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Overexpression of Lysyl Oxidase to Increase Matrix Crosslinking and Improve Tissue Strength in Dermal Wound Healing

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Abstract—In this study, we aimed to increase crosslinking in collagen and elastin in the extracellular matrix through overexpression of lysyl oxidase (LO) in order to improve mechanical strength in dermal wounds during healing. We had used a gene activated matrix (GAM) approach to locally deliver plasmid DNA (pDNA) complexed with polyethylenimine (PEI) in collagen gels at the wound site for localized and sustained transfection of cells involved in the healing process. We first demonstrated *in vitro* that PEI-pDNA complexes in collagen gels could be taken up and expressed by cultured fibroblasts for at least 20 days. *In vitro* studies showed that fibroblast-seeded GAMs with the LO transgene exhibited over a 3-fold increase in mechanical strength as compared with a green fluorescent protein (GFP)-transgene control. Addition of an inhibitor of LO abolished this increase. We applied this system in a rat dermal wound healing model and showed that treatment with LO-producing GAMs led to significantly enhanced mechanical strength of the wound site.

Keywords—Wound healing, Gene therapy, Mechanical strength.

INTRODUCTION

Chronic wounds such as diabetic foot ulcers, pressure ulcers, and venous ulcers account for approximately 70% of all skin wounds.[20] Diabetic foot ulcers alone account for 800,000 chronic wounds in the United States each year, with annual treatment costs of more than $1-billion.[20] A wound is considered chronic if it does not heal in a timely manner or, more commonly, if the healing process does not result in appropriate mechanical integrity, such that the site is not able to remain in a healed state.[18] Approaches to improve the mechanical strength of wounds have generally focused on increasing the deposition of matrix proteins such as collagen through local delivery of growth factors[16] or nitric oxide.[3] In the current study, we have investigated an alternative and potentially synergistic approach to enhance crosslinking of structural matrix proteins, collagen and elastin, at the wound site.

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Dermal wound repair is a complex process that can be divided into three phrases: an initial inflammatory phase, followed by formation of granulation tissue by proliferating fibroblasts and then remodeling of extracellular matrix (ECM) proteins such as collagen. The newly deposited ECM proteins are initially randomly oriented in the granulation tissue matrix and later are broken down and reorganized by proteinases secreted by fibroblasts. Lysyl oxidase (LO) then catalyzes the formation of covalent crosslinks between collagen fibers which subsequently become organized into thick bundles oriented perpendicular to the edges of the wound.[5]

LO is secreted as a 50 kDa proenzyme and then proteolytically cleaved to the 32 kDa active enzyme.[25] It catalyzes inter- and intra-molecular crosslinking in collagen and elastin by oxidatively deaminating lysyl residues in these proteins into peptidyl aldehydes.[13] These aldehydes can then spontaneously condense with one another to yield a variety of covalent cross-linkages. LO has been shown to be regulated by a variety of factors including serum conditions,[9] transforming growth factor-$\beta 1$[2] and shear stress.[1] A commonly used inhibitor of LO is β-aminopropionitrile (BAPN).[22] Other inhibitors include diamines, heparin, amino nitrites, semicarbazides and hydrazines.[10,26]

In this study, we investigated the effect of overexpression of LO on the mechanical strength of newly formed tissue in the wound site through increased cross-linking activities. To achieve overexpression of LO in the wound site, we employed a system that could locally deliver the LO gene for sustained uptake and expression by local cells. Matrices known as gene-activated matrices, or GAMs, are capable of retaining plasmid DNA (pDNA)[7,14] and thus allow local cells to migrate through the matrix, gradually take up the retained pDNA and express the protein of interest. This can lead to continual and prolonged production of proteins in a localized tissue site. PEI is a polycation capable of interacting with and thus, compacting DNA. This protects DNA from degradation by nucleases and increases transfection efficiency. We hypothesized that fibroblasts would migrate into implanted GAMs, take up the PEI-pDNA complexes

and express LO over an extended period of time. This in turn would increase the degree of crosslinking in the newly formed tissue and lead to enhanced mechanical strength. We showed that LO-producing GAMs made of collagen could be strengthened *in vitro* and we demonstrated the same efficacy of these LO-producing GAMs in a dermal wound healing model *in vivo*.

