Pulsed radio frequency energy field treatment of cells in culture results in increased expression of genes involved in the inflammation phase of lower extremity diabetic wound healing.

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Abstract:
Type 2 diabetes is becoming an increasingly prevalent disease in the elderly population leading to acute and chronic wounds. We have used a biophysical modality for treatment of chronic diabetic foot ulcers. The treatment uses pulsed radio frequency energy (PRFE), which significantly increases the proliferation of human dermal cells in vitro and has been successfully used to promote the healing of chronic wounds in vivo.

Wound healing is a complex process involving a programmed sequence of gene expression that is highly regulated temporally and spatially. We use microarray technology to assess the effect of PRFE treatment on the expression of genes involved in the inflammatory stage of wound healing, as evidence suggests chronic wounds in diabetics may be stalled in this stage of the wound healing process.

Using this technique, we found that PRFE treatment of cultured human dermal fibroblasts and human epidermal keratinocytes led to a rapid increase in the transcript levels of numerous genes involved in the inflammatory stage of wound healing. This finding may provide new insight regarding the 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, inflammation, cytokines, matrix metalloproteinases, tissue inhibitors of metalloproteinases, reactive oxygen species.

Introduction
A significant complication associated with diabetes is the development of chronic, non-healing wounds. The most common chronic wound that occurs in diabetic patients is the foot ulcer. Diabetic foot ulcers occur in approximately 15% of diabetic persons and are an important component cause in approximately 85% of all non-traumatic limb amputations in that population. In addition to the obvious detrimental effects on a patient’s quality of life that these wounds present, the treatment of these ulcers represents a significant financial burden. In 2001, diabetic foot ulcers and limb loss cost an estimated $10.9 billion and, with the epidemic rise of diabetes in the American population, these costs will continue to escalate unless more effective wound treatments are identified and developed.

One promising treatment for the care of chronic wounds is that of pulsed radio frequency energy (PRFE). We have developed a high frequency PRFE device that in clinical studies has been shown to promote the healing of chronic wounds that were otherwise unresponsive to standard of care treatment. Further studies to understand the molecular mechanisms underlying these effects will facilitate optimization of treatment parameters for enhanced effectiveness.
Normal wound healing is complex, involving tightly regulated progression through several wound-healing stages: clot formation, inflammation, new tissue formation, and remodeling. The progression through these stages is well orchestrated with unique molecular and cellular requirements. Many cell types participate, and hundreds of genes are up- and down-regulated over the course of the repair process.

An efficient way to obtain large-scale gene expression data is by microarray analysis. Microarray technology can be used to monitor the expression levels of thousands of genes at a given time in a given cell population, thus making it an invaluable tool for understanding underlying molecular mechanisms that contribute to complex processes such as wound healing. Here, we use microarray technology to assess the effect of PRFE treatment on the expression of genes involved in the inflammatory stage of wound healing using human epidermal keratinocytes (HEK) and dermal fibroblasts (HDF) in culture, as evidence suggests that many long-standing, difficult-to-heal wounds may be chronically stalled in the inflammatory phase of the wound healing process. Following PRFE treatment, a rapid increase in transcript levels of a number of inflammation-related genes occurred, including a number of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), as well as cytokines and other genes involved in the inflammatory phase of wound healing.

This data provides evidence that PRFE treatment can have a large-scale impact on transcript levels of genes involved in inflammation. Such widespread changes may allow PRFE to modulate this phase of the wound healing process. In the context of the chronic wound this effect could have important implications.

Materials and Methods

Cell culture.

HEK and HDF cells were purchased from Cell Applications, Inc. (San Diego, CA). Routine culture was performed as recommended by the manufacturer in a 5% CO2 humidified incubator at 37°C. For HEK cells, cells were cultured in keratinocyte growth media, supplied by the manufacturer.

PRFE field conditions and treatment.

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. The signal is delivered in 42 µsec pulses with a period of 1 KHz. Cells were placed at a distance of 5 cm from the source during treatment. Treatment was performed at room temperature for 30 minutes, and then cells were returned to the incubator for growth. Cells were harvested for total RNA at the times indicated in the figures. Control cells received no PRFE treatment.

Cell synchronization and cell cycle analysis.

We synchronized and released cells untreated with PRFE in order to characterize the temporal expression associated with fibroblasts and keratinocytes progressing through the cell cycle. We then compared these data to those of synchronized cells treated with PRFE to determine the effects of PRFE on this temporal pattern of gene expression. Cells were synchronized using mevastatin (compactin) according to the method of Keyomarsi et al. Synchronization of the cells was verified using bromodeoxyuridine (BrdU) incorporation and detection with BrdU-specific antibodies, according to the manufacturer’s recommendation and the method of Gratzner et al.
Cells were released from synchronization by providing a 100-fold excess of mevalonate.

