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Original

Reduction of Early Growth Response-1 Gene Expression in Osteoblasts by Hydrogen Peroxide

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Abstract: Bone formation steadily declines with age resulting in a loss of bone mass. Reactive oxygen species (ROS) are thought to be major contributors to the aging process. The zinc finger transcription factor, Early Growth Response-1 (Egr-1) is a potential regulator as a transcription factor of many target genes, and plays a role in cell growth, development, and differentiation. However, the effect of ageing on Egr-1 gene expression has not yet been ascertained. To identify which genes have an altered transcription level associated with bone loss by ageing, the pre-osteoblastic cell MC3T3-E1 was treated with H_2O_2, and gene expression profiles analyzed with gene chip technology using the Affymetrix GeneChip analysis system (Mouse; 34,000 genes). The expression of many genes in MC3T3-E1 was altered significantly with Egr-1 being decreased by H_2O_2. The reduction of Egr-1 mRNA levels was successfully confirmed by reversed transcription polymerase chain reaction (RT-PCR) and real-time PCR. Since it has been reported that Egr-1 plays an important role as a transcription factor for growth factor genes which promote cell proliferation and differentiation of preosteoblastic cells, the reduction of Egr-1 gene expression by H_2O_2 may be involved in the decline of bone formation in the ageing process.

Key words: Early growth response-1, Ageing, Osteoblasts

Introduction

Bone remodeling requires a delicate balance between bone formation and bone resorption, in which bone-forming osteoblasts and bone-resorbing osteoclasts play the central role[1)]. It is well known that bone formation steadily declines with age, resulting in a significant loss of bone mass, possibly due to a decrease in osteoblast proliferating precursors or the number of pre-osteoblasts, or decreased synthesis and secretion of essential bone matrix proteins[2)]. However, the mechanisms of bone formation decline caused by the aging process are not well understood. A current hypothesis states that reactive oxygen species (ROS), produced during normal cellular metabolism, are major contributors to the aging process[3, 4)]. Recent evidence has shown that ROS may be involved in the pathogenesis of bone loss-related diseases, where a marked decrease in plasma antioxidants was found in aged osteoporotic women[5)].

We previously studied the effects of ROS on bone nodule formation activity in the osteoblastic cell line MC3T3-E1, and found that bone nodule formation was significantly reduced by H_2O_2 treatment[6)]. However, the regulatory mechanisms leading to the observation could not be elucidated, as it is necessary to use a molecular biological approach to comprehensively study the effects of ROS on bone forming activity in osteoblasts.

Recent genome science technology to analyze and compare the expression of genes, DNA microarray systems have became an increasingly used for large number of random screening of mRNA transcripts. In the present study, part of a broader investigation of ageing, we analyzed gene expression in MC3T3-E1 cells whose expressions had been altered by H_2O_2. We used the Affymetrix GeneChip system and identified a significant decrease of the early response gene-1 (Egr-1) gene[7)]. To confirm the GeneChip data, reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analysis of Egr-1 gene expression with or without treatment of H_2O_2 in MC3T3-E1 cells were carried out.

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Materials and Methods

Cell culture and H_2O_2 treatment

MC3T3-E1 cells were purchased from Riken Cell Bank (Ibaraki, Japan). They were seeded at 1 x10^4 cells/well in a 24-

well culture plate (Corning, Grand Island, NY, USA) and grown in α-MEM containing 5 mM of HEPES supplemented with 50 μg/ml of gentamycin sulfate (Sigma, St. Louis, MO, USA), 0.3 μg/ml of amphotericin B (Flow Laboratories, Blacksburg, VA, USA), 100 units/ml of penicillin G potassium (Sigma), and 10% fetal bovine serum, supplemented with 50μg/ml ascorbic acid and 10 mM β-glycerol phosphate. Cells were cultured in a humidified atmosphere containing 95% air and 5% CO_2 at 37℃. After the cells grew and reached confluent stage, they were treated with 400 μM of H_2O_2 for 3 hrs according to the method of previous report[5]. Cells were then washed and incubated with the medium described above.