MATERIALS AND METHODS

Cell Culture

Human dermal fibroblasts (HDFs) were obtained from Clonetics (San Diego, CA) and were maintained in low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM, Sigma) that contained 10% fetal bovine serum (FBS, Whitaker), 2 mM of L-glutamine, 1 unit/mL of penicillin, and 1 μg/mL of streptomycin (GPS, Sigma). Cell number was determined using a Coulter counter (Beckman Coulter, Multisizer^{TM} 3).

Plasmid DNA

Full-length cDNA coding sequences of green fluorescent protein (GFP) and rat LO were cloned into a commercially available mammalian expression vector, PLE-N1 (Clontech, CA). *Escherichia coli* (*E. coli*) was transformed with the vector and the vector was amplified by culturing *E. coli* overnight. Plasmid DNA was extracted by Plasmid Midi Kit (Qiagen).

Assembly of GAMs

PEI-pDNA Complexes

PEI solution (MW = 25,000, Aldrich) was added dropwise to of 50 μg pDNA at an N/P ratio of 7.5:1. The mixture was then incubated for 30 min at room temperature. End pDNA concentration was 16.7 μg/mL of collagen gel.

Collagen Gels with PEI-pDNA Complexes and Cells

Collagen gels were fabricated by mixing bovine dermal collagen (3 mg/mL, Vitrogen), phosphate-buffered saline (PBS) and NaOH (0.1 N) at 8:1:1 ratio respectively. Human dermal fibroblasts were mixed with PEI-pDNA and collagen at a density of 1.5×10^6 cells/mL. The resultant mixture was placed in cell culture wells and incubated for 1.5 h at 37°C. DMEM was then added to each well. Samples were then transferred to an incubator (37°C, 5%CO_2). For the naked DNA control, the same procedure was followed except that pDNA was not coupled to PEI.

In Vitro Assessment of LO-producing GAMs

To confirm cell viability in the GAM system, samples were rinsed for 10 min with PBS (1 ml/gel). 1.13 μL of 4 mM calcein AM and 2.25 μL of 2 mM ethidium homodimer (Live/Dead Kit, Molecular Probes, OR) was added to 4.5 mL PBS. Each gel was incubated with 1 ml of this solution for 30 min. Living cells were stained green when calcein AM was cleaved by esterases and dead cells with compromised membranes were stained red with the membrane-impermeant nucleic acid dye, ethidium homodimer-1. Samples were rinsed with PBS for 5 min and imaged for fluorescence (Ziess Axiovert 135). Viability was accessed by dividing the number of green cells by total number of cells in different views of the gels ($n = 5$) and the result was converted to a percentage.

Immunostaining for proliferating cell nuclear antigen (PCNA) was performed to confirm that the GAM system was conducive to cell proliferation. Samples were cryosectioned to 30 μm and fixed in cold dry acetone for 10 min. Samples were then incubated with 0.3% H_2O_2 in 70% methanol for 30 min and followed by incubation in 10% serum in PBS for 10 min. Sections were incubated with mouse anti-PCNA (1:10, Zymed Laboratories Inc.) overnight at 4°C and then with peroxidase-rabbit anti-mouse IgG (1:10, Zymed Laboratories Inc.). Finally, sections were incubated with DAB substrate solution for 5 min, counterstained in hematoxylin for 2 min and mounted in aqueous mounting medium. The fraction of proliferating cells was obtained by dividing positively stained cells by total number of cells in different views of the gel sections ($n = 5$) and this in turn was converted into a percentage.

Duration of expression of protein in GAMs was investigated using GFP. GAMs were formed as described above with the transgene encoding for GFP and fibroblasts embedded. Expression of GFP by fibroblasts was observed with fluorescence microscopy (Carl Zeiss Inc, Germany) 10, 20 and 30 days after the assembly of the system.

