Adenosine deaminase mRNA expression is regulated posttranscriptionally during differentiation of HL-60 cells

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ABSTRACT

The expression of the enzyme adenosine deaminase (ADA) decreases in the course of the differentiation of the human promyelocytic leukemic cell line HL-60, dependent on the pathway chosen. Differentiation to monocytes as induced by the phorbol ester TPA leads to a 50% reduction of enzyme activity. Induction to myeloid cells as induced by DMSO has a slower and less extensive (75% remaining activity) effect. The reduction in ADA enzymatic activity is preceded by a 5-10 fold reduction in ADA-specific mRNA which is also more rapid during TPA-induced differentiation. In contrast, c-myc mRNA expression is both in TPA- and DMSO-induced differentiation reduced to less than 5 % of its initial level within 4h. Nuclear run-on analysis revealed that the reduction of c-myc-mRNA expression during both TPA- and DMSO-induced differentiation could be ascribed to the abolition of transcription of the third exon, whereas no change in the transcription of the first exon could be observed. No change could be detected in the rate of transcription of either the 5' and 3' parts of the ADA gene during TPA- and DMSO-induced differentiation, indicating that the expression of the ADA gene in HL-60 is controlled at a posttranscriptional level.

INTRODUCTION

Adenosine deaminase (ADA: EC 3.5.4.4) catalyses the irreversible deamination of adenine nucleosides to their respective inosine nucleosides. The mammalian enzyme is a soluble monomeric protein with a molecular weight of ca 42 kDa (1). This value is in good agreement with that predicted from the nucleotide sequence of cloned full-length functional cDNA's (2,3). Different tissues show a great variation in ADA activity, ranging from 1 to 800 mIU/mg protein (4). Low levels of ADA activity are found in red blood cells, lung, liver and fetal placenta. The highest levels of ADA activity are found in representatives of the T-cell lineage in which ADA concentrations are also subject to developmental control; activities peak at the cortical thymocyte stage of T-cell development (5). In gastrointestinal tissues a developmental control of ADA activity has also been demonstrated. Thus, although ADA activity is found in every tissue examined thusfar, its expression is subject to a strong

tissue- and differentiation specific regulation. Several clinical syndromes, which are associated with a significant aberration of ADA expression have been described. Out of these, the autosomal recessive form of severe combined immune deficiency (ADA⁻SCID) disease has been studied most extensively (6, see references 7-9 for reviews). It is striking that the T-lymphocytes, the cells believed to be primarily affected in ADA⁻SCID patients, express the highest levels of ADA activity during their differentiation in normal individuals.

Other clinical syndromes associated with an abnormal ADA expression are: an autosomal dominant form of hemolytic anemia, which manifests itself by a 40- to 70-fold overproduction of ADA in the red blood cells only (10,11); Diamond Blackfan syndrome for which a 5- to 10-fold elevation of ADA activity in the red blood cells of patients has been reported (12), and AIDS, for which recently also a small but significant elevation of ADA activity in patient red blood cells has been observed (13).

Thusfar it is unknown how or at what level the strong tissue- and developmental specific expression of ADA is regulated. Our understanding of this regulation will probably be essential in order for the treatment of ADA⁻SCID patients by gene therapy to be successful.

In order to study the expression of ADA in a well defined model system we chose to investigate the expression of the ADA gene during differentiation of the human promyelocytic HL-60 cell line (14). These HL-60 cells can be induced to differentiate by treatment with certain chemicals (15,16).

In this paper, we show that ADA activity is repressed during differentiation of HL-60 cells. This suppression of ADA activity is preceded by a decrease in ADA-specific mRNA. Nuclear run-on assays demonstrated that this decrease is not caused by a fall in the rate of transcription of the ADA gene. Consequently, the expression of the ADA gene during differentiation of HL-60 cells must be regulated at a posttranscriptional level.

MATERIALS AND METHODS

Cell lines and plasmids. HL-60 promyelocytic leukemic cells were obtained from Dr. P.I. Schrier (Dept. of Clinical Oncology, Leiden University) and grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal-calf serum. The human-c-myc genomic clone (17) was a gift of Dr. S. Cory (P.O. Royal Melbourne Hospital, Victoria 3050, Australia). The human ADA cDNA clone PLL was described previously by Valerio et al. (18).

