Regulation of 5-lipoxygenase and 5-lipoxygenase-activating protein expression in HL-60 cells

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5-Lipoxygenase performs the first two enzymic reactions in the biosynthetic pathway for the leukotrienes. We have utilized HL-60 cells to study the mechanisms regulating expression of 5-lipoxygenase and the recently described 18 kDa membrane protein, 5-lipoxygenase-activating protein (FLAP). Differentiation of HL-60 cells into granulocyte-like cells with dimethyl sulphoxide (Me₂SO), retinoic acid or dibutyryl cyclic AMP (Bt₂-cAMP) resulted in a 2–3-fold increase in 5-lipoxygenase enzyme activity and a 4-fold increase in leukotriene B₄ synthesis. Differentiation of HL-60 cells into monocyte-like cells with 1,25-dihydroxyvitamin D₃ induced 5-lipoxygenase activity 5-fold, but leukotriene B₄ production was only increased 2-fold. Differentiation of HL-60 cells with phorbol 12-myristate 13-acetate (PMA) into macrophage-like cells failed to induce 5-lipoxygenase or leukotriene B₄ production. Examination of the levels of the transcript encoding 5-lipoxygenase and FLAP demonstrated that differentiation of HL-60 cells into granulocytes resulted in a co-ordinate induction of 5-lipoxygenase and FLAP mRNA. In

contrast, differentiation of HL-60 cells into monocytes resulted in discordant regulation of 5-lipoxygenase and FLAP mRNA. Treatment with 1,25-dihydroxyvitamin D₃ resulted in a 6-fold increase in 5-lipoxygenase mRNA and a 1.3-fold increase in FLAP mRNA, while treatment with phorbol ester did not induce 5-lipoxygenase mRNA but did increase FLAP mRNA 2-fold. The transcriptional rate of the 5-lipoxygenase and FLAP genes did not change upon Me₂SO or 1,25-dihydroxyvitamin D₃ treatment, suggesting that the increase of the mRNA coding for these proteins was not due to transcriptional activation of their respective genes. The mRNA half-life for 5-lipoxygenase did not change significantly upon treatment with Me₂SO or 1,25-dihydroxyvitamin D₃. The FLAP mRNA half-life increased from approx. 3.5 h to 4.5 h in cells treated with either Me₂SO or 1,25-dihydroxyvitamin D₃. These data suggest that expression of 5-lipoxygenase and FLAP is controlled by a post-transcriptional event other than stabilization of the mRNA.

INTRODUCTION

Leukotrienes are a family of arachidonic acid metabolites with potent biological activities. The peptidyl leukotrienes, leukotriene C₄, leukotriene D₄ and leukotriene E₄, are potent smooth muscle contractile agents, while leukotriene B₄ is a leukocyte chemo-attractant. 5-Lipoxygenase performs the first two enzymic reactions in the biosynthesis of the leukotrienes, i.e. oxidation of arachidonic acid at C-5 to form 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), followed by dehydration of 5-HPETE to form 5,6-oxido-7,9,11,14-eicosatetraenoic acid (leukotriene A₄). Leukotriene A₄ is further converted either to the peptidyl leukotriene leukotriene C₄ via a conjugation reaction with glutathione, or to leukotriene B₄ by a stereospecific hydrolysis by leukotriene A₄ hydrolase. 5-Lipoxygenase requires Ca²⁺ for maximal enzyme activity and ATP further enhances the 5-lipoxygenase enzyme activity in the presence of Ca²⁺ [1–4]. In addition to requiring Ca²⁺ for enzymic activity, Ca²⁺ promotes translocation of 5-lipoxygenase from the cytosol to a cellular membrane fraction [5–7]. Translocation of 5-lipoxygenase from the cytosol to cell membrane fractions correlates with the synthesis of leukotrienes [6]. Studies utilizing the leukotriene synthesis inhibitor MK-886 provide additional evidence that translocation from the cytosol to the cell membrane is required for leukotriene synthesis. MK-886 inhibited translocation of 5-lipoxygenase from the cytosol to the membrane, resulting in a decrease in leukotriene production without directly inhibiting 5-

lipoxygenase [8,9]. MK-886 was found to bind to an 18 kDa membrane protein, termed 5-lipoxygenase-activating protein (FLAP), which has been cloned and sequenced [8,10]. DNA transfection experiments demonstrated that both 5-lipoxygenase and FLAP were required for synthesis of leukotrienes [10].

