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Effects of cerium oxide nanoparticles on the growth of keratinocytes, fibroblasts and vascular endothelial cells in cutaneous wound healing

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ABSTRACT

Rapid and effective wound healing requires a coordinated cellular response involving fibroblasts, keratinocytes and vascular endothelial cells (VECs). Impaired wound healing can result in multiple adverse health outcomes and, although antibiotics can forestall infection, treatments that accelerate wound healing are lacking. We now report that topical application of water soluble cerium oxide nanoparticles (Nanoceria) accelerates the healing of full-thickness dermal wounds in mice by a mechanism that involves enhancement of the proliferation and migration of fibroblasts, keratinocytes and VECs. The Nanoceria penetrated into the wound tissue and reduced oxidative damage to cellular membranes and proteins, suggesting a therapeutic potential for topical treatment of wounds with antioxidant nanoparticles.

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1. Introduction

Impaired wound healing and its medical complications result in a large burden of morbidity and mortality worldwide. Wound repair requires the proliferation and migration of fibroblasts and keratinocytes which re-establish the normal cellular and extracellular matrix composition of skin, and the growth of vascular endothelial cells (VECs) to form new blood vessels that supply nutrients to the skin cells [1]. The ability of all three cell types to restore skin integrity and function is compromised in non-healing wounds, and one factor that contributes to such impaired wound healing is sustained oxidative stress [2]. Cells within and surrounding wounds experience elevated levels of superoxide, hydrogen peroxide and nitric oxide, and proteins modified by nitration and the lipid peroxidation product 4-hydroxynonenal. Levels of endogenous antioxidants such as glutathione and vitamin E are diminished under conditions that impair wound healing including aging and diabetes [3]. The potential for exogenous antioxidants to enhance wound healing has been tested in only a few studies using animal models with variable results [4–7].

The antioxidant capacity of cerium oxide nanoperticles (Nanoceria) has been explored recently; nanoceria were reported to scavenge superoxide radical [8,9], hydrogen peroxide [10], hydroxyl radical [11] and nitric oxide radical [12]. Therefore, Nanoceria have been tested in biological systems wherein they can protect tissues against radiation induced damage [13], protect against laser-induced retinal damage [14], increase life span of photoreceptor cells [15], reduce spinal injury [16], reduce chronic inflammation [17] and promote angiogenesis [18]. In this study we explore the potential therapeutic effect of Nanoceria in an animal model of wound healing.

2. Materials & methods

2.1. Synthesis and characterization of nanoceria

Cerium oxide nanoparticles were synthesized using wet chemistry methods as described previously [19]. Briefly, stoichiometric amounts of cerium nitrate hexahydrate (99.999% pure from Sigma Aldrich) was dissolved in deionized water. The solution was oxidized using excess hydrogen peroxide. After the synthesis of nanoparticles, the pH of the solution was maintained below 3.0 using 1 N nitric acid

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to keep the Nanoceria in suspension. The size and morphology of the nanoceria were evaluated using high resolution transmission electron microscopy (HRTEM), with FEI Tecnai F30 EDX analyzer. X-ray photon spectrometer 5400 PHI ESCA (XPS) with Mg-Kα X-radiation (1253.6 eV) at a power of 350 W was used to analyze the surface property and oxidation state of Nanoceria. X-ray diffraction (XRD) was performed using monochromatized CuKα radiation.

