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Up-regulation of human deoxyribonuclease II gene expression during myelomonocytic differentiation of HL-60 and THP-1 cells☆

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Abstract

Several recent studies have suggested that intracellular deoxyribonuclease II (DNase II) is responsible for the degradation of DNA from apoptotic cells that are engulfed by macrophages. In this study, we studied DNase II expression during the phorbol 12-myristate-13-acetate (PMA)-induced differentiation of HL-60 and THP-1 cells. Basal levels of DNase II mRNA and protein were low, with expression being up-regulated approximately 15- and 7-fold, respectively, in HL-60 and THP-1 cells 72 h after PMA treatment. Nuclear run-on and luciferase reporter assays showed that transcription of DNase II gene was increased in PMA-treated cells. Together, these results demonstrate that DNase II gene transcription is increased during myelomonocytic differentiation, resulting in increased levels of mRNA and protein. This increase in DNase II levels in differentiated HL-60 and THP-1 cells suggests that it may play an important role in macrophages.

Keywords: DNase II; HL-60; THP-1; Macrophage; PMA

Deoxyribonuclease II (DNase II) (EC 3.1.22.1) is an acid endonuclease that hydrolyzes DNA to 3′-phosphoryl oligonucleotides [1]. Active DNase II occurs mainly in lysosomes [2]. The mature enzyme consists of three non-identical subunits derived by proteolysis from a single large precursor protein [3,4]. Site-directed mutagenesis has shown that two N-glycosylation sites (N^{86} and N^{266}) are required for generation of the mature enzyme [5]. Although knowledge about DNase II structural and enzymatic properties is increasing, its intrinsic physiological significance has not been elucidated until recently. It has now been shown to be involved in the degradation of genomic DNA during apoptosis and lens cell differentiation [6,7]. In addition, Krieser and Eastman [8] demonstrated that, in CHO cells, overexpression of DNase II protein results in cell apoptosis. However, Tsukada et al. [9] found, in CA1 neuronal cells, that DNase II is involved in necrosis, but not in apoptosis. Although DNase II has long been proposed to play a scavenging role in phagosomes [10], evidence to support this hypothesis was obtained only recently. Two recent studies suggest that macrophage DNase II is responsible for DNA degradation in apoptotic cells that are engulfed by macrophages [11,12]. Kawane et al. [13] generated DNase II knockout mice and showed that DNase II deficiency results in the death of the embryos due to severe anemia and also demonstrated that liver macrophages lacking DNase II cannot eliminate nuclear DNA expelled from erythroid precursor cells, thus, inhibiting erythrocyte maturation. These observations indicate that macrophage DNase II may play a pivotal role in the elimination of "unwanted" DNA in vivo.

HL-60 and THP-1 are human leukemia cell lines, which, upon PMA stimulation, differentiate to a macrophage-like state, with this system therefore mimicking monocyte/macrophage differentiation [14,15]. Results from several laboratories [16–18] indicate that these cells may serve as a model system for studying the gene expression during differentiation of monocytes to macrophages. Since macrophages are recognized as

☆ *Abbreviations:* DNase II, deoxyribonuclease II; PMA, phorbol 12-myristate-13-acetate; SRED, single radial enzyme diffusion method; $rHis_6$-DNase II, recombinant $(His)_6$-DNase II.

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"professional" phagocytes, and DNase II in macrophages is suggested to be responsible for clearance of "unwanted" DNA in vivo, it is feasible to hypothesize that higher DNase II is essential for the phagocytic functions of macrophages.

In the present study, we measured DNase II expression in HL-60 and THP-1 cells before and after PMA treatment and found that expression was up-regulated, following PMA-induced differentiation of both cell lines, with this being in part at the transcriptional level.

Materials and methods

Cell culture. Cell lines HL-60 and THP-1 obtained from the American Type Culture Collection (Rockville, MD, USA) were grown at 37 °C in 5% CO_2 in RPMI 1640 medium (Biochrom AG, Berlin, Germany) containing 20% (HL-60 cells) or 10% (THP-1 cells) fetal calf serum (Biological Industry, Israel). To induce differentiation, HL-60 and THP-1 cells were cultured for different times in the presence, respectively, of 30 and 160 nM phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO, USA). Cell adhesion, cell morphology, and expression of the surface marker, CD14, were used to monitor macrophage differentiation.

