ARTICLE

Supplementary information

ECM-mimicking nanofibrous scaffold enriched with dual growth factor carrying nanoparticles for diabetic wound healing

Amritha Vijayan [a,b,§] Nanditha C.K [a,b,§] G.S. Vinod Kumar*

Experimental

Material and methods

Bovine serum albumin, collagen, chitosan, PLGA (50;50, MW 30,000-60,000), VEGF, bFGF and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma, Polyethylene glycol (MW 1500), heparin, DMSO, Trifluoroacetic acid (TFA) and acetone was from MERK and Hexafluoro-2-propanol was from Spectrochem, Fetal bovine serum (FBS) was purchased from Pan America, ELISA kit from R & D Biosystems and DMEM live/dead assay kit were from Invitrogen.

Synthesis of PEG aldehyde

PEG aldehyde was synthesized by the oxidation of PEG 1500 with DMSO and acetic anhydride as reported earlier.[1] 22.5 g of PEG was dissolved in a mixture of 27ml DMSO and 3ml chloroform and acetic anhydride, in the ratio of 12:1. PEG was added and kept for overnight stirring. PEG aldehyde was precipitated with excess diethyl ether. The precipitate was dissolved in chloroform and re-precipitated twice with diethyl ether.

Synthesis of PEG aldehyde cross-linked BSA nanoparticle

PEG aldehyde cross-linked Bovine serum albumin (BSA) nanoparticles were prepared by desolvation method using ethanol as a desolvating agent. BSA was dissolved in water to which ethanol was added under constant stirring. PEG aldehyde was added to BSA in the ratio 0.625:1 and left to react for 2 hours. The remaining ethanol was evaporated using rotor-evaporator and the solution was lyophilized.

Heparin-binding to BSA nanoparticle and its quantification

Heparin was bound to BSA nanoparticles by incubating it with heparin (10 mg) in the presence of EDC:NHS (0.6mmol: 1.2mmol) in MES buffer of pH 5.5 for 4 hours. Quantification of heparin functionalized on the surface of BSA nanoparticle was done by toluidine blue assay.[2] Briefly, 1mg of BSA-Heparin nanoparticle was suspended in a Toluidine blue solution (0.005%, w/v) containing 0.2 wt% NaCl and 0.02 N HCl. The suspension was vortex vigorously for 30 seconds, followed by centrifugation at 12,000 rpm for 10 min. The absorbance of the supernatant was determined at 590 nm using plate reader. Absorption background from the control samples consisting of BSA nanoparticle alone was subtracted from the test samples. The amount of heparin incorporated in the final particles was derived from a series of standards at a heparin concentration.

Growth factor binding to the BSA nanoparticles

10 mg of heparinized BSA nanoparticles were taken in 1 ml of PBS, and 200 µl of growth factor cocktail containing 100ng of VEGF, and 100ng of bFGF was added to it and incubated for 4 hours at room temperature. The supernatant was collected, and ELISA was performed to calculate the binding efficiency of growth factors to the heparinized BSA nanoparticles.

Characterization of the nanoparticles
Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was used to examine the morphology of the nanoparticles prepared in this study. TEM micrographs of nanoparticle samples deposited on copper grids were obtained with a JEOL 1011 (Japan).

Dynamic Light Scattering (DLS)

The hydrodynamic diameter of BSA nanoparticles and growth factor bound BSA nanoparticles were measured by dynamic

light scattering (DLS) method using Delsa™ Nanoparticle size analyzer (Beckman Coulter, Inc.) instrument.

Preparation of PLGA-Collagen-Chitosan nanofiber scaffold

For the nanofiber preparation, PLGA and collagen were dissolved in Hexafluoroisopropanol (HFIP) and chitosan was dissolved in HFIP: TFA (90:10). The solutions are then blended in the ratio 74:25:1. The solutions were electrospun at a flow rate of 0.2 ml/ h, at a voltage of +12 kV and the tip to collector distance of 12 cm. For the preparation of growth factor containing nanoparticle loaded scaffolds, the nanoparticle was dispersed in PBS, sonicated and added to the PLGA- collagen-chitosan electrospinning scaffold and lyophilized prior to use.

SEM Analysis

The morphology of electrospun PLGA-Collagen-Chitosan fibers was observed by scanning electron microscopy (SEM) (Hitachi) at an accelerated voltage of 10 or 15 kV. Before SEM, the samples were sputter-coated with gold.