The ability of fibroblasts to infiltrate into GAMs was assessed. Collagen gels were fabricated by mixing bovine dermal collagen (3 mg/ml, Vitrogen), DMEM, PBS and NaOH (0.1 N) at 8:2:1:1 ratio respectively. 2 ml of this collagen solution was placed in culture wells and incubated for 1.5 h at 37°C. 1.5×10^6 of HDFs were then seeded on top of the resultant gels and allowed to migrate into collagen gels over 3 days. The percentage of cells that had migrated into the gels was determined by subtracting the number of cells on top of gels obtained by trypsinization and collected from media after 3 days from the total number of cells seeded on top of the gels at the beginning of the experiment.

A flurometric peroxidase assay was performed to confirm enhanced bioactivity of LO in LO-producing GAMs. H_2O_2 is a product of LO deamination reaction and in this assay, homovanillic acid (HVA, Sigma) was converted to a fluorescent product in the presence of H_2O_2 (excitation: 325 nm, emission: 425 nm). 1ml of conditioned media

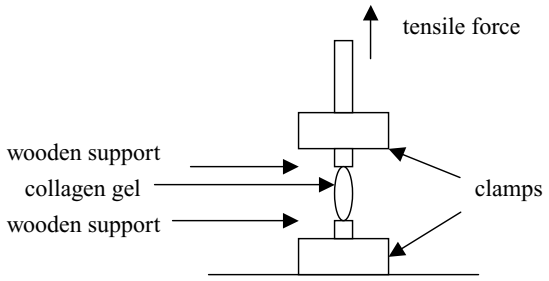

FIGURE 1. Collagen gels were attached to balsa wood using a cyanoacrylate glue to mediate the clamping of gels. Tensile testing was performed using Vitrodyne V1000 with a strain rate of 100 μm/sec using a 150 g loading cell.

was collected from GAMs (assembled as described above) at various time points : 0, 1, 2, 3, 4 weeks ($n = 4$). 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 (Sigma, 10 mg/ml in 0.1 M of K-phosphate buffer, pH 6.0), 100 μl of LO substrate (Aldrich,1,5—diaminopentane, 100 mg/ml) and 20 μl of HVA (Sigma, 50 mg/ml). 100 μl of BAPN (Sigma, 50 mg/ml), a lysyl oxidase inhibitor, was used as a negative control. All reactions then were incubated while shaking at 37°C for 1.5 h and placed on ice to stop the reaction for subsequent fluorometric measurement.

In vitro mechanical testing of GAMs was performed to evaluate the effect of overexpression of LO on the mechanical strength of collagen-based GAMs. Fibroblasts-seeded collagen gels were fabricated as described above. After gelation, complete media was added and the seeded gels ($n = 3$) were moved to a cell culture incubator where they were cultured for 3 weeks. 150 μg/ml of BAPN (Sigma), a specific inhibitor of LO, was added to one set of wells ($n = 3$) as a control. The collagen gels were attached to balsa wood using a cyanoacrylate glue to mediate the clamping of gels. Tensile testing was performed using Vitrodyne V1000 with a strain rate of 100 μm/sec using a 150 g loading cell (Fig. 1). Elastic moduli and tensile strengths were determined from stress-strain plots.

In Vivo Wound Healing Model in Rats

A rat skin wound healing model was used to assess the efficacy of LO-producing GAMs. Male Sprague-Dawley rats ($n = 4$) were obtained (375–400 g; Charles River Laboratories, Wilmington, MA). Under isofluorane anesthesia, the dorsal region of each rat was shaved and two full thickness 3 cm incisions were made approximately 1.5 cm away from median line, GAMs with either the LO or GFP pDNA (without cells) were made in rectangular chambers one day in advance as described above. Dimensions of the GAMs were 2 mm × 20 mm × 2 mm. Pre-made GAMs were placed into the wound sites (each rat with one control GFP-GAM and one experimental LO-GAM) followed by suturing with polypropylene 5–0 sutures (PROLENE, Ethicon) in an interrupted fashion with approximately 2 mm separation between sutures.