Acid DNase activity assay. Acid DNase activity was assayed using the single radial enzyme diffusion (SRED) method according to Yasuda et al. [19] with some modifications. Briefly, the cells were lysed in acid DNase assay buffer [0.1 M sodium acetate buffer (pH 4.7) containing 20 mM EDTA] with sonication and then centrifuged for 15 min at 10,000*g*. The supernatant of cell extracts (1 μg protein) was applied to wells in an 1% agar plate, which consisted of 5 μg/ml salmon sperm DNA and 0.25 μg/ml ethidium bromide in acid DNase assay buffer. Purified porcine spleen DNase II (provided by Dr. T.H. Liao, National Taiwan University) was used as the standard.

Western blot analysis. To produce antibody against human DNase II, a cDNA fragment was amplified by PCR with two specific primers (forward primer 5′-TCGGATCCCATATGCTGACCTGCTACGGG GAC-3′ and reverse primer 5′-GCGGATCCTTAGATCTTATAAGC TC- 3′), covering the mature protein without the sequence coding for the signal peptide. Sequences of restriction enzymes *Nde*I and *Bam*HI (underline) were introduced into forward and reverse primers, respectively, to facilitate cloning. The amplified cDNA was, after digestion with *Nde*I and *Bam*HI, ligated into the pET-15b vector (Novagen, Madison, WI, USA) to construct the pET$(His)_6$-DNase II expression plasmid. After verified by restriction mapping and DNA sequencing, the plasmid was transformed into *Escherichia coli* BL21(DE3) (Novagen). The recombinant $(His)_6$-DNase II ($rHis_6$-DNase II) was expressed and purified following manufacturer's instruction (Novagen). The $rHis_6$-DNase II was isolated further from SDS–PAGE, then mixed with adjuvant, and injected into New Zealand white rabbit. The antiserum was passed through a protein A column (Pierce, Rockford, IL, USA) to isolate IgG. The antiserum and IgG recognized purified $rHis_6$-DNase II and porcine spleen DNase II [3] but the pre-immune serum did not. Cell lysates (75 μg protein/lane) were separated by 10% SDS–PAGE and transferred to a PVDF membrane, which was then blocked overnight at 4 °C with blocking buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl containing 5% fat-free milk). The blots were then incubated at room temperature sequentially for 1 h with the purified anti-$rHis_6$-DNase II IgG and then for 40 min with peroxidase-conjugated goat anti-rabbit immunoglobulin (Amersham Biosciences, Little Chalfont, Buckinghamshire, England), with bound second antibody being visualized by chemiluminescence using the ECL Western blot detection reagent (Amersham Biosciences) according to manufacturer's instructions. The signal was quantified by densitometry.

RNA isolation and RNase protection assay. Total cellular RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform method [20] and 30 μg of total cellular RNA was used in the RNase protection assay using a kit from Ambion (Austin, TX, USA). To generate radiolabeled antisense RNA probes, a full-length DNase II cDNA was cloned from Hep G2 cells by RT-PCR and the 290 bp *Eco*RI–*Hin*dIII DNase II cDNA fragment (nucleotides 577–866) was subcloned into the pGEM-7Z vector (Promega, Madison, WI, USA) and transcribed in vitro using Sp6 RNA polymerase (Promega) in the presence of [α-^{32}P]CTP (NEN Life Science Products, Boston, MA, USA). The protected fragments were detected by denaturing PAGE, followed by autoradiography and quantification of the bands on a phosphoimager. 36B4 was used as internal control [21].

Nuclear run-on assay. Nuclear run-on assays were performed using nuclei from resting HL-60 cells and HL-60 cells cultured with 30 nM PMA for 72 h, as described above. For each experiment, 2×10^7 nuclei were used. The final nuclear pellet was resuspended in 100 μl transcription reaction buffer (50 mM Tris–HCl, pH 8.3, 20% (v/v) glycerol, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of ATP, UTP, and GTP and 100 μCi of [α-^{32}P]CTP) and incubated for 30 min at 30 °C and then RNA was isolated as described above. The labeled RNA was hybridized to antisense RNAs, immobilized on a nitrocellulose (Hybond N+) membrane (Amersham Biosciences).