Growth factor release kinetics

To assess the growth factor release kinetics, VEGF and bFGF coated BSA nanoparticle embedded scaffolds were used. The scaffolds weighing 10 mg were loaded into 100 kDa dialysis membrane and placed in the 100ml of PBS solution with pH 7.4. Samples were then placed on an orbital shaker at 37°C. At predetermined time points, 100 µl of PBS solution was collected and the release profiles of VEGF and bFGF were analyzed using ELISA following the manufacturer's instructions (R and D Biosystems). The loading efficiency of VEGF and bFGF on the nanoparticles was determined by measuring the concentration of the growth factors in washing solutions collected during the nanoparticle formation process.

Cellular assays

HaCaT cells (human keratinocyte cell line) were purchased from National Centre for Cell Science (NCCS), Pune. HaCaT cells were grown in DMEM supplemented with 10% FBS and 1% antibiotics. On reaching 70% confluence, the cells in tissue culture flasks were trypsinized with PBS solution containing 0.25% trypsin and 0.03% EDTA.

Cell Viability Assay

MTT3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was performed to assess the cell viability. HaCaT Cells were seeded onto a 96 well plate (2×10^3cells/well) and incubated under standard growth conditions for 24 hours. Media treated with only growth factors (GF – VEGF and bFGF 100ng/ml each), BSA-GF nanoparticles (GF-NP - 500µg/ml), Nanofibers (NF), BSA-GF embedded nanofibers (GF-NP-NF), for different time periods (24,48, and 72 h), were collected and added to wells containing cells and incubated for 48 hours. After 48 hours, the cells were incubated for 4 h with 10% v/v MTT (5 mg/ml dissolved in PBS pH 7.4). The media was then removed and the formazan crystals thus formed were dissolved in isopropyl alcohol. Absorbance was measured at 570 nm using BioradiMark™ Microplate Absorbance Reader. Percentage viability was calculated using the formula: [(Avg. OD of test / Avg. OD of control) × 100].

Scratch Wound Assay

The HaCaT cells were grown to form a confluent monolayer in 12 well culture plates and were then scratched with 2.5 µL pipette tip. A uniform cell-free zone was created in each well. The wells were washed with PBS multiple times to remove cellular debris. Media treated with BSA-GF nanoparticles (GF-NP -500µg/ml), Nanofibers (NF), BSA-GF embedded nanofibers (GF-NP-NF) samples with the same concentration as cell viability assay were added to the wells and incubated for 24 hours. The cell culture wells were observed at 0 hours and 24 hour time points after injury and images were taken and the wound closure (diameter in µm) between wound edges was calculated with a computer-assisted analysis system (Olympus CellSens). The percentage wound closure was calculated using the formula: [% Wound closure = (Pre-migration)$_{diameter}$ − (Migration)$_{diameter}$ / (Pre-migration)$_{diameter}$ ×100]

Live/Dead Assay

A live/dead assay was performed to evaluate cell viability and proliferation of keratinocyte cells when treated with a BSA-GF embedded nanofiber system. On a 12-well plate serum-free medium containing the samples (Groups: BSA-GF nanoparticles (GF-NP - 500µg/ml), Nanofibers (NF), BSA-GF embedded nanofibers (GF-NP-NF) was added to appropriate group wells and HaCaT cells (1×10^5cells/well) were seeded on it and was incubated for 3 days. The cells were then labelled with calcein AM and ethidium homodimer-1 to distinguish the population of live cells. Images were acquired using a laser confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany).

Animal Studies

All animal experiments were approved by the institutional animal ethics committee at Rajiv Gandhi Centre for Biotechnology. Reference number: IAEC/285/GSV/2015).

Diabetes induction by streptozocin injection

Diabetes was induced in male Swiss albino mice, 6-8 weeks of age, weighing 25-30 g using a single dose of 180mg/ kg streptozotocin (STZ) injection as per standardized protocol.[3,4] After 48h whole-blood glucose was determined using blood glucose monitoring system (Accu-Chek). STZ-treated mice with whole-blood glucose levels higher than 300 mg/dL were considered diabetic.

