

Original Article

Reactive oxygen species (ROS) induces distinct gene expression in human fibroblast like synoviocytes (HFLS)

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Adhesive capsulitis of the shoulder is a common cause of pain that occurs during shoulder movement, thereby restricting shoulder rotation in clinical practice. Although most patients respond to pain relief treatment (NSAID or corticosteroids) by improving their range of motion, it remains poorly understood without any definitive treatment algorithm. In addition to immune cells, synoviocytes, chondrocytes and osteoblasts in the joint are known to produce pro-inflammatory mediators such as reactive oxygen species (ROS), inflammatory cytokines and lipid mediators, presumably contributing to the pathogenesis of osteoarthritis (OA) and adhesive capsulitis. Although inflammation and also fibrosis are proposed to be the basic pathological changes of a frozen shoulder, there is a lack of information regarding the downstream targets of the pro-inflammatory ROS signaling pathway in the synoviocytes and also how these ROS targets are modulated at the transcription level by a corticosteroid - dexamethasone. In this study, we used human fibroblast like synoviocytes (HFLS) to characterize the signaling targets of ROS by employing a human DNA microarray tool and studied the role of dexamethasone in this process. Our data suggest that several genes such as FOS, FOSB and NFkBIZ, which are known to be involved in pro- or anti- inflammation response, are modulated at the transcription level by ROS and dexamethasone.

Key words: synoviocytes, reactive oxygen species, dexamethasone, adhesive capsulitis, DNA microarray

Introduction

Primary frozen shoulder (FS) is known to be a painful contracture of the glenohumeral joint that arises spontaneously without an obvious preceding event [1].

Although the cause of frozen shoulder still remains unclear, molecular biological studies demonstrated that inflammation and fibrosis are the basic pathological changes of FS [2]. This seems to be related to an increased amount of collagen, fibrotic growth factors such as TGF-beta, and inflammatory cytokines like tumor necrosis factor-alpha and interleukins. Additionally, B and T lymphocytes and macrophages appear to be associated with FS [1].

Multi-factorial etiology is reported in osteoarthritis to include oxidative stress. The overproduction of ROS may regulate an intracellular signaling processes, chondrocyte senescence and apoptosis, extracellular matrix (ECM) synthesis and degradation along with synovial inflammation and ultimately fibrosis [3, 4].

Investigation of the rotator interval capsule and coracohumeral ligament obtained from FS patients disclosed active fibroblastic proliferation accompanied by some transformation to myofibroblasts. However, at least with inflammation and synovial involvement in a resemblance to Dupuytren's disease [5,6], fibroblast-like synoviocytes (FLS) are a special cell type located inside joints in the synovium and are presumably involved in chronic inflammatory diseases including rheumatoid arthritis [7].

Current medication for FS patients includes either nonsteroidal anti-inflammation drugs (NSAID) or corticosteroids such as dexamethasone (Dexa). Injections of corticosteroids can provide rapid pain relief in the short-

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term (particularly in the first 6 weeks), but long-term outcomes are known to be close to other treatments including placebos [8].

Nevertheless, there is a dearth of reports about ROS signaling and the role of anti-inflammation drugs in this process in the synoviocytes. We aimed to investigate this relationship using DNA microarray and RT-PCR methods in hydrogen peroxide-treated and/or dexamethasone-treated synoviocytes.

Materials and Methods

Cell culture and reagents

Human fibroblast-like synoviocytes (HFLS), which are derived from normal healthy human synovial tissue, were purchased from Cell Applications Inc. (Sandiego, CA). They were cultured in HFLS media (Cell Applications Inc.) at 37 °C in a humidified atmosphere containing 5% CO₂. Dexamethasone (Dexa) was purchased from Sigma Chemical Co. (St Louis, MO) and a medical grade H₂O₂ was purchased in a local pharmacy.