HL-60 growth and differentiation HL-60 cells were seeded at 5×10^5 cells/ml with or without 1.2% (v/v) DMSO or 10 ng/ml TPA. The relative maturity of

HL-60 cells was evaluated by direct inspection of their morphology (TPA induction) or by their ability to reduce nitroblue tetrazolium (NBT) (DMSO induction). TPA-induced HL-60 cells exhibit a drastic change in their morphology within 24 h and, moreover, 60-80% of the cells become adherent in a typical induction experiment as described previously by Rovera et al. (16). Reduction of NBT by HL-60 cells untreated or treated with DMSO was determined as described by Lucas et al. (19). In a typical experiment, 30-40% of the HL-60 cells will become NBT-reduction-positive within 24 h compared to 5-15% for untreated HL-60 cells. 72 h after addition of DMSO the percentages run up to 70-90% NBT-reduction-positive cells.

Quantitation of ADA enzyme activity, RNA isolation and Northern blot analysis

Specific ADA activity was determined as described previously (20). Total RNA was isolated by the LiCl/urea precipitation method essentially as described by Auffray and Rougeon (21). Agarose gel electrophoresis, transfer onto nitrocellulose and hybridization were performed as described by Schrier et al. (22).

In vitro transcription on isolated nuclei (run-on assay). Preparation of nuclei and elongation of nascent transcripts were carried out as described previously by Vaessen (23) with one modification: preparation and washing of nuclei was carried out in buffers containing 50 mM KCl instead of 150 mM KCl to avoid irreversible clotting of nuclei. Equivalent amounts of labeled RNA from the various differentiation stages of the HL-60 cells were treated for 10 min at 65°C in hybridization buffer containing 40% formamide, 20mM PIPES (pH 7.5), 5% SDS, 0.5 M NaCl, 0.01% PPI and 200 g/ml E.coli tRNA. The RNA was subsequently hybridized in the same buffer to 1-2 ug of phage M13 single-stranded DNA dot-spotted onto nitrocellulose. Hybridization was for 48-72 hours at 42°C. The filters were washed in 0.1xSSC/0.1% SDS up to 60°C.

RESULTS

ADA activity is suppressed during differentiation of HL-60 cells.

To examine the expression of the human ADA enzyme during differentiation of HL-60 cells, HL-60 cultures were induced to differentiate by the addition of the chemicals TPA or DMSO. DMSO induces HL-60 cells to differentiate along the myeloid lineage while treatment with TPA induces differentiation along the monocytic lineage (15,16). Cells were isolated at different times after addition of the inducing chemicals and their specific ADA activity was determined (fig. 1).

FIGURE 1 : ADA activity during differentiation of HL-60 cells.
HL-60 cells were seeded at a density of 5×10^5 cells/ml with or without the addition of 1.2%(v/v) DMSO or 10ng/ml TPA. At the indicated times samples were taken and ADA activity and protein content of the samples were determined as described under Materials and Methods.

The data show that in the course of the differentiation induced by both chemicals the ADA activity decreases to 75- 50% of the initial value, but with different kinetics. The decline was somewhat steeper and continued further in the presence of TPA. The fact that the ADA activity dropped during differentiation of HL-60 cells prompted us to investigate whether an artificial reduction of ADA activity by treatment with deoxycoformycin (DCF), a very specific and potent inhibitor of ADA (22), could trigger HL-60 differentiation. In HL-60 cells treated with DCF at a concentration that effectively blocked ADA activity to undetectable levels, neither the morphological properties nor the ADA or *c-myc* mRNA levels showed any change up to 48 h after addition of DCF. Treatment of HL-60 cells with DCF in combination with deoxyadenosine did not trigger any observable difference in HL-60 cells, either (data not shown).

FIGURE 2 : Northern blot analysis of ADA-, c-myc- and γ -actin RNA of HL-60 cells induced by DMSO. HL-60 cells were seeded at a density of 5×10^5 cells/ml and grown for the indicated time in the presence of 1.2%(v/v) DMSO. Total RNA was extracted as described under Materials and Methods. 40- μ g samples were processed for northern blotting as described under Materials and Methods. The same filter was screened subsequently with a nick-translated ADA cDNA probe, a c-myc genomic probe (EcoRI-ClaI fragment covering the third exon) and a mouse γ -actin cDNA probe. After autoradiography, the filter was stripped by treatment for 30 minutes in H_2O at $70^\circ C$ before hybridization of the next probe was performed.

Suppression of ADA activity is preceded by a decrease of ADA mRNA levels during differentiation of HL-60 cells.