cDNA clones for human and rat 5-lipoxygenases have been isolated [11–13]. Human 5-lipoxygenase cDNA encodes a 674-amino-acid protein with a molecular mass of 78 kDa. 5-Lipoxygenase contains two domains which exhibit similarity to a 17-amino-acid consensus sequence found in Ca²⁺-dependent membrane binding proteins such as lipocortin and endonexin [13]. Human 5-lipoxygenase is encoded for by a gene greater than 80 kb in length, comprising 14 exons, which is processed to a mature mRNA of 2.5 kb in length [14]. The expression of 5-lipoxygenase is restricted primarily to haematopoietic cells, with granulocytes and monocytes expressing the highest level of 5-lipoxygenase enzyme activity and mRNA. In contrast to the data demonstrating that expression of 5-lipoxygenase is limited to only a few cell types [13], the putative promoter region of 5-lipoxygenase contains promoter sequences typically found in many housekeeping genes, suggesting that expression of 5-lipoxygenase would be constitutive [14]. The promoter region for 5-lipoxygenase has been further characterized by cloning 5'-flanking sequences to a reporter gene [15]. These studies demonstrate the presence of two positive enhancer elements which function in both HL-60 and Hela cells. In addition a modest enhancement by phorbol 12-myristate 13-acetate (PMA) was

Abbreviations used: Bt₂cAMP, N⁶,2'-O-dibutyryl-adenosine cyclic AMP; FLAP, 5-lipoxygenase-activating protein; GTP[S], guanosine-5'-(γ-thio)triphosphate; 5-HETE, 5(*S*)-hydroxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid; 5-HPETE, 5(*S*)-hydroperoxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid; LBK AP, liver/bone/kidney form of alkaline phosphatase; Me₂SO, dimethyl sulphoxide; NBT, Nitro Blue Tetrazolium; PMA, phorbol 12-myristate 13-acetate.

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identified. Hela cells do not normally express 5-lipoxygenase; therefore these studies failed to identify the mechanism by which 5-lipoxygenase is preferentially expressed in leukocytes.

The human gene for FLAP has also been cloned and sequenced [16]. Like 5-lipoxygenase, FLAP is encoded by a relatively large gene, of greater than 31 kb, consisting of five exons and four introns. Previous studies suggest that, like 5-lipoxygenase, FLAP is expressed primarily in cells of haematopoietic origin [13,16]. However, unlike 5-lipoxygenase, FLAP gene has a TATA-like box upstream of the transcription initiation site. In addition, a putative AP-2 binding site and a binding site for the glucocorticoid receptor are in the 5'-flanking sequences of the FLAP gene [16]. Sequences within the first 700 nucleotides of the 5'-flanking sequences were identified which result in tissue-specific expression of the FLAP gene.

HL-60 cells are a human promyelocytic leukaemia cell line which may be induced to differentiate either into monocytic/macrophage-like cells by phorbol esters or 1,25-dihydroxyvitamin D₃ or into granulocyte-like cells by dimethyl sulphoxide (Me₂SO), retinoic acid or dibutyryl cyclic AMP (Bt₂-cAMP) [17]. It has been previously reported that, upon differentiation into granulocytes with Me₂SO, 5-lipoxygenase enzyme activity and mRNA are induced in HL-60 cells [11,18]. More recent data demonstrated that differentiation of HL-60 cells with Me₂SO will also induce FLAP, with similar kinetics as for 5-lipoxygenase [19]. To gain further insight into the mechanisms which regulate 5-lipoxygenase expression, we have utilized HL-60 cells as a cellular model system for the control of 5-lipoxygenase expression. In addition, regulation of FLAP in differentiated HL-60 cells was compared with 5-lipoxygenase expression to determine if the two gene products are co-ordinately regulated as a function of various inducing agents.

