Evaluation of Differentially Expressed Genes by Shear Stress in Human Osteoarthritic Chondrocytes In Vitro

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Background: The pathogenesis of osteoarthritis is related to abnormal mechanical stresses that alter cartilage metabolism and chondrocyte survival. Among the mechanical stresses, shear stress is held responsible for the development of arthritis.

Methods: Monolayer cultures of human osteoarthritic chondrocytes were subjected to fluid-induced shear stress in vitro. A cDNA microarray technology was used to screen the differentially regulated genes and quantitative real-time polymerase chain reaction (Q-RT-PCR) was used to confirm the results. The significance of the expression ratio for each gene was determined on the lowest associated false discovery rate calculated from the changes of gene expression in relation to the standard deviation of repeated measurements for that gene.

Results: Exposure of human osteoarthritic chondrocytes to shear stress (0.82 Pa) for 2 hours differentially regulated 373 and 227 clones in two independent microarray analyses with at least a 1.7-fold change. By comparing the differentially regulated clones, 14 upregulated and 6 downregulated genes were identified. Many of the differentially expressed genes were related to cell proliferation/differentiation (TGF-β, acidic FGF), cell survival/apoptosis (CYP1B1, BCL2L3, TNFRSF11B, chemokine ligands, ADM), and matrix homeostasis (DCN, SDC2, MGP, WISP2).

Conclusion: The gene expression patterns following shear stress show a high similarity to the gene expression in the reparative process of osteoarthritis chondrocytes. Using microarray analysis, this study suggests a close interaction between shear stress and the pathogenesis of osteoarthritis.

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Key words: osteoarthritis, chondrocytes, microarray, shear stress

Articular cartilage is subjected to various mechanical loadings during daily activity. Within the joint, chondrocytes are exposed to either compressive hydrostatic pressure or deviatoric

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stresses such as shear stress.[1] Shear stress participates in extracelluar matrix homeostasis and increases the release of proinflammatory mediators that are postulated to be related to the development of arthritis.[2-5] Shear stress also induces nitric oxide release and influences cell survival in monolayer chondrocyte cultures.[2,6,7] The cellular mechanisms underlying the responses of articular chondrocytes to mechanical stresses are important for understanding the pathogenesis of arthritis. However, the mechanotransduction pathways remain unclear.

DNA microarray technology has been enthusiastically integrated into biomedical research because of its high throughput analysis of differentially expressed gene profiles in many aspects.[8-11] It has been proven to be a powerful tool for assessing gene expression patterns in articular cartilage.[12-14] Few studies have investigated gene expression patterns of osteoarthritic chondrocytes following shear stress. The purpose of this study was to analyze how shear stress alters gene expression in human osteoarthritis chondrocytes with the use of cDNA microarray. We used quantitative real-time polymerase chain reaction (Q-RT-PCR) to verify the differential expression of selected genes identified from the microarray analysis.

METHODS

Cell culture and shear stress

Osteoarthritic cartilage specimens were collected following total knee arthroplasty from regions of the joint surface as described previously.[7] The chondrocytes were dissociated and then plated at 2.6 x 10^4 cells/cm^2 in DMEM containing fetal bovine serum (FBS), gentamicin, and ascorbic acid (25 μg/ml) (Sigma Chemical, St. Louis, MO, U.S.A.). High density monolayer cultures with serum-free medium were exposed to shear stress at a rotating velocity of 100 rpm (0.82 Pa) for 2 h similar to a previously described setting.[2,3,7] Control cells were not exposed to shear stress but were maintained under identical culture conditions. After loading, the cells were maintained under the same culture conditions for a constant period of 24 h to allow post-loading cellular processing.