Quantitation of bone-nodule formation

For quantitation of bone-nodule formation, MC3T3-E1 cells were cultured for 21 days in the medium, after which the contents of each well was fixed for 15 minutes in 10% neutral buffered formalin and stained using a von Kossa technique.

RNA preparation

Total cellular RNA was isolated from MC3T3-E1 cells after treatment with or without H_2O_2 using an acid guanidinium thiocyanate-phenol-chloroform extraction method[8].

Gene Chip analysis

Antisense complimentary RNA (cRNA) derived from double strand complimentary DNA was labeled in the presence of biotinylated deoxyribonucleotide triphosphate derivatives to produce cRNA probes. The probes were fragmented and hybridized onto the GeneChip array (Affymetrix, Santa Clara, CA, USA; Mouse Genome 430 2.0, 34,000 genes). Washing and staining was performed by a GeneChip Fluidics station 450 (Affymetrix). Chip performance, background levels, and the presence or absence of signals was assessed using Microarray Suite software (Affymetrix). The presence or absence of signals was re-evaluated and intensity normalization was performed across arrays. Data analysis was performed using the GeneChip Expression Analysis software (Affymetrix) and GeneSpring software (Silicone Genetics, Redwood, CA, USA).

RT-PCR and real time PCR Analysis

RT-PCR and real-time PCR reactions were carried out using a DNA thermal analyzer (Rotor-Gene™ 6000; Corbett Life Science, Corbett Robotics Inc. San Francisco, USA). For RT-PCR, PCR products were electrophoresed on 1.5% agarose gel, followed by staining with ethidium bromide to examine the size of PCR products. Each assay was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. For real-time PCR, a SYBR Green Premix Ex Taq™ PCR kit (Takara Bio, Shiga, Japan) was used. PCR was started with an initial incubation at 95℃ for 15 minutes to activate the Taq DNA polymerase followed by 94℃ for 15 seconds, 56℃ for 30 seconds, and 86.4℃ for 30 seconds, for 40 cycles. The fluorescent signals were measured at the end of each elongation step and the beginning points of their exponential curves were determined for conversion of the cycle number into the amount of PCR. To calculate gene expression fold changes, the initial template concentration was derived from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the real time PCR reaction. After the final PCR step, the temperature was gradually raised to 95℃ while monitoring the fluorescent signals in order to form the melting curves and check the specificity of the PCR amplification. Values were calculated as means ± standard deviation (SD). Comparisons were made between two groups using a Student's t-test.

The DNA primer sequences were 5'-ctccttcagcacctcaactg -3' (the forward primer for Egr-1 gene); 5'-atctcttccctcctgtgctt -3' (the reverse primer for Egr-1 gene), (expected product size=135 bp); 5'-atcaccatcttccaggag-3' (the forward primer for GAPDH); and 5'-atggactgtggtcatgag-3' (the reverse primer for GAPDH gene), (expected product size=318 bp).

Results

As shown in Figure 1, bone nodule formation was significantly reduced in MC3T3-E1 cells following treatment with H_2O_2.

Following from this finding, we isolated RNA from both H_2O_2-treated and untreated cells, and examined differential gene expression using the Affymetrix GeneChip system. The scatter plot of gene expression levels in MC3T3-E1 from both H_2O_2-

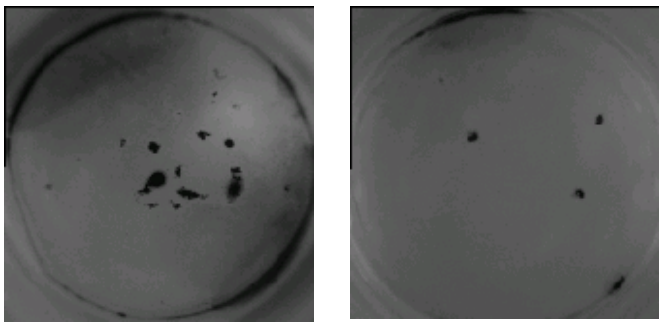

Figure 1 Effects of H_2O_2 on bone nodule formation by MC3T3-E1 cells.
(A) Untreated MC3T3-E1 cells (control). (B) H_2O_2-treated MC3T3-E1 cells.