MATERIALS AND METHODS

Cell culture

HL-60 cells obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Irvine Scientific, Irvine, CA, U.S.A.). Cultures of HL-60 cells were found to change in their responsiveness to Me₂SO differentiation with respect to leukotriene B₄ induction after 3–4 months in culture. A single cell clone (H1) was obtained from the parent HL-60 cell culture by performing limiting dilution twice, resulting in the isolation of several subclones of the HL-60 cell line. Clones were screened for induction by Me₂SO of leukotriene B₄ synthesis. HL-60 clone H1 induced 5-lipoxygenase enzyme activity and leukotriene B₄ for greater than 12 months, and was used for the experiments described in this paper.

Leukotriene measurements

The quantity of leukotriene B₄ released from cells was determined in the cell supernatants by radioimmunoassays using commercially available radioimmunoassay kits (New England Nuclear, Boston, MA, U.S.A.). Following differentiation, cells were washed twice with Dulbecco's phosphate-buffered saline and resuspended at a concentration of 1×10^7 cells/ml in Dulbecco's phosphate-buffered saline supplemented with 1 mg/ml fatty-acid-free BSA. The cells were equilibrated at 37 °C for 5 min, 10 μ M calcium ionophore A23187 was added and the cells were incubated for an additional 15 min at 37 °C. Cells were removed from the supernatant by centrifugation at 5000 *g* for 2 min. The amount of leukotriene B₄ produced in 100 μ l of cell supernatant

was determined by radioimmunoassay as described by the manufacturer.

Lipoxygenase products synthesized in HL-60 cells were also analysed by reverse-phase h.p.l.c. Cells were washed twice in Dulbecco's phosphate-buffered saline, resuspended at 2.5×10^7 cells/ml, divided into 1 ml aliquots and equilibrated at 37 °C for 5 min. Calcium ionophore (A23187) was added to the cells at a final concentration of 5 μ M and the cells were incubated for an additional 10 min at 37 °C. Leukotriene production was terminated by the addition of EDTA to the cells to give a final concentration of 4 mM, followed by 1 ml of cold ethanol. The cell extracts were acidified by the addition of 10 μ l of formic acid and the aqueous phase was extracted twice with 1 ml of chloroform. The chloroform extracts were combined and dried under nitrogen. The extracts were dissolved in 60 μ l of methanol and the leukotrienes were analysed by reverse-phase h.p.l.c. using a C₁₈ Novapak column (3.9 mm $\times$ 150 mm). The material was separated by elution of the column with 57% methanol/0.1% acetic acid, pH 5.4, for 10 min, followed by a linear gradient from 57% methanol to 60% methanol over 5 min at a flow rate of 1.2 ml/min. Leukotrienes and dihydroxyeicosatetraenoic acid (di-HETE) products were detected by absorbance at 270 nm. Identification of the lipoxygenase products was determined by co-elution with authentic standards.