To investigate whether the observed reduction in ADA enzymatic activity is the consequence of a reduction of the steady-state level of ADA- specific mRNA, we analysed the ADA-mRNA content of HL-60 cells during TPA- and DMSO-induced differentiation. At various times after induction of differentiation by TPA or DMSO, total RNA from the HL-60 cells was isolated by the LiCl/urea method. The RNA samples were quantitated by measuring the A_{260} and by running samples in agarose test gels and subsequent staining with EtBr. Equal amounts of RNA were analysed for ADA mRNA content by Northern transfer and hybridization to nick-translated ADA-specific probes. To check whether the same amount of intact RNA was indeed present in every lane, the filters were also screened with nick-translated γ -actin-specific sequences (fig. 2 and 3). As an internal control for the successful induction of differentiation, the same filters were also hybridized with nick-translated c-myc-specific sequences. It has been reported previously that c-myc mRNA expression is strongly repressed during differentiation of HL-60 (25,26). The relative amount of c-myc mRNA present 4 h after induction of differentiation by each chemical was

FIGURE 3 . Northern blot analysis of RNA of HL-60 cells induced by TPA HL-60 cells were seeded at a density of 5×10^5 cells/ml and were grown for the indicated time in the presence of 10 ng/ml TPA. Isolation of RNA and Northern blot analysis were essentially as described in figure 2.

estimated to be less than 5% of the initial level (see fig. 2 and 3). The relative amounts (in comparison with uninduced HL-60 cells) of ADA mRNA present at various times were estimated by visually comparing autoradiograms of different exposures to the same filters and by densitometric scanning. The values obtained were standardized to those for γ -actin mRNA (fig. 4). It is evident that the ADA steady-state mRNA levels are repressed during differentiation of HL-60 cells. However, in contrast to the reduction of the *c-myc* mRNA level, the rate and extent of decrease of ADA mRNA is dependent on the inducing agent used, i.e. it is faster and more extensive along the monocytic lineage, as induced by TPA. This difference is in agreement with the aforementioned difference in downregulation of the enzyme activities. We therefore conclude that the downregulation of ADA activity is determined by repression of ADA mRNA levels.

Modulation of ADA expression is not at the level of transcription initiation.

In order to analyse whether control of expression of ADA mRNA during differentiation of HL-60 is exerted at the transcriptional level we measured the relative transcription rate of the ADA gene by the nuclear run-on assay in isolated nuclei of HL-60 cells which were either uninduced, or had been induced 24h before by TPA or DMSO. In this nuclear run-on assay, transcripts that already have initiated *in vivo* will be elongated *in vitro* in the presence of 32 P-UTP. The labelled RNA's are isolated and hybridized to excess single-stranded (ss) DNA fragments immobilized on nitrocellulose filters. The

MYC and ADA mRNA levels during HL-60 differentiation

FIGURE 4 : Relative levels of c-myc- and ADA RNA during differentiation of HL-60. Autoradiographic bands corresponding to c-myc RNA (2.3 kb), to ADA RNA (1.5 kb) and to γ -actin RNA (1.8 kb) shown in figure 2 and 3, were subjected to scanning densitometry and the values obtained standardized to those for γ -actin were plotted against the time. The values shown are relative to those determined for the uninduced HL-60 cells.

amount of radioactivity bound by the ss DNA fragments is a measure of the density of RNA polymerase molecules active on the corresponding gene at the time of cell lysis because under these condition new RNA transcripts are not initiated. The density of the bound polymerase molecules depends on the rate of initiation of transcription and therefore the amount of radioactivity bound is a measure of the level of transcription initiation of the gene in question at the time of cell lysis (27). To avoid interference of possible anti-sense transcriptions, the DNA fragments of interest were subcloned in either orientation into bacteriophage M13 and ss DNA's representing sense as well as antisense strands were used as probes. The data discussed here were obtained in four separate run-on assays. Furthermore, sarkosyl (0.5% w/v), added to exclude rigorously the possibility of reinitiation did not have any influence on the measured relative transcription rates. The results of the nuclear run-on assay (fig. 5) reveal that, as expected, there is no difference in the relative rate of transcription in the sense direction of the

