Distinct Temporal Patterns of Defensin mRNA Regulation During Drug-Induced Differentiation of Human Myeloid Leukemia Cells

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Defensins are microbicidal peptides and the principal constituents of neutrophil primary granules. They are presumed to play a prominent role in innate host defenses. We examined defensin mRNA levels during drug-induced differentiation of the promyelocytic leukemia cell line, HL-60. Transcription was restricted to promyelocyte, myelocyte, and very early metamyelocyte stages of the granulocytic pathway. Complete downregulation occurred during late granulocytic maturation or early during phorbol ester-promoted differentiation along the monocyte/macrophage lineage. Retinoic acid (RA) was the strongest inducer of defensin mRNA accumulation, even at doses too low to effect morphologic changes; the initial (first 48 hours), gradual increase resulted from transcriptional activation and was enhanced by granulocyte colony-stimulating factor. In contrast, addition of hybrid polar compounds led to a transient, drug-specific downregulation within the same time period, apparently by means of selectively induced, biphasic degradation of transcripts. Subsequent increase in transcript levels was faster and more pronounced with hexamethylene bisacetamide, relative to dimethyl sulfoxide (DMSO). DMSO-promoted effects were strikingly different in serum-free medium or in the presence of the tyrosine kinase inhibitor, genistein. Under these conditions, and although differentiation was unaffected, early defensin mRNA downregulation was final. The effect did not occur with RA and expression of other myeloid-specific genes was also unchanged. Addition of selected cytokines caused a similar "dip," only at earlier times and uncoupled from differentiation. Tumor necrosis factor-α markedly induced defensin levels after 2 days in previously untreated HL-60 cells, but inhibited expression in RA-differentiated cells. These results begin to detail a complex regulation of defensin mRNA synthesis with both spatial and temporal control elements, and a unique modulation by chemical agents, cytokines, and serum-factors.

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NEUTROPHILS FUNCTION as an important line of defense against microbial invasion. A two-prong tactic is used for killing ingested microbes: reactive oxidants and polypeptide antibiotics. Upon activation, the oxygen-dependent system generates a rapid set of metabolic events, referred to as respiratory burst, leading to the production of oxidized halogens and radicals harmful to bacteria and fungi. In addition, cytotoxic proteins, peptides, and digestive enzymes are stored in cytoplasmic granules and, whenever required, are rapidly released into phagolysosomes where they contribute to destruction of microorganisms. Defensins are the major components of this oxygen-independent system and account for a considerable percentage of total granular protein. These peptides are 29 to 33 amino acids in length and characterized by a conserved cysteine backbone. In addition to killing various bacteria, fungi and enveloped viruses, extra-phagosomal activities such as mammalian cell cytotoxicity, chemotaxis for monocytes, interaction with ACTH-receptors, and inhibition of protein kinase C have been reported.

Besides their presence in myeloid white blood cells of some (but not all) mammalian species, defensin-like peptides have also been detected in various anatomical locations where direct exposure to potentially harmful microbes is likely to occur, namely epithelial linings of the tongue, respiratory tract, and gut. Four different defensin isoforms, also termed HNP-1 to 4, have been isolated from human neutrophils. Defensin 4 is approximately 50-fold less abundant and, aside from the conserved disulfide array, differs substantially from its analogs. The cDNAs encoding all four defensins have been characterized.

The study of expression and regulation of genes that are specific for the functional lines of myeloid white blood cells is relevant for understanding the complex process of differentiation and may contribute to probe the molecular basis of myeloid disorders. Previous studies have indicated that defensin transcripts are present in undifferentiated bone marrow (BM), in the blood of patients with myelogenous leukemia, but not in normal peripheral blood (PB) cells. Supposedly, synthesis occurs during myeloid cell maturation and the peptides are stockpiled for later use, when fully differentiated neutrophils have entered the bloodstream and encounter invading microorganisms. More specifically, in situ hybridization analysis of BM and leukemic cell populations indicated the presence of defensin mRNA, predominantly in promyelocytes and myelocytes, and to a lesser extent in myeloblasts and metamyelocytes. Thus, transcription seems restricted to a narrow window in granulocytic differentiation. Consistent with these findings is the reported presence of defensin mRNA in the HL-60 human promyelocytic leukemia cell line, but apparent absence from myeloblastic, erythroid precursor and T-cell lines.

HL-60 cells can be chemically induced to differentiate along several lineages and to various extents of maturity. Thus, this cell line may provide a potential in vitro system to study the molecular mechanisms of differentiation-stage specific gene regulation. However, it should be recognized that some molecular events during drug-induced differentiation may perhaps not entirely reflect those occurring during normal granulopoiesis. Nevertheless, as this appears to be the only experimental route for study of defensin regulation
in a cellular model, effects of the various agents, and components of the culture medium, should be documented first. In addition, such information may contribute to a better understanding, mechanistically, of particular chemotherapeutic interventions. Only sketchy data, conflicting in part, are available on the changes of defensin steady state mRNA levels in HL-60 cells, treated to differentiate with dimethyl sulfoxide (DMSO) or retinoic acid.13,17,20

In the current study, defensin transcriptional regulation (and mRNA accumulation) during chemically induced differentiation was fine-analyzed. We have investigated drug-specific phenomena, and the effects of selected cytokines and serum components. We show that defensin gene expression is lineage-specific and, although generally maturation stage-dependent as well, mRNA steady-state levels can be uncoupled from morphologic differentiation. Distinct molecular facets of retinoid and hybrid polar compound action are discussed in this context. We speculate that drug-promoted defensin regulation might involve phosphorylation dependent events in general and, more specifically, protein tyrosine kinases in the case of DMSO.