2.2. Cell cultures

Human keratinocyte cells were obtained from ATCC and grown in keratinocyte medium containing 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Invitrogen). Skin tissue explants established from young adult mice were used to harvest fibroblasts. Dermal tissue specimens were cut into ~3–5 mm pieces. These fragments were placed on the surface of 100 mm Petri dishes for 40–50 min to allow adherence of the tissue to the culture surface. 10 ml of DMEM with 20% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin (pH 7.6; 37 °C), was gently added to the culture dishes. Cultures were maintained in a humidified incubator at 37 °C in a 5% CO_2/95% air atmosphere. Cultures were passaged on reaching 75–80% confluence, using 0.05% trypsin/EDTA (Invitrogen) and the medium was changed every two days. Cells were used at passage 4 or 5 for cell migration and proliferation assays in order to minimize the influence of genetic alterations and senescent changes. Human microvascular endothelial cells (HMEC-1 cells) were kindly provided by Fransisco Candal (Centers for Disease Control, Atlanta, GA). The latter cells are of human dermal microvascular origin and retain the morphologic, phenotypic, and functional features of normal human microvascular endothelial cells [20]. HMEC-1 cells were maintained in MCDB 131 formula (Invitrogen) supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 1 mg/ml hydrocortisone and 10 mM L-glutamine.

2.3. Full-thickness skin wounds and quantification of healing

These methods were similar to those described previously [7]. All experiments were performed using 3–4 month-old male C57BL/6 mice. Mice were anesthetized using 2% vaporized inhaled isoflurane and the dorsal skin was cleansed with Betadine. Two full-thickness wounds were created in the skin on the back of each mouse using a 4 mm diameter biopsy punch (Miltex Instrument, York, PA, USA) and a biotome (Acu Punch, Acuderm Inc., Fort Lauderdale, FL, USA). Mice were treated with vehicle (10 μl of deionized water) or 10 μl of a 10 μM solution of Nanoceria applied directly to the wound site once daily. Some mice in each group were euthanized on days 1, 3, 5, 8 and 13 post-wounding, and skin tissue samples from the wound site were collected from all of the mice for histological and biochemical analyses. Some mice from each treatment group ($n = 6$) were evaluated daily for 13 days following wounding. Digital photographs of the injury site were taken with a standard-sized dot placed beside the wound; wound size was expressed as the ratio of the wound area to the dot measurement. Measurements of wound length and width were obtained using a caliper. The first post-incision wound measurement was made on day 0. The measurements were done without knowledge of the treatment history of the mice. Wound area was calculated using digital planimetry; linear healing progress was determined using the standard formula [7].

2.4. Histological examination

Biopsy specimens that included the central part of the wounds (days 1, 3 5, 8 and 13) were obtained perpendicularly to the dorsal midline from mice for light microscopy. Skin specimens were fixed in formalin, dehydrated through a graded ethanol concentration series, cleared in xylene, and embedded in paraffin wax. Sections were cut at 5 μm thickness using a vibratome, and were stained with hematoxylin and eosin. The histomorphometric method was an adaptation of the point-counting procedure. The counting of mononuclear inflammatory cells and blood vessels was performed at a total magnification of 200 in 3 random fields per section limited to the wounded area. Images were acquired using a Nikon Eclipse 80i microscope and images were analyzed using IP lab software (BD Biosciences Bioimaging, Rockville, MD). After acquiring transmitted light images, a 252-square graticule was superimposed on the screen over the wounded site to facilitate counting. A standard histologic grading system was used to evaluate cellular aspects of the wound healing process. All the slides were evaluated by a veterinary pathologist (S. C.) in a blinded manner.

2.5. Immunoblot analysis

Proteins were extracted from skin tissue samples using T-PER tissue protein extraction buffer with protease inhibitor cocktail (Sigma). Methods for protein quantification, electrophoretic separation of proteins, and transfer of the proteins to nitrocellulose membranes have been described previously [21]. Membranes were incubated in blocking solution (1% BSA in Tween Tris-buffered saline; TTBS) overnight at 4 °C followed by a 1 h incubation in primary antibody diluted in blocking solution at room temperature. Membranes were then incubated for 1 h in secondary antibody conjugated to horseradish peroxidase and bands were visualized using a chemiluminiscence detection kit (ECL, Amersham). The primary antibodies were a mouse monoclonal selective for proteins that are covalently modified by HNE on lysine residues [22] and a β-tubulin antibody (Sigma).