Skin samples were excised from rats after 31 days and cut into strips with a cross sectional area of 2 mm × 6 mm and subjected to tensile mechanical testing on an Instron Series 3340 (Instron Corporation, Canton, MA). Tensile testing was performed at a strain rate of 417 μm/sec using a 51 kg load cell. The experimental setup was similar to the mechanical testing of collagen gels except no wooden supports were used. For histological evaluation, tissue samples were fixed in 10% formalin, paraffin embedded, sectioned and stained with Masson's Trichrome.

Our animal protocols followed the guidelines of the animal oversight regulations and were approved by the animal welfare committee.

Statistical analysis

All data are presented as mean ± 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

In Vitro Assessment of LO-producing GAMs

Cell Viability

To confirm that our system is conducive to cell survival, we performed a viability assay in the presence of PEI-pDNA complexes. PEI-pDNA complexes were embedded in GAMs at a concentration of 16.7 μg pDNA/mL of GAM at an N/P ratio of 7.5:1. The calcein AM/ethidium homodimer staining showed that approximately 96% of fibroblasts were viable after being embedded in GAMs for 2 days in the presence of PEI-DNA complexes in GAMs. For our system to work effectively, it is also important that fibroblasts maintain their ability to replicate inside of the GAMs. PCNA staining showed that approximately 21% of fibroblasts were in S-phase of mitosis, indicating good proliferation (Fig. 2).

Duration of Protein Expression Duration in GAMs

The ability of fibroblasts to continually take up and express the protein of interest was confirmed by monitoring the expression of GFP by fibroblasts in GAMs loaded with GFP transgene at day 10 and day 20 after assembly of the system. We observed GFP-transfected fibroblasts in the GAMs both at day 10 and day 20 (Fig. 3) but not at day 30. Since expression lasted for at least 20 days and typical

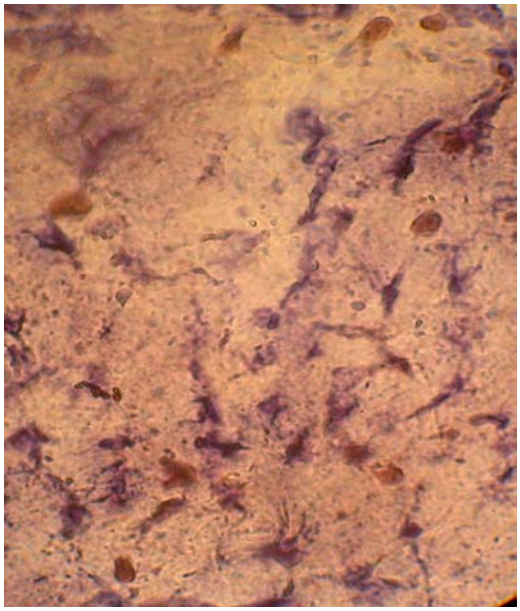

FIGURE 2. PCNA staining of fibroblasts on sections of GAMs. An average of approximately 21% of fibroblasts were stained indicating fibroblasts were able to proliferate in the presence of PEI-pDNA complexes.

expression duration of protein through PEI-aided transfection is about 3–5 days, the PEI-pDNA complexes were gradually being taken up and expressed by the fibroblasts embedded in the GAM. This experiment was performed in the presence of serum, suggesting that PEI is able to protect the pDNA from degradation by nucleases in the serum.

Infiltration of Fibroblast into GAMs

When seeded on top of GAMs, approximately 98% of fibroblasts migrated into the GAMs over 3 days. Staining of cells showed that they homogeneously populated the GAMs after migrating into them. This experiment suggests that local fibroblasts could migrate into GAMs in an *in vivo* wound healing scenario.

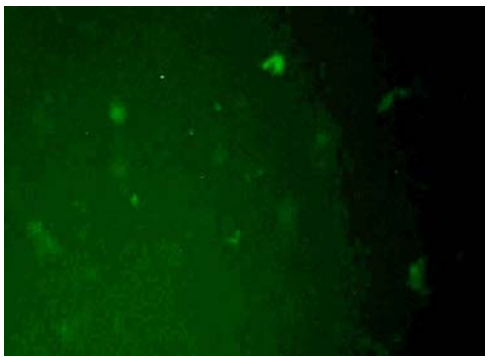

FIGURE 3. Expression of GFP by fiborblasts in GAM loaded with GFP transgene at day 20. This shows that GAM was able to retain PEI-pDNA complexes for gradual uptake and expression of transgene by embedded cells.

