation of collagen gels

A sterile solution of bovine dermal collagen type I (Vitrogen 100, Collagen Corp.) was dissolved in 0.01*N* of HCl. Ten× phosphate-buffered saline (0.2*M* of Na_2PO_4, 1.3*M* of NaCl, pH 7.4), 0.1*N* of NaOH, and the collagen solution (3 mg/mL) were mixed in a 1:1:8 ratio and seeded with VSMCs (10^6 cells/mL). The cell/collagen mixture was brought to a final concentration of 2 mg of collagen/mL by adding DMEM and allowed to gel at 37°C for 1 h. DMEM was added to each gel in the wells, and the plates were transferred to a 37°C, 5% CO_2 environment. The culture medium was supplemented with 5 μg/mL of ascorbic acid and 5 μg /mL of $CuSO_4$.

Immunohistochemistry of collagen gels

Gels were fixed in 10% buffered formalin overnight, and frozen sections (14 μm) were prepared. Immunohistochemistry for desmosine was performed with the affinity-purified anti-desmosine monoclonal antibody described above diluted in blocking buffer (1.5% goat serum in PBS) at 1:100. A primary antibody detection kit (Anti-rabbit VECTASTAIN Elite ABC System kit, Vector Laboratories) consisting of goat anti-rabbit biotinylated secondary antibody was utilized at a dilution of 1:5000. Detection of the secondary antibody was carried out using an avidin/biotinylated horseradish peroxidase (HRP) complex. Sections were incubated for 8 h with the primary antibody and for 30 min with secondary antibody. An endogenous peroxidase quenching step was per-

formed with 0.3% H_2O_2 for 30 min prior to incubation with the HRP complex.

Three washes with PBS were carried out between each successive incubation step. 3,3′-diaminobenzidine (DAB) was used for secondary antibody detection, forming a dark gray precipitate. Sections stained in the absence of primary antibody were used as negative controls. Images of the immunostained sections were captured and analyzed, as described above.

Mechanical testing

Dynamic mechanical testing was conducted to evaluate the stiffness and strength of VSMC-seeded gels. Collagen gels ($n = 5$) were prepared, as described above, at a total collagen/cell solution volume of 5 mL in a rectangular shape. The gels were cultured for a 1-week period, during which they contracted in volume and were free-floating in the culture dish. After this culture period, the engineered tissues were subjected to mechanical testing. The gels were cut into a smaller size with 75 × 25-mm dimensions. Balsa wood with a thickness of 1/16″ was attached to each gel end using cyanoacrylate glue (Loctite) to attach the gels to the clamps for tensile testing.

Dynamic tensile testing was performed using a Vitrodyne V1000. Tensile testing was performed with a strain rate of 100 μm/s using a 150-gram loading cell. Stress calculations were based on measured loads and cross-sectional areas. Cross-sections were determined using a digital caliper. The elastic modulus was calculated as the slope of stress versus strain plot while the ultimate tensile strength is represented by the highest stress point achieved.

Statistical analysis

All data are presented as means ± standard deviation (SD). Analyses of data were performed using two-tailed, unpaired t tests. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

LO expression

Our goal in this study was to implement gene therapy as a tool to optimize the performance of mechanically challenged engineered tissue. Rat aortic smooth muscle cells (SMCs) were liposomally transfected with lysyl oxidase full cDNA. Northern analysis confirmed increased levels of lysyl oxidase mRNA in the LO-transfected SMCs as compared to mock-transfected cells [Fig. 1(A)]. LO protein expression was confirmed by Western blotting [Fig. 1(B)].