Enzyme measurements

5-Lipoxygenase activity was measured in 8000 *g* supernatants of HL-60 cells. Cells were washed twice with Dulbecco's phosphate-buffered saline (calcium- and magnesium-free) prechilled at 4 °C, sonicated (10 $\times$ 1 s bursts, 30% power; Branson model 450 sonifier) in 20 mM bistris/HCl, pH 7.2, 10 mM NaCl, 2 mM EGTA, 0.1 mM leupeptin and 10 μ g/ml aprotinin. The homogenate was centrifuged at 8000 *g* for 20 min at 4 °C. 5-Lipoxygenase enzyme activity was determined using 100 μ g of the 8000 *g* supernatant using a modification of the assay described by Hogaboom et al. [3]. The final reaction mixture contained 50 mM bistris/HCl, pH 7.0, 1 mM ATP, 0.5 mM CaCl₂, 0.5 mM EGTA, 1 μ M guanosine 5'-[γ -thio]triphosphate (GTP[S]) and 18 μ M [¹⁴C]arachidonic acid (55 mCi/mmol; New England Nuclear) in a total volume of 200 μ l. The reaction mixture was incubated for 15 min at 28 °C. Reactions were terminated by the addition of 400 μ l of cold acetone containing 1 mg/ml prostaglandin B₂. Protein was precipitated by incubation at –20 °C for 20 min, followed by centrifugation at 18000 *g* for 10 min. Arachidonic acid metabolites were extracted by the addition of 800 μ l of ethyl acetate followed by 400 μ l of ethyl acetate. The ethyl acetate extracts were pooled and the samples dried *in vacuo*. The 5-lipoxygenase products 5-HETE and 5-HPETE were quantified by h.p.l.c. separation on a C₁₈ Hypersil column (4.5 mm $\times$ 200 mm; Hewlett Packard, Avondale, PA, U.S.A.) using a Beckman System Gold h.p.l.c. system, either by u.v. absorbance at 234 nm using a model 161 detector or by radioactivity using a model 171 detector with a 300 μ l solid scintillate cell as described by Hogaboom et al. [3].

cDNA and genomic clones

The human 5-lipoxygenase cDNA clone [11] was kindly provided by Dr. Richard Dixon (Merck Sharp Dohme Laboratories, West Point, PA, U.S.A.). Human *c-myc* genomic clone pH5R-1 was obtained from the American Type Culture Collection. A 3'-specific probe to human *c-myc* mRNA was prepared by subcloning the *Clal*–*EcoRI* fragment [20] into pUC18.

A partial FLAP cDNA clone was isolated from HL-60 cells

differentiated with Me_2SO for 3 days by a two-step PCR procedure. Total cellular RNA (1 μg) was reverse-transcribed as described by Kingston [21]. The first PCR reaction was performed using primers designated to hybridize to human FLAP mRNA at -10 to $+15$ (5'-AGTATCAATCATGGATCAAGAACT-3') and $+485$ to $+509$ (5'-CCAACCCCATATTCAGCAGAGAGTT-3'). The reaction mixture contained 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl_2 , 0.01% (w/v) gelatin, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 1 μM of each primer and 2.5 units of *Taq* polymerase in a final volume of 100 μl . A total of 30 cycles were performed; 90 °C for 1 min, 50 °C for 2 min, and 70 °C for 2.5 min per cycle. The PCR product from the first reaction was amplified as described above using new primers containing the restriction enzyme sites for *Eco*R1 (5'-CCGGAATTCCGTTCTGGAGTATCAATCATGG-3') and *Pst*I and *Kpn*I (5'-AGCTGCAGCGGGTACCCGAGAGTTAGGAAATGAGAA-3'). The second PCR product was cloned into pUC19 using *Eco*RI and *Pst*I restriction sites.

DNA segments of interest corresponding to regions of the human 5-lipoxygenase gene [19] containing introns E and M were amplified from human genomic DNA by PCR techniques [22]. Oligonucleotides flanking intron E in the fifth and sixth exon of the human 5-lipoxygenase gene (5'-CCTGTTCATCAACCGCTTCAT-3' and 5'-CTGAGCTGCCGCTCCAGGCT-3' respectively) and the 13th and 14th exons of the human 5-lipoxygenase gene (5'-CATGCGAGCCCCGCCACCGACT-3' and 5'-TAGTAATATGGCAGCTGCTT-3' respectively) were utilized as PCR primers. PCR fragments were blunt-end-ligated into pGEM vectors (Promega Inc, Madison, WI, U.S.A.). Colonies containing recombinant plasmid were selected and sequenced by the dideoxy method [23].

