Influence of 660 and 830 Nm Laser Irradiation on Genetic Profile of Extracellular Matrix Proteins in Diabetic Wounded Human Skin Fibroblast Cells

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Abstract. The extracellular matrix (ECM) provides tissue structural integrity and its synthesis plays a crucial role in wound healing. Impaired wound healing ensues following the destruction of the ECM, or the inhibition of its synthesis. An imbalance in ECM synthesis and degradation is seen in patients with diabetes. This has led to the development of novel therapies that aim to decrease ECM destruction and increase its synthesis. Photobiomodulation (PBM) has been shown to speed up the healing of these slow-to-heal wounds, and numerous studies are being conducted to determine the underlying molecular cause. This study aimed to ascertain the effect of laser irradiation at a wavelength of 660 or 830 nm at a dose of 5 J/cm^2 on the genetic expression profile of genes involved in ECM proteins. cDNA was reverse transcribed from total isolated RNA and used in real-time qualitative polymerase chain reaction (qPCR). Genes concerned with the basement membrane, collagen and ECM structural constituents, and ECM protesases and inhibitors were evaluated. Results showed that a similar genetic profile (although not identical) was seen post irradiation. PBM was able to reduce the expression of ECM proteases and increase mRNA levels of ECM proteins. This study fortifies the notion that PBM stimulates cellular activity and can influence ECM matrix synthesis and degradation during wound healing. This study has also shown that PBM is able to stimulate cells at a genetic level.

Introduction

The process of wound healing is a highly complex, well-orchestrated process which involves a variety of cells, cytokines and growth factors all aimed at reversing the loss of tissue structural integrity, usually by replacement with scar-forming connective tissue. Wound healing proceeds through 4 phases, namely homeostasis, inflammation, proliferation and remodeling. Extracellular matrix (ECM) synthesis plays a crucial role in all phases of wound healing, and provides structural integrity. Impaired wound healing ensues following the destruction of the ECM, or the inhibition of its synthesis. Diabetes Mellitus (DM) has been declared as a global burden, with 415 million cases (adults aged 20-79) worldwide in 2015, and a further estimated 193 million undiagnosed cases. The estimated number of people with DM on the African continent in 2015 was at 14.2 million, and is thought to increase to 140.2 million by 2040 [1]. It was also estimated that at the end of 2015 there would be 5 million deaths worldwide related to DM at a cost of between USD673 billion and USD1,197 billion in healthcare. To put this into perspective, there was only 1.5 million deaths related to

HIV/AIDS, 1.5 million related to tuberculosis and 0.6 million related to malaria [1]. Patients with DM commonly develop a number of life threatening health conditions, and repeatedly suffer from non-healing, chronic and frequently debilitating lower limb ulcers, which often necessitate amputation.

A number of underlying pathologies contributes to the impaired wound healing seen in diabetes. These include, but not limited to, increased oxidative stress, advanced glycation end products, inflammation, and infection, and decreased immunity, angiogenesis, circulation and fibroblast migration, as well as a disruption in the ECM, hypoxia, ischemia and neuropathy. In diabetic wound healing there is a disruption in the formation and synthesis of the ECM, and is commonly due to a disruption in the interaction between ECM proteins and growth factors, as well as impaired fibroblast migration and proliferation [2]. Collagen is one of the most important proteins found in the ECM and during wound healing there is a carefully controlled balance between its degradation and synthesis. Degradation of collagen and other ECM proteins by proteases is essential to remove cellular debris and during remodeling. In DM there is an imbalance between the class of proteases referred to as Matrix metalloproteinase (MMP) and their inhibitors, tissue inhibitor metalloproteinases (TIMPs). The decrease in collagen seen in diabetes may be as a result of decreased synthesis and/or enhanced metabolism [3]. Chronic diabetic foot ulcers have presented with elevated MMP activity, with a 30- to 60- fold increase in MMP-2 and -9 [4, 5]. Other studies have shown an increase in MMP-8 and -9 [6].

