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Original Contribution

MicroRNA expression changes during human leukemic HL-60 cell differentiation induced by 4-hydroxynonenal, a product of lipid peroxidation

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ABSTRACT

4-Hydroxynonenal (HNE) is one of several lipid oxidation products that may have an impact on human pathophysiology. It is an important second messenger involved in the regulation of various cellular processes and exhibits antiproliferative and differentiative properties in various tumor cell lines. The mechanisms by which HNE affects cell growth and differentiation are only partially clarified. Because microRNAs (miRNAs) have the ability to regulate several cellular processes, we hypothesized that HNE, in addition to other mechanisms, could affect miRNA expression. Here, we present the results of a genome-wide miRNA expression profiling of HNE-treated HL-60 leukemic cells. Among 470 human miRNAs, 10 were found to be differentially expressed between control and HNE-treated cells (at $p<0.05$). Six miRNAs were down-regulated (miR-181a*, miR-199b, miR-202, miR-378, miR-454-3p, miR-575) and 4 were up-regulated (miR-125a, miR-339, miR-663, miR-660). Three of these regulated miRNAs (miR-202, miR-339, miR-378) were further assayed and validated by quantitative real-time RT-PCR. Moreover, consistent with the down-regulation of miR-378, HNE also induced the expression of the SUFU protein, a tumor suppressor recently identified as a target of miR-378. The finding that HNE could regulate the expression of miRNAs and their targets opens new perspectives on the understanding of HNE-controlled pathways. A functional analysis of 191 putative gene targets of miRNAs modulated by HNE is discussed.

Small noncoding RNA molecules, called microRNAs (miRNAs), were first discovered in the early 1990s by Ambros, Ruvkun, and co-workers, while studying development in the nematode *Caenorhabditis elegans* [1,2]. MiRNAs are phylogenetically conserved genes that act as negative regulators of gene expression at the posttranscriptional level [3,4]. The mechanism by which they work, referred to as "RNA interference," is similar in plants and animals, implying that they are involved in fundamental cellular processes [3,4]. To date, more than 500 human miRNAs have been identified [5]; each of them may control hundreds of target genes to regulate 30% or more of all protein-coding genes [6]. Mature forms of miRNAs are single-stranded RNAs of 18–24 nt in length and their maturation consists of an extensive posttranscriptional modification. Most miRNA genes are transcribed by RNA polymerase II, the same RNA polymerase that transcribes protein-coding genes [7], but some microRNAs in repetitive regions of the genome may be transcribed by RNA polymerase III [8]. Several microRNA primary transcripts encode only a single mature miRNA, whereas other loci contain clusters of miRNAs that seem to be produced from a single primary transcript [5]. Approximately half of all human miRNA genes are contained within the introns of protein-coding genes, whereas others reside in intergenic regions, in the exons of untranslated genes [9]. Control of miRNA transcription is incompletely understood. However, they are likely regulated by transcriptional elements identical to those of protein-coding genes [10], as was demonstrated for the oncogenic transcription factor c-myc, able to induce the expression of the miR-17-92 cluster by recognizing an upstream E-box element [11].

MicroRNA long primary transcripts (several thousands nucleotides), termed pri-miRNAs, contain one or more local hairpin structures. These hairpins are processed in the nucleus by the RNase III-type enzyme Drosha to produce a 70- to 100-nt precursor miRNA (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm and cleaved by cytoplasmic RNase III-Dicer into a 22-nt miRNA duplex: one strand (miRNA*) of the short-lived duplex is usually degraded, whereas the other strand, which serves as the mature miRNA, is incorporated into the RNA-induced silencing complex and drives the selection of target mRNAs containing antisense sequences [3,12].

To understand miRNA biological function, identification of miRNA targets is critical and it has become one of the most active research fields in biology. The development of computational algorithms leads to the prediction of many targets regulated by miRNA [5,13,14], but only a few of them have been verified. By silencing various target mRNAs, miRNAs have key roles in cellular processes, including diffe-

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rentiation, proliferation, and death [15,16]. In addition to critical functions during normal development and cellular homeostasis, it has become evident that abnormalities in miRNA activity may contribute to human diseases. In particular, an important role for miRNAs in the development of cancer has emerged [17,18]. Moreover, it has been suggested that miRNA expression profiles may enable classification of tumors, highlighting the potential of miRNA profiling in cancer diagnosis [19,20].

In recent years, it has become evident that 4-hydroxynonenal (HNE), one of the most intensively studied among lipid peroxidation products, is an important second messenger involved in the regulation of various cellular processes, such as signaling, the cell cycle, and, more generally, the response of target cells [21,22]. HNE has antiproliferative and differentiative properties toward various tumor cell lines [23–27]. In human leukemic HL-60 cells HNE exerts its action by modulating the expression of c-myc and c-myb oncogenes [28,29], some cyclins (cyclin D1, D2, A) [30], and the E2F4 transcription factor [31]. The role of HNE in controlling the replicative potential of cells was recently extended, showing its ability to inhibit telomerase activity and hTERT expression in HL-60, U-937, and ML-1 leukemic cell lines [26]. The mechanisms by which HNE affects cell growth and differentiation are only partially known. In view of the ability of miRNAs to regulate cell proliferation, we postulated that HNE, in addition to other mechanisms, could also affect miRNA expression. In this paper, the genome-wide miRNA expression profile in HNE-treated HL-60 leukemic cells has been investigated and new perspectives on HNE-controlled pathways are discussed.