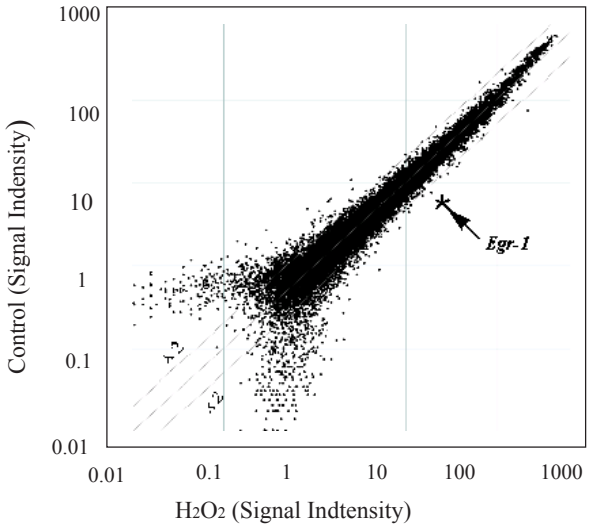

Figure 2 The scatter plot of gene expression levels in H_2O_2-treated and untreated MC3T3-E1 cells after 6 hrs.

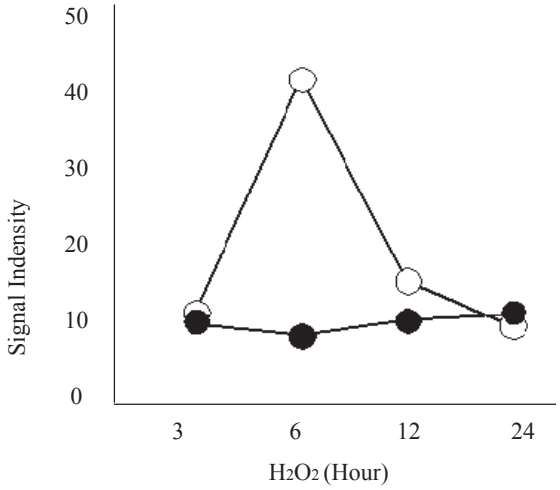

Figure 3 Egr-1 mRNA levels after H_2O_2-treatment. Raw signal intensity of mRNA levels from Affymetrix GeneChip analysis were shown. Open circle, Control; close circle, H_2O_2-treated.

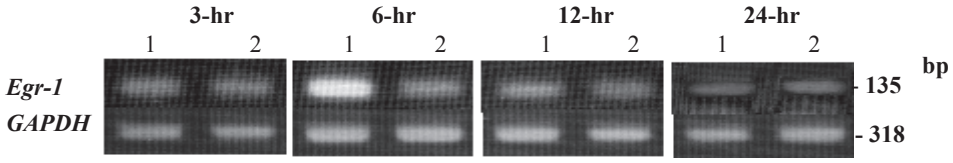

Figure 4 RT-PCR analysis of Egr-1 mRNA levels in MC3T3-E1. An ethidium bromide-staining pattern of the amplified PCR products using agarose gel electrophoresis is shown. 1, Control; 2, H_2O_2.

treated and untreated cells after 6 hrs is shown in Figure 2. H_2O_2-treatment altered the gene expressions of many genes in MC3T3-E1 when compared with a control. Among the altered genes, Egr-1 expressed mRNA level 5 times lower in H_2O_2-treated MC3T3-E1 compared to the control. Figure 3 summarizes the signal intensity of Egr-1 gene expression during time after H_2O_2-treatment. Egr-1 signal increased at 6-hr and decreased at 24-hr in non-treatment. Egr-1 signal was reduced at 6-, 12- hr in H_2O_2-yreated MC3T3-E1.

