Gene expression changes in diabetic wound healing as induced by photobiostimulation *in vitro*

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Abstract. Diabetes Mellitus (DM) is a complex metabolic disorder resulting in hyperglycaemia. Impaired wound healing is a serious complication of diabetes, and is a severe public health problem. Photobiostimulation is a non-invasive form of treatment known to enhance healing of such wounds using low energy lasers. This study investigated the changes in extracellular matrix (ECM) gene expression in diabetic wounded fibroblasts *in vitro* after photobiostimulation at 830 nm. Normal (N-unstressed), normal wounded (NW-stressed) and diabetic wounded (DW-stressed) fibroblasts were incubated for 48 h after irradiation using a continuous wave diode laser at a wavelength of 830 nm with 5 J/cm^2. Non-irradiated cells (0 J/cm^2) were used as controls. The gene expression profile (84 genes) was assessed using an ECM real-time reverse transcription polymerase chain reaction (RT-PCR) array with the appropriate controls included. Sixty one genes were significantly regulated (55 up-regulated and 6 down-regulated) in N-cells; 40 genes (20 up-regulated, and 20 down-regulated) in NW-cells; and 42 genes (9 up-regulated and 33 down-regulated) in DW-cells. Several genes were down-regulated in DW-cells as compared to N- and NW-cells. Photobiostimulation modulated the expression of important genes in wound healing, including cell adhesion molecules, integrins, ECM proteins, proteases, and inhibitors involved in the ECM. An in depth comprehension of the molecular and biological processes may create an improved therapeutic protocol for the treatment of diabetic wounds.

1. Introduction

Diabetes Mellitus (DM) is a complex metabolic disorder resulting in hyperglycaemia (high glucose). Complications of diabetes and other non-healing ulcers remain a major concern in public health. Global statistics show that millions of people suffer from this disease, with a prevalence of 15% with diabetic foot ulcers and 3% with lower limb amputation [1]. Wound healing involves a series of biological processes that must occur appropriately within a given time, and necessitates the interaction of various cells and growth factors to ensure proper healing [2-4]. Fibroblasts are very important in wound healing; they maintain homeostatic balance, and through cell proliferation, differentiation and extracellular matrix (ECM) development stimulates the process of tissue repair by synthesising collagen around the matrix [5,6]. Chronic ulcers may result from an imbalance between ECM formation and degradation [7]. Hyperglycaemia can affect these cellular and biological activities through oxidative stress or alkaline glycation end (AGE) products which in turn affect changes in genes encoding for ECM proteins and proteases [8], leading to impair wound healing [9]. Several investigations have shown diabetes to affect gene expression as well as wound healing [10,11]. Photobiostimulation is a non-invasive form of light treatment known to enhance healing of wounds. Photobiostimulation at various wavelengths and dosages is known for its stimulatory effect, and enhances wound healing in animal and clinical studies, as well as cell cultures [12-14]. Literature has shown that photobiostimulation is able to effect the expression of genes in animal and cell culture models [15-17]. In order for these biological processes to be achieved, wavelengths within the visible and Near Infra-Red (NIR) spectrum are implemented during treatment with a power output of 10-200 mW. This treatment is acceptable in many clinical practices however disputes on protocol and treatment specifications, for different models, create room for more research [13]. The purpose of this study was to evaluate the expression of extracellular matrix genes in diabetic wounded cells after photobiostimulation with 830 nm light.

2. Materials and methods

2.1. Cell Culture and Laser Irradiation

Isolated human skin fibroblasts (Academic ethics Committee, Clearance Reference Number: 01/06, University of Johannesburg) were used to create various models, namely normal (N-unstressed, Figure 1A), normal wound (NW-stressed, Figure 1B) and diabetic wounded (DW-stressed). Cells were cultured via standardised protocols [18]. Approximately 6 x 10^5 cells was re-suspended in growth media and seeded in 3.4 cm diameter culture dishes. Using media with a basal concentration of 5.6 mM/L D-glucose, an *in vitro* diabetic model was established by adding 17 mM/L D-glucose [19]. The Council for Scientific and Industrial Research (CSIR) / National Laser Center (NLC) of South Africa provided the lasers. The stressed wounded models were created by introducing a central scratch on a confluent monolayer of cells using a 1 ml sterile disposable pipette (Figure 1B) [20,21] and left to stabilise at 37^0C in 5% CO_2 for 30 min, after which laser irradiation was performed at 830 nm (Fremont, California, USA, RGBlase, TECIRL-70G-830SMA or FC-830-300-mm2-sma-1-0) (continuous wave; 98 mW; 9.1 cm^2; 10.76 mW/cm^2; 5 J/cm^2; duration of irradiation 7 min 43 s). Non-irradiated (untreated, 0 J/cm^2) N-cells were used as controls for irradiated N- and NW-cells, while non-irradiated (untreated, 0 J/cm^2) DW-cells were used as controls for irradiated DW-cells. This was due to significant differences in gene expression between non-irradiated NW- and DW-cells. Post irradiation cells were incubated for 48 h. Total RNA was isolated and quantified, and 1 µg was reverse transcribed into cDNA and the gene profile was determined making use of the human ECM and adhesion molecules RT² Profiler™ real-time reverse transcriptase polymerase chain reaction (RT-PCR) Array (SA Biosciences, PAHS-0132Z) [15]. The statistical analysis was achieved on three repeats of each sample.