Materials and methods

Cells and culture conditions

HL-60 leukemic cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2–air using RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics, and 10% fetal calf serum (FCS) (Biochrom AG Seromed, Berlin, Germany). Growth rate and cell viability were monitored daily by the trypan blue exclusion test (Sigma–Aldrich SpA, Milano, Italy).

Cell treatments

HNE (Calbiochem, La Jolla, CA, USA) was added to the cell suspension (200,000 cell/ml) at the final concentration of 1 μM. The aldehyde was added at regular intervals of time (45 min) up to 10 treatments (the overall time of exposure to the aldehyde was 7.5 h). No washing steps were performed. This experimental procedure was used because 1 μM HNE disappears form the culture medium within 45 min and must be repeatedly added to activate the differentiation program in HL-60 cells [23].

MicroRNA microarray hybridization and data analysis

MiRNA expression profiling was carried out on an Agilent human microRNA microarray (G4470A; Agilent Technologies, Palo Alto, CA, USA). This microarray consists of 60-mer DNA probes synthesized in situ, which represent 470 human and 64 human viral microRNAs from the Sanger database (release 9.1). One-color miRNA labeling, hybridization, and washing were performed in accordance with the manufacturer's procedure. Images at 5-μm resolution were generated by the Agilent scanner and the Feature Extraction 9.5 software (Agilent Technologies) was used to obtain the microarray raw data. Microarray data were analyzed using the GeneSpring GX software (Agilent Technologies). Data files were preprocessed using the GeneSpring plug-in for Agilent Feature Extraction software results. Data transformation was applied to set all the negative raw values at 0.01, followed by on-chip and on-gene median normalization. A filter on low gene expression was used so that only the probes expressed (flagged as present) in at least one sample were kept; the probes that do not change between samples, identified as having an expression value across all samples between the median ± 1.5, were removed. Then, samples were grouped in accordance with their status and compared. Differentially expressed genes were selected as having a 1.5-fold expression difference between their geometrical mean in the two groups. Analysis of variance (ANOVA) was followed by the application of the Benjamini and Hoechberg correction for false-positive reduction. Differentially expressed genes were employed for cluster analysis of samples, using the Pearson correlation as a measure of similarity.

Real-time RT-PCR

The three independent experiments of HNE-treated (sample) versus untreated (control) HL-60 cells were analyzed using quantitative reverse transcription PCR (qRT-PCR), which was performed to validate microarray data for three miRNAs: miR-202, miR-339, and miR-378. As a normalization control, RNU6B was employed. Kits were purchased from Applied Biosystems (Monza, MI, Italy) and used in accordance with the manufacturer's instructions. RNAs were diluted at 2 ng/μl in DEPC water and 5 ng was used as template in each reverse-transcription reaction. The subsequent quantitative PCR was carried out in triplicate for each sample for both the RNU6B control and each miRNA. The reaction was performed in a Bio-Rad C1000 thermal cycler, equipped with the CFX96 optical module, using the following conditions: 10 min at 95 °C for the activation of the enzyme, followed by 40 cycles consisting of 15 s at 95 °C and 1 min at 60 °C. The level of miRNA expression was measured using the C_t (threshold cycle), the fractional cycle number at which the fluorescence of each sample passes a fixed threshold. The relative fold change of miRNAs was determined with the $2^{-\Delta\Delta Ct}$ method, in which $\Delta\Delta C_t = (C_{t\ miR} - C_{t\ RNU6B})_{sample} - (C_{t\ miR} - C_{t\ RNU6B})_{control}$.

Preparation of total extracts and Western blot analysis

Cells (10×10^6) were washed twice in cold PBS, pH 7.4. Total extracts were prepared by lysis in a buffer containing Tris–HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, and 0.05% aprotinin. Insoluble proteins were discarded by high-speed centrifugation at 4 °C. Protein concentration in the supernatant was measured in triplicate using the commercially available Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, MI, Italy). Ten micrograms of protein was separated by electrophoresis in 9.3% polyacrylamide gels (Bio-Rad Mini Protean II system). Proteins were electroblotted to nitrocellulose membranes (GE Healthcare, Milano, Italy) using the Biometra-Fast-Blot, a semidry blotting apparatus (400 mA, 30 min) (Biometra, Goettingen, Germany). Membranes were blocked overnight at 4 °C in Tris-buffered saline containing 5% milk plus 0.5% Tween 20 and then incubated at room temperature with primary anti-SUFU sc-28847 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase-conjugated secondary (Bio-Rad Laboratories) antibodies. Detection was carried out by enhanced chemiluminescence according to the manufacturer's protocol (Amersham–Pharmacia Biotechnologies Italia, Cologno Monzese, Italy). Densitometric analysis was performed using a software program (Multi-Analyst, version 1.1; Bio-Rad Laboratories). All results were standardized using the signal obtained with β-actin.

Gene target analyses

Computational identification of the putative miRNA targets was performed using PicTar (http://pictar.bio.nyu.edu) [13], TargetScan