MATERIALS AND METHODS

Materials. Recombinant human interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were purchased from PharMingen (San Diego, CA). Actinomycin D (ActD) was obtained from Calbiochem (San Diego, CA), phorbol 12-myristate 13-acetate (PMA) and Genistein were from LC Laboratories (Woburn, MA). Recombinant human granulocyte colony-stimulating factor (G-CSF) was a generous gift from Amgen (Thousand Oaks, CA). All other chemicals and proteins were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

Cell lines and culture conditions. The human promyelocytic leukemia cell line HL-60, the myeloblastic leukemia cell line KG-1, the monocytic cell line U-937, and erythroleukemic cell line K-562 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The retinoic acid-resistant cell line HL-60R was kindly provided by Dr S.J. Collins (Fred Hutchinson Cancer Center, Seattle, WA), and the lymphoblastoid cell line SK-CML8-BN12 and T-cell line J-LB 1 by, respectively, Drs Bernd Gansbacher and David Golde (Memorial Sloan-Kettering Cancer Center). HL-60, HL-60R, and U937 were grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO BRL, Gaithersburg, MD). The retinoic acid-resistant cell line HL-60R was kindly provided by Amgen (Thousand Oaks, CA). All other chemicals and proteins were purchased from Sigma (St Louis, MO), unless otherwise indicated.

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step guanidinium method as described,22 electrophoresis was performed under denaturing conditions in a 1% agarose-formaldehyde gel (loading 8 μg of RNA sample per lane) and transfer of the RNA to Hybond-N' membranes (Amersham Corp, Arlington Heights, IL) was done for 4 hours in 0.5 mol/L NaOH. After 2 hours of prehybridization in 50% formamide (containing 10% polyethylene glycol 6000, 3.5% SDS, 150 mmol/L sodium phosphate buffer, pH 6.8, 250 mmol/L NaCl, 1 mmol/L EDTA, and 2× Denhardt's solution) hybridization was performed in the same buffer overnight at 65°C. Blots were then washed for 15 minutes in 2× SSC (0.3 mol/L NaCl, 0.03 mol/L Na-citrate × 2 H2O, pH 7.5), 5 mmol/L sodium phosphate buffer, 1% SDS, 0.02% tetrasodium pyrophosphate at room temperature, followed by two washes at 70°C (30 minutes each) in the same solution but containing only 0.1× SSC, and a final wash without SSC for 5 minutes. The blots were exposed to Hyperfilm-MP (Amersham Corp) with intensifying screen for 4 to 24 hours at −80°C. Quantitation and normalization of RNA levels in Northern blots were performed as previously described. Briefly, bands were scanned from autoradiographs using the charged coupled device (CCD) camera of the BioImage Visage 110 computerized imaging system (Millipore Biologic Products, Ann Arbor, MI) and normalized to ribosomal RNA levels determined from ethidium bromide-stained gels in the same way. Quantitation was performed using the BioImage Whole Band Analysis subroutines. Lanes were defined, bands quantified, and integrated optical density (IOD) values determined. Ribosomal RNA bands were analyzed after the gray scale values of the negatives were inverted using the Image Processor.

Ribonuclease protection assay. Labeling of the RNA antisense probes specific for defensin 4 and defensins 1 and 3 with [α-32P]UTP was done as described for Northern blots except that the transcription was started with 1 μg of template DNA. Hybridization of probe, and sample RNA, and RNase digestion were performed using the ribonuclease protection assay kit (RPA II; Ambion Inc, Austin, TX). Briefly, after DNaseI incubation, the labeled transcripts were purified by electrophoresis in an 8 mol/L urea/6% acrylamide gel and full-size transcripts eluted with 300 μL elution buffer for 2 hours at 37°C. Probe (1 × 105 cpm) and sample RNA (5 μg) were combined, lyophilized, and redissolved by thoroughly vortexing in 20 μL hybridization buffer. After heating at 85°C for 3 minutes, hybridization was performed overnight at 45°C. Single stranded RNA was digested with 200 μL diluted RNase solution (1:80, 1:100, and 1:150 for each sample) for 30 minutes at 37°C. Because the expected protected fragments were only 61 and 63 bp in length, 200 μL ethanol (in 0.3 mol/L sodium acetate, pH 5.2, and two washes with 70% EtOH. The samples were dried and redissolved in RNA loading buffer for 2 hours at 37°C. The reaction was stopped by phenol extraction (Tris-saturated, pH 8.5), 0.2 mol/L EDTA, heated to 65°C for 5 minutes and chilled on ice. Oligo(dT) (0.3 pg, Pharmacia Biotech, Piscataway, NJ) was added, allowed to hybridize for 10 minutes at room temperature and neutralized with 100 μL N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid, pH 7.4, 0.2% SDS, 10 mmoVL EDTA) at 5 × 106 cpm/mL. The RNA solution was mixed with an equal volume of hybridization buffer (TES sample buffer plus 0.6 mol/L NaCl, 2× Denhardt's solution, 500 μg/mL Escherichia coli tRNA) and hybridized at 60°C for 48 hours to template cDNA immobilized on Hybond N' membranes. As template, the same defensin- (HNP-1B) and GAPDH-cDNA clones that were used for Northern hybridizations were employed. After hybridization, the membranes were washed twice for 15 minutes at room temperature with 2× SSC, 0.1% SDS, twice for 1 hour at 60°C in 0.1× SSC, 0.1% SDS and exposed to Hyperfilm-MP. For preparation of the membrane, template cDNA (10 μg) was linearized, denatured with 0.2 mol/L NaOH (final concentration) for 30 minutes at room temperature and neutralized with 10 vol 6× SSC. The DNA was transferred to the membrane using a slot-blot apparatus (MiniBlot-S; Millipore, Bedford, MA) and baked for 2 hours at 80°C. Prehybridization was performed overnight at 60°C in hybridization buffer.