Reporter gene construction and transient transfection. A region containing the DNase II gene promoter spanning nucleotides −1875 to +72 was PCR-amplified using the forward primer, 5′-ACGCGTTTAG TTTGGGGAAGGGCTA-3′, and the reverse primer, 5′-CTCGAGCT GCTATGGGGCTGAGATCC-3′, based on the genomic DNA sequence of human DNase II from GenBank (Accession No. AB031422); the underlined bases were introduced to provide a *Mlu*I site in the forward primer and a *Xho*I site in the reverse primer. The PCR-amplified DNA fragment was checked by sequencing and then ligated into the *Mlu*I–*Xho*I sites of the pGL3-basic *Photinus pyralis* luciferase reporter vector (Promega). Recombinant plasmids were extracted and purified by alkaline lysis and CsCl gradient ultracentrifugation [22]. Transfection was carried out by the DEAE–dextran procedure [17]. Briefly, cells (2×10^7) were resuspended for 15 min at 37 °C in 1 ml of 25 mM Tris–HCl buffer, pH 7.4, 5 mM KCl, 0.7 mM $CaCl_2$, 137 mM NaCl, 0.6 mM Na_2HPO4, and 0.5 mM $MgCl_2$ containing 5 μg test plasmid DNA and 5 μg pRL-TK plasmid DNA (*Renilla reniformis* luciferase under the control of the thymidine kinase promoter as a transfection efficiency control; Promega) and 50 μg/ml DEAE–dextran (Sigma, St. Louis, MO, USA). After incubating the cells for 30 min at 37 °C with 100 μM chloroquine, they were resuspended in RPMI containing 20% FCS and cultured for another 48 h in the presence or absence of PMA. At the end of the reaction, 100 μl passive lysis buffer (dual-luciferase reporter assay system; Promega) was added to the cells and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) and a TopCount microplate scintillation and luminescence counter (Packard Instrument, Meriden, CT, USA). Promoter activity was evaluated as relative light units, defined as the ratio of the light intensity produced by *Photinus* luciferase (test plasmid) to that produced by *Renilla* luciferase (control plasmid).

Results

Acid DNase activity is up-regulated in PMA-treated HL-60

In the continuous presence of 30 nM PMA, more than 90% of HL-60 cells became adherent to the plastic dish within 24 h and gradual increase of CD14, a macrophage surface marker, positive cells. Acidic DNase

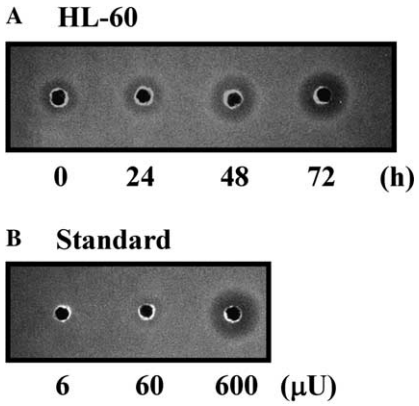

Fig. 1. Acid DNase activity assays in HL-60 cells. Acid DNase activity was measured by the single radial enzyme diffusion (SRED) method as described in the Materials and methods. HL-60 cells were treated with 30 nM PMA to induce macrophage differentiation. (A) Cells were harvested after 0, 24, 48, or 72 h of PMA treatment and 1 μg total lysate protein was loaded in each well; (B) 6–600 μU of purified porcine DNase II was used as a standard.

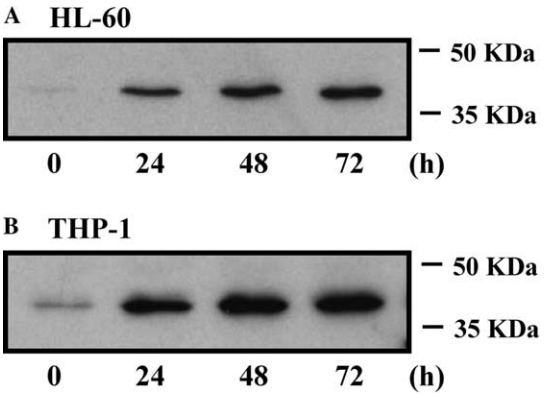

Fig. 2. Western blot analysis of DNase II in HL-60 and THP-1 cells. HL-60 (A) or THP-1 (B) cells were induced to differentiate using PMA and harvested after 0, 24, 48, or 72 h of PMA treatment and 75 μg total cell lysate was added to each lane of an SDS gel. Western blot analysis was performed as described in the Materials and methods. Similar results were obtained in at least two independent experiments for each cell line.

activity, determined by the SRED method, was low in untreated HL-60, but increased in a time-dependent manner during treatment with 30 nM PMA (Fig. 1).

PMA induces expression of DNase II protein in HL-60 and THP-1 cells

To differentiate DNase II from other DNase II-like enzymes, antibody against recombinant DNase II expressed in *E. coli* was produced. Western blot analysis (Fig. 2A) showed that levels of DNase II protein, barely detectable in untreated HL-60, increased up to 17-fold at 72 h after exposure to PMA. A similar, but lower, increase was seen using THP-1 cells (5-fold increase) (Fig. 2B).

