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Functional and mechanistic investigation of Shikonin in scarring

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ABSTRACT

Scarring is a significant medical burden; financially to the health care system and physically and psychologically for patients. Importantly, there have been numerous case reports describing the occurrence of cancer in burn scars. Currently available therapies are not satisfactory due to their undesirable side-effects, complex delivery routes, requirements for long-term use and/or expense. *Radix Arnebiae* (*Zi Cao*), a perennial herb, has been clinically applied to treat burns and manage scars for thousands of years in Asia. Shikonin, an active component extracted from *Radix Arnebiae*, has been demonstrated to induce apoptosis in cancer cells. Apoptosis is an essential process during scar tissue remodelling. It was therefore hypothesized that Shikonin may induce apoptosis in scar-associated cells. This investigation presents the first detailed *in vitro* study examining the functional responses of scar-associated cells to Shikonin, and investigates the mechanisms underlying these responses. The data obtained suggests that Shikonin inhibits cell viability and proliferation and reduces detectable collagen in scar-derived fibroblasts. Further investigation revealed that Shikonin induces apoptosis in scar fibroblasts by differentially regulating the expression of caspase 3, Bcl-2, phospho-Erk1/2 and phospho-p38. In addition, Shikonin down-regulates the expression of collagen I, collagen III and alpha-smooth muscle actin genes hence attenuating collagen synthesis in scar-derived fibroblasts. In summary, it is demonstrated that Shikonin induces apoptosis and decreases collagen production in scar-associated fibroblasts and may therefore hold potential as a novel scar remediation therapy.

1. Introduction

Scarring is a significant medical burden; financially to the health care system and physically and psychologically for patients. Elective and trauma operations in the Western world result in 100 million patients acquiring scars per annum, many of whom require considerable ongoing treatment. Of this number, an estimated 11 million are keloid scars and 4 million are burn scars, with 70% of these burns occurring in children [1]. The cost of treatment for scars has been estimated to be at least $4 billion per annum in the US [2]. In addition, many adverse effects, such as loss of function, restriction of tissue movement and itching are experienced by people with scars [3]. While research effort has been directed at the development of improved approaches to remediate scars, currently available therapies are not satisfactory due to their undesirable side-effects, complex delivery routes, requirements for long-term use and/or expense [4,5]. Therefore, a scar remediation therapy that is simple to use and has minimal side-effects and cost is of great importance for both clinicians and patients.

Abbreviations: αSMA, alpha-smooth muscle actin; Bcl-2, B-cell lymphoma 2; Caspases, Cysteine-dependent aspartate-directed proteases; COL1A1, collagen type I gene; COL3A1, collagen type III gene; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; Erk1/2, extracellular signal-regulated kinase 1 and 2; FCS, fetal calf serum; HSF, hypertrophic scar-derived human skin fibroblasts; JNK1/2, c-Jun N-terminal kinase 1 and 2; KC, human skin keratinocytes; NaOH, sodium hydroxide; p38α/β, mitogen-activated protein kinase 14 α and β; p-p38α/β, phospho-p38 α and β; p-Erk1/2, phospho-Erk 1 and 2; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SDS–PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of the mean; SFM, serum free medium; TUNEL, Terminal deoxynucleotidyl Transferase dUTP nick end labelling.

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In any phase of wound healing the dynamic balance between synthesis and degradation of the extracellular matrix (ECM) is important to sustain the structural integrity of the healing tissue [6]. Some individuals, however, experience the phenomenon whereby large amounts of collagen are continually produced; resulting in hypertrophic or keloid scars. It is thought that an abnormally large number of fibroblasts accumulating in the wound site, or excessive deposition of collagen, caused by either increased production of collagen by existing cells or the presence of an increased number of cells, are the main reasons underlying scar formation and maintenance [7]. Keratinocytes also play an important role in scar formation. They communicate with dermal fibroblasts via paracrine mechanisms, affecting physiological responses in the fibroblasts, including those associated with scarring [6]. For example, Duinslaeger et al. demonstrated that factors secreted by keratinocytes *in vitro* inhibited collagen lattice contraction by skin fibroblasts [8].

Chinese herbal medicine has been used and developed continuously for thousands of years. Recently, increasing attention has been focused on Chinese herbal medicine practices in Western counties. The United States is now the biggest importer of Chinese herbal medicines, spending US$7.6 billion in 2010 [9]. In a recent study two-thirds of the Australian population self-reported they had used some form of Chinese herbal medicine in the past year [9]. However, in many cases research is clearly required to ensure and validate the safety, efficacy and quality of the Chinese herbal medicines [9].