RNA preparation

Total RNA was extracted with Tri-Reagent (Sigma) and the RNA quality was checked by an RNA LabChip read on a Bioanalyzer 2100 (Agilent, Santa Clara, Calif, U.S.A.). Total RNA (100 μg) was reverse transcribed and labeled at final concentrations of 500 μM dATP, 500 μM dGTP, 500 μM dTTP, 100 μM dCTP, 10 mM DTT, 1 x first strand buffer, 400 U SuperScript II, 40 U RNase inhibitor, and 45 μM of Cy3- or Cy5-dCTP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Microarray analysis

Microarray experiments were performed with Genomic Medicine Research Core Laboratory (GMRCL) microarrays[15] which contained 7334 sequence-verified clones (Incyte Genomics, Palo Alto, Calif. U.S.A.). All analyses were done in duplicate and dye swap experiments were used. Hybridization was performed on an automated slide processor (ASP, Amersham Pharmacia Biotechnology, Buckinghamshire, Sunnyvale, U.K.) for 16 h before scanning with a ChipReader (Virtek, Waterloo, Ontario, Canada).

Data analysis

GenePix Pro 4.1 (Axon Instruments, Inc., Natick, Calif, U.S.A.) and MATLAB 6.0 (MathWorks, Inc., Natick, Mass, U.S.A.) were used for the analysis. Global normalization for signal values of all genes on the array was used. Clustering analysis was performed with the use of a binary agglomerative hierarchical average linkage algorithm. Gene clusters were viewed with TreeView software.[16] The significance of the expression ratio was estimated using SAM software (Significance Analysis of Microarrays).[17] For each gene, significance was determined on the lowest associated false discovery rate calculated from the changes of gene expression in relation to the standard deviation of repeated measurements for that gene.

Quantitative real-time PCR

Six differentially expressed genes were selected for validation analysis using Q- RT-PCR with the HT 7900 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.). Gene-specific

TaqMan probes (TaqMan®Gene Expression Assays, Applied Biosystems, Foster City, CA, U.S.A.) were used for signal analysis. House-keeping GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was used as the internal control for relative gene expression quantitation.

RESULTS

Global analysis of gene expression in osteoarthritic chondrocytes regulated by shear stresss

The purpose of our study was to compare the gene expression in osteoarthritic chondrocytes exposed to shear stress. We could identify 373 clones and 227 clones that were differentially expressed by two separate analyses. Hierarchical clustering analysis showed these genes could be classified into different groups according to their expression patterns. (Fig. 1).

Putative shear stress-responsive genes

All microarray results were pooled and the lowest associated false discovery rate approach discovered 20 genes that included 14 upregulated genes (Table 1) and 6 downregulated genes (Table 2).