RNase H digestion. Total RNA (10 μg/lane) was supplemented with 1 mmol/L EDTA, heated to 65°C for 5 minutes and chilled on ice. Oligo(dT) (0.3 μg, Pharmacia Biotech, Piscataway, NJ) was added, allowed to hybridize for 10 minutes at 25°C, and samples adjusted to 50 mmol/L KCl and hybridization continued for an additional 10 minutes at 25°C. The samples were then diluted 1:1 with 2× RNase H digestion buffer (80 mmol/L Tris-Cl, pH 8.0, 8 mmol/L MgCl2, 2 mmol/L EDTA, 0.06 mg/mL bovine serum albumin [BSA]) and digestion was started by the addition of 0.5 U RNase H (Pharmacia). Incubation was done at 37°C for the indicated time. The reaction was stopped by phenol extraction (Tris-saturated, pH 7.6) (Chloroform (1:1) followed by precipitation with 2.5 vol 96% EtOH/0.3 mol/L sodium acetate, pH 5.2, and two washes with 70% EtOH. The samples were dried and redissolved in RNA loading buffer for agarose-formaldehyde gels (50% glycerol, 1 mmol/L EDTA, pH 8, 0.4% bromophenol blue, 0.4% xylene cyanol).

Cell synchronization and cell cycle analysis. Exponentially growing cells (1 × 106) were treated with 4 μg/mL aphidicolin (APH) for 24 hours. APH was then removed by three washes with fresh medium and cell growth continued in complete medium. After various times of incubation in complete medium, cells were removed for RNA extractions (1 × 107) and cell cycle analysis (1 × 107). For cell-cycle analysis, cells were washed once in 1× PBS and resuspended in 100 μL of PBS. While vortexing, 1 mL ice-cold ethanol (−20°C) was added dropwise and the now fixed cells stored overnight at 4°C. For staining, cells were washed once in PBS, resuspended in 200 μL PI-mix (50 μg/mL propidium iodide, 700 μg/mL DNase-free RNase in 1× PBS), and incubated for 1 hour at room
temperature. Cell-cycle analysis was done on a FACStar cytofluorometer (Becton Dickinson, San Jose, CA). Data were acquired on a FACScan (Becton Dickinson) using LYSYS II software, and analyzed with MULTICYCLE software (Phoenix, San Diego, CA).

RESULTS

Lineage and differentiation-stage specific transcription of defensin genes in myeloid cells. Levels of defensin mRNA in various myeloid leukemic cell lines were analyzed by Northern blotting. The hybridization probe (RNA) was derived from the full-length HNP-1 cDNA clone and detected 0.66-kb transcripts corresponding to the three defensins, HNP-1, 2, and 3. In agreement with an earlier report, we found defensin transcripts in uninduced HL-60 cells and, although varying, the basal levels were usually quite low. After treatment of the cells for 4 days with either RA or DMSO, which both are known to induce granulocytic differentiation,21 defensin transcript levels had increased, with RA being the more effective agent by far (Fig 1). DMSO-promoted induction is rather subtle (twofold to sixfold on average after 4 days). Transcription of the X-CGD gene, which encodes the heavy-chain subunit of the phagocyte cytochrome b and is part of the membrane-associated NADPH oxidase, was used as a molecular marker for the acquisition of functional maturity.22 Low X-CGD mRNA levels were observed in immature cells; treatment with RA or DMSO for 4 days caused a significant increase. Induction of monocytic differentiation with PMA22 resulted in the loss of detectable defensin mRNA levels within 1 day. In contrast, steady-state levels of X-CGD mRNA reached a maximum at that time point, to then decrease gradually over a 4-day period and return to basal values. Because many studies on gene expression have been done in serum-free growth media, we cultured HL-60 cells in defined medium for four weeks (“HL-60ITS”) to investigate if serum factors had any influence on defensin regulation after induction. Although HL-60ITS cells showed higher constitutive defensin mRNA levels, RA treatment still resulted in further upregulation. In contrast, treatment with DMSO led to a complete disappearance of the message within 4 days (Fig 1). Although the X-CGD levels in DMSO-treated HL-60ITS cells seem to reflect a strong maturation, only moderate induction was observed with RA. Northern blot analysis of RNA from a retinoic acid-resistant HL-60 subclone (HL-60R) with either the defensin or X-CGD probe did not result in any signal, neither before nor after treatment with RA or DMSO. Analysis of the myeloblastic cell line KG-1 suggested that the earliest stage of myeloid blood cell differentiation during which the defensin and X-CGD genes are transcribed is in promyelocytic cells. Moreover, both messages cannot be induced by either DMSO or RA in this early stage of maturation. In keeping with the PMA-promoted downregulation, the lack of a measurable signal in U937 cells, a monoblast-like lymphoma cell line, further showed that defensin transcription is restricted to the granulocytic pathway (Fig 1). Defensin transcripts were also absent (as assessed by Northern blotting) in megakaryoblastic (MO7 cell line), erythroleukemic (K562), Epstein-Barr virus (EBV)-transformed B-lymphoblastoid (SK-CML8-BN12) and HTLV V-II infected T-cells (J-LB I) (data not shown). Exposure of the HL-60R, KG-1, U937, MO7, K562, BN12, and J-LBI blots for up to a week still did not show any signals. Hybridization with a GAPDH probe indicated equal RNA loading in all the lanes of the different experiments.

