Pulsed radio frequency energy field treatment of cells in culture: Increased expression of genes involved in angiogenesis and tissue remodeling during wound healing.

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Abstract:

The treatment of chronic lower extremity wounds can represent a challenging medical complication in patients with underlying conditions associated with impaired wound healing, particularly when wounds are refractory to traditional wound care management methods. One promising treatment for the care of chronic wounds is pulsed radio frequency energy (PRFE) therapy. In clinical studies PRFE has been shown to promote the healing of wounds otherwise unresponsive to standard of care treatment, including chronic lower extremity wounds in diabetic patients. In a previous report we found that PRFE regulates groups of genes directly involved in the inflammation phase of wound healing, an effect that may promote wound progression from inflammation to subsequent stages of wound repair. This process may likely be impaired in chronic diabetic wounds. Here we use a similar approach to assess the impact of PRFE on genes involved in angiogenesis and tissue remodeling. Using microarray analysis, we found that PRFE treatment of human keratinocyte and fibroblast cell lines leads to an increase in transcript levels of multiple factors involved in these processes, including growth factors, extracellular matrix proteins and their receptors, cyclins, transcription factors, and DNA replication factors. Based on these results we propose a model to describe molecular mechanisms underlying PRFE treatment and the promotion of wound healing in chronic wounds.

Key words: diabetic foot ulcers, wound healing, microarrays, programmed gene expression, extracellular matrix, growth factors, angiogenesis, tissue remodeling, radio frequency

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Abbreviations: reactive oxygen species (ROS), cyclooxygenases (COX), heme oxygenase (HO), nitric oxide synthase (NOS), matrix metalloproteinases (MMPs), carbon monoxide (CO), insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), transforming growth factor 1 beta (TGF-1β), extracellular matrix (ECM), glycosaminoglycans (GAG)
Introduction

Normal wound healing occurs in several progressive, overlapping stages, beginning with clot formation. This is followed by inflammation, new tissue formation and finally tissue remodeling. Multiple cell types are involved in the process, and genetic control of events is regulated in a temporal, spatial, and cell-type specific manner.\(^1,^2\) While normal progression of wound healing follows this general pattern, dysregulated wound repair frequently occurs in patients with diabetes\(^1,^3,^4\) and peripheral artery disease (PAD),\(^5\) resulting in chronic, non-healing wounds. These often show signs of chronic inflammation without normal progression to later wound healing stages, a possible indication of inappropriate stalling of the wound in the inflammatory phase of wound repair.

Often refractory to standard of care treatment, chronic lower extremity wounds represent a challenging medical complication. With almost 8% of the American population estimated with diabetes,\(^6\) and PAD affecting 12-20% of the US population age 65 and over,\(^7\) the development and implementation of effective therapies to treat such wounds is a critical medical need. Pulsed radio frequency energy (PRFE) has been shown to promote the healing of chronic wounds that were otherwise unresponsive to standard of care treatment,\(^8,^9\) including chronic lower extremity wounds in diabetic patients. Although the use

of this biophysical treatment paradigm has been shown to be an effective treatment modality for pain and edema and soft tissue wounds, little is known about the biological mechanisms underlying these effects.

By performing microarray analysis of PRFE-treated human dermal fibroblasts and keratinocytes in culture, we previously found that PRFE treatment results in increased expression of many genes involved in the inflammatory stage of wound repair in a temporally regulated manner, including the upregulation of cytokines, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs).\(^10\) Here, we evaluate the impact of PRFE treatment on the expression of genes involved in angiogenesis and tissue remodeling. We have found that PRFE treatment is followed by a rapid increase in transcripts encoding factors involved in these processes, including cyclins, growth factors, cell adhesion-related proteins, and DNA replication factors.

These results suggest that PRFE regulates a sequence of gene expression events that lead to healing of chronic wounds in the diabetic patient by promoting progression of the chronic wound beyond chronic inflammation to angiogenesis and tissue remodeling.

Materials and Methods

Cell culture. Human epidermal keratinocyte (HEK) and human dermal fibroblasts (HDF) were purchased from Cell Applications, Inc. (San Diego, CA), and cultures maintained as previously described.\(^10\)

PRFE field conditions and treatment. PRFE treatment was performed by exposing cells to

the signal from a PRFE device (Provant\(^{®}\), Regenesis Biomedical, Inc.), which emits a 27.12 MHz radio frequency (RF) signal. Energy parameters and treatment conditions used were as previously described.\(^10\) Control cells received no PRFE treatment.
Cell synchronization and cell cycle analysis.

Cell synchronization studies were performed as previously described. Briefly, cells were synchronized using mevastatin (compactin), to synchronize cells in early G1 phase of the cell cycle. 30 minutes prior to exposure to PRFE, cells were released from the G1 by treating with a 100-fold excess of mevalonate. Cell synchronization was verified using bromodeoxyuridine (BrdU) incorporation and detection with BrdU-specific antibodies (Roche) as previously described.