PMA induces DNase II mRNA expression in HL-60 and THP-1 cells

When DNase II mRNA levels were measured using the RNase protection assay in HL-60 and THP-1 cells before and after PMA treatment, levels in HL-60 cells

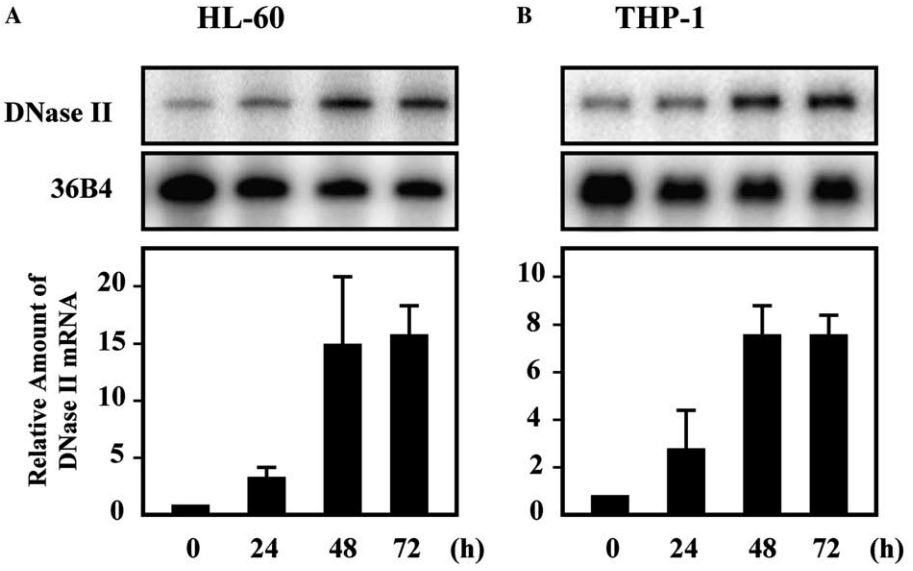

Fig. 3. DNase II mRNA levels in HL-60 and THP-1 cells before and after PMA treatment. Total RNA from HL-60 (A) or THP-1 (B) cells was isolated after 0, 24, 48, or 72 h of PMA treatment and DNase II mRNA was detected by the RNase protection assay as described in the Materials and methods. The upper panels show representative RNase protection assay results. Lower panels: levels of DNase II mRNA were normalized to those for 36B4 and the results were expressed relative to those in the non-treated control (0 h; relative value = 1); the results are means ± SD of three independent experiments..

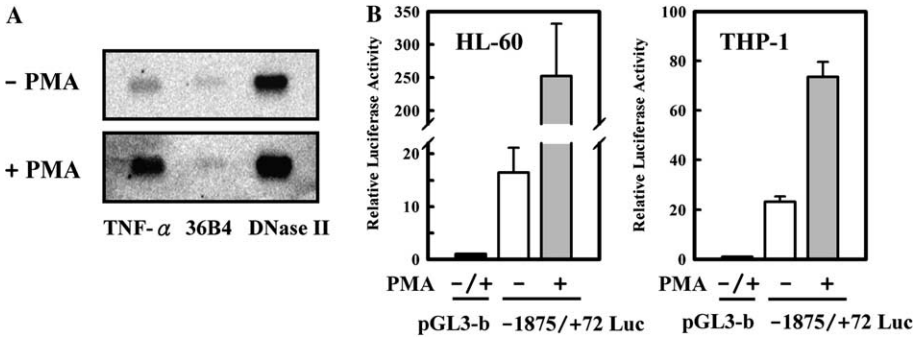

Fig. 4. Nuclear run-on and luciferase reporter assays. (A) Nuclear run-on assay. Nuclei were isolated from control and 72 h PMA-treated HL-60 cells. Identical results were obtained in two separate experiments. (B) Luciferase reporter assays. Five micrograms of pRL-TK and 5 μg of either pGL3-basic or (−1875/+72) DNase II–luciferase were co-transfected into HL-60 (left panel) or THP-1 (right panel) cells using the DEAE–dextran method. The cells were then treated with (+) or without (−) PMA for 48 h and luciferase was assayed as described in the Materials and methods. Firefly luciferase activity was normalized to *Renilla* luciferase activity and expressed relative to that of cells transfected with pGL3-b. The results are means ± SD of three independent experiments.

increased 4-fold after 24 h, and peaked at approximately 14-fold at 48–72 h, of treatment (Fig. 3A). A similar, but lower, increase was seen in PMA-treated THP-1 cells (Fig. 3B).