Steady-state levels of defensin mRNA during induced granulocytic differentiation of HL-60 cells. To further investigate (1) possible drug-specific effects and (2) the putative correlation with HL-60 cell differentiation, time-course analysis of defensin mRNA steady-state levels was performed. Addition of RA affected defensin transcript levels in a dose-dependent manner (Fig 2A). Ten nanomolar of RA, which had no visible effect on morphologic differentiation after 4 days and only low effects on functional maturation (as assessed by NBT-reduction and X-CGD expression),
elicited a near 20-fold increase of defensin transcripts on day 2. With 1 \( \mu \)mol/L RA, a steady increase of defensin mRNA levels was observed, ranging from about 7-fold induction after 24 hours to 80-fold on day 4. Cessation of growth on day 3 was accompanied by significant maturation changes. Cells exhibiting metamyelocytic characteristics increased from 14% on day 2 to 59% on day 4; 35% of the cell population had a banded or segmented neutrophil phenotype on day 4 of the treatment. Between experiments, the efficacy of RA to induce terminal differentiation was not always the same. This is best illustrated by the two representative data sets shown in Fig 3 (A and B). In one experiment (Fig 3A), RA induced a relatively fast differentiation with 64% promyelocytes and myelocytes, 27% metamyelocytes and 9% banded/segmented neutrophils on day 2. Although this distribution had barely changed at day 4, and the levels of the X-CGD message also remained the same, defensin mRNA levels had doubled (from 30- to 60-fold over control levels). Steady-state levels of myeloperoxidase (MPO) transcripts, on the other hand, were stable for the first 2 days to then decrease about 10-fold by day 4. In another experiment (Fig 3B), a dramatic increase in differentiation occurred between days 2 and 4 (86% metamyelocytes and 11% banded/segmented neutrophils at day 4) with concomitant further upregulation of X-CGD mRNA levels but, surprisingly, a substantial downregulation of defensin transcripts. In this particular case, MPO transcript levels decreased more rapidly, to about 40% of basal values after the first day and to 10% on day 3. Thus, our findings on temporal defensin expression patterns during myeloid granulocytic differentiation corroborate earlier studies, using in situ hybridization, indicating that defensin transcription peaks at the stage of promyelocytes and myelocytes.\(^{17-19}\)

When HL-60 cells were stimulated with hybrid polar compounds (DMSO, HMBA, DMF), expression patterns of defensin mRNA were more varied (Fig 2B). Within 24 hours, continuous exposure to DMSO caused an initial decrease not only in the level of transcript but also of its size. This is in contrast with GAPDH message levels, which remained unchanged (data not shown). At 48 hours, the original larger
During the early phase (0 to 48 hours) of induced granulocytic differentiation of HL-60 cells, the X-CGD mRNA steady-state levels and morphologic changes of the cell population during treatment. The key to evaluate the degree of differentiation is given in Materials and Methods.

An almost reverse situation was observed for MPO and 3). An almost reverse situation was observed for MPO. By comparison, HMBA seemed to be more potent in its ability to induce defensin mRNA (Fig 2B). The initial decrease in transcript levels and size paralleled the DMSO-induced pattern but was then followed by a sixfold increase over basal levels after 2 days, to remain unchanged until day 4. The combined treatment with RA/DMF, previously established to result in maximal granulocytic differentiation, consists of a 24-hour pretreatment with RA followed by addition of DMF. Not surprisingly, defensin mRNA levels were elevated during the first 24 hours, to then decrease 24 hours after DMF addition (Figs 2B and 3C). However, in contrast to DMSO- or HMBA-promoted “late” induction, transcript levels after 4 days of RA/DMF treatment had decreased to an almost zero value. At this time point, over 60% of the cell population had reached the stage of banded segmented neutrophils. From the combined results it follows that, once past the initial (>24 hours) downregulation of defensin steady-state mRNA levels by hybrid polar compounds in HL-60 cells, highest transcript levels are generally associated with the late myelocyte, perhaps very early metamyelocyte stage (eg, HMBA-treated cells at days 2 through 4) to then decrease and sometimes disappear entirely during more advanced maturation (eg, RA/DMF-treated cells on day 4). In contrast, a very simple relationship exists between X-CGD mRNA steady-state levels and morphologic differentiation; past 24 hours of treatment, transcript accumulation is strictly correlated with a more mature phenotype (Figs 2B and 3). An almost reverse situation was observed for MPO transcripts; here levels decreased gradually, but quite substantially, between the promyelocytic and neutrophilic stages.

Because the changing pattern of defensin transcript levels during the early phase (0 to 48 hours) of induced granulocytic differentiation of HL-60 cells appeared to be drug-specific, we performed kinetic fine-analysis for that particular time-window. With DMSO, the reduction in amount and size (from 660 to about 530 nucleotides) of defensin transcripts occurred gradually to reach a lower level at 24 hours, that remained for at least 6 hours; larger size transcripts then began to reaccumulate between 36 and 48 hours (Fig 4A). A similar pattern of downregulation within 24 hours was observed after addition of HMBA but, in contrast to DMSO, reaccumulation started without any delay between 24 hours and 27 hours, and peaked at 48 hours to further remain unchanged. No biphasic pattern was observed during RA-induced differentiation (Fig 5, upper row); instead, defensin transcript levels always increased steadily during the first 48 hours.

Transcriptional and posttranscriptional mechanisms regulate defensin mRNA levels in differentiating HL-60 cells. To investigate whether drug-dependent accumulation of defensin transcripts is the result of transcriptional activation or enhanced stabilization, HL-60 cells were treated with ActD (Fig 5). Regardless of whether the cells had been exposed to RA and ActD simultaneously, pretreated with RA for 9 hours before ActD addition, or treated with ActD alone (HL-60 RA-uninduced cells; data not shown), message levels decreased by about one third within 3 hours before disappearing altogether at 6 hours. With DMSO, a similar pattern was observed (data not shown). Therefore, the data indicate that defensin mRNA is rather short-lived in HL-60 cells and that neither RA nor DMSO treatment change this significantly. To determine whether the RA-promoted increase in defensin mRNA was dependent on protein synthesis, a similar experiment as above, but this time using cycloheximide (CHX), was performed (Fig 5, rows 4 and 5). With CHX alone (RA-untreated cells), the control levels of defensin mRNA remained constant for about 24 hours and then decreased, and the cells started to die after 12 hours (data not shown).
shown). After combined RA/CHX treatment, no upregulation could be observed but, instead, loss of message occurred between 15 and 24 hours; similar kinetics were obtained when cells were pretreated with RA before CHX addition. Because CHX had an identical effect on defensin mRNA levels in DMSO-treated cells (data not shown), one could speculate that protein synthesis might be required for induced accumulation of transcripts. However, considering the rather slow nature of the induction process, we cannot exclude that the observations are the simple consequence of all cellular processes just grinding to a halt.