Photobiomodulation (PBM), also referred to as low-level laser therapy (LLLT), laser therapy or phototherapy, involves the application of low powered light (typically from a laser or light emitting diode, LED) to stimulate cellular processes. It is a non-invasive and non-thermal therapy to treat a wide variety of conditions, including chronic ulcers. Light absorbing structures within the cell, or chromophores, absorb this light (photon energy) which is then converted to chemical energy. It is well accepted that the absorbing chromophore for visible red and near infrared (NIR) light is the mitochondria, the powerhouse of the cell [7]. This leads to an increase in adenosine triphosphate (ATP) which in turn activates a cascade of intracellular signals and messengers, leading to various down-stream effects such as increased proliferation, cellular migration and viability, collagen production, as well as gene transcription [8]. There are several research papers which demonstrate the beneficial effect of PBM in the treatment of diabetic ulcers [9-12].

It was the aim of this study to determine the effect of 660 (visible red) and 830 nm (NIR) at an optimal dose for *in vitro* studies, 5 J/cm^2, on the genetic expression profile of ECM proteins in a diabetic wounded human skin fibroblast cell model.

Materials and Methods

Cell Culture

This study was conducted on WS1 human skin fibroblast cells purchased from the American Type Culture Collection (ATCC, CRL-1502), and received ethical clearance from the Faculty of Health Sciences, University of Johannesburg, Research Ethics Committee (AEC05/01-2011). A diabetic wounded in vitro model was achieved as previously described [13]. Briefly, WS1 cells were continuously grown in supplemented Minimum Essential Medium (MEM) containing and additional 17mM/L D-glucose [13, 14]. For experiments, 6 X 10^5 cells were seeded into 3.4 cm diameter tissue culture plates and incubated at 37°C in 5% CO_2 to allow for attachment. The following morning an in vitro wound model was achieved via the

central scratch method whereby the tip of a sterile 1 mL pipette was used to create a standardized scratch or "wound" down the center of the confluent monolayer, thus creating a cell free zone bordered by cells [13, 15, 16]. Cells were incubated for 30 min pre-laser irradiation.

Laser Irradiation

Diabetic wounded (DW) cells were irradiated with a 660 (visible red) and 830 (NIR) nm diode laser (RGBlase, Fremont, California, USA) with a power output (measured prior to each irradiation using the Fieldmate, 0398D05) of 93 and 98 mW respectively. At the cellular level the laser had a beam spot size of 9.1 cm2, giving a power density of 10.22 and 10.76 mW/cm2 respectively. To achieve a fluence of 5 J/cm2, cells were irradiated for 8 min 9 s for the 660 nm laser, and 7 min 44 s for the 830 nm laser. Cells were irradiated in the dark, from above via fiber optics with the culture dish lid off. Non-irradiated controls (0 J/cm2) were kept in a dark box on the bench.

Gene Expression Profiling

Briefly, total RNA was isolated 48 h post-irradiation making use of the Qiagen RNeasy Mini Kit (74104) on the QIAcube (Qiagen) and 1 µg was reverse transcribed into copy DNA (cDNA) using the Qiagen QuantiTect Reverse Transcription Kit (205311). cDNA was used as a template in qPCR using the SABiosciences human extracellular matrix and adhesion molecules RT2 Profiler PCR Array System (PAHS-01321Z) on the Stratagene MX3000p. A dissociation curve was performed at the end to ensure amplification of a single product. Threshold cycle (Ct) values were exported to the SABiosciences Excel-based Data Analysis Template where results were normalized against an average of the five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, ACTB). To ensure intra-well, as well as inter-plate validity, all positive PCR controls (included in the PCR array) had to have a Ct value of 20 ±2. Expression levels from irradiated cells were first normalized against the housekeeping genes and then calculated relative to non-irradiated control cells according to the $2^{(-\Delta\Delta Ct)}$ method.

Statistical Analysis

Experiments were repeated three times (n=3). The student T-test was performed by the SABiosciences Excel-based Data Analysis Template and reported as significant if $P \leq 0.05$.