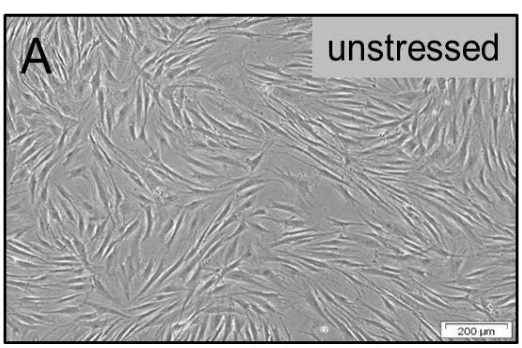

Figure 1A. Micrograph of unstressed fibroblasts seeded to 70-80% confluency.

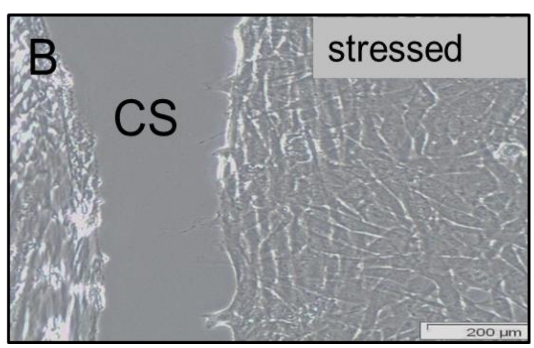

Figure 1B. Stressed fibroblasts created with central scratch (CS).

3. Results The results showed that laser photobiostimulation mediated the expression of 84 genes. Sixty one genes were significantly regulated (55 up-regulated and 6 down-regulated) in N-cells; 40 genes (20 up-regulated, and 20 down-regulated) in NW-cells and 42 genes (9 up-regulated and 33 down-regulated) in DW-cells. There was a significant change in gene expression as compared to the respective controls (Table 1).

Table 1. Summarises the gene expression of N-, NW- and DW-cells. The total RNA from non-irradiated (N- and DW-) and irradiated (N-, NW- and DW-) cells were characterised in triplicates. The gene expression profile is denoted as gene up-regulation in black or gene down-regulation in bold.