To verify whether transcriptional activation is involved in regulating defensin mRNA levels after induction with RA or DMSO, nuclear run-on analyses were performed with untreated and continuously treated HL-60 cells over a 4-day period (Fig 6). Incubation with RA led to a gradual rise of defensin gene transcription, peaking at day 3 and decreasing slightly thereafter; however, transcription rates of the GAPDH gene remained unchanged. Increased transcriptional activity at early time points (~fourfold at day 1) might fully account for the increasing levels of defensin mRNA after RA treatment, and the later decrease in transcriptional rate causes the steady-state levels to decrease accordingly. On the other hand, DMSO treatment of HL-60 cells apparently had the opposite effect on defensin mRNA levels than on transcriptional activity. Although transcript levels decreased drastically during the first 48 hours before reaccumulation (see Fig 2B), transcriptional activity peaked at that very moment before returning to baseline levels on day 4 (Fig 6). These observations can only be explained by assuming that DMSO, and most likely HMBA as well, set off a series of events in HL-60 cells leading to transcriptional activation but, at the same time, cause a very strong destabilization of the defensin message, all 24 hours after adding the drug.

DMSO-promoted, “early” defensin mRNA degradation is clearly a biphasic process. The first step is characterized by a size reduction of about 130 nucleotides, perhaps the result of poly(A)-tail truncation. That premise was verified by RNase H digestion of “big-size” transcripts (eg, from untreated HL-60 cells or from ‘4 day’-DMSO treated cells) after prior duplexing with oligo(dT). As shown in Fig 4B, such treatment gave rise to transcripts about 170 nucleotides smaller in size, that is 40 nucleotides shorter than those observed 24 hours after addition of DMSO. Control experiments where either oligo(dT) hybridization or RNase H had been omitted had no effect on defensin transcripts.
Defensin 1-3 and 4 mRNA levels are both upregulated upon RA-treatment of HL-60 cells. The cDNA clones encoding defensin 1 and 3 differ by just two nucleotides, and defensin 2, which lacks the N-terminal amino acid, is probably encoded by one of these clones as well. Defensin 4 on the other hand, shares about 72% identity with the defensin 1 gene but also differs by an extra 83-base segment. Because it was possible that the probe used for our Northern hybridizations could have detected defensin 4 transcripts as well, and if so, to what extent defensin 4 mRNA would contribute to the signal, synthetic oligonucleotides were prepared that specifically hybridized to either defensin 1-3 or defensin 4. As shown in Fig 7A, the signal obtained with the defensin 1 specific oligonucleotide probe was indistinguishable from that obtained with the original cDNA probe, both after a 24-hour exposure. In contrast, no signal was noticeable with the defensin 4 specific probe, not even after 1 week of exposure. To verify that defensin 4 transcripts were present but, perhaps, at a level too low to be detected by Northern hybridization, we performed ribonuclease protection assays using both oligonucleotide probes (Fig 7B). The expected 63-bp protected defensin 4 fragment was detectable after a 1-week exposure, and about fivefold upregulation was observed on RA induction. Defensin 1 (61-bp fragment), which was upregulated 12-fold after RA treatment, was readily detectable after only 6 hours of exposure. Control experiments with yeast RNA as template confirmed the synthesis of full-length transcripts (134 bp for defensin 1, 136 bp for defensin 4) in the absence of RNAse, and neither of the fragments were not protected.

Effect of cytokines on defensin transcription in HL-60 cells. Our studies on defensin regulation were expanded to include the possible effects of various cytokines, because these are physiologic regulators involved in haematopoiesis, maturation, and control of functional activities of myeloid cells. G-CSF has been reported to stimulate proliferation and differentiation of neutrophil progenitor cells in vitro, and to profoundly affect the functions involved in host defense of mature neutrophils. Although IFN-γ and TNF-α induce the appearance of a monocytic phenotype in HL-60 cells, both factors are able to mediate enhanced host defense against pathogens, not only in macrophages but also in neutrophils. All three factors caused an initial downregulation of defensin transcript levels that then returned to basal levels after 24 hours (Fig 8A). During IFN-γ treatment, mRNA levels remained subsequently unchanged. TNF-α, on the other hand, effected a gradual increase of message, on a par with the levels observed during RA (100 nmol/L) treatments. With G-CSF, levels increased about twofold at 48 to 72 hours, then decreased below basal values after 4 days. Aside from a small increase in NBT reduction detectable after G-CSF (20%) and TNF-α (50%) treatment, no further functional or morphologic (<5% metamyelocytes) maturation was induced by any of the factors (data not shown). However, TNF-α treatment resulted in the appearance of many dead cells as early as day 2. Because G-CSF is known to induce differentiation of HL-60 cells in cooperation with RA, we used this particular combination to further investigate the effect on defensin mRNA regulation. The expression pattern was biphasic, in initially following the linear RA-induced increase up to 48 hours but then following the G-CSF-promoted decline to markedly lower levels on day 4 (Fig 8B). During these first 48 hours, an apparent synergistic effect on defensin mRNA levels was observed. However, major morphologic changes were evident throughout the 4 days of combined treatment (eg, 71% NBT-positive cells at day 4 compared with 24% in case of RA alone), in all probability explaining the diminution of defensin transcripts at the end. Thus, it seems likely that the temporal defensin mRNA pattern in this case reflects, in fact, the more rapid kinetics of differentiation. Interestingly, X-CGD mRNA levels increased at days 2 and 3 but, like defensin, disappeared at day 4 when NBT reduction was maximal (data not shown). Because cytokines have been shown to influence the functions of mature neutrophils, HL-60 cells were treated for 4 days with RA/DMF to induce maximal differentiation and were then cultured, in three separate experiments, in fresh medium containing either one of the described cytokines (no RA/DMF). Only TNF-α had a detectable effect on defensin mRNA levels in “mature” cells that, interestingly, was the opposite of the effect on immature HL-60 cells, ie, levels decreased (Fig 8C). Effects of serum factors on defensin mRNA levels during HL-60 granulocytic differentiation. When comparing defensin transcript levels during granulocytic differentiation of HL-60 cells grown in serum-supplemented or -free (HL-60TS) medium, it was obvious that DMSO-treated HL-60TS cells did not express any message on day 4 (Fig 1). To further investigate this phenomenon, HL-60TS cells were induced to differentiate along the granulocytic pathway and the defensin expression pattern was more closely monitored.
over time (Fig 9). Although DMSO induced a complete and final downregulation of defensin mRNA after 48 hours, HMBA and RA caused expression patterns comparable with those observed in cells grown in serum-supplemented medium, ie, message levels were up at day 4. In contrast, the X-CGD levels in HL-60ITS cells closely resembled the patterns that were obtained in the presence of serum and each of the inducers, including DMSO (Fig 9). Morphologic changes induced by the drugs were also the same in the presence and absence of serum (data not shown). It should be noted that for DMSO-promoted long-term downregulation of defensin transcripts to occur, HL-60 cells must be cultured in serum-free medium for at least several weeks, for it was not observed with cells that had been serum starved for only 24 hours (data not shown). It has been reported that activation of tyrosine kinases is an early response to the addition of DMSO to HL-60 cells.40 Because many serum factors are also known to act through tyrosine kinases, we then used.