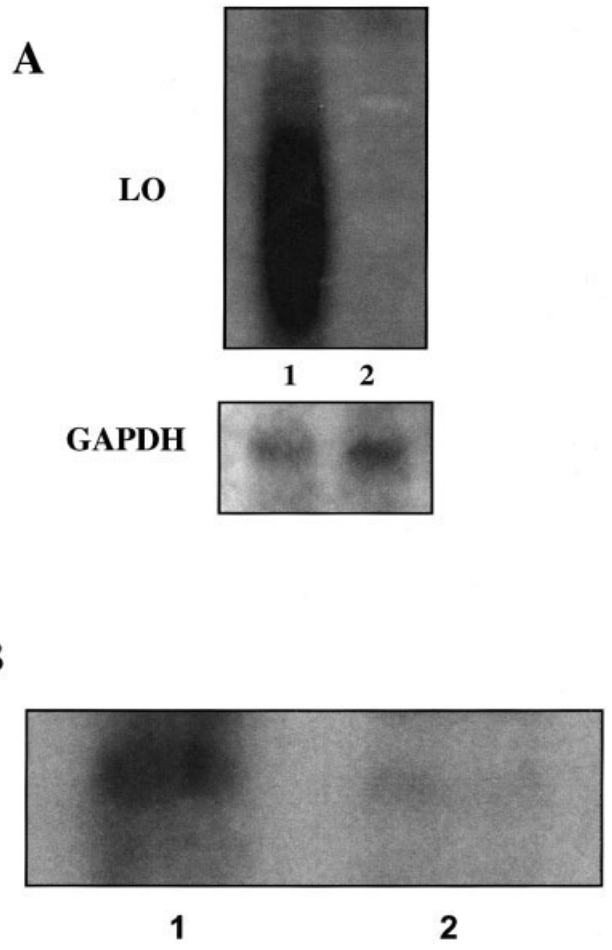

Figure 1. Analysis of lysyl oxidase expression: (A) Northern blot of (1) LO-transfected- and (2) mock-transfected SMCs; (B) Western blot of (1) LO-transfected and (2) mock-transfected SMCs.

LO activity and desmosine levels

Lysyl oxidase activity was evaluated in the conditioned media of transfected cells. Transfection with LO resulted in an increase in LO enzymatic activity, as determined by fluorescent substrate assay 7 days post-transfection (Fig. 2(A)]. The addition of BAPN, a LO inhibitor, to the reaction decreased LO activity below levels observed in mock-transfected cells.

At week 3 post-transfection, the desmosine content of LO-transfected SMCs was approximately two-fold higher than it was in mock-transfected cells (Fig. 2(B)]. Addition of BAPN significantly reduced desmosine levels in the ECM produced by LO-transfected SMCs to levels equal to those in mock-transfected cells. After 2 weeks in culture, immunostaining for desmosine suggested that there was a marked increase in the amount of desmosine present in the matrix from the LO-transfected cells [Fig. 3(A)] compared to the amount in the mock-transfected cell matrix [Fig. 3(B)]. These results were

further confirmed with gray-scale levels of LO-transfected cell matrix, which showed significantly higher values compared to mock cells and BAPN-treated cells [Fig. 2(C)].