2.6. Immunofluorescence

Tissue samples were embedded in Optimal Cutting Temperature (OCT) compound and frozen. Sections (6 μm diameter) were cut with a cryostat and fixed in acetone. Subsequently sections were blocked with 10% goat serum before being incubated with rabbit anti-α smooth muscle actin (1:200; Abcam) and mouse anti-nitrotyrosine (1:200; Zymed) overnight at 4 °C. After being washed, the sections were incubated in anti-rabbit or anti-mouse IgG conjugated to Alexa 568 and 488 (1:200 dilution), respectively for 45 min at room temperature. Sections were counterstained with Hoechst 33342 (Invitrogen) visualized under a Nikon Eclipse 80i microscope. Using calibrated images the total area of positive pixel intensity was measured and analyzed with two-way ANOVA (Newman–Keuls post-hoc tests for pair-wise comparisons) using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego CA, USA.

2.7. Endothelial cell scratch wound healing assay

HMEC-1 cells, human keratinocytes and mouse fibroblasts were seeded into 60 mm plates and grown to confluency. After 24 h of serum starvation (DMEM supplemented with 1% FBS), cells were treated with either vehicle or Nanoceria (500 nM, 1 and 10 μM). The cell monolayer was then damaged by scratching with a sterile 200 μl pipette tip. Cells were then cultured for additional period of 24 h in a serum-free basal medium in the continued presence of vehicle or Nanoceria. Cells were then fixed in a solution of 4% paraformaldehyde in PBS and stained with crystal violet. Cells in the injury area were visualized under phase-contrast optics (10× objective) and the number of cells which had migrated into the initially cell-free scratch area was counted.

2.8. Endothelial tube formation and chemotaxis cell migration assays

HMEC-1 cells (1×10^3 cells/well) were dispensed to Matrigel-coated 8-well chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY, USA) in 125 ml of EGM-2 medium containing either vehicle or Nanoceria (500 nM, 1 and 10 μM) and incubated for 18 h. The cells were then visualized by microscopy and tube formation was quantified as described previously [7]. Analysis of migration of keratinocytes, fibroblasts and HMEC-1 cells was performed using Transwell membrane filters (Corning, Costar) containing a polycarbonate filter with 8 mm pores. The bottom chamber was filled with complete growth medium containing chemo-attractant growth factors. Cells (5×10^4 in 100 μl) were seeded into each transwell with EGM containing 0.2% fetal bovine serum with vehicle or Nanoceria (500 nM, 1 and 10 μM) and allowed to migrate for 6 h. At the end of the incubation, non-migrated cells remaining in the transwell insert were removed. The migrated cells (on the outer bottom of the transwell) were fixed with methanol and stained with hematoxylin and eosin. The stained cells were counted in 5 or more random 100× fields. Each experiment was performed in triplicate, and the experiment was repeated twice. Growth correction was not applied because a significant increase in the cell number did not occur during the incubation period of 6 h.

2.9. Cell proliferation assay

The proliferation of cultured endothelial cells, keratinocytes and fibroblasts was measured using a colorimetric assay. Cells (1×10^4) were incubated with either vehicle or Nanoceria (500 nM, 1 and 10 μM) for 24, 48 or 72 h. Ten microliters of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide solution (R&D Systems Inc. Minneapolis, MN) was added to each well and the cells were incubated for a further 4 h at 37 °C. After the cells were washed 3 times with PBS (pH 7.4), the insoluble formazan product was dissolved by incubation with 100 μl detergent for 2 h. The absorbance of each well was measured on an enzyme-linked immunosorbent assay (ELISA) micro-plate reader at 570 nm. Each experiment was performed in quadruplicate. The cell proliferation rate was calculated as absorbance Nanoceria-treated cultures/absorbance control cultures ×100.

2.10. Statistical analyses

All values are expressed as the mean and SEM. Data were analyzed by ANOVA with Newman–Keuls post-hoc tests for pair-wise comparisons using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

3. Results

Shape, size and physiochemical properties of nanoparticles are very important for biological applications, and we therefore engineered nanoparticles within a size range of 3–5 nm (Fig. 1A), with agglomeration size ≤50 nm (data not shown). The selected area

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