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**Fig 7.** Specific detection of defensins 1/3 and defensin 4 transcripts in HL-60 cells. According to the alignment presented by Palfree et al.,19 positions 296 through 358 of defensin 4 and positions 304 through 364 of defensin 1/3 shared the lowest sequence homology and were therefore chosen to design isoform-specific synthetic oligonucleotides that could distinguish between defensin 1/3 and defensin 4. (A) Northern blot analysis of untreated and RA-treated HL-60 cells. As probes we used: top row, the 0.45-kb Sph I-EcoRI fragment derived from the HNP-1B cDNA clone;17 middle row, synthetic oligonucleotide specific for defensin 1; bottom row, synthetic probe specific for defensin 4. Exposure time was 1 week for defensin 4 compared with 24 hours for defensin 1 and defensins 1/3. (B) RNase protection assay was used to specifically detect defensin 1/3 (61 bp) and defensin 4 RNA (63 bp) in untreated and RA-treated cells. Yeast RNA was used as control for RNase digestion. Hybridization of the probes to yeast RNA without any RNase confirmed the transcription of full-length RNA probes, including vector sequences in the polylinker region (134 bp for defensin 1, 136 bp for defensin 4). Exposure time was 6 hours for defensin 1 and 1 week for defensin 4.
DEFENSIN mRNA IN HL-60 CELLS

Fig 8. Effect of cytokines on defensin transcription in HL-60 cells. (A) Cells were cultured in the presence of G-CSF (1,000 U/mL), IFN-γ (100 U/mL), or TNF-α (100 U/mL) for 4 days and, at indicated time points, samples for RNA extraction were taken and analyzed by Northern blotting. Autoradiograms obtained after hybridization with the defensin probe were analyzed by densitometry, normalized for gel-loading and IOD-values expressed as x-fold induction of control levels (arbitrary value of 1). (B) Procedures described in (A) also apply to this figure. Concentrations of inducers were 100 nmol/L (RA) or 1,000 U/mL (G-CSF), or both. (C) Cells were treated for 3 days with 100 U/mL TNF-α (undiff/TNF-α) or pretreated with RA/DMF for 4 days before the medium was replaced by TNF-α-containing (no RA/DMF) medium (diff/TNF-α).

The specific tyrosine kinase inhibitor, genistein, to investigate if such factors might be involved in regulating defensin levels during DMSO treatment. The results are summarized in Fig 10, where steady-state levels of defensin mRNA in HL-60 cells are compared over a 4-day period, after addition of DMSO, in the presence or absence of serum and genistein. Downregulation by DMSO in the absence of serum could be brought about equally well in the presence of serum by simultaneous addition of genistein. Either observation cannot be simply explained by assuming enhanced cell differentiation as this did not occur (data not shown). Moreover, genistein alone (in serum-containing medium) did not downregulate defensin levels, and neither did this kinase inhibitor have any attenuating effects on RA-dependent defensin mRNA induction (data not shown). Finally, genistein did not adversely affect any of the drug-induced X-CGD mRNA regulatory patterns in HL-60 cells. On the contrary, DMSO-dependent X-CGD expression in serum-supplemented medium is actually enhanced by genistein 48 hours after drug addition (Fig 10).

DISCUSSION

Defensins are peptide antibiotics stored in the primary granules of human neutrophils. These peptides are synthesized during cell maturation and accumulate for later use when fully differentiated neutrophils have entered the blood stream. Consistent with this model, defensin transcripts have been found in the human promyelocytic cell line HL-60, and yet only sketchy data are available on expression patterns during further differentiation. Here we have fine-analyzed defensin mRNA steady-state levels and transcriptional regulation during chemically induced differentiation of HL-60 cells.

Our data confirm that defensin transcription is indeed limited to the granulocytic pathway, beginning at the promyelocytic stage, and that it is quickly turned off when cells differentiate towards the monocyte/macrophage lineage. Although it has been reported recently that myeloblastic KG-1 cells did respond to RA with morphologic maturation, but without NBT reduction we were unable to induce such morphologic changes with neither RA nor DMSO, nor did the cells express any defensin or X-CGD mRNA. On the other hand, the apparent absence of either transcript in the monocytic cell line U937 is in accordance with the marked downregulation of steady-state levels, after 4 days, in PMA-treated HL-60 cells. Interestingly, a different temporal pattern of expression was observed for the two genes during early monocytic differentiation. Although defensin downregulation is immediate and final, X-CGD mRNA levels are actually elevated after 24 hours, to disappear thereafter. However, despite what seems like a monocytic lineage-dependent block in transcription, inducibility of the X-CGD gene in monocytes/macrophages, in response to cytokines or LPS treatments in vitro, has been observed.54 Similarly, lung macrophages from adult rabbits, but not peritoneal macrophages or monocytes, contain defensin transcripts and peptide, more abundantly so in fact than in similar neonatal cells; and injection with complete Freund adjuvant increased peptide levels even further.43,44 Thus, differentiation independent regulatory mechanisms must be recognized.