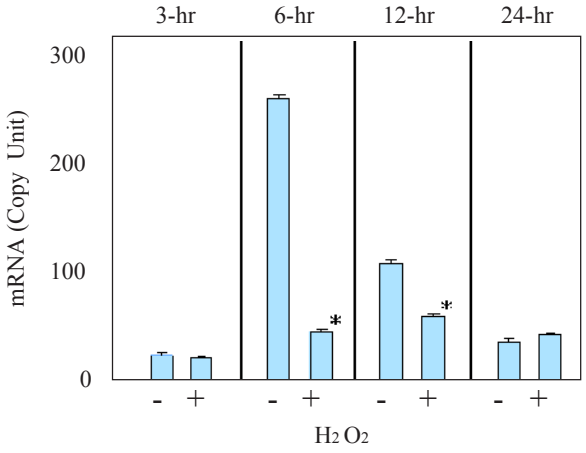

Figure 5 Real-time analysis of Egr-1 mRNA levels in MC3T3-E1. Results were expressed as mRNA copy unit by normalization to a housekeeping gene GAPDH. Differences between H_2O_2 treatment and control were determined using Student's t-test. *, $p<0.001$, n=3.

End-point RT-PCR was carried out to confirm the reduction of Egr-1 mRNA levels. Figure 4 shows that a significantly lower level of Egr-1 mRNA band was detected in MC3T3-E1 cultured with H_2O_2 when compared with control. In contrast, mRNA levels of GAPDH, the housekeeping control, showed no change between H_2O_2 treatment and non-treated in MC3T3-E1 cells.

Further experiments to determine the quantitative values altered in the Egr-1 gene expression levels were performed using real-time PCR. The data was converted to mRNA copy unit, and is shown in Figure 5. A significant reduction of Egr-1 mRNA levels was observed after 6 and 12 hrs after treatment with H_2O_2.

Discussion

In the present study, we attempted to identify the effects of H_2O_2 on gene expression in MC3T3-E1 cells using gene chip technology. Based on Affymetrix GeneChip results, we selected the gene Egr-1, which showed a 5–fold reduction in intensity of mRNA level at 6 hr following H_2O_2 treatment. To confirm the gene chip analysis result, we used endpoint PCR and real-time compared to that in non-treated cells.

Many attempts have been made to discover genes essential for

growth, proliferation, or differentiation using genome science technology.The expression of a zinc finger-encoding gene, Egr-1, was found during growth and differentiation. Egr-1 is an early growth response gene that displays *fos*-like induction kinetics in fibroblasts, epithelial cells, and lymphocytes following mitogenic stimulation. Sequence analysis of Egr-1 cDNA predicts a protein with three DNA binding zinc fingers, and Egr-1 mRNA levels increase dramatically during cardiac and neural cell differentiation[9,10]. Research has been directed towards the function of Egr-1 in growth and proliferation, the induction of Egr-1 was demonstrated as a transcriptional regulator, and a direct role of Egr-1 in controlling proliferation has been proposed in many type of cells [11].

The Egr-1 gene, therefore, functions as a convergence point for many signaling cascades. Egr-1 is thought to couple extracellular signals to long-term responses by altering gene expression of Egr-1 target genes. The inducible expression of Egr-1–dependent genes have been identified as PDGF-A, PDGF-B, TGF-ß1, and FGF-2[12]. These gene products are important for the enhancement of the healing process in bone fractures[13]. There have been no investigations in the effect of H_2O_2 on the Egr-1 gene expressions, considering the function of Egr-1 gene expression in osteoblasts for bone formation, several interesting findings have been reported. Egr-1 gene expression was enhanced by low-intensity pulsed ultrasound[14], by a short pulse of mechanical force[15], and by extracellular ATP[16], which are all well known factors stimulating bone formation. While acute metabolic acidosis, which is known as an inhibitor of bone formation, reduced the induction of osteoblastic Egr-1 and type 1 collagen gene expression[17]. Taken together with the finding presented here, the reduction of Egr-1 gene expression by H_2O_2 may be involved in bone loss during the aging process.

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