PMA induces DNase II gene transcription

To determine whether PMA-induced DNase II expression at the transcriptional level, we performed a nuclear run-on assay and transient expression analysis using a DNase II–luciferase reporter gene construct. In the run-on assay, HL-60 cell nuclei were incubated with [α-^{32}P]CTP for 30 min at 37 °C and then newly synthesized [α-^{32}P] labeled RNA was isolated and hybridized to membrane-bound DNase II, TNF-α, and 36B4 antisense RNAs; synthesis of DNase II and TNF-α mRNA was found to be significantly increased by PMA treatment, whereas 36B4 mRNA levels were unaffected (Fig. 4A). For the transient expression analysis, a 1947 bp DNA fragment (nucleotides −1875 to +72) of the human DNase II gene was cloned upstream of the luciferase gene and transfected into HL-60 and THP-1 cells. Following PMA treatment, the relative luciferase activity showed a 14-fold increase in promoter activity in HL-60 cells and a 4.5-fold increase in THP-1 cells (Fig. 4B). These results demonstrate that DNase II is up-regulated at the transcriptional level during the PMA-induced differentiation of HL-60 and THP-1 cells.

Discussion

In the present study, we demonstrated that DNase II was up-regulated when HL-60 and THP-1 cells were exposed to PMA, which is known to activate protein kinase C and stimulate monocytic differentiation [23,24]. We have also observed an increase in DNase II when peripheral blood monocytes are induced to differentiate by granulocyte/macrophage colony-stimulating factor (data not shown). And that mouse peritoneal macrophages and the macrophage cell line, RAW264.7, have a 10- to 20-fold higher DNase II activity per unit of total cellular protein than most Balb/c mouse tissues and several non-macrophage cell lines, such as HL-60, HepG2, 293T, and CHO cells (data not shown). These results demonstrate that DNase II activity is higher in macrophages and therefore suggest that DNase II may play an important function in macrophages. Macrophages are ''professional'' phagocytes. Recent results [11,12] suggest that phagocytes are responsible for the rapid removal of apoptotic cells. Following recognition of so-called ''eat me'' signals on the surface of apoptotic cells, the dying cells are engulfed by phagocytes [25] and their DNA digested by lysosomal acid DNase(s), probably DNase II [11].

As shown in Fig. 1, the SRED method showed an increase in DNase II activity in HL-60 cells stimulated with PMA; however, this method cannot rule out activities contributed by DNase II-like acid DNase(s), such as L-DNase II [26], Xib [27], DLAD [28], and DNase IIβ [29]. To distinguish DNase II from other DNases, a specific rabbit antibody against recombinant DNase II expressed in *E. coli* was raised to detect DNase II protein and was shown on Western blots to detect a ~38 kDa DNase II protein. Using this antibody, it was possible to demonstrate that DNase II protein was up-regulated during the PMA-induced differentiation of HL-60 and THP-1 cells, while the RNase protection assay showed a parallel increase in DNase II mRNA levels.

The DNase II promoter, which is TATA-less and GC-rich, is characterized as a housekeeping gene [30], but our nuclear run-on and reporter gene assay results showed that transcription of the DNase II gene was up-regulated by PMA. Several potential binding sequences for Sp-1, GATA-1, and MZF1 have been identified in

the proximal region of the promoter [30]. Sp1 is a ubiquitous transcription factor that binds mainly to GC/GT-box promoters [31]. Sakamoto and Taniguchi [32] demonstrated that the binding of Sp1 to the PMA-responsive element in the interferon-γ receptor 1 chain promoter results in increased transcription of the gene in PMA-treated THP-1 cells; Sp1 is also essential for the PMA-mediated induction of lysosomal acid lipase [17] and acid sphingomyelinase [33] in these cells. Three potential Sp1 sites, located at −43, −74, and −135 of DNase II promoter, may be important for PMA induction of DNase II expression. Although our results show that DNase II expression is mainly regulated at the transcriptional level, additional post-transcriptional mechanisms, such as mRNA stability, cannot be excluded.

We conclude that expression of DNase II is upregulated during the PMA-induced differentiation of HL-60 and THP-1 cells and that this occurs mainly at the gene transcription level. These results suggest that DNase II may play an important role in macrophages, presumably in the scavenging of unwanted DNA.

Acknowledgments

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