Fluorometric Peroxidase Assay for monitoring LO activity

Lysyl oxidase activity was enhanced at each time point in LO-producing GAMs as compared with control ($p < 0.001$, Fig. 4). LO activity in conditioned media collected from LO-producing GAMs increases from week 0 to week 3 and eventually decreases at week 4 whereas that from control GAMs increases steadily but to lesser extent from week 0 to week 4. The increase in biochemical activity showed that the LO produced was in its bioactive form. The activity of LO decreased after 3 weeks suggesting that the transfection is of a transient nature. The addition of BAPN, a LO inhibitor, to the reaction decreased LO activity to levels similar to controls.

In Vitro Mechanical Testing of Collagen Gels

Fibroblast-loaded GAMs with LO transgene or GFP transgene were cultured for 3 weeks and subjected to tensile mechanical testing. Results of mechanical testing showed that the elastic modulus of LO-producing GAMs was 5170 Pa $\pm$ 2443 Pa as compared with 1477 Pa $\pm$ 209 Pa of the GFP control ($p < 0.03$). Addition of BAPN as a LO inhibitor reduced the elastic modulus of LO-producing GAMs to 2443 Pa $\pm$ 1089 Pa while having minimal effect on the elastic modulus of control GAMs ($p < 0.03$). Ultimate tensile strength (UTS) of LO-producing GAMs was found to be 2879 Pa $\pm$ 676 Pa as compared with 824 Pa $\pm$ 77 Pa of the GFP control GAMs (Fig. 5 and Fig. 6). Addition of BAPN reduced ultimate tensile strength of LO-producing GAMs to 1696 Pa $\pm$ 563 Pa while again, having no effect on control GAMs ($p < 0.003$). Again, when BAPN was added to the culture media, the increases in elastic modulus and UTS were abolished.

In Vivo Wound Healing Model in Rats

We investigated the effect of this gene delivery system in a wound healing model *in vivo*. Full-thickness incisions were made through the dorsal skin in rats and GAMs were inserted into the wound pockets prior to suturing. Skin specimens at the wound site were taken from the rats after one month and subjected to tensile mechanical testing. Mechanical testing showed that rat skin treated with GAMs with LO transgene had an UTS of 34.8 kPa $\pm$ 5.1 kPa as compared with 20.0 kPa $\pm$ 4.0 kPa of the control skin ($p < 0.001$; Fig. 7). GAMs were totally resorbed one month after implantation. Histological sections with Masson's Trichrome stain showed that rate of healing of both LO-treated and control wounded skin was the same as both had undergone complete re-epithelialization (Fig. 8).

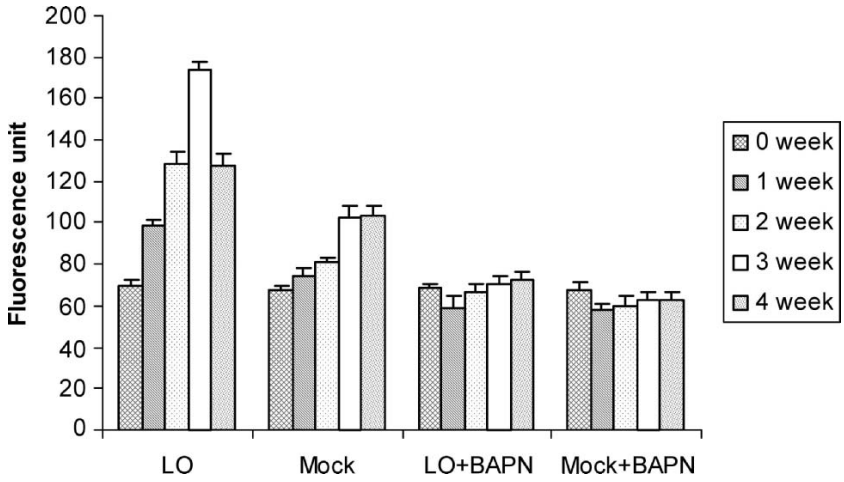

FIGURE 4. A fluorescent substrate assay for monitoring LO bioactivity at different time points. LO activity was enhanced in LO-producing GAMs as compared with control GAMs at each time point (p-value <0.001, $n=4$).