Results and Discussion

Disruption to the ECM is a common underlying pathology seen in chronic diabetic ulcers. Research has shown that PBM in the visible red and NIR region of the spectrum is able to modulate the imbalance in the synthesis of the ECM, and stimulates the production of collagen [17]. In this study, diabetic wounded human skin fibroblast cells (WS1) were irradiated at a fluence of 5 J/cm2 at a wavelength of 660 nm (visible red) or 830 nm (NIR). The gene expression profile of ECM proteins was determined 48h post-irradiation (Table 1). PBM typically includes wavelengths of between 500 and 1,100 nm and a fluence of 1 to 4 J/cm2 using lasers with output powers of 10 to 90 mW [18]. A fluence of 5 J/cm2 was selected as it has been shown to stimulate wound healing in earlier studies which made use of the same cell models [13, 17, 19, 20]. The choice of wavelength in treatment is largely dependent on penetration depth of the different wavelengths, with longer wavelengths

penetrating deeper into tissue, as well as the desired effects and target chromophores. The visible red (e.g., 660 nm) and infrared portions of the electromagnetic spectrum (e.g., 800 to 900 nm) have been shown to be highly absorbent in living tissues [21] and seem to provide the best results [22].

Diabetic wounded cells irradiated with a wavelength of 660 nm exhibited a total of 22 genes which were significantly down-regulated, while 10 were significantly up-regulated. The same cells irradiated with 830 nm exhibited a total of 22 significantly down-regulated genes, and only 4 significantly up-regulated genes. The functional groupings (basement membranes, collagens and ECM structural constituents, ECM proteases, ECM protease inhibitors and other ECM molecules) can be seen in Table 1. With the exception of a few genes, diabetic wounded WS1 cells irradiated at either 660 or 830 nm showed almost an identical gene profile, with a number of the same genes being significantly up- or down-regulated. The following genes were significantly down-regulated with both wavelengths: LAMA1, LAMB3, SPARC, COL5A1, COL6A1, COL7A1, COL12A1, COL16A1, FN1, KAL1, ADAMTS1, MMP1, MMP2, MMP8, MMP14, MMP16, SPG7, THBS1, and SPP1. The following genes were significantly up-regulated at both wavelengths: COL11A1, COL14A1 and ADAMTS8.

The ECM is a highly dynamic structure which provides extracellular structural support to cells, and is constantly being remodeled. The ECM is comprised of two classes of macromolecules, namely proteoglycans (PGs) which form a hydrated gel, and fibrous proteins which are collagen, elastin, fibronectin and laminin [23]. Collagen constitutes the most important fibrous protein in the ECM and are responsible for tensile strength, control cell adhesion, support chemotaxis and migration, and direct tissue development. Elastin interacts with collagen, and offers tissue elasticity. Fibronectin (FN) directs the organization of the ECM and plays a crucial role in mediating cell attachment and function [23]. Laminin forms part of the basement membrane and is involved in cellular attachment, shape and movement. The synthesis and degradation of the ECM are both important during wound healing. ECM degradation is necessary for cellular migration and is carried out by MMPs and other proteases. In recent years, new studies have also shown that MMPs play a role in regulating extracellular tissue signaling networks [24]. Due to their destructive nature, the activity of these ECM proteases have to be controlled. MMPs are controlled by TIMPs.

The failure of diabetic ulcers to heal has been linked to decreased ECM synthesis, and or increased ECM degradation [3]. In a study conducted by Liu and colleagues, it was found that high wound fluid concentrations of MMP-9 and high MMP-9–to–TIMP-1 ratios was a predictor of poor wound healing in diabetic foot ulcers [25]. In addition to increased MMP-9, other studies have also found increased levels of MMP-2 and -8 [4-6]. PBM has been shown to stimulate cells and wound healing, and has aided in the healing of chronic diabetic ulcers [8-13]. Aparecida Da Silva and co-workers irradiated diabetic induced male Wistar rats (660 nm, 4 J/cm2) and evaluated MMP-2 and -9, and Type I and III collagen 24 h post-irradiation [25]. The results showed that PBM significantly lowered MMP-2 and -9 expression as well as accelerate the production of collagen and increase the total percentage of collagen type III in diabetic animals [25]. Results from this study showed a significant reduction in mRNA in ECM proteases a disintegrin and metalloproteinase with thrombospondin motifs I (ADAMTS-1), MMP-1, -2, -8, -12, -14, and -16, and a significant increase in TIMP-1 in diabetic wounded cells irradiated with 660 nm. There was also a significant up-regulation in COL11A1, COL14A1 (coding for

