Micro-RNA-21 is Associated with Nitrogen Mustard Treated Skin Inflammation on Mouse Keratinocyte Pam212 Cell

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Introduction

MicroRNAs (miRNAs) have appeared as key gene regulators that control inflammation, autoimmune disease, and skin barrier regulator in keratinocyte. (1-3)

A variety of wound healing- related miRNAs have been identified and their misregulation likely contribute to wound pathologies.(4)

Nitrogen Mustard (NM) is a blistering agent, or vesicants, that has been used in chemical warfare and induce inflammation and skin injury. (5-10)

It was suggested that different cellular mechanism and molecular pathways are responsible for damages to body tissues such as DNA damages, oxidative stress, apoptosis, and inflammation in NM driven skin injury. (11-13)

Intoxication of a mustard is associated with oxidative stress which is caused by an imbalance between production of oxidants and antioxidants in the lung and respiratory tract. (10)

It has been thought that oxidative stress is a primary event triggering the inflammatory cascade and tissue injury. (10)

Hypothesis and Purpose

Micro-RNA-21 plays a key role in skin inflammation which results from oxidative stress due to nitrogen mustard exposure.

By understanding cellular/molecular mechanisms of the underlying skin blistering and injury process used on animal or skin samples. We can develop better biomarkers to understand disease pathogenesis and develop countermeasures by targeting key molecules that protect the body from harm when exposed to Nitrogen Mustard. Herein, we try to understand the gene expression (transcriptomic level) strategy if nitrogen exposure mediated skin inflammation would be regulated by microRNAs and unfold its target molecules in mouse keratinocyte cell.

Materials And Methods

Cell Culture
Mouse keratinocyte Pam 212 cells were gifted from Dr. Laskin (Rutger's University, NJ). Pam 212 cells were cultured in complete culture medium consisted of DMEM supplemented with heat inactivated10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Chemicals
Nitrogen mustard (mechlorethamine hydrochloride, Sigma-Aldrich, St. Louis, MO) was purchased from Sigma-Aldrich (St Louis, MO). SB203580 was purchased from purchased from Promega (Madison, WI), and primer gene of miR21 was purchased from Qiagen (Valencia, CA). All other chemicals were from Sigma-Aldrich (St Louis, MO)

Total RNA isolation and RT-PCR
RNA extraction from NM- treated Pam212 cells were performed by TRI reagent (Sigma Aldrich, St Louis, Mo) according to the manufacturer's protocol.

cDNA preparation and Semi-Quantitative real-time PCR
Gene of interest which was listed in the table was examined with specific primers using a CFX 96 real time PCR instrument (BioRad, Hercules, CA, USA). RNA samples were reverse transcribed to synthesize first stand cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA), then analyzed by real-time PCR using specific primers and the double stand iQ SYBR Green supermix dye (2X) (BioRad, Hercules, CA, USA), according to protocols by manufacturer. All reactions were performed with two step method as following; a hot-start pre-incubation step of 3 min at 95oC, followed by cycles of 10 sec. at 95oC and 30 sec. at 55oC. mRNA gene expression was quantified by using the comparative CT method following as manufacturer's protocol. Expression of each gene was normalized to GAPDH mRNA level (ΔCt) and relative to the appropriate internal control (ΔΔCt) expressed as fold induction or decrease of control. For reporting, representative data are mean ± S.E.M of three independent experiments.

Measurement of microRNA by QRT-PCR and cell transfection with siRNA tool
The siRNA for microRNA (miR)-21 and scrambled siRNA as negative control were synthesized by eurofins MWG operon (Huntsville, AL). The siRNA oligonucleotide sequence were used as followings; siRNA for miR-21, 5'-UCA ACA UCA GUC UGA UAA GCU A-3'; siRNA for negative control, 5'-UUG UAC ACA AAA GUA CUG-3'. For performing cell transfection of siRNA of miR-21 or scrambled siRNA to each well, 5 µl of each siRNA (20 µM) were added into 100 µl Opti-MEM solution (Invitrogen, CA), 5 µl of lipofectamine 2000 (Invitrogen, Carlsbad, CA) into 100 µl of Opti-MEM solution and then mixed following by standing for 5 min under biological hood. After 10 min later, add the mixture of siRNA for each miR-21, or scrambled as negative control to each well and continued cell culture for 6 hr before changing Opti-MEM medium to 10% DMEM medium. For detection miR change in response to NM (10 µM) for 1 hr exposure in siRNA transfected Pam212 cells, cells were harvested and isolated RNA on 48 h after transfection for 48h and do preformed miRNA gene amplification of targeted one by QRT-PCR and analytical software (Bio-Rad, CA) following manufacturer's protocol. To amplify miR-21, we used to the oligonucleotide of each miR-21 primer described as followings; miR-21 5'- TAC CAC CTT GTC GGA TAG C-3'; universal primer 5'-GCG AGC ACA GAA TTA ATA CGA C-3'; U6 5'-CGCTTCGGCAGCACATATACTA-3' (F), 5'-CGCTTCACGATTTTGCGTGTCA-3'(R) as control gene.