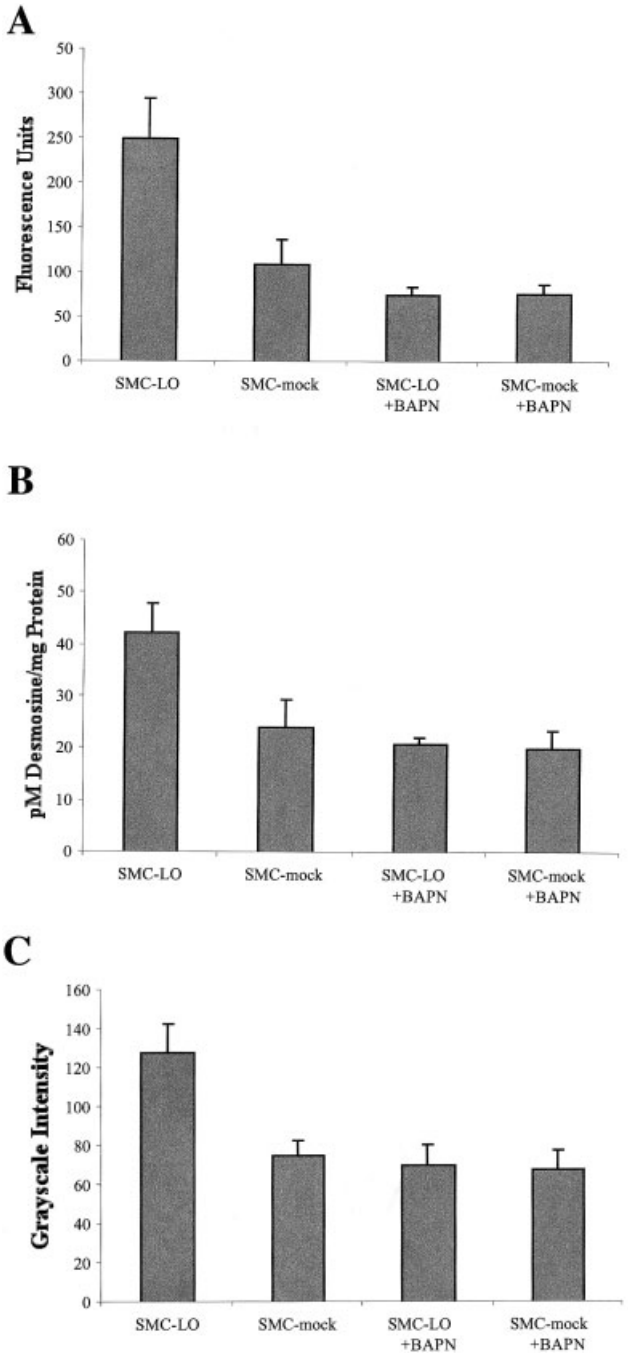

Figure 2. Analysis of LO activity: (A) relative lysyl oxidase activity for LO- or mock-transfected SMCs using a fluorescent substrate assay ($p < 0.03$, $n = 5$ per group); (B) desmosine levels in LO- or mock-transfected SMCs cultured in the presence or absence of BAPN [results are expressed as pmol desmosine/mg of protein ($p < 0.001$, $n = 6$ per group)]; (C) gray-scale histogram analysis of anti-desmosine immunostained transfected VSMCs ($p < 0.04$, $n = 5$ per group).

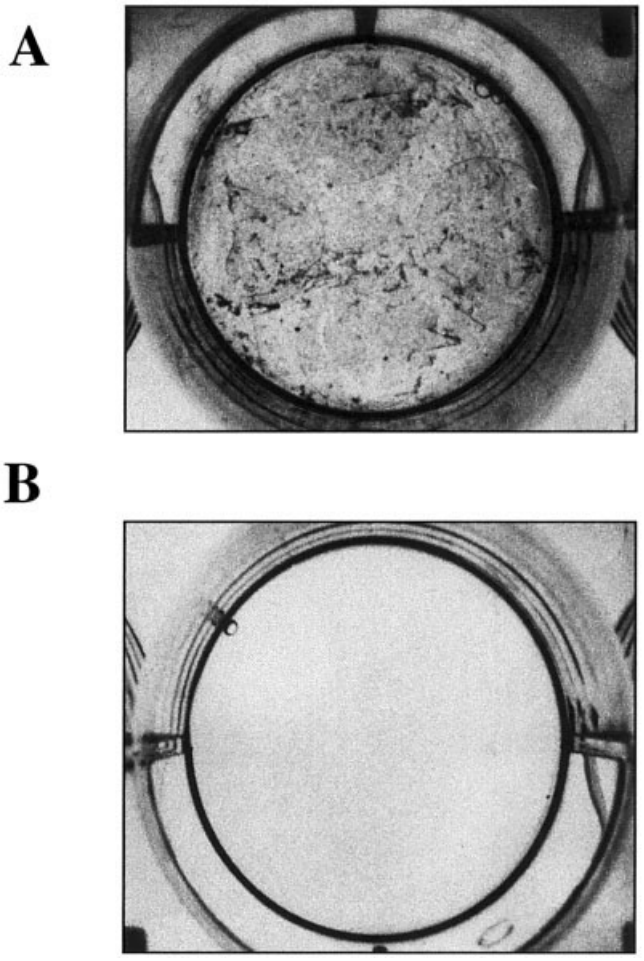

Figure 3. Immunohistochemical staining for desmosine in transfected VSMCs 14 days post-transfection: (A) LO-transfected VSMCs showed more staining, indicating more ECM crosslinking as compared to (B) mock-transfected cells.

Cell proliferation

The effect on SMC proliferation of elevating LO activity was determined in order to isolate the source of changes in mechanical properties. The proliferation values of transformed SMCs were compared at day 8 post-transfection. As shown in Figure 4, LO expression decreased the final cell number by approximately 40% ($p < 0.05$). Interestingly, the addition of BAPN did not alter the proliferation of LO-transfected SMCs to levels seen in non-LO-expressing SMCs. This may be because of the inverse correlation between LO mRNA levels and cell proliferation.[19] BAPN does not affect LO message levels; it affects only protein activity.