collagen type XI and XIV respectively) and LAMA3 (Laminin, Alpha 3). Interestingly, there was also a significant up-regulation in ADAMTS8, MMP3, MMP7, MMP9, MMP11 and MMP13. However, despite this upregulation, it should be remembered that proteases are needed for ECM remodeling, and qPCR was done 48 h post-irradiation, also TIMP1 expression was up-regulated. Irradiation at 830 nm showed a significant reduction in ECM protease mRNA levels ADAMTS-1, MMP-1, -2, -2, -8, -14 and -16, while ADAMTS-8 was upregulated. Expression of type XI and XIV collagen was significantly upregulated.

Conclusion

Numerous biostimulatory effects of PBM has been demonstrated in several in vitro and in vivo studies, and the question is no longer whether it has an effect on the human cell, but rather what is the underlying mechanism of action? There is a rise in the number of studies being done on the influence of red and NIR laser light on biological systems, however there is not enough progress being made in the field of diabetes. If one looks at the literature, PBM is a non-invasive, non-thermal phototherapy with no reported side-effects when used at the optimal parameters, so why is this therapy not being used more frequently in the treatment of diabetic foot ulcers? As declared by the International Diabetes Federation, diabetes is one of the largest global health emergencies of the 21st century, and in light of the ever present threat of ulcers, infection, and amputation, and increasing incidence of diabetes, new improved therapies and the fortification of PBM in wound healing research deserves better attention.

Diabetic wounded WS1 cells irradiated at a fluence of 5 J/cm2 at a wavelength of either 660 (visible red) or 830 nm (NIR) showed a similar gene profile, with a number of genes significantly up- or –down regulated. It should be remembered that the gene profile of any cell type will differ depending on the stage of wound healing (and hence the time RNA is isolated post-irradiation), and even though there was an up-regulation in some proteases, these are essential for remodeling and breaking down old collagen and ECM proteins and making way for stronger, new collagen, as well as aiding in cellular migration. This study fortifies the notion that PBM stimulates cellular activity and can influence ECM matrix synthesis and degradation during wound healing. This study has also shown that PBM is able to stimulate cells at a genetic level.

Table 1. Gene expression profile of extracellular matrix proteins in diabetic wounded human skin fibroblast cells irradiated with 5 J/cm^2 at 660 or 830 nm. Expression levels from irradiated cells were first normalized against the housekeeping genes and then calculated relative to non-irradiated control cells according to the 2^(-ΔΔCt) method. Fold-change (2^(-ΔΔCt)) was calculated; if the fold-change is greater than 1, then the result is reported as fold up-regulation, and if the fold-change was less than 1, then the result is reported as a fold down-regulation.