Total RNA isolation. Cells were treated with PRFE field, or left as untreated controls, and total RNA was isolated according to the method of Chomczynski and Sacchi. Cells were harvested for total RNA at the times indicated in the figures.

cDNA array analysis. Microarrays and reagents were purchased from BD Biosciences-Clontech (Palo Alto, CA). In general, each sample combined at least 3 plates. Total RNA (1-2 ug) was labeled with 32P-dATP using reverse transcriptase (RT) from Clontech (Palo Alto, CA) as described by the manufacturer, purified by column chromatography, and used as probe for cDNA array hybridization.

Gene expression analysis. Autoradiographs were scanned at 200 dpi and analyzed using Atlas Image software (BD Biosciences-Clontech, Palo Alto, CA). Two reference genes, GAPDH and ribosomal protein L13a, were used to normalize the cDNA array expression profiles. Further gene expression analysis was performed with Gene Linker software (Predictive Patterns Software, Canada).

Reverse transcription (RT) and polymerase chain reaction (PCR). Cells were treated with PRFE and RNA isolated as described above. Reverse transcription of the total RNA was performed using the SuperScript First Strand system from Invitrogen (Carlsbad, CA), following the manufacturer’s instructions and as previously described. cDNA was amplified by polymerase chain reaction (PCR). PCR products were electrophoresed on 2% agarose gels, photographed, and specific DNA fragments quantitated using Scion Image.

Results

The effect of PRFE on the expression of genes involved in angiogenesis and tissue remodeling in HDF and HEK cells. The impact of PRFE treatment on the expression of genes involved in angiogenesis and wound remodeling was assessed using microarray analysis of cultured human dermal fibroblasts (HDF) and human epidermal keratinocytes (HEK). Relative transcript levels of factors involved in these processes were determined at multiple time points following PRFE treatment using cDNA microarray analysis and confirmed by RT-PCR. Microarray results were grouped according to gene product functionality using the following groups: (A) transcription factors, (B) cyclins and their related kinases, (C) DNA synthesis proteins, (D) cell adhesion-related proteins, and (E) growth factors and their receptors. In both cell types, PRFE treatment resulted in an increase in relative transcript levels for multiple factors in each functional group, detected as early as 5 and 15 minutes after PRFE treatment for HDF cells (Fig. 1) and HEK cells (Fig. 2), respectively.
Figure 1: Functional grouping of microarray analysis of gene expression after PRFE treatment of HDF cells. Gene lists were developed to place expression data in functional groups. Expression groups related to gene products involved in angiogenesis and tissue remodeling are shown. (A) Transcription factors, (B) cyclins and their related kinases, (C) DNA synthesis proteins, (D) cell adhesion-related proteins, and (E) growth factors and their receptors. Expression levels are relative to the zero hour control.
Figure 2: Functional grouping of cDNA array analysis of gene expression after PRFE field treatment of HEK cells. Expression groups related to gene products involved in angiogenesis and tissue remodeling are shown. (A) Transcription factors, (B) cyclins and their related kinases, (C) DNA synthesis proteins, (D) cell adhesion-related proteins, and (E) growth factors and their receptors. Expression levels are relative to the zero hour control.
Next, to further characterize the effect of PRFE on the expression of factors involved in these processes, the effect of PRFE on gene expression in synchronized cells was examined. HDF and HEK cells were synchronized in G1, followed by release and subsequent microarray analysis at multiple time points. For PRFE-treated cells, PRFE treatment was performed 30 minutes after release from the G1 block. In both cell types, PRFE treatment led to a robust increase of transcripts in all functional groups (Fig. 3), most notably at 15 minutes in synchronized HDF cells, and at 5 hours in synchronized HEK cells, following PRFE treatment.

To understand the overall effect of PRFE on temporal gene expression, cDNA arrays were used to obtain expression data for 1,176 genes in synchronized HDF and HEK cells following PRFE treatment. Data was clustered by time point, regardless of functional group, for control and PRFE-treated synchronized cells. Microarray analysis revealed that the majority of expression changes occurred more rapidly in PRFE-treated cells compared to control cells in both cell types (HEK cells, Fig. 4; HDF cells, data not shown), though typically later in synchronized HEK cells than that seen for synchronized HDF cells. In synchronized HEK cells, a large induction of gene expression occurred five hours following PRFE treatment (Fig. 4, cluster C-PRFE treated) followed by another large-scale induction of gene expression eight hours after treatment (Fig. 4, cluster D-PRFE treated). In the untreated cells more than 80% of the genes were expressed at the eight hour time point (Fig. 4, cluster D-control), with very little expression changes occurring at earlier time points for the genes analyzed.
Finally, PRFE treatment led to an increased rate of progression to S phase by 6-8 hours in synchronized HDF cells, and by 4 to 6 hours in synchronized HEK cells relative to synchronized control cells (data not shown). This is in agreement with rapid transcript level increases noted for factors involved in cell proliferation in both cell types following PRFE treatment.