Figure 5 : In vitro transcription assay on nuclei of uninduced as well as TPA- or DMSO-induced HL-60 cells. Preparation of nuclei, elongation of nascent RNA, isolation of labelled RNA, hybridization and washing was performed as described under Materials and Methods. In panel A the same amount of labelled run-on RNA from I, HL-60; II HL-60 grown for 24 hrs in the presence of 1.2%(v/v) DMSO; III HL-60 grown for 24 hrs in the presence of 10 ng/ml TPA, were hybridized against 1-2 μ g single stranded DNA's dot spotted onto nitrocellulose. The ss M13 clones contain either strand (as indicated by sense(s)- and antisense(as)-direction) of the following DNA fragments; γ -actin, a 1.1 Kb PstI mouse cDNA insert, myc1, a 862-bp PvuII genomic fragment including the first exon subcloned from the hu-c-myc clone, myc3 a 1.3 kb EcoRI-ClaI genomic fragment including the third exon subcloned from the hu-c-myc clone, ADA BA a 400-bp PstI cDNA fragment subcloned from the ADA cDNA clone PLL spanning exon 1-5 and ADA BE a 300-bp PstI fragment subcloned from the ADA cDNA clone PLL representing exon 12 and a minor part of exon 11. ss M13 mp11 DNA was used as a negative control and was dot-spotted in 2 concentrations, s= 1 μ g and as= 2 μ g. In panel B the myc1, myc3, ADA BA and ADA BO fragments are schematically depicted relative to their genomic organization.

control gene (γ -actin), the steady-state mRNA levels of which are the same before and after induction. No signal in the antisense direction could be detected. The transcription initiation rate of the c-myc gene as measured by the amount of radioactive RNA bound to c-myc first-exon probes shows no decrease. Here we did detect transcription in the antisense direction but this was not subject to any significant modulation during differentiation, either. However, during differentiation, we found a strong decrease in the

rate of transcription of the third c-myc exon in the sense direction, which explains the decrease in mature c-myc mRNA observed during differentiation by TPA and DMSO. This decrease can already be observed 4h after addition of the inducing chemicals (data not shown). Moreover, even in the uninduced cells, transcription of the third exon is less than that of the first exon. These phenomena have been reported previously by Bentley and Groudine (28) and Eick (29), who ascribe these phenomena to a block in elongation and intragenic pausing respectively. In contrast to the c-myc gene no differences in the relative rate of transcription of the entire ADA gene during TPA- and DMSO-induced differentiation could be detected as measured by the amount of radioactivity bound by 5' and 3' probes (fig. 5). No transcription in the anti-sense direction was detected. From the above observations we conclude that the 8-10 fold reduction of the steady-state levels of ADA mRNA during differentiation of HL-60 is not regulated similarly to the reduction of the c-myc gene. The reduction of the ADA mRNA is not caused by a modulation in the rate of transcription initiation of the ADA gene but rather must be regulated mainly at some posttranscriptional level.

Discussion

The data presented in this paper demonstrate that the expression of the ADA gene is modulated during differentiation of HL-60 cells. Differentiation leads to a reduction in the expression of the ADA gene at both the enzymatic and mRNA levels. The kinetics of suppression differ depending on the inducing chemical used. The suppression of ADA is more rapid and more extensive during the differentiation towards monocytes induced by TPA. In contrast c-myc expression measured at the RNA level dropped to less than 5% within 4h after initiation of differentiation by both TPA and DMSO. The fact that the choice of inducing agent differentially affects the expression of the ADA gene, but not that of the c-myc gene indicates in our view that ADA is modulated as a consequence of the differentiation pathway chosen and not as a mere consequence of entering a resting state. Since the expression of c-myc is invariant throughout the cell cycle (30, our own unpublished results) we consider the observed drop in c-myc expression during differentiation of HL-60 cells a sign that most of the cells become committed to leave the cell cycle and enter the terminal differentiation phase with kinetics more or less similar for TPA- and DMSO-induced differentiation. We therefore conclude that differentiation into monocytes leads apparently to a different regulation of ADA expression than differentiation into granulocytes.

Run-on experiments reveal that there is no discernable difference in the rate of transcription of the ADA gene during differentiation of HL-60 cells. Thus, the observed 8-10 fold down-regulation of expression of the ADA gene during TPA- and DMSO-induced differentiation of HL-60 cells is not the consequence of regulation of transcription of the ADA gene but of a posttranscriptional control mechanism. This contrasts to the expression of the *c-myc* gene for which abolition of transcription of the third exon can be held responsible for the observed downregulation during differentiation of HL-60. This phenomenon has been reported previously by Bentley and Groudine (28), who determined the transcription rate of the *c-myc* gene in HL-60 2½ days after induction by retinoic acid, as well as by Eick et al. (29), who determined the rate 1 hour after induction of differentiation by DMSO. Here we report that induction of HL-60 cells by TPA, inducing monocytic differentiation, also leads to a block in elongation abolishing transcription of the third *c-myc* exon.

