Effect of Polyhydrated Ionogen (PHI) on Viability and Matrix Metalloproteinase Levels in Cultures of Normal and Diabetic Human Dermal Fibroblasts

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INTRODUCTION

It has been postulated that elevated levels of matrix metalloproteinases (MMPs) and reactive oxygen species detected in fluids and biopsies of chronic wounds contribute to the failure of wound to heal by degrading essential growth factors, receptors and extracellular matrix proteins. This stimulated the development of a formulation containing metal ions (polyhydrated ionogen, or PHI) and citric acid that reduced reactive oxygen species in cultures of polymorphonuclear leukocytes (J Wound Care 12:10, 2003). The effect of PHI on gene expression, including synthesis of MMPs, was studied in cultures of dermal fibroblasts from normal and diabetic patients.

MATERIALS AND METHODS

Fibroblast Cultures

Cultures of dermal fibroblasts were established from punch biopsies taken from the thigh of normal volunteers and from the base of chronic foot ulcers of diabetic patients. Cells were grown in defined culture medium (DMEM) supplemented with 10% calf serum.

Effect of PHI on Fibroblast Viability

Cultures of human dermal fibroblasts were seeded in 96 well plates and grown to approximately 80% confluency in complete medium (DMEM supplemented with 10% calf serum) then medium was removed and replaced with complete medium containing PHI at concentrations of 0%, 0.02%, 0.04%, 0.08%, 0.16%, 0.32%, 0.63%, 1.25%, 2.5%, 5.0%, 10% (W/V). After 24 hours of incubation, cell number and metabolic activity were measured using a soluble tetrazolium dye (MTT) and an electron coupling reagent as described by Promega Corp (Madison, WI) .

Microarray Analysis of Gene Expression in Fibroblasts Incubated with PHI

Cultures of normal and diabetic fibroblasts were grown to initial confluency in complete medium containing 10% serum in T-75 culture flasks then the medium was removed and replaced by complete medium with or without 0.125% PHI. After 30 hours of incubation, total RNA was isolated using the RNeasy protocol (Qiagen, Inc., Valencia, CA). Briefly, cells were lysed in RLT buffer, lysate solutions were centrifuged through QIAshredder columns, the solutions were applied to RNeasy mini silica-gel membrane columns, DNA was removed with on-column DNase I digestion, and total RNA was eluted from the mini column with RNase-free water. The quality of each clean RNA sample was assessed with a RNA 6000 Nano Chip on a Bioanalyzer (Agilent Technologies, Palo Alto, CA) (Figure 1).

Five µg aliquots of RNA were used as templates for cDNA synthesis with the Superscript Choice System kit. (Invitrogen Life Technologies, Gaithersburg, MD). Briefly, first strand synthesis was primed with a T7-(dT)24 oligonucelotide primer containing a T7 RNA polymerase promoter sequence on the 5' end (Genset Oligos, La Jolla, CA). Second strand products were cleaned with the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) and used as templates for in vitro transcription with biotin-labeled nucleotides (Bioarray High Yield RNA Transcript Labeling Kit, Enzo Diagnostics, Farmindale, NY). The copy RNA (cRNA) products were cleaned with the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) and 20 µg aliquots were heated at 94˚C for 35 min in fragmentation buffer.

Fifteen µg of cRNA from each sample were hybridized for 16 hr at 45˚C to an Affymetrix U133 2.0 plus array. After hybridization, each array was stained with a streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, Oregon), washed and scanned with a Genearray Scanner (Agilent Technologies, Palo Alto, CA). Genes that were not expressed under any of the four experimenal condition (absent on all four chips) were removed from further analyses. Signal intensities for the remaining genes were variance normalized, and genes with the greatest variation (± 3 SD) in signal values were selected and normalized signals values were analyzed by both 2-way hierachical clustering and K-Means cluster analysis.

RESULTS

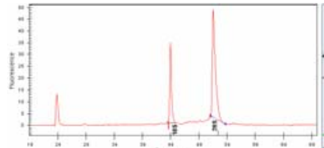


Figure 1. BioAnalyzer Evaluation of RNA
A sample of total RNA isolated from culture of dermal fibroblasts established from a chronic foot ulcer of a diabetic patient. The ratio of the 18S and 28S ribosomal RNA peaks and the lack of multiple smaller peaks indicate the RNA is intact.