Further analysis of how defensin expression might be tied to defined stages of granulopoiesis, promyelocytic and beyond, clearly showed high transcript levels in late promyelocyte, myelocyte, and perhaps very early metamyelocyte cell populations, and a total absence from actual metamyelocytes and banded/segmented neutrophils, at least in the HL-60 model system that was used. Early elevated steady-state levels are the direct consequence of an increased transcriptional activity; stabilization of the message is not obvious and
deemed unlikely. Within the framework of induced HL-60 differentiation, regulation of the related defensin 1/3 and defensin 4 genes seem to occur synchronously with the important distinction, however, that the defensin 4 message is significantly less abundant at all times. This observation was of defensin HNP-4 against E coli exceeds that of the other reason for the low expression of defensin 4 is unknown. However, it is noteworthy that the specific antibiotic activity of defensin HNP-4 against E coli exceeds that of the other human isoforms about 100-fold and, in addition, HNP-4 has been shown to exhibit other physiologic activities such as binding to ACTH-receptors and activation of L-type Ca" channels. Granulopoietic stage-specific expression has not entirely surprising in view of the earlier reported defensin synthesis, or further upregulation of the levels in the original "producer" cells, or both. Whatever the scenario, (enhanced) transcriptional activation must be recognized as the underlying mechanism. However, even though our studies were concerned with this specific aspect of regulation, it should also be recognized that additional levels of control to producing functional defensins may exist further downstream, eg, translation, correct processing, and accumulation in granules.

As for defensin mRNA regulation, drug-specific temporal patterns were quite evident. RA consistently induced defensin transcript accumulation far better than hybrid polar compounds did, a capacity that was to some extent uncoupled described for neutrophil elastase gene expression. Others, such as myeloblastin/PR-3 and MPO (and this study) are expressed earlier, during the myeloblast/promyelocyte stage, or are restricted to promyelocytes only, as cathepsin G for example. Genes for the secondary granule proteins, lactoferrin and transcobalamin, are transcribed at the myelocyte and metamyelocyte stages, albeit never observed in the HL-60 system. Therefore, it is rather unlikely that synthesis of all granule constituents is subject to identical regulatory mechanisms; the molecular components may be largely overlapping perhaps, but unique elements must exist for each. Even more so for transcription of the X-CGD gene (the product of which is thought to accumulate in specialized neutrophilic vesicles, albeit not in HL-60 cells) that was found to increase steadily as granulocytic maturation proceeds, with the highest steady-state mRNA levels in the most advanced stages of HL-60 cell differentiation (and this study) or in mature neutrophils. Although an unmistakable trend in temporal defensin mRNA profiles was observed between comparable experiments, somewhat puzzling were the variations in basal levels and induction (both in terms of kinetics and magnitude of the response) noted throughout this study. It has been reported that individual untreated HL-60 cells may contain greatly variable amounts of defensin peptide (as assessed by immunostaining). From these findings it can be extrapolated that a similar distribution may exist at the mRNA level as well, although no experimental proof is currently available. Therefore, measured steady-state kinetics may represent either recruitment of additional cells to defensin transcription, or further upregulation of the levels in the original "producer" cells, or both. Whatever the scenario, (enhanced) transcriptional activation must be recognized as the underlying mechanism. However, even though our studies were concerned with this specific aspect of regulation, it should also be recognized that additional levels of control to producing functional defensins may exist further downstream, eg, translation, correct processing, and accumulation in granules.
from differentiation as treatment with suboptimal concentrations of RA, unable to effect a morphologically differentiated phenotype, upregulated defensin mRNA as well, albeit to a lesser extent. Also, 2 mmol/L HMBA caused more pronounced defensin induction over a 4-day period than 160 mmol/L DMSO, even though no major differences in HL-60 cell maturation could be discerned. Convincing evidence exists that RA-promoted terminal differentiation of HL-60 cells is mediated through the retinoic acid receptor-α (RARα).69 However, the relatively late induction by RA argues against direct activation of the defensin gene by ligand-mediated RAR binding to specific sequences in its control region. Furthermore, no such RA response elements (RAREs) of strict consensus could be identified within the hematopoietic defensin genes, at least not in the 1.2 kb of upstream sequence and in the first intron sequence (about 1.4 kb) available in the published literature.60,61 Therefore, transcriptional activation of defensin genes after addition of RA to HL-60 cells is more likely controlled by a combination of "other," as yet unidentified factors, at least some of which must be induced and/or posttranslationally activated in response to retinoids. Consistent with this view is the inability of RA to effect defensin expression in an RA-resistant subclone of HL-60 (HL-60R), a cell line that harbors a point mutation in the RARα gene.62 However, quite unexpected was the failure to promote defensin and X-CGD gene activation (and morphologic maturation) in (or of) HL-60R cells with hybrid polar compounds as well. Whether the cells carry another, as yet unrecognized, defect or whether RA and DMSO/HMBA-induced defensin regulation share common elements that involve RARα activity remains to be determined.

Hybrid polar compounds have long been recognized as agents that induce terminal differentiation of transformed cells, including HL-60.63 As the precise molecular mechanisms underlying induction remain unresolved, it is not clear if a single, universal pathway is used by different compounds, DMSO and HMBA for instance. In this study, we came upon a striking, but highly reproducible, difference between the latter two chemicals in their effects on HL-60 cells, namely the timing of a very pronounced "dip" in the early (0 to 48 hours) steady-state kinetics of defensin mRNA regulation. This early downregulation of message levels, accompanied by an apparent reduction in transcript size, is the sole result of induced degradation as defensin transcriptional rates are actually up at this point. In contrast, no detectable destabilization of the defensin message occurred during or after RA-dependent transcriptional activation. Upon what can only be explained as "restored" stability of defensin mRNA, and as the result of continuing intensified synthesis, new larger sized transcripts begin to appear 27 or 36 hours after the original addition of, respectively, HMBA or DMSO to the cells. Consequently, kinetics of defensin transcript accumulation were noticeably faster in the presence of HMBA. This was initially paralleled by an earlier appearance of metamyelocyte cell characteristics upon exposure to that compound.