DNA microarray

Illumina Human HT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA), which includes about 47,000 genes, was used to analyze the gene expression profile of the HFLS cells. After stimulation with H₂O₂ for 2 hrs, total RNAs were extracted with RNA Blood Mini Kit (QIAGEN, Hilden, Germany), and equal amount of total RNAs was analyzed for differentially expressed genes by the gene chip and normalized by MAS5 normalization (Macrogen, Seoul, Korea).

Reverse Transcriptase (RT) PCR

HFLS cells were seeded in 100 mm dish at the density of 5x10⁶/dish and after overnight, treated with H₂O₂ (10 mM final concentration) and/or Dexa (1 μM) for 2 hrs. Total RNAs were extracted with RNA Blood Mini Kit (QIAGEN) before cDNA synthesis with AccuPower rocket script RT/PCR premix (Bioneer, Daejeon, Korea) and 10 ng of total RNA and 10 μM of 5' and 3' primers (each) in a total volume of 20 μL. PCR reaction was proceeded as follows; cDNA synthesis for 42°C/40 min, predenaturation for 95°C/5 min, followed by 35 cycles of

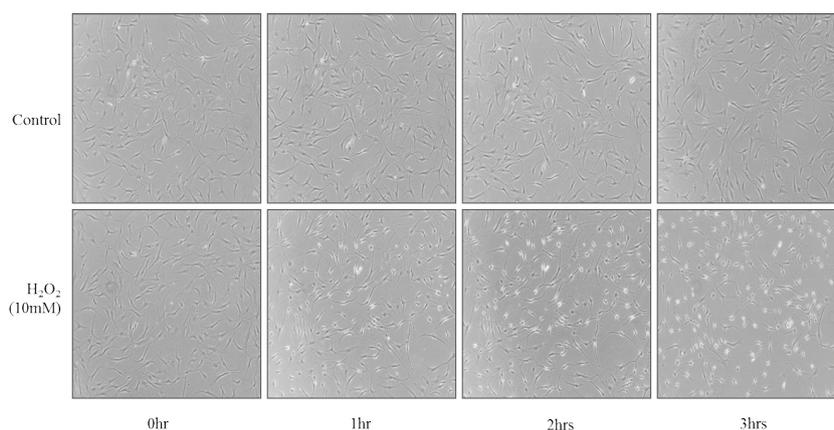


Fig. 1. Morphological change of HFLS after treatment with H₂O₂ over the 3 hour course. HFLS cells were plated in 6 well plate at a cell density of 5x10⁴ and after overnight, they were treated with 10mM H₂O₂ for 0, 1, 2, and 3 hrs before imaging with Olympus IX71 microscope

Table 1. List of genes that are differentially expressed in the DNA microarray

NCBI No.	Gene (Homo sapiens)	Gene symbol	Fold Change
NM_005252.2	Fos proto-oncogene, AP-1 transcription factor subunit	FOS	15.6
NM_006732.1	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	9.4
NM_015675.2	Growth arrest and DNA-damage-inducible, beta	GADD45B	2.5
NM_002228.3	Jun proto-oncogene, AP-1 transcription factor subunit	JUN	2.2
NM_002648.2	Pim-1 oncogene	PIM1	1.8
NM_006622.2	Polo-like kinase 2	PLK2	1.7
NM_000399.2	Early growth response 2, transcript variant 1	EGR	1.6
NM_001005474.1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta, transcript variant 2	NFKBIZ	1.6

denaturation for 95°C/30 sec, annealing for 54°C/30 sec, extension for 72°C/30 sec. 10 ul of PCR reaction was run on 1.5% agarose gels for electrophoresis and stained with ethidium bromide staining for image analysis.

Results

H₂O₂ induces morphological change of HFLS cells over 3 hrs

In order to investigate whether H₂O₂ may induce any cell shape changes or apoptotic cell death over time, the HFLS cells were treated with 10 mM H₂O₂ for various lengths of time: from 1 to 3 hrs. As reported previously, 10 mM H₂O₂ induced a morphological change as early as 1 hr after treatment and later cell death. Since a high concentration of H₂O₂ is known to induce cell death [9], we chose this concentration of H₂O₂ in the subsequent DNA microarray experiment to identify cell stress response against pro-inflammatory ROS.