The upregulated genes are genes related to

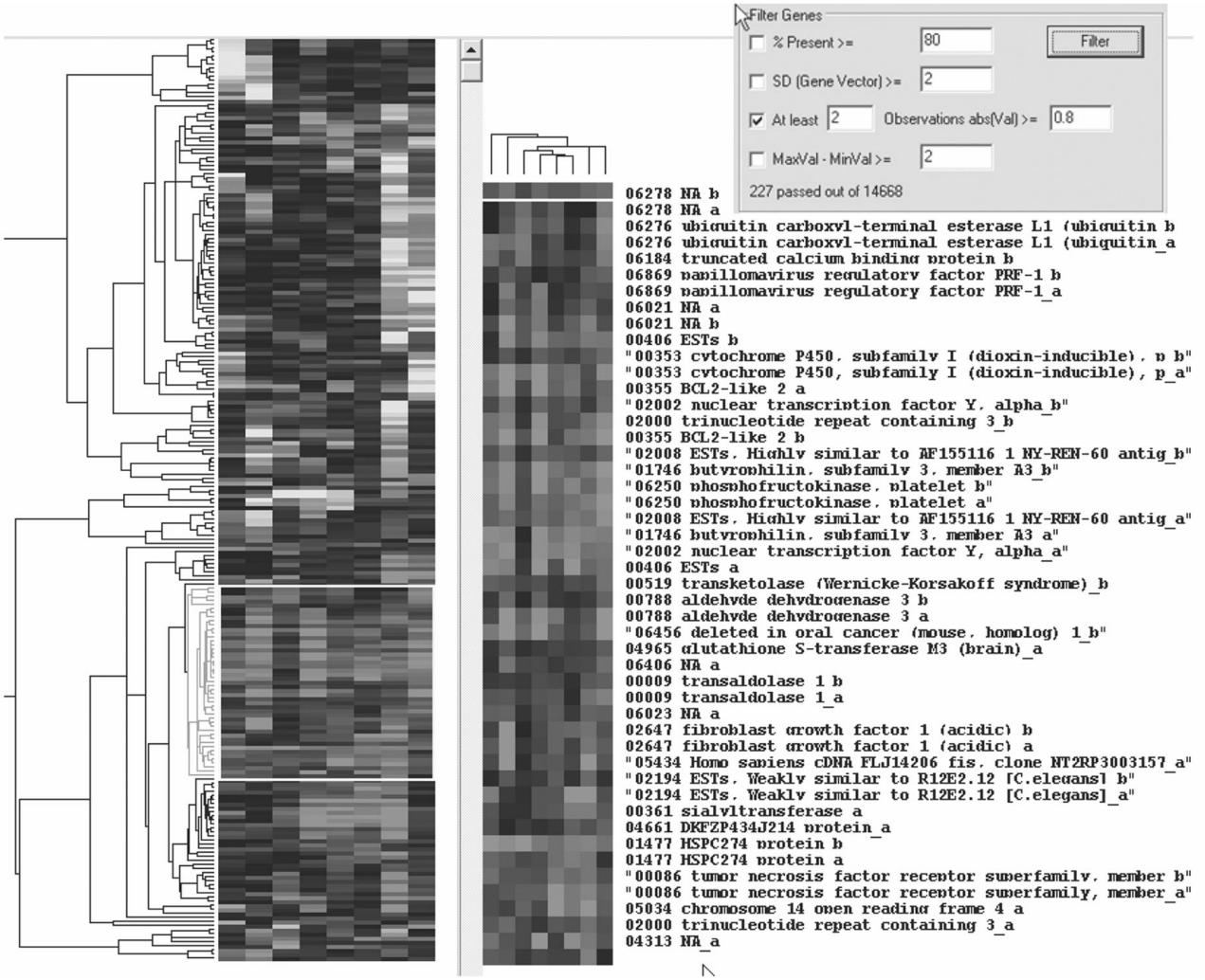

Fig. 1 An example of hierarchical clustering analysis from 8 donors.

Table 1. Upregulated Genes by Shear Stress in Osteoarthritic Chondrocytes

Gene title	Gene symbol	Fold change	Standard error	Accesion no.
Aldehyde dehydrogenase, dimeric NADP-preferring	ALDH3A1	2.7	0.9	NM_000691
Butyrophilin, subfamily 3, member A3	BTN3A3	2.6	1.1	NM_006994
Cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	2.6	0.6	NM_000104.2
Trinucleotide repeat containing 4	TNRC4	2.4	0.4	NM_007185.3
Phosphofructokinase, platelet	PFKP	2.4	0.3	NM_002627
Nuclear transcription factor Y, alpha	NFYA	2.4	0.3	NM_002505
Tumor necrosis factor receptor superfamily, member 11b	TNFRSF11B	2.2	0.4	NM_002546
Fibroblast growth factor (acidic)	FGF1	2.1	0.6	NM_000800
Ubiquitin carboxyl-terminal esterase L1	UCHL1	2.0	0.3	NM_004181
Transforming growth factor, beta-induced, 68KDa	TGFB1	1.9	0.7	NM_000358
BCL 2-like 2	BCL2L2	1.8	0.3	NM_004050
Isocitrate dehydrogenase 2 (NADP+), mitochondrial	IDH2	1.5	0.6	NM_002168
Decorin	DCN	1.4	0.2	NM_001920
Syndecan 2	SDC2	1.3	0.3	NM_002998

Table 2. Downregulated Genes by Shear Stress in Osteoarthritic Chondrocytes

Gene title	Gene symbol	Fold change	Standard error	Accesion no.
Chemokine (C-X-C motif) ligand 5	CXCL5	–3.4	0.9	NM_002994
Chemokine (C-X-C motif) ligand 1	CXCL1	–2.3	0.2	NM_001511
Interferon-stimulated protein, 15 KDa	ISG15	–2.1	0.6	NM_005101
Matrix Gla protein	MGP	–1.9	0.4	NM_000900
Adrenomedullin	ADM	–1.8	0.6	NM_001124.1
WNT1 inducible signaling pathway protein 2	WISP2	–1.6	0.7	NM_003881

stress and energy metabolism (ALDH3A1 and IDH2), cell survival (CYP1B1 and BCL2L2), growth factors (acidic FGF and TGF-β), a transcription factor (NFYA), alternative splicing of mRNA (TNRC4), osteoprotegrin (TNFRSF11B), small leucin-rich proteoglycans (decorin and syndecan), and others (BTN3A3, PFKP, UCHL1).