**cDNA array analysis**

Cells were treated with PRFE field, and total RNA was isolated as described above. In general, each sample combined at least 3 plates. Total RNA (1-2 µg) was labeled with ³²P-dATP using reverse transcriptase (RT) from Clontech (Palo Alto, CA) as described by the manufacturer.

**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 analysis of the expression levels of the genes was performed with Gene Linker software (Predictive Patterns Software, Canada). Genes were clustered into expression sets using a K-means algorithm that measures distance based on Pearson correlation.

**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. 1-5 µg of total RNA was used for reverse transcription (RT). 5µl of the RT reaction was used for PCR, which was performed using the following reaction times and temperatures: denaturation for 1 minute at 94°C, annealing for 2 minutes at 55-58°C, elongation for 2 minutes at 72°C. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Gels were then photographed and specific DNA fragments were quantitated using Scion Image. Synchronization of the cells was verified using BrdU incorporation and detection with BrdU-specific antibodies, according to the manufacturer’s (Roche) recommendations.

**Results**

The effect of PRFE on the expression of genes involved in the inflammatory phase of wound healing in HDF and HEK cells. Two cell types essential to the wound healing process are fibroblasts and keratinocytes.⁷,¹²-¹³ To determine the effect of PRFE treatment on gene expression in these cell types, cultured human dermal fibroblasts (HDF) and human epidermal keratinocytes (HEK) were PRFE- or mock-treated. Resulting changes in gene expression were assessed by microarray analysis at multiple time points post-treatment using cDNA arrays enriched for genes involved in the wound healing process, providing expression data for 1,176 known genes. The expression results were clustered into five expression groups using a K-means algorithm that measures distances based on a Pearson correlation. We found expression groups of (A) immediate early (5 minute expression), (B) early (15 minute expression), (C) intermediate (180 minute expression), (D) late 1 (300 minute expression), and (E) late 2 (480 minute expression) (where time denotes time post PRFE treatment) in HDF cells (Fig. 1), and similar such expression groups in HEK cells as well (data not shown). These groupings are similar but more specific than the classical definition of gene expression groups described by Nathans.¹⁴ The results suggest that PRFE treatment of HDF and HEK cells stimulates a programmed pattern of gene expression, including genes implicated in the wound healing process.

Next, we grouped gene expression results according to gene product functionality using the following inflammation-related groups: (A) matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), (B) interleukin (IL)- and interferon (INF)-related genes, and (C) tumor necrosis factor (TNF)-related genes. Expression matrixes of functional gene groupings are shown in figures 2 and 3 for HDF and HEK cells, respectively.
PRFE treatment resulted in the increased expression of several matrix metalloproteinases (MMPs) as well as several of their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), in both HDF (Fig. 2A) and HEK cells (Fig. 3A). Expressional changes occurred rapidly, as early as 5 and 15 minutes post PRFE treatment. In addition, in HEK cells a second wave of gene expression occurred after one hour, possibly due to activation by cytokines induced by PRFE (Fig. 3A). PRFE treatment also led to changes in the expression of numerous interleukin-, interferon-, and tumor necrosis factor (TNF)-related genes, in both HDF (Fig. 2B and Fig. 2C) and HEK (Fig. 3B and Fig. 3C) cells.

Finally, in order to characterize the temporal expression of inflammation-related genes in fibroblasts and keratinocytes, HDF and HEK cells were synchronized in G1, followed by release and subsequent microarray analysis at multiple time points. To determine the effects of PRFE treatment on this temporal pattern of gene expression, we performed the same experiment with PRFE treatment of the synchronized cells 30 minutes after release from G1. With regards to gene expression changes, PRFE treatment of synchronized HDF cells resulted in a large burst of upregulated gene expression at 15 minutes post PRFE treatment, whereas control cells showed a maximum increase in gene expression at 5 hours (data not shown). A comparison of the two data sets showed that, of the 512 genes upregulated at 5 hours in the control cells, 74% were upregulated at 15 minutes in PRFE-treated cells. This suggests that, in synchronized HDF cells, there is an immediate induction of genes as a result of PRFE treatment. Furthermore, within this same group of 512 genes, 59% are upregulated again at 5 hours in the PRFE-treated cells. This suggests that synchronized HDF cells that have been PRFE treated continue progression through the normal cell cycle after this initial burst of gene expression.

This difference in the timing of the onset of upregulated gene expression between PRFE treated cells and untreated cells is easily seen when the microarray data is divided into functional expression groups as well (Fig. 4). This is most clearly illustrated for the MMP and TIMP functional group (Fig. 4B). Taken together, both the HDF and HEK synchronized cell data suggest that PRFE treatment leads to an earlier induction of genes many of which are involved in the regulation of the inflammation phase of wound healing.