Analysis of the ECM

Characterization of the extracellular matrix of transformed SMCs is critical for confirming that the changes in mechanical properties are due to crosslink-

ing rather than to compositional changes within the ECM. Eight days post-transfection, both LO- and mock-transfected SMCs exhibited similar quantities of the major ECM components [Fig. 5(A)]. The presence of the LO inhibitor did not alter the levels of ECM deposited. Neither did the composition of the ECM differ between LO- and mock-transfected SMCs. Relative amounts of collagen (collagenase-sensitive), elastin (elastase-sensitive), and glycoproteins (trypsin-sensitive) levels were similar among all groups, as shown in Figure 5(B).

Desmosine content in collagen gels

Desmosine density in collagen gel constructs was assessed to confirm increased crosslinking of newly deposited ECM proteins in these tissue-engineered constructs. Desmosine immunohistochemical staining of LO and mock SMC-populated gels revealed evident differences in staining intensity for desmosine crosslinkage at 1 week (Fig. 6). Gels populated with LO-transfected SMCs had elevated desmosine density [Fig. 6(A)]. The addition of BAPN reduced desmosine levels to that seen in mock samples [Fig. 6(C)].

Mechanical effects of LO crosslinking

Lysyl oxidase- or mock-transfected SMCs were seeded into collagen gels and cultured for 1 week. We utilized this tissue engineering model system to assess the effects of LO since this model has been widely employed for vascular tissue engineering.[8] LO-seeded gels appeared to be less contracted and compacted as compared to mock-seeded gels. Collagen gels seeded with LO-transfected SMCs had an elastic modulus of approximately 75 kPa while mock controls and BAPN-treated samples were at or below 40 kPa [Fig. 7(B)]. Ultimate tensile strength was increased as well, with LO transfectants having approximately double the strength of mock controls [Fig. 7(C)].

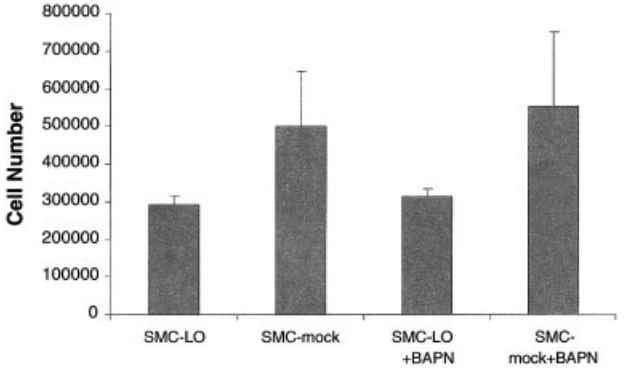

Figure 4. Proliferation of LO- and mock-transfected SMCs cultured in the presence or absence of BAPN 8 days post-transfection. A significant difference was found between LO- and mock-transfected SMC proliferation ($p = 0.001$, $n = 6$ per group).

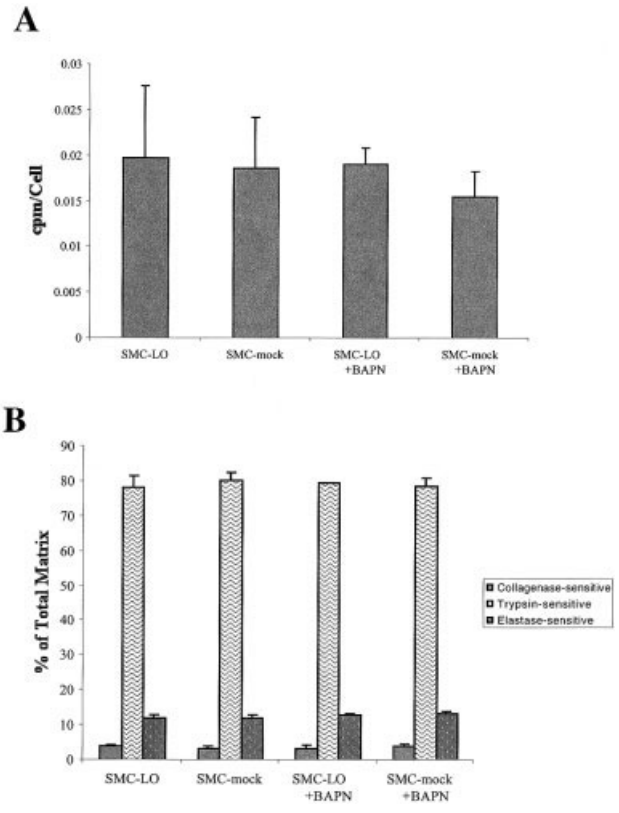

Figure 5. (A) Matrix production of LO- and mock-transfected SMCs in the presence or absence of BAPN. The results were determined as total radioactivity in the ECM (sum of all digests) divided by the final cell number ($n = 3$ per group). (B) Compositional analysis of extracellular matrix polypeptide material elaborated by LO- and mock-transfected SMCs in the presence or absence of BAPN. The results are reported as percentages of total matrix sensitive to collagenase, trypsin, and elastase, which yield relative amounts of collagen, glycoproteins, and elastin, respectively. The levels of collagen, elastin, and glycoproteins were similar in all groups ($n = 3$ per group).