The down-regulated genes include genes related to development, immune function (CXCL5, CXCL1), cell differentiation/survival (ADM), protein polyubiquitination/degradation (ISG15), signal transduction regulatory functions, and matrix home-

ostasis (MGP and WISP2).

Validation of differential gene expression by Q-RT-PCR

The patterns of gene expression in microarray analysis were confirmed by Q-RT-PCR in selected genes (Table 3). In osteoarthritic chondrocytes, both the microarray analysis and Q-RT-PCR analysis showed the energy metabolic gene IDH2, small leucine-rich proteoglycans DCN and SDC2 and the cell survival related gene BCL2L2 were upregulated by shear stress. It was also noted the extracellular matrix homeostasis genes including the MGP and type II collagen gene were down-regulated by shear stress in osteoarthritic chondrocytes.

DISCUSSION

To our knowledge, the use of microarray technology to study thousands of genes in human osteoarthritic chondrocytes perturbed by shear stress has not been reported before. However, we regard the present investigation using microarray technology as an attempt to identify differentially expressed genes that might be overlooked in other studies. In this study, we were able to identify 20 genes that were differentially regulated by shear stress. These significantly altered genes included inflammatory cytokines, and genes involved in cell proliferation and differentiation, energy metabolism, cell survival, and extracellular matrix protein homeostasis.

The genes associated with extracelluar matrix protein homeostasis identified in this study include acidic FGF, TGF-β, small leucin-rich proteoglycans (decorin and syndecan), matrix Gla protein, and WISP2. TGF-β and acidic FGF are involved in the pathogenesis of arthritis at the cellular level and in animal models.[18-20] In cartilage exposed to high mechanical stresses, the expressions of TGF-β and small leucin-rich proteoglycans were significantly upregulated.[21-23] Shear stress has also been suggested to participate in human osteoarthritic cartilage metabolism and progression of joint destruction.[2,7] The matrix Gla protein is a regulatory protein for bone morphogenetic protein-2 which in turn is important for chondrogenic phenotype expression.[24] WISP2 encodes proteins of the WNT1 inducible signaling pathway and has been found to be closely related to the pathogenesis of inflammatory arthritis and the modulation of bone turnover.[25] The effects of shear stress on osteoarthritic chondrocytes analyzed by high-throughput microarray technology demonstrated that shear stress is closely related to cartilage matrix protein homeostasis. In addition, the expressions of energy metabolism -related genes ALDH3A1 and IHD2 were also increased in this study. This suggests a stress-related process of energy metabolism might also be involved in shear stress perturbed osteoarthritis.[26] In the reparative process of osteoarthritis, the altered gene expression also involves growth factors, small leucin-rich proteoglycans, and energy metabolism. The effects of shear

Table 3. Results of Q-RT-PCR in Selected Genes

TaqMan Probe	Accession no.	ABI Assay ID.	Fold change ± SD by Q-RT-PCR	Fold change by Microarray
Isocitrate dehydrogenase 2 (NADP+), mitochondrial	NM_002168	Hs00158033_m1	5.3 ± 2.9	1.5
Decorin	NM_001920	Hs00266491_m1	2.0 ± 1.3	1.4
Syndecan	NM_002998	Hs01081432_ml	2.2 ± 1.3	1.3
BCL2-like2	NM_004050	Hs00187848_m1	1.5 ± 0.6	1.8
Matrix Gla protein	NM_000900	Hs00179899_m1	–1.9 ± 0.5	–1.9
Collagen type II*	X16711.1	Hs00264051_m1	–2.2 ± 0.7	NA