The mechanism of drug-induced transient degradation of defensin transcripts is not entirely clear, even more so because ATTTA motifs, known to influence rapid transcript turnover,64 are absent from the 3' untranslated region in defensin mRNA. It has been reported that the poly(A)-tail of mRNAs, in conjunction with its binding protein (PABP), has a major impact on stability by protecting the message from cellular nucleases.65 Depletion of PABP has been shown to result in truncation of the poly(A)-tract and subsequent complete degradation of globin and c-myc transcripts.66,67 The apparent biphasic decay of defensin mRNA (small reduction in size followed by degradation) is curiously reminiscent of these earlier findings. Moreover, we have shown by RNase H digestion that the "early" reduction in size (−130 bases) is indeed most likely the result of partial loss of the poly(A)-tail (about 170 nucleotides long in case of defensin transcripts). The remaining 40 nucleotides are the minimally required length for a single PABP to bind and stabilize the entire transcript66,67; therefore, messages of this size may transiently accumulate. Upon further truncation, the residual PABP dissociates and the rest of the transcript gets degraded by endonucleases. Hence, we speculate that DMSO/HMBA could initiate some events leading to poly(A) degradation and temporary loss or sequestration of PABP in HL-60 cells within the first 24 to 36 hours. The question then arises if this could be the result of a regular cellular process. Hybrid polar compound-induced terminal cell differentiation is thought to be a cell-cycle dependent process. Specifically, HMBA has been shown to cause murine erythroleukemia cells to arrest in G1 phase and to modulate factors regulating G1-S phase progression.65 In view of these data, we examined steady state defensin mRNA levels in HL-60 cell populations that had been synchronized in separate cell-cycle phases. No significantly lower levels were associated with G1 phase (data not shown), indicating that the HMBA-promoted decay of defensin mRNA is not the simple result of cells accumulating in a particular phase of the cell cycle where specific degradation is customary during regular growth.

Upon addition of selected cytokines (G-CSF, IFN-γ, and TNF-α; this study) and LPS (S.H. and P.T., unpublished observation, 1994) to the HL-60 culture medium, a "hybrid-polar-compound-like" drop in early defensin mRNA kinetics also occurred; at present it is not known whether this was the result of compromised transcript stability. However, the timing of this event was decidedly different from either the DMSO- or HMBA-induced decrease in steady-state kinetics, in that it transpired early on, between 6 and 24 hours. Once defensin levels were back to baseline values, no significant further changes occurred, except for TNF-α–treated cells, where an increase was noted. The particular effects of selected cytokines on defensin regulation were neither associated, nor heralded, measurable morphologic and functional differentiation, nor did X-CGD mRNA levels change during treatment. This intriguing molecular facet of cytokine and hybrid polar compound action on HL-60 cells is not shared by RA or phorbol esters. In fact, when added simultaneously to the culture medium, RA overrides the G-CSF–promoted early effect on defensin mRNA. Instead, this par-
ticular combination synergistically induces defensin mRNA over the first 48 hours, apparently the effect of a more pronounced differentiation. This is perhaps most simply explained by assuming that (putative) mRNA degradation cannot keep pace with a highly accelerated transcription. Anyway, the inducer-specificity of “early” defensin mRNA regulation must be inherent in common, or overlapping, signaling events. Therefore, it is fascinating that treatment of HL-60 cells with G-CSF, IFN-γ, TNF-α, and DMSO, but not RA and phorbol esters, has been reported to trigger a rapid, stable phosphorylation on Ser and Tyr of a 75-kD protein.40 Cytokine-induced signaling is known to involve phosphorylation steps, for example by JAK kinases in case of G-CSF and IFN-γ40 and a ceramide-activated kinase in response to TNF-α.48 Similarly, tyrosine protein kinase activity was shown to be stimulated by DMSO.70

While still largely speculative at the present time, the contention that (tyrosine) protein kinases might be involved in regulating defensin gene expression during drug-induced HL-60 cell differentiation was corroborated some by the observation that the DMSO-promoted effects were strikingly different in serum-free medium or in the presence of genistein, a tyrosine kinase inhibitor. Under those conditions, even though differentiation was apparently unaffected, the typical early downregulation was not followed by any visible reappearance of defensin transcripts. This finding was entirely specific for the effect of DMSO on accumulation of defensin transcripts as it did not occur with RA, and neither was X-CGD expression affected. Interestingly, the usual reappearance of defensin transcripts after 48 hours is not impeded in HMBA-treated cells cultured under serum-free conditions. Whether this latter observation is correlated in any way to the relatively faster kinetics (compared with DMSO) of G-CSF and IFN-γ65 and a ceramide-activated kinase in response to TNF-α.66 Studies to expand our understanding of drug-induced signaling leading to defensin gene expression, and to resolve the putative role(s) of protein kinases in this pathway, are ongoing.

ACKNOWLEDGMENT

The authors are grateful to Dr S.J. Collins (Fred Hutchinson Cancer Center, Seattle, WA) for providing the retinoic acid-resistant cell line HL-60R and to Drs Bernd Gansbacher and David Golde (Memorial Sloan-Kettering Cancer Center) for providing the cell lines SK-CML8-BN12 and J-LBL. We also thank Dr Stuart Orkin (Children’s Hospital, Boston, MA) for the X-CGD probe. Thanks are extended to Tom Delohery for his assistance with flow cytometry and Jeanne Kwik from the Sloan-Kettering Microchemistry Core Facility for oligonucleotide synthesis.

REFERENCES

25. Royer-Pokora B, Kunzel LM, Monaco AP, Goff SC, Newburger PE, Buehler RL, Cole FS, Curnute JT, Orkin SH: Cloning of...


Distinct temporal patterns of defensin mRNA regulation during drug-induced differentiation of human myeloid leukemia cells

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