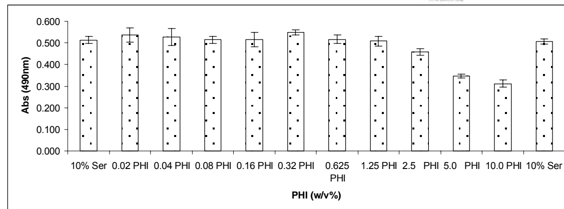
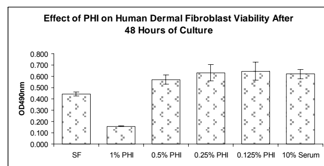


Figure 2. Effect of Wide Range of PHI Concentrations on Fibroblast Viability
Human dermal fibroblasts from a chronic foot ulcer of a diabetic patient were seeded into 96 well plate and cultured with PHI at the indicated concentrations for 48 hours then MTT was added and the numbers of cells were measured by absorbance at 490 nm. PHI showed no toxicity from 0.02% to 1.25%, but higher concentrations 2.5%, 5%, and 10% reduced cell numbers.



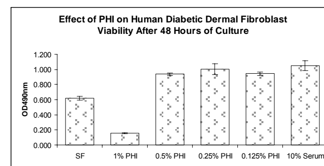
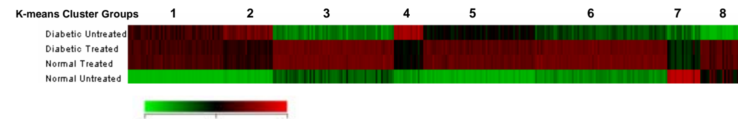
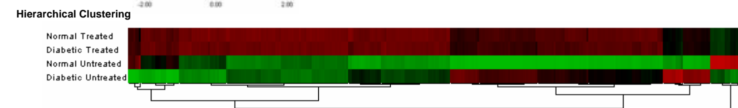
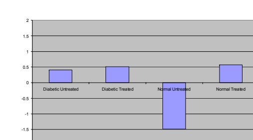


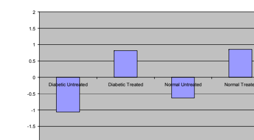
Figure 3. Effect of Narrow Range PHI on Fibroblast Viability
Fibroblasts from a diabetic patient or normal volunteer were seeded into 96 well plate and cultured with PHI at the indicated concentrations for 48 hours then MTT was added and the numbers of cells were measured by absorbance at 490 nm. PHI showed no toxicity at 0.5% or lower concentrations.



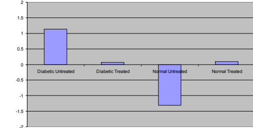




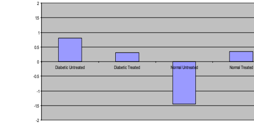
Cluster 2
chloride intracellular channel 4
fibrillin 1 (Marfan syndrome)
thrombospondin 1
tissue inhibitor of metalloproteinase 1
tissue inhibitor of metalloproteinase 3



Cluster 4
collagen type I, alpha 1 chain
collagen type III, alpha 1 chain
collagen type XII, alpha 1 chain
connective tissue growth factor (CTGF)
lysyl oxidase-like 2



Cluster 5
insulin-like growth factor binding protein 7
matrix metalloproteinase 1 (interstitial collagenase)
matrix metalloproteinase 3 (stromelysin 1, progelatinase)



Cluster 8
chemokine ligand 6 (GCP 2)
fibronectin 1
matrix metalloproteinase 2 (gelatinase B)

CONCLUSIONS

- PHI has no toxicity at ≤ 0.5% in cultures of fibroblasts from normal or diabetic patients.
- PHI induced similar changes in patterns of gene expression in normal and diabetic fibroblasts
- PHI decreased MMP-2 mRNA and increased TIMP mRNAs levels, similar to results from clinical studies
- Gene expression patterns are different in fibroblast cultures from normal and diabetic patients

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