Statistical Analysis
All Data are presented as the mean ± Standard Deviation (SD). Statistical Analyses were determined by one-way ANOVA or student t-test as analysis of variance was used for Q-PCR analysis. For miRNA gene expression analyses compared to control, a p value of < 0.05 was considered statistically significant after applying two-tailed student T-test or Mann–Whitney U test.

ACKNOWLEDGEMENTS

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Results

Effect of SB203580(MAPK inhibitor) on HO-1 mRNA expression on Nitrogen mustard treated Pam212 cells

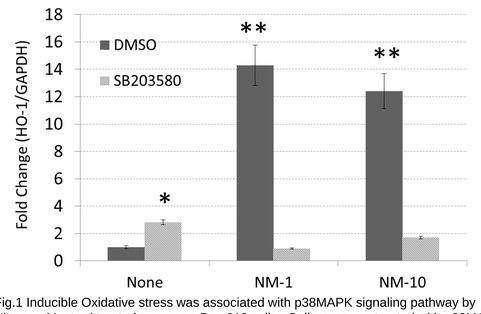


Fig.1 Inducible Oxidative stress was associated with p38MAPK signaling pathway by Nitrogen Mustard treated on mouse Pam212 cells. Cells were pre-treated with p38MAPK inhibitor (SB203580, 10 µM) for 30 min and then treated different of nitrogen mustard (NM; 0, 1, and 10 µM) for 24h. Total RNA was used to prepare cDNA and a expression of Heme Oxygenase 1 (HO-1) mRNA as marker for inducible oxidative stress as a cellular response was measured by QPCR. Data are represented as mean ± SD. **p<0.01, *P<0.05

Effect of Nitrogen mustard on microRNA 21 expression on Mouse keratinocyte Pam212 cells

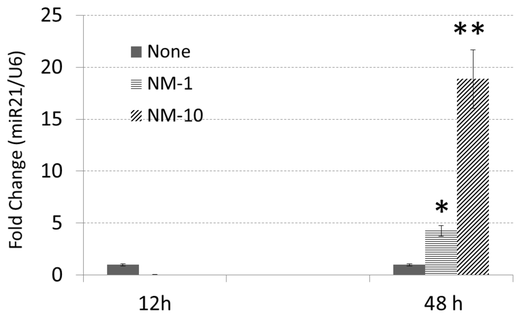


Fig.4 microRNA 21 was increased by nitrogen mustard exposure on mouse keratinocyte Pam212 cell. After screening microRNAs expression profile utilized miRNA finder in PCR array format, miRNA 21 was chosen and validated the expression patter on time- and dose-response curve using U6 as control gene. Nitrogen mustard (NM; 0, 1, and 10 µM) induce microRNA 21 in dose- and time-dependent manner on Pam212 cells. Data was represented as mean ± SD. **p<0.01, *P<0.05 (vs. Control)

Effect of Nitrogen mustard on inflammatory chemokines expression on Pam212 cells for 48h

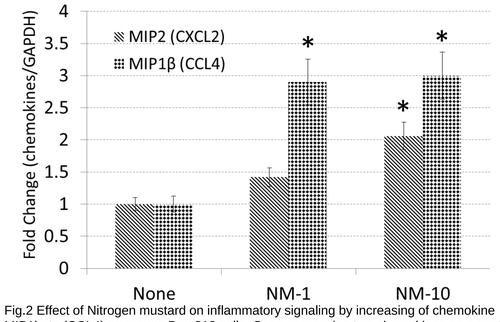


Fig.2 Effect of Nitrogen mustard on inflammatory signaling by increasing of chemokine MIP1beta (CCL4) on mouse Pam212 cells. Gene expression two chemokines as a marker of skin inflammation was examined utilize QPCR method using SYBR dye. Nitrogen mustard (NM) altered expression of chemokines (i.e., CCl4 and CXCL2) in dose-dependent manner on Pam212 cells. Data was shown as mean ± SD *P<0.05 (vs. control)

Effect of SB 203580 (p38 MAPK inhibitor) on microRNA 21 expression on Pam212 cells

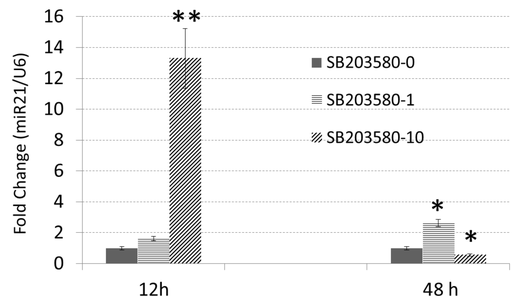


Fig.5 Induction of microRNA 21 was associated p38MAPK signaling pathway on nitrogen mustard treated mouse keratinocyte Pam212 cell. Cells were pre-treated p38MAPK inhibitor (SB203580, 10 µM) 30 min before treatment of nitrogen mustard (NM; 0, 1, and 10 µM) and measured the microRNA 21 mRNA expression for 12 h and 48h. Total RNA was isolated and prepared microRNA cDNA use QIAGEN kit and gene expression of microRNA 21 was quantitated with U6 as internal control gene utilize SYBR fluorescence dye in QPCR. Data are represented as mean ± SD. **p<0.01, *P<0.05 (vs. Control)