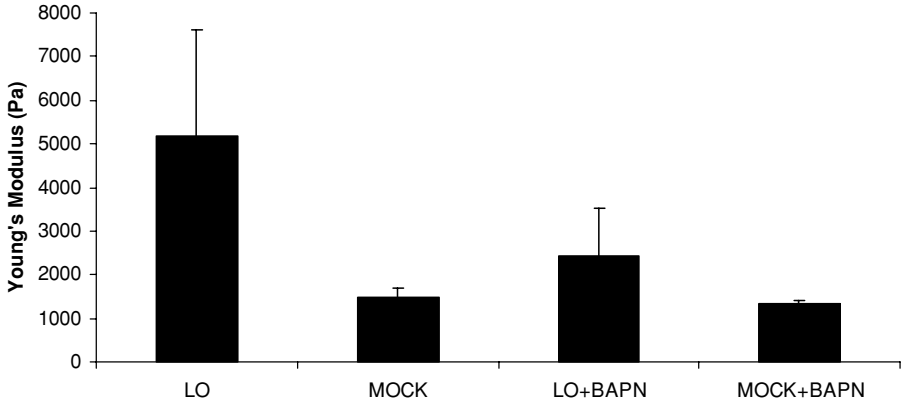

FIGURE 5. Comparison of Young's Modulus of fibroblast-loaded GAMs with LO transgene and GFP transgene. GAMs with LO transgene showed more than a 3-fold increase in Young's Modulus as compared with GAMs with GFP transgene. The addition of BAPN abolished the increase in Young's Modulus in GAMs with LO transgene indicating that the increase was due to the catalytic action of LO. (p-value <0.03, $n=3$ per group).

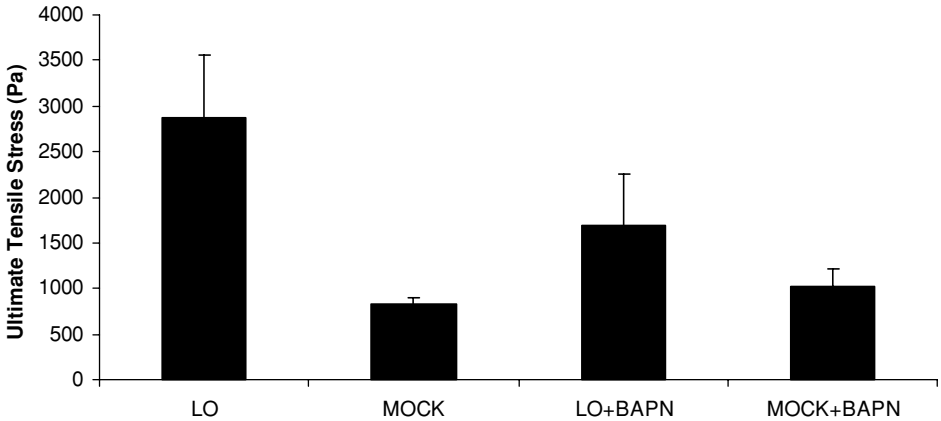

FIGURE 6. Comparison of UTS of fibroblast-loaded GAMs with LO transgene and GFP transgene. GAMs with LO transgene showed more than a 3-fold increase in UTS as compared with GAMs with GFP transgene. The addition of BAPN abolished the increase in UTS in GAMs with LO transgene indicating that the increase was due to the catalytic action of LO. (p-value <0.003, $n=3$ per group).