Differential expression of H₂O₂ responsive genes

After HFLS cells were treated with normal growth media (control) or 10 mM H₂O₂, total RNAs were extracted and analyzed with Illumina HumanHT-12 v4 DNA array chip containing about 47,000 genes. From the DNA array data, we found that 106 and 32 genes were up-regulated by more than 1.5 folds and 2 folds respectively, whereas 135 and 20 genes were down-regulated in H₂O₂ treated cells compared to those of control cells (result not

shown). From the up-regulated genes, we chose 12 genes which include FOS, FOSB, GADD45B, JUN, PIM1, PLK2, NFKBIZ, OSR2, EGR2, LOC338758, GDF15 and LOC387763, whose functions are reported or unknown for further analysis by RT-PCR. Out of 12 genes, we could not identify a PCR amplified band with PCR primers specific for LOC338758, GDF15 or LOC387763 cDNAs (result not shown).

Since we noticed that the tubulin controls were differentially expressed by 50% between mock (-) control and H₂O₂ treated samples, we chose the sum of 28S- and 18S-rRNA signal as an internal loading control (supplementary result). After compensation of the two rRNA signals between the two samples, the expression levels of 9 genes were adjusted accordingly, as shown in Fig. 2B.

From these analyses, FOS, FOSB, NFKBIZ and OSR2 appear to be transcriptionally up-regulated by more than 50% in the H₂O₂ samples, whereas JUN, PIM, PLK2 and EGR2 exhibited a modest up- or down-regulation in the H₂O₂ samples.

Modulation of H₂O₂ responsive gene expression by Dexa

Based on the data in the above, we sought to study whether a Dexa, one of the most commonly prescribed corticosteroids for pain and osteoarthritis, can modulate the expression of the ROS responsive genes. For this purpose, HFLS cells were treated with ethanol (mock), H₂O₂, Dexa or H₂O₂ + Dexa for 2 hrs and subjected to RT-PCR analysis. As shown in Fig. 3A, Dexa appears

Table 2. List of primers for RT-PCR analysis

Gene symbol	Sequences of primers (5' - 3')	Location	Size of PCR products(bp)
FOS	CCAACCTGCTGAAGGAGAAG	556	233
	GCTGCTGATGCTCTTGACAG	788	
FOSB	AGGAAGAGGAGAAGCGAAGG	458	225
	CTTCGTAGGGGATCTTGCAG	682	
GADD45B	GTGTACGAGTCGGCCAAGTT	118	249
	GACCAGGAGACAATGCAGGT	366	
JUN	GAGCGGACCTTATGGCTACA	66	192
	CCGTTGCTGGACTGGATTAT	257	
PIM1	CAGAGTGGATCCGCTACCAT	629	226
	TGGATTTCTTCGAAGGTTGG	854	
PLK2	CAGCAGATTGGGGATGCTAT	1315	203
	TTGGTGACCCACTGAAATGA	1517	
EGR2	ATTCTGAGGCCTCGCAAGTA	958	259
	TTCGGCCACAGTAGTCACAG	1436	
NFKBIZ	GCCTAATTCAAATGGGAGCA	1490	201
	TCAACCGATACTGCAAGCTG	1690	

to down-regulate the expression of FOSB, PIM1 and PLK2, while it increases the expression level of NFKBIZ irrespective of H₂O₂. Meanwhile, the expression of JUN seems to be inhibited only when HFLS cells were treated with both H₂O₂ and Dexa.

Discussion

Main pathology of primary adhesive capsulitis has been reported to be an inflammatory contracture of the shoulder joint capsule resulting from synovitis and subsequent fibrosis [1]. Although several biomarkers including TGF-β, PDGF, TNF-α, IL-1β and COL3A1 are demonstrated to be associated with the synovial hyperplasia and capsular fibrosis of the affected tissues, their precise role in the pathological process is not clear [10,11].