Effect of Nitrogen mustard on cell differentiation use Krt10 mRNA expression on Pam212 cells

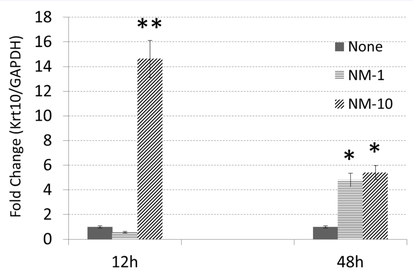


Fig.3 Nitrogen mustard affect cell proliferation on mouse keratinocyte Pam212 cells. Cells were treated different doses of nitrogen mustard (NM; 0, 1, and 10 µM) and measured the krt10 mRNA expression as a marker on cell proliferation for indicated time period (12 h and 48h). Total RNA was isolated and prepared cDNA use commercial kit and gene expression was measured by SYBR fluorescence dye in QPCR. Data are represented as mean ± SD. **p<0.01, *P<0.05 (vs. Control)

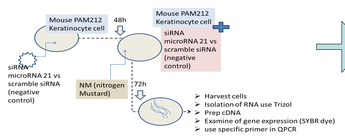
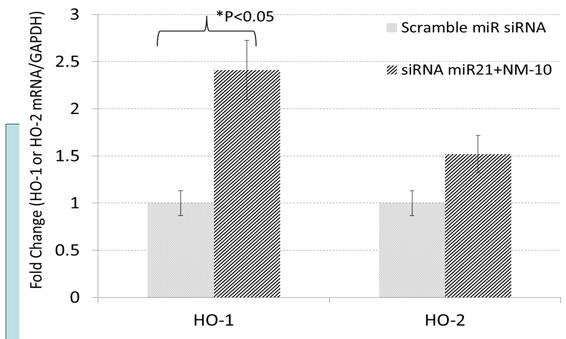
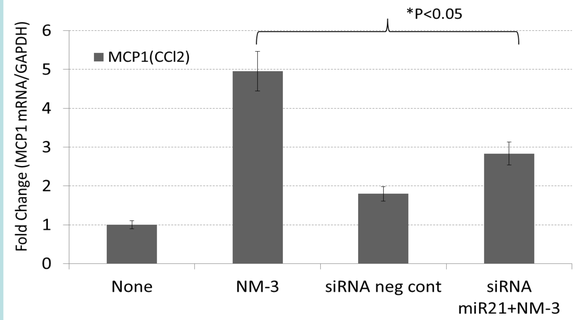


Fig.6 microRNA 21 modulate the oxidative stress and inflammatory chemokines on mouse keratinocyte Pam212 cell. Cells were transfected siRNA miRNA 21 and negative siRNA control to Pam212 cells for 48h and following by treated cells with NM (0, 10 µM) for 24h. Total RNA was isolated and validated the expression patter specific gene primer to HO-1 mRNA vs HO-2 mRNA (a), MCP-1 mRNA (b), and MIP1alpha mRNA (C). Data was shown as mean ± SD. **p<0.01, *P<0.05 (vs. Control)

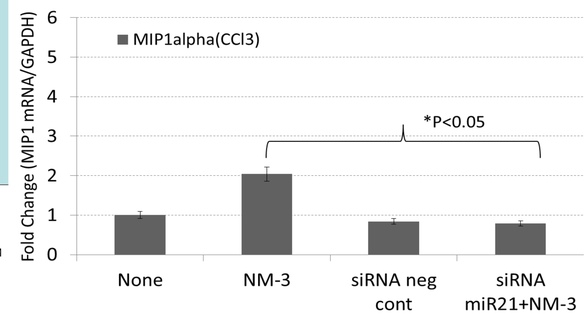
(A) Effect of siRNA microRNA-21 on HO-1 mRNA expression on Pam212 cells



(B) Effect of siRNA microRNA-21 on MCP1 mRNA expression on Pam212 cells



(C) Effect of siRNA microRNA-21 on MIP1 mRNA expression on Pam212 cells



Conclusion

1. Nitrogen mustard induces oxidative stress by altering HO-1 mRNA expression along with p38MAPK-dependent signaling pathway on keratinocyte pam212 cell.
2. Alteration of chemokines genes (i.e., MIP2 and MIP1 beta) is associated in the NM mediated skin inflammation using QPCR method. Keratinocyte cell proliferation was altered by induction of krt10 mRNA expression on dose-time-dependent manner.
3. We detected microRNA-21 was inducted by NM exposure on pam212 cell and its regulation is modulated under p38MAPK inhibitor (SB203580) presence.
4. Functional analysis utilizes siRNA tools, we confirmed that microRNA-21 is a key regulator on skin inflammation by mitigating inflammatory cytokines (i.e., MCP1 and MIP1alpha), not HO-1 mRNA expression.

Taken together this data suggest that, microRNA -21 plays a key role on regulation of skin inflammation due to NM-driven oxidative stress evoked on mouse keratinocyte Pam212 cellular model.

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