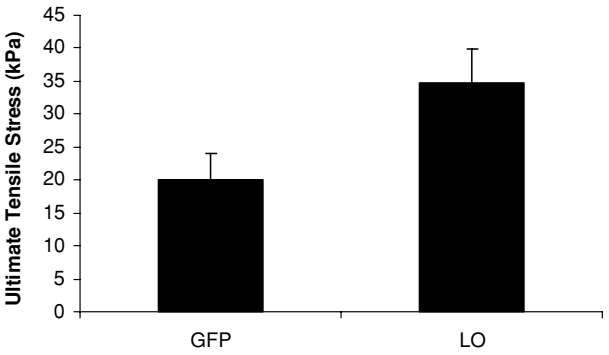

FIGURE 7. Comparison of UTS of rat skin treated with LO-producing GAMs and GFP-producing GAMs one month after surgery. Rat skin treated with LO-producing GAMs showed an average UTS of 34.8 kPa as compared with 20.0 kPa of that treated with GFP-producing GAMs (p-value <0.001, $n=4$ per group).

DISCUSSION

LO was chosen as the protein of interest in this study due to its ability to strengthen the ECM produced by cells through catalyzing the formation of crosslinks[6] in ECM structural proteins, namely collagen and elastin. Apart from the wound healing application which we investigated in this study, this property of LO might be useful in many tissue engineering applications where improved tissue mechanical properties are required. In this study, we demonstrated that mechanical strength of collagen scaffolds could be enhanced through overexpression of LO.

We have shown in this study that local gene delivery was achieved through a collagen-based GAM and high efficacy of the system was demonstrated both *in vitro* in a collagen gel model and *in vivo* in a wound healing model. Mechanical strength of collagen gels was enhanced when PEI-pDNA complexes coding for LO were embedded with fibroblasts as compared with control. In the wound healing model, wounded skin treated with GAMs loaded with PEI-pDNA complexes coding for LO showed superior mechanical strength over control wounded skin after one month of injury and implantation of GAMs.

Studies have shown that collagen-based GAMs were able to retain pDNA.[4] Naked pDNA on its own has very low transfection efficiency due to degradation by nucleases as well as repulsive ionic interaction with cell membrane.[15] Polycations have been shown to greatly increase transfection efficiency.[19,21,24] PEI is a polycation widely used for gene therapy purposes.[12,23,27] It is capable of interacting with and compacting DNA via ionic interaction. This protects DNA from degradation by nucleases and increases transfection efficiency via favorable ionic interaction with cell membrane. Transfection with such PEI-pDNA complexes is of a highly transient nature. Introducing these complexes in GAMs might increase their duration of expression

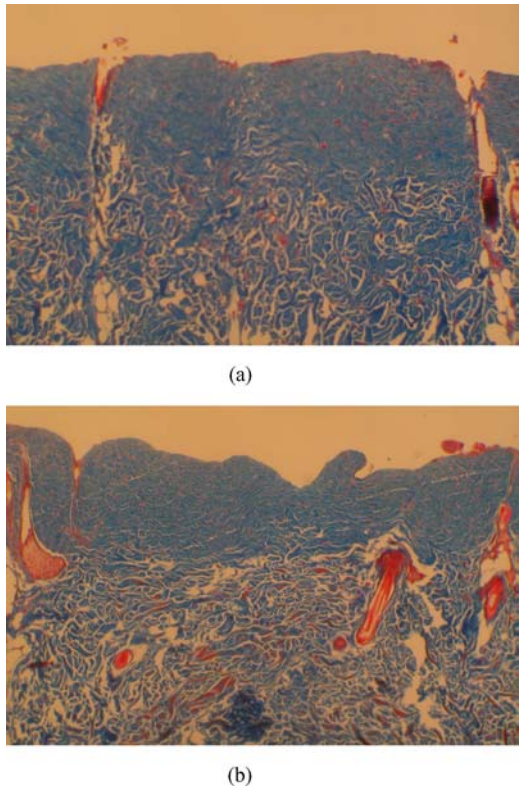

FIGURE 8. Masson's Trichrome stain on sections of rat skin treated with formalin. 7(a) control skin 7(b) LO-treated skin. Collagen stained for blue and fatty tissue stained for red. Healing rates are identical in both control-treated skin and LO-treated skin.

at a tissue site as compared with using naked pDNA. The success of our *in vitro* study performed in the presence of serum showed that PEI indeed was able to protect pDNA from such enzymatic degradation. Although PEI has been shown to be an effective agent for non-viral transfection, the presence of free PEI in the cellular nucleus may interfere with transcriptional and translational processes and thus induce cell death.[11] In one study, an N/P ratio of 16.7 was shown to have cytotoxic effect on COS-1 cells.[8] We have shown that fibroblasts were both viable and proliferating in the presence of PEI-pDNA complexes at the concentrations used in this study.