Recent studies have identified both oxidative stress and ROS to be significantly associated with osteoarthritis (OA) progression [12, 13]. Therefore, unraveling ROS signaling is proposed to offer a valuable perspective for exploration of potential therapeutic strategies in the treatment of OA [3]. However, the role of ROS signaling in adhesive capsulitis remains unknown and in this study, we attempted to characterize the role of oxidative stress in a human synoviocyte cell line (HFLS) using H₂O₂.

HFLS cells showed a morphological change at 10 mM of H₂O₂ starting from 1 to 3 hrs and thus, we choose this condition (10 mM and 2 hr) in the subsequent DNA microarray. To chart an expression profile of whole human genome, we used an Illumina HumanHT-12 v4 Expression BeadChip covering 25,000 annotated genes. Out of 106 genes, which exhibited an increased expression in H₂O₂-treated HFLS by more than 1.5 folds, we chose 12 genes to confirm the expression by using the reverse transcriptase PCR method and could get the PCR products from 9 genes. The 3 exceptions were (GDF15 (NM_004864.1), LOC338758 (XM_931359.2), and LOC387763 (XM_941665.2)). This RT-PCR analysis revealed that ROS stress could elicit a differential expression of novel genes such as FOS, FOSB, PIM1, NFKBIZ, PLK2 and OSR2 by about 1.5 folds. On the other hand, GADD45B, JUNB and PLK2 turned out to be unresponsive to H₂O₂ stimulation at the transcription level (Fig. 2A and 2B).

FOS and FOSB constitute AP1 transcriptional regulator involved in inflammatory bone and skin [14, 15] and rapidly is up-regulated by inflammatory cytokines such as TNF and IL-6 [16]. In contrast, Pim1 kinase is reported to have both pro- and anti-inflammatory activities depending on cellular backgrounds and the nature of stimuli [17,18], NFKBIZ is a nuclear inhibitor of NFκB that

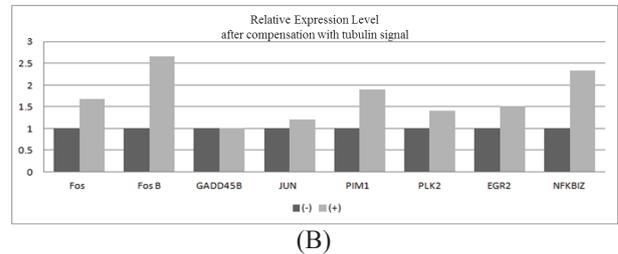
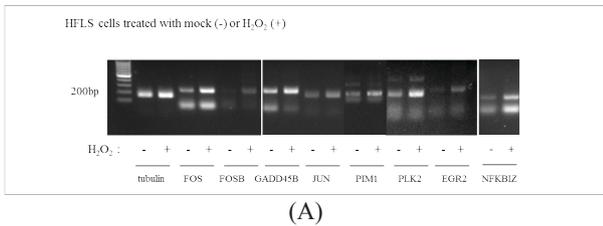


Fig. 2. Differential expression of H₂O₂ responsive genes in HFLS Cells. HFLS cells were grown in normal growth media before addition of either plain media or 10 mM of H₂O₂ for 2 hrs. Thereafter, total RNAs were extracted for RT-PCR using specific primers for the genes screened from DNA microarray analysis. Tubulin was included as an internal control of equal loading of total RNA samples. (A) The signal intensity, in each lane on the agarose gel containing the PCR product of the expected size, was quantitated by Image Lab 5.0 and presented as graph (B).