Key (Black-Up-regulation and Bold-Down-regulation)	**Normal**	**Normal Wounded**	**Diabetic Wounded**
	Cell adhesion molecules participates in cell migration, cell proliferation, survival and differentiation		
Transmembrane Molecules	CD44, CDH1, HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGB2, ITGB3, ITGB4, MMP14, MMP15, MMP16, NCAM1, PECAM1, SELE, SELL, SELP, SPG7, **ITGB5**	ITGA5, ITGA6, ITGA7, ITGA8, ITGAV, ITGB2, ITGB4, MMP15, PECAM1, SELP, **HAS1, ITGA2, ITGAL, ITGB1, SGCE, VCAM1**	MMP15, SELE, SELL, SGCE, VCAM1, **CD44, HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA5, ITGAV, ITGB1, ITGB3, MMP14, SPG7**
Cell-Cell Adhesion	CD44, CDH1, COL14A1, COL6A2, ICAM1, ITGA8, **COL11A1**	ITGA8, **COL14A1, VCAM1**	COL11A1, COL14A1, VCAM1, **CD44, COL6A2, CTNND1**
Cell-Matrix Adhesion	ADAMTS13, ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGB2, ITGB3, ITGB4, THBS3, **ITGB5**	ITGA5, ITGA6, ITGA7, ITGA8, ITGAV, ITGB2, ITGB4, **ITGA2, ITGAL, ITGB1, SGCE, SPP1**	**CD44, ITGA1, ITGA2, ITGA3, ITGA5, ITGAV, ITGB1, ITGB3, SPP1, THBS3**
Other Adhesion Molecules	CNTN1, COL5A1, COL6A1, COL7A1, COL8A1, CTNNA1, FN1, KAL1, LAMA1, LAMA2, LAMA3, LAMC1, THBS2, CLEC3B, VTN, **VCAN, CTGF**	COL12A1, COL5A1, KAL1, LAMA3, CLEC3B, **VCAN, CTGF, CTNND2, LAMA1, LAMA2, LAMC1, TNC**	VCAN, **COL12A1, COL16A1, COL5A1, COL6A1, COL7A1, CTNNB1, FN1, KAL1, LAMA1, LAMA3, LAMB3, THBS1**
	Extracellular Matrix maintains skin integrity and homeostasis and relates with several structural and extracellular proteins		
Basement Membrane Constituents	LAMA1, LAMA2, LAMA3, LAMC1	COL7A1, SPARC, **LAMA1, LAMA2, LAMC1**	**COL7A1, LAMA1, LAMA3, LAMB3**
Collagens and ECM Structural Constituents (Synthesis of ECM)	COL5A1, COL6A1, COL7A1, COL8A1, FN1, KAL1, **COL11A1, COL1A1**	COL12A1, COL1A1, COL5A1, KAL1, **COL14A1**	COL11A1, COL14A1, **COL12A1, COL16A1, COL5A1, COL6A1, COL6A2, COL7A1, COL14A1, FN1, KAL1**
ECM Proteases (MMPs responsible for ECM degradation)	ADAMTS1, ADAMTS13, ADAMTS8, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP2, MMP7, MMP8, MMP9, **MMP1**	MMP15, MMP2, MMP8, TIMP1, **MMP1, MMP10, MMP12, MMP3**	ADAMST8, MMP15, **ADAMST1, MMP1, MMP14, MMP2, MMP3, MMP8, SPG7**
ECM Protease Inhibitors	COL7A1, KAL1	KAL1	**COL7A1, KAL1, THBS1**
Other ECM Molecules	HAS1, THBS2, CLEC3B, VTN, **VCAN, CTGF**	CLEC3B, **VCAN, CTGF, ECM, SPP1**	**HAS1, SPP1**

4. Discussion and Conclusion

Photobiostimulation affects various cellular responses both *in vivo* and *in vitro*, as well as gene expression [15-17,22,23] This study focused on the gene expression profile of normal, normal wounded and diabetic wounded human skin fibroblast cell models. The explanation behind these findings could be the change in the respiratory chain of mitochondria to release energy in the form of adenosine tri phosphate (ATP) as well as reactive oxygen species (ROS) leading to the release of transcription factors stimulating gene transcription and eventually ECM formation, cell proliferation, collagen production, growth factor production, as well as the expression of various proteins [24]. Wound healing is impaired in diabetes due to the poor synthesis of growth factors and proteins [11,25,26], as well as cell-ECM interaction [27]. In this study, the main components affected by photobiostimulation in the ECM and adhesion molecules are the cell adhesion molecules (Integrins, Cadherins, Immunoglobulins and Selectins) as well as ECM molecules (Fibronectin, Collagen, Laminin, Matrix Metalloproteinases (MMPs) and their inhibitors). This study showed that 55 genes in N-cells were significantly up-regulated, while only 20 genes in NW-cells and nine genes in DW-cells were up-regulated. Genes in DW-cells were significantly down-regulated possibly as a result of the ECM dysregulation due to hyperglycaemia. In addition, the inflammatory process is delayed enhancing the release of proteases such as MMPs promoting ECM degradation and vital growth factors and receptors responsible for wound healing. The dysregulation may also prevent integrins from binding with fibronectin, and hence impairing migration [28,29]. According to Matsumoto and colleagues [30] a concentration of 1.5% glucose impaired wound healing in mesothelial cells. Their study showed that photobiostimulation in a paracrine or autocrine fashion regulates gene expression in the ECM in isolated skin fibroblasts *in vitro*. Similarly, Peplow and colleagues [23] reviewed 17 papers on gene expression in human and animal cell cultures and confirmed that photobiostimulation at green, red, and NIR stimulated gene expression, even though further work needs to be done to elucidate its cellular effects. In addition, McDaniel *et al.*, [31] exposed human skin fibroblasts to multiple wavelengths (590/870 nm LED array) and concluded that simultaneous exposure can affect cell metabolism as well as gene expression. They suggested that gene expression patterns in fibroblasts could be influenced by adjusting the wavelength intensities in both visible and NIR treatments.

In conclusion, photobiostimulation modulates gene expression of cell adhesion molecules, integrins, ECM proteins, proteases, and inhibitors involved in the ECM at a wavelength of 830 nm. This study confirms that hyperglycaemia is responsible for impaired wound healing in cell cultures *in vitro*. Furthermore an in depth comprehension of the molecular and biological processes may create an improved therapeutic protocol for diabetic wounds and future studies conducted on gene modulation and their receptors for improve understanding.

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