DISCUSSION AND CONCLUSIONS

Many tissue-engineering applications are dependent on constructs with mechanical properties adequate to withstand loads that will be experienced after implantation. The mechanical properties of tissue equivalents may be improved by increased crosslinking of extracellular matrix components, namely, collagen and elastin. Glycation, the nonenzymatic crosslinking of proteins by reducing sugars, previously has been proposed as a strategy for enhancing the stiffness and strength of tissue equivalents.[8] However, there are reports of detrimental effects of glyca-

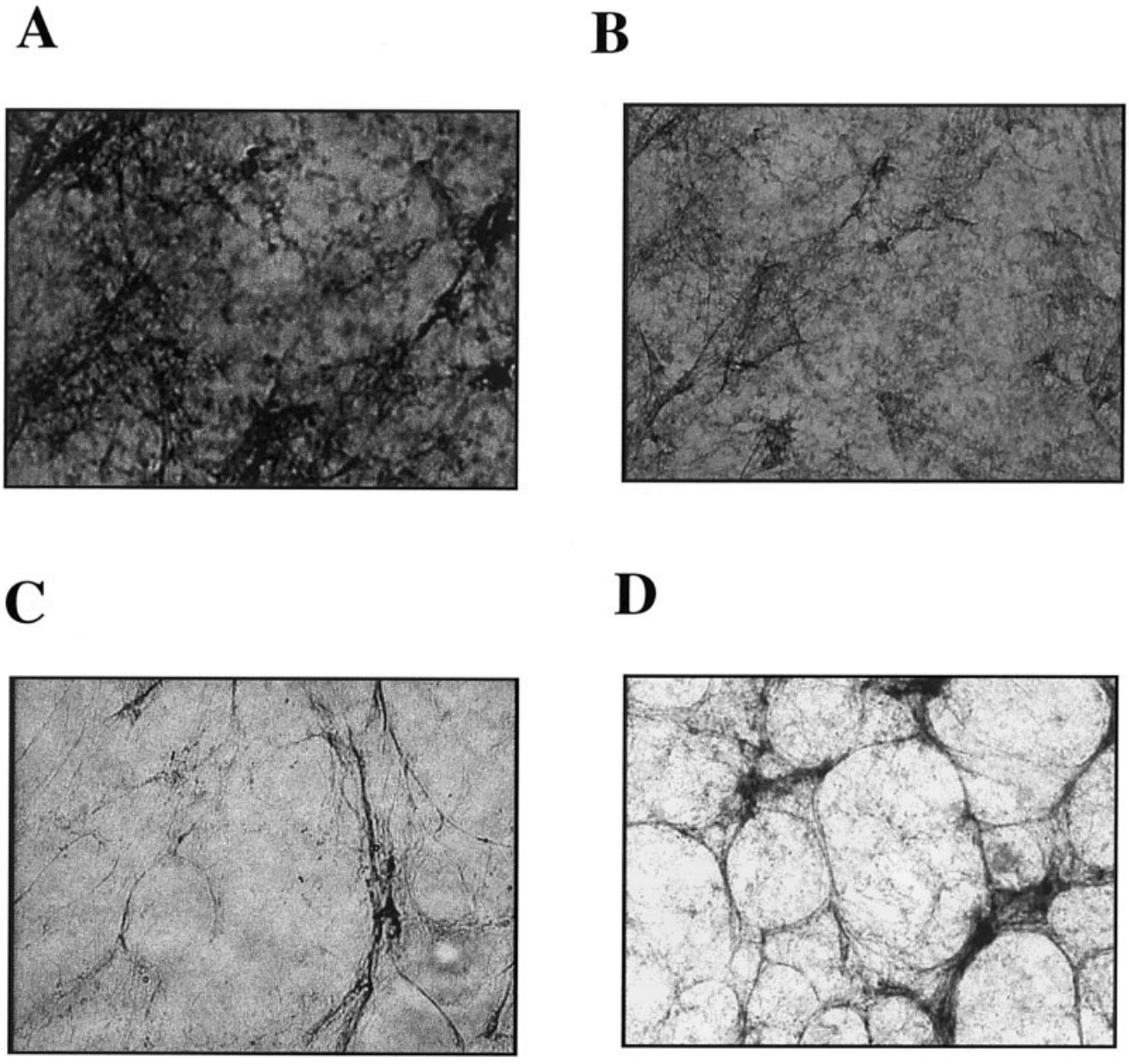

Figure 6. Anti-desmosine immunostained sections of SMC-populated collagen gels cultured for 1 week: (A) LO-populated; (B) mock-populated; (C) LO-populated in the presence of BAPN; and (D) mock-populated in the presence of BAPN. Original magnification 400×.

tion products.[20] Also, there are concerns about the stability of glycation after removal of ribose from the culture environment.

Tissue transglutaminase may be useful for enzymatic crosslinking of the ECM, but there are concerns about the role of this enzyme in apoptosis.[21] Lysyl oxidase crosslinking of the ECM may be an alternative effective strategy for altering the mechanical properties of engineered tissues. We have chosen gene therapy as the method of introducing LO because of the high cost of protein purification and issues with long-term delivery. Due to our research interests in the tissue engineering of vascular grafts (TEVG) and the importance of tissue mechanical properties in vascular tissue engineering, vascular smooth muscle cells were chosen as the LO delivery vehicle.

Arterial occlusive disease remains the leading cause of death in western countries and often requires vascular reconstructive surgery.[22] For small diameter vascular graft applications (i.d. < 6 mm), such as coronary artery bypass grafting, autologous tissue currently is the only suitable biomaterial. Unfortunately, approximately 60% of patients undergoing vascular surgery do not have a suitable vessel for grafting.[23]

Vascular tissue engineering may help to address this critical need. Most approaches to TEVG design have focused on generating the equivalents of the arterial media, composed of SMCs and matrix proteins, and the intima, a monolayer of nonthrombogenic endothelial cells. TEVGs have been constructed using collagen gels,[8,24,25] synthetic polymer scaf-