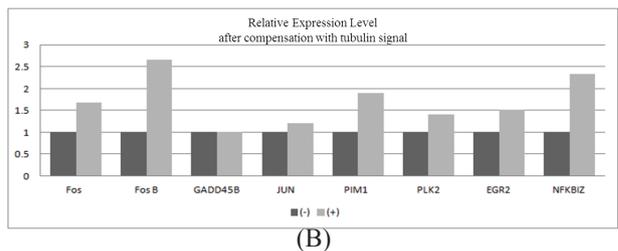
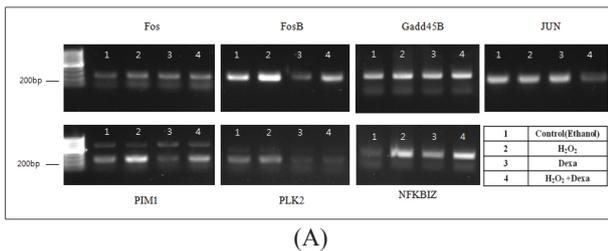


Fig. 3. Effect of Dexa on the H₂O₂ responsive gene expression. HFLS cells were grown in normal growth media before they are simulated with either plain media, 10 mM H₂O₂, and/or 1 uM Dexa for 2 hrs. Subsequently, total RNAs were extracted and used for RT-PCR using gene specific primers before agarose gel run (A). Individual signal intensity in (A) was quantified by Bio Lab 5.0, and the signal in each control lane was used as a reference for equal loading before presenting as graphs in B.

is induced by lipopolysaccharide or interleukin-1, and its role in the suppression of atopic dermatitis was recently demonstrated [19, 20]. PLK2 appears to be involved in high glucose induced inflammation in podocytes [21], while OSR2, a zinc finger transcription factor involved in mouse development and joint formation, does not have any apparent role in inflammation [22,23]

Corticosteroids are often recommended for adhesive capsulitis and osteoarthritis to control inflammation, pain and to inhibit tissue destruction [24]. However, corticosteroids in adhesive capsulitis are reported to confer a protective effect only in the short-term - less than 12 weeks [25, 26]. We studied whether a corticosteroid-Dexa could modulate the expression of the ROS target genes in the above. Compared to FOS, the expression of pro-inflammatory FOSB, PIM1 and PLK2 appeared to be inhibited by 1 μ M of Dexa, whereas the expression of GADD45 and JUN was inhibited only when both ROS stress and Dexa were present. NFKBIZ expression increased by more than 2 folds in the presence of Dexa and it will be interesting to study whether this NFkB inhibitor plays a critical role in anti-inflammatory activity of Dexa in adhesive capsulitis.

Our data in this study present novel biomarkers, which are known to be involved in pro- or anti-inflammatory cellular signaling, and future study is necessary to identify their respective or collective role in adhesive capsul-

litis.

Acknowledgements

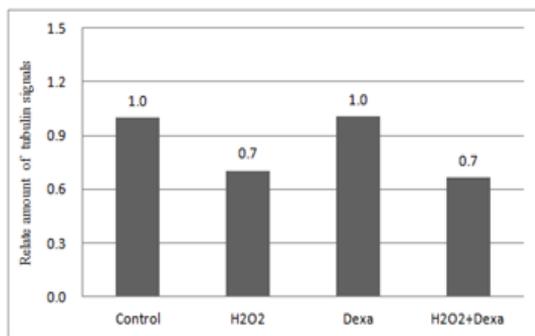
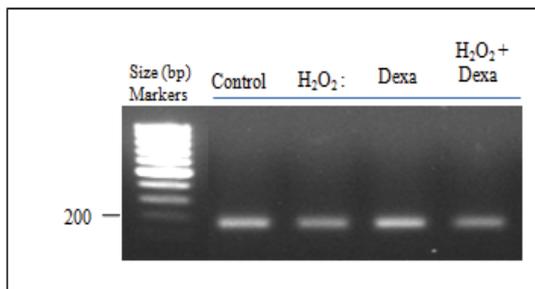
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Supplementary data for Fig. 2 & 3. A 200 ng of total RNAs was electrophoresed in agarose gel and the relative signal intensity of tubulin in each sample was quantified by Image Lab 5.0 (Biorad, USA). After compensation of H₂O₂, Dexa and H₂O₂/Dexa signals to that of control, individual gene expression was presented in Fig. 2 and Fig. 3.

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