Although polycation-aided transfection can enhance transefection efficiency, the expression of the transgene is of a transient nature since the transgene is not incorporated into the cell genome. Transfection with PEI would typically lead to expression of proteins for 3–5 days. Since we observed expression of proteins at least 20 days, this suggests that GAMs were able to retain PEI-pDNA complexes for gradual uptake by the embedded cells, thus prolonging the expression duration. Permanent gene expression is undesirable in many applications since prolonged delivery of certain growth factors or proteins might have adverse

effects leading to pathological conditions. GFP transfected cells were observed 10 days and 20 days after being embedded into GAMs but not after 30 days. This shows that gene expression in this system is of a transient nature. We had used GFP transgene to monitor duration of expression since antibody for LO was not commercially available. We wanted to confirm that fibroblasts were able to gradually take up PEI-pDNA complexes and express the transgene encoded.

To further confirm the expression of LO, we used a fluorometric assay to monitor lysyl oxidase activity, making use of H_2O_2, a byproduct in its catalytic reaction. We had shown that LO activity in conditioned media collected from LO-producing GAMs was enhanced significantly at each time point as compared with control and the addition of BAPN abolished this increase in activity. The steady and small increase in LO activity in control GAMs over 4 weeks may be due to proliferation of fibroblasts in the GAMs. However, LO activity increases only up to 3 weeks in LO-producing GAMs and decreases afterwards, though the initial increase was much greater than that seen in control GAMs. Since the expression of protein in our GAM is of a prolonged but still transient nature, it might be suitable for various clinical applications.

Our *in vivo* study suggested that LO-producing GAMs might be able to strengthen newly formed tissue after injury. The effect of overexpression of LO and the ability of fibroblasts to migrate into implants was confirmed by our *in vitro* studies. An important application of this system could be for diabetic patients. In many diabetic patients, wound repair is severely impaired. The newly formed tissue in the wound site usually lacks mechanical integrity and the wound would very often reopen when subjected to tearing forces which are inevitable in certain parts of the body. Our LO-producing GAM system might provide a solution to this by locally delivery LO transgene for extended crosslinking activity which in turn might confer enhanced mechanical strength of the newly formed tissue. Moreover, prior studies had shown that transfection with LO did not alter proliferation and ECM composition, and overexpression of LO led to increase levels of desmosine (a crosslink product of elastin) in the ECM.[6] It is probable that the increase in mechanical strength observed was due to the catalytic action of LO on ECM proteins. LO was shown to induce directional migration of fibroblasts[17] and it is uncertain whether this contributes to the enhanced mechanical strength observed.

Apart from the particular application in this study, this GAM system can be implemented in a wide variety of applications to enhance tissue mechanical properties. The procedure to fabricate the GAMs used in this study was very simple as compared with other similar gene delivery systems. A great advantage of these GAMs is that they can be formulated to include multiple which could lead to advances in tissue engineering and other applications. The GAM technology is also advantageous in cases where proteins undergo complex processing when synthesized by cells as compared with exogenously delivered recombinant proteins. In the GAM system, bioactive factors are continually being produced by local cells for replenishment. Moreover, collagen is biodegradable and would disintergrate over time in the host body if being implanted. In this study, the collagen-based GAMs implanted in rats completely disintegrated after one month when the rats were sacrificed. The rats did not have any adverse reaction to the implanted GAMs indicating that these biodegradable materials might be safe to be used in humans. The GAM system is versatile, cost-effective and can be implemented in many applications which require different factors.

CONCLUSION

We demonstrated the efficacy of this GAM system for overexpression of LO both *in vitro* and *in vivo*. Enhanced mechanical strength was achieved in a collagen gel model as well as a wound healing model in rat skin. This may have application in treatment of poorly healing wounds.

ACKNOWLEDGMENTS

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