*: Expression levels of type II collagen were analyzed by Q-RT-PCR but the fold change was not available by microarray analysis.

stress on osteoarthritic chondrocytes in this study showed a high similarity to the reparative process found in osteoarthritis.[18-25]

In previous studies, shear stress was associated with the processes of degeneration, ossification, and cell survival in chondrocytes.[1,7] Chondrocyte apoptosis has been demonstrated as a major determinant of cartilage damage in osteoarthritis.[27,28] In this study, shear stress also altered the expression of cell differentiation and survival-associated genes including CYP1B1, BCL2L2, TNFRSF11B, chemokine ligands, and ADM. CYP1B1 is a senescence-associated gene in normal human oral keratinocytes.[29] BCL2L2 is involved in the regulation of chondrocyte apoptosis in osteoarthritic cartilage,[30] in the epiphyseal growth plate,[31] and in shear stress perturbed osteoarthritic chondrocytes.[7] Osteoprotegrin (TNFRSF11B), a member of the TNF-decoy receptors, together with nuclear factor-kappa B, is involved in bone and joint disease.[32] Chemokines not only associate with cell survival but also play fundamental roles in homeostasis, articular chondrocyte hypertrophic differentiation, and arthritis.[33,34] Adrenomedullin (ADM) prevents synoviocyte apoptosis in inflammatory arthritis and may be involved in the process of de-differentiation in chondrocytes.[35,36]

In this study, we could identify groups of genes that were differentially expressed in osteoarthritis chondrocytes by shear stress using high throughput microarray analysis. However it should be emphasized that osteoarthritis cartilage tissue has inherent heterogeneity and the gene expression patterns associated with individual variations are expected to be complex. Furthermore, this study used the lowest false discovery rate approach based on the global normalization of all genes on the chip to identify differentially expressed genes. It is very likely that many differentially regulated genes by shear stress could be overlooked. Some of the undiscovered genes might have important physiologic functions related to shear stress.[2,3,6,7] In addition, the roles of many shear stress- regulated genes (BTN3A3, TNRC4, PFKP, NFYA, UCHL1, and ISG15) identified in this study are not clear. Nevertheless, shear stress in this study differentially regulated the expression levels of genes related to cell proliferation, differentiation, survival, and matrix homeostasis in osteoarthritis chondrocytes. The gene expression patterns following shear stress show a high similarity to the gene expression in the reparative process of osteoarthritis chondrocytes. Using microarray analysis, this study suggests a close interaction between shear stress and the pathogenesis of osteoarthritis.

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剪應力對人類退化性關節軟骨基因調控之研究

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背 景： 關節炎的病理成因與不正常的機械應力作用在軟骨細胞、影響軟骨的代謝與細胞的存活有關。在許多的機械應力之中，剪應力被認爲是造成關節炎主要的原因。

方 法： 人類關節軟骨細胞在單層細胞培養的條件下施予剪應力。被剪應力所調控的基因則以 DNA 微陣列以最低相關僞發現率 (基因表現量之改變相對於重複試驗的標準差) 加以分析，並以定量反轉錄聚合鍊反應加以確認。

結 果： 以 0.82 Pa 的剪應力作用在人類關節炎軟骨細胞 2 小時，在兩次獨立的 DNA 微陣列分析可以發現各有 373 與 227 個基因改變量大於 1.7 倍。綜合分析發現有 14 個基因是有意義的增高，有 6 個基因則有意義的降低。這些被調控的基因包括與細胞生長分化有關者 (如 TGF-β, acidic FGF)，與細胞存活凋亡有關者 (CYP1B1, BCL2L3, TNFRSF11B, cehmokine ligands, ADM)，以及軟骨細胞基質代謝平衡有關者 (DCN, SDC2, MGP, WISP2)。

結 論： 軟骨細胞經過剪應力的刺激後，基因表現的型態與關節炎軟骨細胞的修復過程相類似。利用 DNA 微陣列分析，這個研究支持剪應力與關節炎的成因有很密切的相關。
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關鍵詞： 關節炎、軟骨細胞、微陣列、剪應力

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