Gene	*Gene Name*	*660 nm*		*830 nm*	
		Fold Up- or Down-Regulation 2^(-ΔΔCt)	*T-Test*	*Fold Up- or Down-Regulation 2^(-ΔΔCt)*	*T-Test*
		Basement membrane			
COL4A2	Collagen, type IV, alpha 2	-1.01	0.966	-1.81	0.103
COL7A1	Collagen, type VII, alpha 1	-2.78	0.017	-5.19	0.005
LAMA1	Laminin, alpha 1	-1.67	0.014	-2.02	0.004
LAMA2	Laminin, alpha 2	1.17	0.397	1.26	0.179
LAMA3	Laminin, alpha 3	2.25	0.031	1.36	0.332
LAMB1	Laminin, beta 1	-1.05	0.609	1.13	0.308
LAMB3	Laminin, beta 3	-1.64	0.010	-3.78	0.002
LAMC1	Laminin, gamma 1	-1.85	0.008	-1.29	0.153
SPARC	Secreted protein, acidic, cysteine-rich	-1.52	0.050	-2.18	0.004
		Collagen & ECM			
COL1A1	Collagen, type I, alpha 1	-1.15	0.498	-1.53	0.089
COL4A2	Collagen, type IV, alpha 2	-1.01	0.966	-1.81	0.103
COL5A1	Collagen, type V, alpha 1	-1.80	0.036	-3.09	0.009
COL6A1	Collagen, type VI, alpha 1	-1.57	0.015	-6.06	0.002
COL6A2	Collagen, type VI, alpha 2	-1.72	0.014	-4.92	0.092
COL7A1	Collagen, type VII, alpha 1	-2.78	0.017	-5.19	0.005
COL8A1	Collagen, type VIII, alpha 1	-1.41	0.271	-4.04	0.065
COL11A1	Collagen, type XI, alpha 1	3.68	0.002	3.21	0.003
COL12A1	Collagen, type XII, alpha 1	-2.69	0.003	-5.95	0.000
COL14A1	Collagen, type XIV, alpha 1	1.24	0.023	2.19	0.000
COL15A1	Collagen, type XV, alpha 1	-1.49	0.082	1.23	0.089
COL16A1	Collagen, type XVI, alpha 1	-1.65	0.001	-1.44	0.009
FN1	Fibronectin 1	-1.71	0.008	-5.08	0.001
KAL1	Kallmann syndrome 1 sequence	-2.44	0.000	-4.62	0.001
		ECM Proteases			
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-2.01	0.003	-6.93	0.002
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	2.75	0.005	2.09	0.015
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	-1.09	0.589	-1.12	0.492
MMP1	Matrix metallopeptidase 1	-2.61	0.002	-3.57	0.005
MMP2	Matrix metallopeptidase 2	-1.85	0.002	-2.77	0.000
MMP3	Matrix metallopeptidase 3	2.42	0.009	-1.85	0.049
MMP7	Matrix metallopeptidase 7	1.86	0.024	1.40	0.066

Gene	Gene Name	660 nm		830 nm	
		Fold Up- or Down-Regulation 2^(-ΔΔCt)	*T-Test*	*Fold Up- or Down-Regulation 2^(-ΔΔCt)*	*T-Test*
		Basement membrane			
MMP8	Matrix metallopeptidase 2	-1.59	0.029	-2.89	0.002
MMP9	Matrix metallopeptidase 9	1.47	0.009	-1.16	0.435
MMP10	Matrix metallopeptidase 10	1.28	0.271	-1.70	0.066
MMP11	Matrix metallopeptidase 11	1.71	0.025	1.28	0.263
MMP12	Matrix metallopeptidase 12	-1.91	0.005	1.33	0.067
MMP13	Matrix metallopeptidase 13	1.89	0.034	1.10	0.606
MMP14	Matrix metallopeptidase 14	-2.28	0.016	-15.63	0.000
MMP15	Matrix metallopeptidase 15	1.24	0.157	1.55	0.022
MMP16	Matrix metallopeptidase 16	-2.28	0.003	-1.87	0.017
SPG7	Spastic paraplegia 7	-2.08	0.005	-7.92	0.000
		ECM Protease Inhibitors			
KAL1	Kallmann syndrome 1 sequence	-2.44	0.001	-4.62	0.001
THBS1	Thrombospondin 1	-2.85	0.001	-3.63	0.000
TIMP1	TIMP metallopeptidase inhibitor 1	1.47	0.046	1.07	0.688
TIMP2	TIMP metallopeptidase inhibitor 2	-1.07	0.788	-1.26	0.449
TIMP3	TIMP metallopeptidase inhibitor 3	1.05	0.825	1.42	0.247
		Other			
VCAN	Versican	-1.04	0.775	1.59	0.015
CTGF	Connective tissue growth factor	1.10	0.521	1.40	0.121
ECM1	Extracellular matrix protein 1	-1.35	0.076	1.11	0.554
HAS1	Hyaluronan synthase 1	-1.14	0.544	-3.04	0.013
SPP1	Secreted phosphoprotein 1	-1.74	0.009	-2.12	0.003
TGFB1	Transforming growth factor, beta-1	-1.27	0.099	1.05	0.742
THBS2	Thrombospondin 2	-1.24	0.088	-1.30	0.310
THBS3	Thrombospondin 3	1.03	0.794	-1.81	0.013
CLEC3B	C-type lectin domain family 3, member B	1.14	0.749	2.71	0.070
TNC	Tenascin C	1.18	0.492	1.27	0.178
VTN	Vitronectin	1.20	0.240	-1.15	0.193

Table 1-2.

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