Like ADA, other so-called housekeeping genes, namely those for chicken thymidine kinase (*ctk*) and dihydrofolate reductase (*dhfr*), are also regulated at a level other than that of transcription initiation (31,32). For the *ctk* gene, 'run-on' experiments revealed no detectable difference in *ctk* transcription in isolated nuclei derived from dividing or non-dividing cells containing high or low levels of steady-state *ctk* mRNA. For the *dhfr* gene, 'run-on' experiments exhibited no variation in transcription in isolated nuclei derived from a methotrexate-resistant cell line with a 500-fold amplification of the *dhfr* gene, whether it was growth-stimulated or not. Growth stimulation leads to a 3-5-fold increase in *dhfr* mRNA levels (32). Subsequently it became apparent that the *dhfr* mRNA levels are regulated by transcript stabilization within the nucleus (33)

The housekeeping genes just mentioned share some striking similarities: first, of course, the fact that they are housekeeping genes, secondly, they are all involved in the nucleoside metabolism and, thirdly, they have promoters that share remarkable sequence homologies and which apparently do not contribute to their expression regulation by means of modulating transcription initiation in the above-mentioned cases. Instead of TATA- and CAAT-boxes these genes have promoters that are extremely GC-rich and that contain so-called GC-rich motifs (34,35). These GC-rich motifs have homology with the core Sp1-binding sequence (36). For the *dhfr* promoter these motifs have been shown to be able to bind Sp1 in vitro which leads to an elevation of the transcription initiation rate in an in vitro transcription system (36).

Figure 6. The C_pG doublet island in the 5' region of the ADA-gene.

The ADA gene is shown on the top line. The organization of the ADA gene is shown in the top line where numbered tick marks denote the 12 exons, according to the sequence data of Wiginton (38). Vertical bars on the three remaining lines represent the number of C_pG, C_pC and G_pC doublets present within 100 base-pairs. C_pG is 4-5 fold underrepresented throughout the entire gene, except in the island region (bp 3500-4000) where its frequency increases about 5-10 fold. C_pC and G_pC distributions are also shown to emphasize the relative deficiency of C_pG outside the C_pG island found in the 5' region of the ADA-gene. The histograms were generated on an ATARI ST computer.

Furthermore, these GC-rich promoters have a significantly higher C_pG- versus G_pC-doublet-ratio than found in the remainder of the human genome. It has been suggested that this is a consequence of a constant protection against cytosine-methylation, normally selecting against C_pG-doublets in the eukaryotic genome, by transcription factors constantly binding tightly to these G+C-rich islands (37). The ADA promoter region also displays a significant higher C_pG-ratio than the remainder of the gene (see fig 6). Thus the picture emerges that the promoters of housekeeping genes are always protected against cytosine-methylation by transcription factors and that the levels of their expression during differentiation or growth stimulation are subsequently regulated at a posttranscriptional level. Furthermore, it is noteworthy that individuals can exist without any functional ADA- and that cells derived from ADA-deficient patients can be grown in culture. Recently we have found ADA deficiency in an ADA⁻SCID patient caused by a homozygous 3.2 Kb deletion including the promoter and the first exon. No ADA specific mRNA is present in cells of this patient and therefore this will be the very first example of a real null allele for ADA (manuscript in preparation). These data suggest that ADA may not at all be strictly required for growth, differentiation and maintenance of many if not most cells including cells of the myeloid lineage

except for the T-lymphocytes. Because on the other hand no cell type of a healthy individual thusfar examined does not posses at least a basal expression of ADA we would like to propose that if it is not absolutely essential to shut off transcription of a particular gene during differentiation it is perhaps advantageous to the organism to allow a basal transcription level and modulate the steady-state levels of mRNA posttranscriptionally. Future experiments hopefully will shed more light on the nature of the posttranscriptional control mechanisms that determine the large variety of expression levels of the ADA-gene.

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Abbreviations: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; DMSO, dimethylsulfoxide; IU, international units; PPI, pyrophosphate; kb, kilo-base-pairs.

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