**Figure 4: cDNA array analysis of synchronized HEK cells treated with a PRFE field.**
HEK cells were synchronized using mevastatin treatment for 36 hours. Cells were released from cell cycle block using a 100X excess of mevalonate. Expression matrices of cDNA array data of synchronized cells, both control and PRFE field-treated. Each set of data was divided in four groups based on expression. Data is expressed relative to zero hour control.
Discussion

Keratinocytes and fibroblasts play important functions during wound repair, impairments of which are associated with delayed or chronically stalled wound healing in many metabolic disorders. To understand the mechanisms underlying PRFE therapy-mediated effects during wound healing, we used a microarray approach to look at the effect of PRFE on the expression of factors involved in the wound healing process in human keratinocyte and fibroblast cell lines. In a previous article we showed that PRFE treatment upregulated mRNA levels of numerous factors important for the inflammatory phase of wound healing. In this report, we focus on the effects of PRFE treatment on factors involved in subsequent stages of the wound healing process.

The effect of PRFE on factors involved in the proliferative and tissue remodeling phases of wound healing.

Angiogenesis, the formation of granulation tissue, and tissue remodeling are complex processes involving participation of numerous factors and signaling pathways. Here, we find that mRNA levels of many of these factors are upregulated following exposure to PRFE. In both cell types examined, PRFE treatment resulted in increased mRNA levels for multiple subunits of integrins (Fig. 1D, Fig. 2D, Fig. 3A), cell surface receptors that interact with components of the extracellular matrix (ECM) and surface molecules on other cells. Directly involved in cell motility, integrins also regulate a number of cell processes such as proliferation and survival. During wound healing, they are important for re-epithelialization, angiogenesis, and wound contraction, and binding to their ligands can mediate cell-signaling events important for expression of other factors involved in wound repair.

Transcript levels of multiple cyclins and DNA replication factors, factors that regulate cell division, were also upregulated following PRFE treatment in both cell types, with particularly rapid and synchronous increases following treatment of synchronized HDF cells (Fig. 3C). During wound repair, properly controlled cell proliferation is necessary for the formation of new tissue, and proliferation defects in keratinocytes and fibroblasts have been reported in diabetic foot ulcers and chronic venous leg ulcers. Specific cyclins are critical to progression through checkpoints in the cell cycle, and cell synchronization studies suggest that PRFE exposure increases the rate of progression though the cell cycle.

Dysregulation of wound healing in metabolic disorders.

Impaired wound healing is a frequent complication of conditions with underlying metabolic dysfunction, such as in diabetes, metabolic syndrome, and PAD. In such cases, numerous physiological factors are thought to contribute to dysregulated wound repair. In diabetes, this includes impairments related to growth factor production, angiogenesis, quality of granulation tissue formation, keratinocyte and fibroblast proliferation and migration, and extracellular matrix (ECM) deposition. In addition, lack of synchrony within the wound can also occur, with progression through different phases of wound repair occurring at different rates throughout the wound, or non-progression through the wound healing cycle and wound stalling in a chronic inflammatory state (Fig. 5). The underlying cause of such disorders is a topic of intense research, and several hypotheses have been proposed. Disruptions in insulin and leptin signaling are hallmarks of several metabolic disorders, and disruption of these pathways has important systemic effects on metabolism. Dysregulated wound healing associated with these disorders is thought to be primarily due to these widespread metabolic abnormalities.
However, abnormal repair is likely confounded by the fact that both leptin and insulin signaling pathways appear to function directly in wound repair as well.31-34

In conclusion, we have shown that PRFE can mediate robust, and often synchronous, mRNA upregulation for a wide range of factors involved in the wound healing process. Since improper wound healing in metabolic disorders is multifactorial, therapies that facilitate wound healing by promoting multiple wound repair pathways, as evidence suggests for PRFE therapy, have great potential as treatment options for chronic wounds.

![Figure 5: A cyclic model for wound healing.](image)

For complete healing of acute or chronic wounds, the cellular mechanisms involved can be modeled in stages progressing through a cycle. In patients with chronic wounds due to diabetes, peripheral vascular disease, or improper off-loading leading to pressure ulcers, wounds may be blocked or stalled in inflammation5,28,29 PRFE, negative pressure wound therapy (NPWT), and compression (for Venous ulcers) may accelerate wound healing by inducing genes which allow for the transition of the wound through the stall point promoting transition to angiogenesis and granulation tissue formation and the completion of wound healing.10,35,36
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