Radix Arnebiae (*Zi Cao*), a Chinese herbal medicine, has been clinically used to treat burns in China for thousands of years [10–12]. Shikonin (SHI), a natural naphthoquinone isolated from *Zi Cao*, has been demonstrated to possess various biological activities, such as anti-tumourigenic, anti-oxidant, anti-bacterial and anti-inflammatory properties [13]. The anti-tumourigenic properties of SHI in particular have been intensively investigated; SHI is reported to induce apoptosis in many different cancer cell lines [13–16]. Chang et al. reported SHI induced the up-regulation of mitogen-activated protein kinase 1 (ERK) signalling and down-regulated B-cell CLL/lymphoma 2 (Bcl-2) resulting in reduced viability of 143B osteosarcoma cells [14]. When exposed to SHI, the colorectal cancer cell line, COLO 205, was observed to up-regulate the tumour suppressor protein p53 [13]. Additionally, activation of mitogen-activated protein kinase 8 (JNK), apoptosis-related cysteine peptidase (caspase 3) and mitogen-activated protein kinase 14 (p38) was observed in HL-60 promyelocytic leukaemia cells after treatment with SHI [15,16]. These data provide a clear link between SHI and apoptosis in a range of cancer-related studies. Apoptosis is well described as an intrinsic process during scar tissue remodelling. The number of apoptotic cells is greatly increased following wound closure and remains prevalent during wound maturation; characterised by remodelling of the ECM [7,17]. The biological response of somatic tissues, and skin in particular, to SHI remains poorly understood and uncharacterised. We hypothesised that the anecdotal beneficial scar-remediation effects of Zi Cao results from the SHI component stimulating apoptosis in cells that contribute to scar formation and maintenance and report herein results supporting this concept.

2. Materials and methods

2.1. Shikonin solution preparation

SHI powder was produced by the National Institute for the Control of Pharmaceutical and Biological Products, China, and was gifted by Associate Professor Yonghua Su (Second Military Medical University, Shanghai, China). SHI powder was dissolved in DMSO at 10 mg/mL as a stock solution and stored at −20 °C.

2.2. Skin collection and cell culture

Skin samples were obtained from consenting donors undergoing elective abdominal and breast reduction procedures at St Andrew's Hospital (Brisbane, Australia). Human ethics committee approval was provided by St Andrew's Hospital (2003/46), and by the Queensland University of Technology (3865H).

Primary human keratinocytes (KC) were extracted from native human skin and cultured *ex vivo* in "Green's" medium containing 10% FCS following methods previously described by Rheinwald and Green and others [18,19].

Hypertrophic scar-derived fibroblasts (HSF) and normal fibroblasts (nHSF) isolated from three different patients were purchased from Cell Research Corporation (Singapore). These cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% FCS (Hyclone, Australia) at 37 °C, 5% CO_2/95% air [20].

2.3. Microarray analysis

Total RNA was extracted from HSF and nHSF using the Qiagen RNeasy Mini kit (Qiagen, Australia), as per the manufacturer's protocol. HSF and nHSF were cultured in 25 cm^2 cell culture flasks (Nunc, Roskilde, Norway) at a density of 2×10^5 cell/5 mL in culture media for 24 h. To lyse the cells, 600 μL of buffer RLT plus 6 μL β-mercaptoethanol (Sigma–Aldrich, USA) was added into the cell culture flasks and the cells were detached with a cell scraper and were recovered. Cell lysates were stored at −80 °C until analysis. The HumanHT-12 v3 Expression BeadChip microarray (Illumina, USA), used to determine differentially expressed genes in cultures of HSF and nHSF, was performed using total RNA extracts (above) by the Special Research Facility Microarray Service, University of Queensland. Data analysis was performed using Gene Spring GX 10.0 (Agilent Technologies, USA) and quartile normalization technique was applied. Data were pre-processed using the baseline to median baseline transformation tool with standard filtering options. Changes occurring between sample pairs were manually investigated by fold-change on a gene-to-gene basis. Specifically, only genes with fold-change values greater than ±2 were included.

2.4. Real-time microscopy

Real-time microscopy was used to visualize the response of HSF to SHI treatment over 96 h. In brief, HSF cells (1.6×10^5/2 mL) were plated into 6 well plates and treated with SHI (1, 0.5 μg/mL) treatment 24 h later. Real-time microscopy was then performed with a Leica AF6000 Widefield Microscope (Leica Microsystems) and images were taken every 20 min for 96 h. Still images and movies were prepared using Leica Application Suite software.

2.5. Functional assays in skin cells

The effects of SHI on cell viability and collagen metabolism were investigated using the Alamar Blue and Sirius Red assay techniques, respectively. HSF (2×10^4) and KC (4×10^4) cells were seeded into 48-well plates per well in 500 μL of culture media and cultured for 24 and 48 h, respectively. When cultures were approximately 80% confluent, spent media was removed and replaced with 450 μL of fresh culture medium. Serial dilutions of SHI (0.0625, 0.125, 0.25, 0.5 and 1 μg/mL) were made in a final volume of 50 μL DMSO. Test SHI samples or Control DMSO samples (0.0000625%, 0.000125%, 0.00025%, 0.0005% and 0.001% v/v) in 50 μL were added to respective wells in a final volume of 500 μL.

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