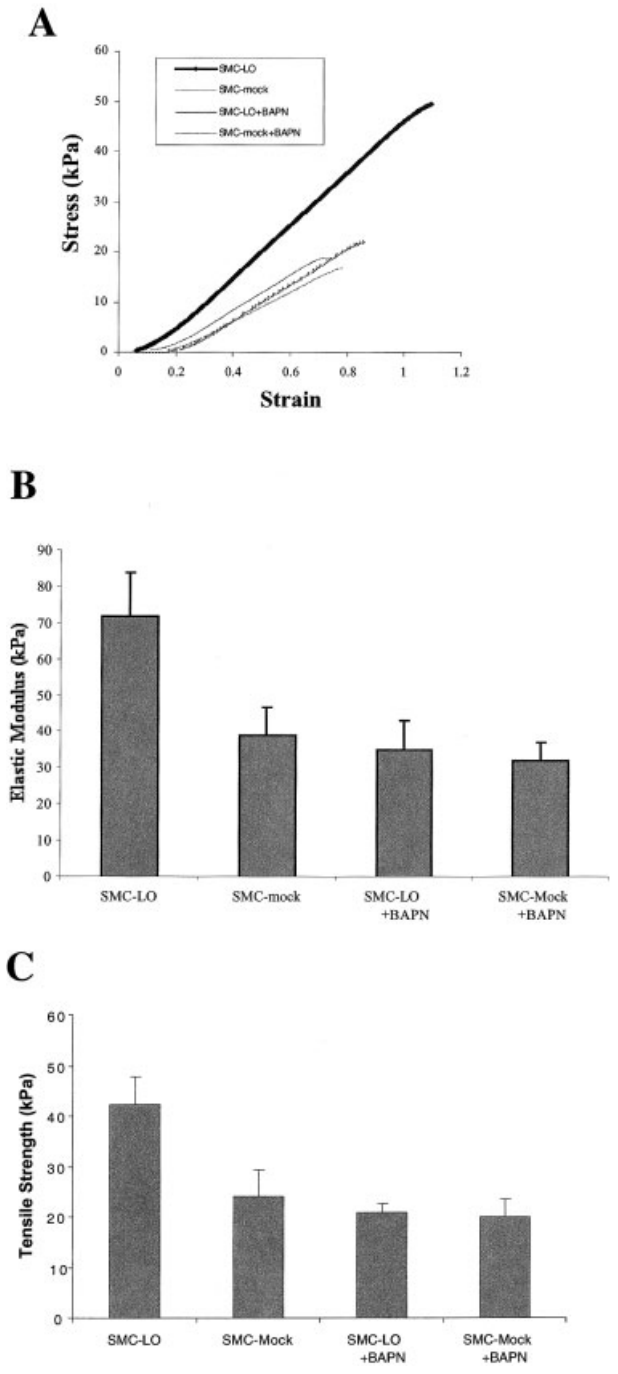

Figure 7. Mechanical properties of collagen gels seeded with the transfected VSMCs and cultured in the presence or absence of BAPN for 1 week. (A) Stress–strain curves of collagen gels seeded with the transfected SMCs. (B) A significant difference was found in the Young's modulus between LO- and mock-transfected SMC-populated collagen gels ($p < 0.03$, $n = 5$ per group). (C) Tensile strength was significantly increased in LO-transfected SMC-populated collagen gels as compared to mock-seeded gels ($p < 0.02$, $n = 5$ per group).

folds,[26–28] or solely the ECM elaborated by cells in culture.[29] In many of the TEVG constructs investigated to date, it has not been possible to achieve mechanical properties similar to arterial tissue, generating concern about the potential for burst failure of these grafts and interest in methods to improve TEVG properties.

This current study has demonstrated the ability of ECM crosslinking by the enzyme lysyl oxidase to improve the mechanical properties of tissue-engineered constructs formed from vascular smooth muscle cells seeded within collagen gels, similar to constructs that have been used in vascular tissue engineering.[8,25] Northern blots, Western blots, and activity assays confirmed increased LO expression and activity in SMCs that were liposomally transfected with the gene for LO. Increased ECM crosslinking was demonstrated by a biochemical assay for desmosine, a product of LO-mediated crosslinking, in the cultured transfected cells. Immunohistochemical staining of both cultured cells and SMC-populated collagen gels revealed higher densities of desmosine. Furthermore, the elastic modulus and ultimate tensile strength of collagen gels seeded with LO-transfected SMCs nearly doubled as compared to mock-seeded gels. These enhanced mechanical properties were due to increased ECM crosslinking rather than to increased amounts of ECM, changes in the ECM composition, or increased cellularity.

The model chosen for this study was based on the collagen gel system for tissue-engineered vascular grafts that has been studied by other groups. Gels were fabricated from 2 mg/mL of pepsin-digested bovine skin collagen and adult rat aorta SMCs at an initial seeding density of 1×10^6 cells/mL, as reported in previous studies by other groups.[8,24,25,30,31]