Excision wound creation

Animals were anesthetized using Isoflurane inhalation anesthesia. The dorsal surface of each animal was shaved and sterilized with 70% ethanol. A full-thickness excisional wound of 8 mm-diameter was created using a sterile biopsy punch. After surgery, mice were randomly divided into wounds treated with GF (Growth factors, VEGF and bFGF - Group 1), GF-NP (Growth factors coated BSA-PEG nanoparticles - Group2), NF (PLGA- Collagen-Chitosan - Group 3), GF-NP- NF (VEGF and bFGF coated BSA-PEG nanoparticles embedded nanofiber scaffold - Group 4) and control groups (0.1M PBS, pH 7.4). The appropriate treatment materials (based on each group) were applied to the wound site. The wounds were sutured along the wound boundary with silicone sheet without covering the wound area to avoid skin contraction after surgery. Wound area monitoring, on the 3rd, 7th and 15th day, was measured by tracing the wound boundaries. Wound contraction (%) was calculated using the following formula:

Wound contraction= (Area (0 day)-Area (n day))/ (Area (0 day)) ×100%

Where "n" represents the day, like 3rd, 7th and 15th.

The healing was evaluated at different time periods and on the 15th day, animals were sacrificed, wound tissue was excised and processed for histopathology and RT PCR.

Histopathology

On day 15, animals were sacrificed; their skin biopsies were collected and fixed in 10% formalin, dehydrated through graded alcohol series, cleared in xylene and embedded in paraffin wax. Sections of 5 μm were cut and stained with hematoxylin, eosin (H&E) and Masson's trichrome staining.

Histomorphometrical analysis of angiogenesis by image analysis

Histological sections stained with H&E were visualized in a Nikon Eclipse 55i microscopic system, Japan. Images were collected using 10X, 20X and 40X magnification objectives. Five fields were randomly selected for every test and control samples. All groups were evaluated for angiogenesis quantification. The Cell Counter ImageJ software was used to count the number of blood vessels and the data was reported as the average number of blood vessels of three samples of each sampling period to both groups (Fig. S1†).

RT PCR

Wounds were harvested on day 15, and healed skin samples were taken and stored in RNA later.[5] The samples were taken, homogenized with trizol and RNA was extracted. Purified RNA was quantified by absorbance spectroscopy at 260/280 nm. After extraction and purification, RNA was converted to cDNA using iScript Reverse Transcription Supermix for reverse transcription then stored at −20°C until use. SYBR green master mix was used as a

DNA binding dye.[6] Quantitative expression levels of mRNA of col I, col III and ki67 were evaluated.

Statistics

The experiments performed in this study were done in triplicate and the data were analyzed using GraphPad Prism™ software (version 6). Statistical analysis was carried out by means of a two-way analysis of variance (ANOVA, non-parametric analysis). Data are shown as the mean ± standard deviation (SD), n= 3, with significance accepted when $p < 0.05$.

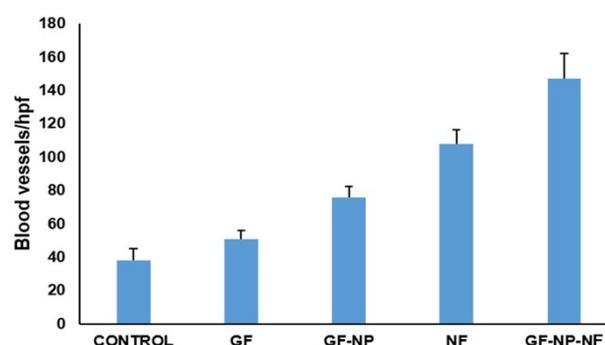### Histomorphometrical analysis of angiogenesis by image analysis

Fig. S1†. Histomorphometrical analysis of angiogenesis. Blood vessels (hpf -High powered field) formation in Day 15 wound biopsy samples (Control, GF, GF-NP, NF, GF-NP-NF).

Notes and references

1. M.Zhang, X.H. Li, Y.D. Gong, N.M. Zhao and X.F. Zhang, *Biomaterials,* 2002, **23**, 2641-2648.
2. F.C.Macintosh, *Biochem. J*, 1941, **35**, 776-782.
3. X.Zhang, T.Cui, J.He, H.Wang, R.Cai, P.Popovics, I.Vidaurre, W.Sha, J.Schmid, B.Ludwig, N.L Block, S.R.Bornstein and A.V.Schally, *Proc. Natl. Acad. Sci.* 2015, **112**, 13651-13656.
4. J.Jeong, M.J. Conboy and I.M. Conboy, *Acta Pharmacol. Sin*. 2013, **34,** 1052.
5. C.M.Coffin, J.A.Holden, J.W.Swimmermann, J.W.Gerwels, B.K.Summers, D.A.Jones and S.A.Leachman, *Mod. Pathol.* 2001, **14**, 116-128.
6. E.D.Ruedrich, M.K.Henzel, B.S.Hausman and K.M. Bogie, *J Biomol. Tech.* 2013, **24,** 181-186.