**Figure 1.** Microarray analysis of PRFE-induced gene expression in HDF cells. Total RNA was isolated from cells at the indicated times after the initiation of PRFE treatment. Equal amounts of RNA were used to synthesize 32P labeled probe. Similar amounts of labeled probe were hybridized to microarray membranes. Autoradiographs were scanned and quantitated. The expression levels are relative to the zero hour control. Expression profiles were placed into five groups (A-E). The colored bar along the right side of the expression matrix reflects the location of each group within the matrix.
Figure 3. Functional grouping of cDNA array analysis of gene expression after PRFE field treatment of HEK cells. Gene lists were developed to place expression data in functional groups. Expression groups related to inflammation-related genes are shown. (A) Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), (B) interleukins (ILs) and interferons (IFNs) and their receptors, and (C) tumor necrosis factors (TNFs) and their receptors. Expression levels are relative to the zero hour control.

Figure 4. Functional expression matrix for synchronized HDF and HEK cells treated with PRFE. Expression groups related to inflammation-related genes are shown. For HDF cells: (A) TNF and their receptors. For HEK cells: (B) matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), (B) interleukins (ILs) and interferons (IFNs) and their receptors, and (C) tumor necrosis factors (TNFs) and their receptors. Data are expressed relative to untreated synchronized control cells.
PRFE field treatment has shown clinical success in promoting the healing of chronic pressure wounds when used as a treatment adjuvant to basic wound care. In an attempt to elucidate possible mechanisms underlying PRFE-mediated effects on wound healing, we used microarray analysis to determine the effect of PRFE field treatment on the expression of a large set of genes involved in the inflammatory phase of wound healing.

The effects of PRFE on expression of genes involved in the inflammatory phase of wound healing. PRFE treatment of HDF and HEK cells led to wide-spread upregulation of transcripts encoding factors important to the inflammatory phase of the wound repair process, including numerous matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), interleukin (IL)-related genes, interferon (INF)-related genes, and tumor necrosis factor (TNF)-related genes, suggesting that PRFE treatment may lead to wide-spread modulation of inflammation.

MMPs function by mediating proteolytic processing (i.e. protein cleavage) of their substrates, and TIMPs function as endogenous inhibitors of MMPs. There are multiple MMPs with different substrate specificities, and a wide range of MMP substrates have been identified, including ECM components, cell adhesion molecules, chemokines and other cytokines, growth factors, as well as other regulatory factors. In wound healing, MMPs and TIMPs are thought to play a role in multiple aspects of the repair process, including inflammation, re-epithelialization, angiogenesis, wound contraction and remodeling. During the inflammatory phase of wound repair, they are involved in the removal of devitalized tissue and the regulation of inflammation through their role in regulating chemokines and other cytokines.

In this study, PRFE treatment of HDF and HEK cells led to the rapid increase in numerous MMP and TIMP-encoding transcripts (HDF cells (Fig. 2) and HEK cells (Fig. 3 and 4)). Given the widespread role of MMPs and TIMPs, this suggests PRFE could have an impact on numerous wound repair processes.

PRFE treatment of HDF and HEK cells also resulted in increased transcript levels encoding several cytokines and cytokine receptors. Cytokines are small, secreted proteins involved in cell-to-cell communication that encompass a variety of mediator families, including ILs, TNFs, and INFs. They mediate their effect by binding receptors on the surface of cells, activating signal transduction pathways and downstream events such as the activation of cytokine-responsive genes. Cytokine-mediated effects are dependent on the microenvironment in which the cytokine is expressed, and can vary depending on other cytokines and regulatory molecules present. In wound healing, cytokines play a role in the inflammatory response by regulating a number of processes including inflammatory cell activation and differentiation, cell recruitment, expression of cytokines and other factors within the wound bed, phagocytosis of devitalized cells and infectious agents, and apoptosis. Following PRFE treatment, transcripts for a number of cytokines and their receptors were upregulated in both HDF cells (Fig. 2B, 2C, and 4A) and HEK cells (Fig. 3B, 3C, 4C, and 4D), suggesting PRFE may function to modulate cytokine and cytokine receptor expression during the wound repair process as well.

From a more general perspective, PRFE field treatment had a dramatic impact on the general timing of transcript level increases, eliciting increases in transcript levels as early as 5 minutes after treatment. For cells entering the synthesis phase of the cell cycle, PRFE field treatment can initiate synchronous and robust activation of entire functional gene programs (Fig. 4).

Relevancy to the chronic diabetic wound. Failure of chronic diabetic wounds to heal is often thought to be associated with a chronic inflammatory state. This is likely the result of multiple contributing factors, including both
In this study we have shown that PRFE field treatment of human dermal fibroblasts and epidermal keratinocytes resulted in robust increases in the levels of numerous transcripts encoding factors involved in the inflammatory phase of the wound healing process. The data provided here suggests potential for large-scale immunomodulation by PRFE treatment via the upregulation of MMPs and TIMPs, as well as numerous cytokines and their receptors. Taken together with results from clinical studies, this data supports a mechanism whereby PRFE field treatment promotes the healing of chronic wounds by facilitating the transition from a chronic inflammation cycle to that of a functional wound healing cycle, a process that in part may involve PRFE-mediated immunomodulation.

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References