Using this system, Hirari and Matsuda[30] previously reported a burst pressure of approximately 110 mmHg, corresponding to a burst strength of 36 kPa. More recently, Girton et al.[31] reported a different mechanism for enhancing tensile stiffness and tensile strength of media equivalents (MEs) using glycation. Their findings included a 16-fold increase for the modulus while tensile strength was increased by only fourfold after a 10-week culture of MEs. The mechanical properties of collagen gels seeded with LO-transfected SMCs nearly doubled after 1 week of culture. SMC cell type, seeding densities, incubation time, and strain rates are some of the variables that make it difficult to directly compare study results to previously published studies, but the current results indicate the promise of lysyl oxidase for manipulation of the mechanical properties of engineered tissues.

Gene therapy methods to increase LO-mediated crosslinking may be applied to many tissue-engineering applications. Nonviral or viral gene delivery methods may be employed,[32] allowing one to manipulate the transfection efficiency and duration of expression in order to achieve the desired properties for a given application. In these studies, liposomal transfection, which provides relatively short-term expression with low efficiency, was sufficient to significantly increase both the modulus and the tensile strength of the tissue

equivalents. For additional control over the system, one also could utilize an inducible promoter.[33] LO-mediated crosslinking also could be used in combination with tissue transglutaminase, glycation, mechanical conditioning, and factors, such as TGF-β, that increase the synthesis of ECM proteins to achieve synergistic effects. This novel approach may benefit many tissue-engineering applications where there are concerns about mechanical failure.

Tim Scott-Burden, deceased, of the Texas Heart Institute, was instrumental in planning this project.

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