RNA isolation and Northern blot analysis

RNA was isolated from HL-60 cells by lysing the cells in 4 M guanidinium isothiocyanate followed by a caesium chloride gradient [24]. Total cellular RNA was separated on 1% agarose gels containing 1.1% formaldehyde, and transferred to nylon membranes. Blots were hybridized to the appropriate cDNA probe labelled with [α - ^{32}P]dCTP by random priming. Specific transcripts were quantified by analysis on a Molecular Dynamics PhosphorImager (Sunnyvale, CA, U.S.A.). mRNA half-life was determined by Northern blot analysis of HL-60 cells treated with 5 $\mu\text{g}/\text{ml}$ actinomycin D for the indicated period of time. To increase the signal of the 5-lipoxygenase mRNA on the blots, single-stranded DNA probes generated by PCR [25] were utilized. Filters were prehybridized for 15 min with QuikHyb (Stratagene, La Jolla, CA, U.S.A.) at 68 °C, then hybridized for 90 min. Transcripts were quantified by analysis on a Molecular Dynamics PhosphorImager.

Nuclear run-off reactions

Nuclei from HL-60 cells were prepared by lysis of the cells in 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 and 0.5% Nonidet P-40 as described by Greenberg [26]. RNA was labelled by incubation for 20 min at 30 °C in 10 mM Tris/HCl, pH 7.4, 80 mM KCl, 5 mM MgCl_2 , 0.1 mM EDTA, 35% glycerol, 1 mM ATP, 1 mM CTP, 1 mM GTP, 200 μCi of UTP (3000 Ci/mmol) 0.5 mM *S*-adenosylmethionine and 0.5 mM dithiothreitol in a final volume of 230 μl . The reactions were terminated and RNA extracted as described by Celano et al. [27]. Equal amounts of radioactivity of ^{32}P -labelled RNA were hybridized to filters containing 10 μg each of the linearized plasmids.

RESULTS

Differentiation of HL-60 cells into granulocytes results in enhanced leukotriene B_4 biosynthesis

It has been previously reported that differentiation of HL-60 cells with 1.2% Me_2SO for 6 days results in increased leukotriene B_4 synthesis and 5-lipoxygenase enzyme activity [18]. To determine if other differentiation agents were also capable of inducing 5-lipoxygenase and leukotriene B_4 synthesis, we analysed 5-lipoxygenase enzyme activity and A23187-induced leukotriene B_4 synthesis in cells differentiated for 5 days with 1.3% Me_2SO , 500 μM Bt_2 -cAMP, 1 μM retinoic acid, 0.1 μM 1,25-dihydroxyvitamin D_3 or 10 nM PMA (Table 1). The percentage of cells which responded to PMA-induced superoxide anion generation, as determined by Nitro Blue Tetrazolium (NBT) reduction, was defined as a marker for cellular differentiation. Differentiation of HL-60 cells into granulocytes by treatment with Me_2SO and Bt_2 -cAMP resulted in a 2-fold increase in 5-lipoxygenase enzyme activity and a 4-fold increase in A23187-induced leukotriene B_4 production. Retinoic acid, which also induces granulocytic differentiation, was less effective at induction of 5-lipoxygenase enzyme activity and leukotriene B_4 production than either Me_2SO or Bt_2 -cAMP. Differentiation of HL-60 cells into monocyte-like cells by treatment with PMA did not significantly change 5-lipoxygenase enzyme activity compared with that of basal cells. Leukotriene B_4 production by PMA-differentiated cells was below the limits of detection of the radioimmunoassay (Table 1). In contrast, differentiation with 1,25-dihydroxyvitamin D_3 , which also promotes differentiation of HL-60 cells into monocytes, resulted in a 5-fold increase in 5-lipoxygenase enzyme activity and a 2-fold increase in A23187-induced leukotriene B_4 synthesis (Table 1). The percentage of cells which were NBT-positive did not correlate with either 5-lipoxygenase activity or leukotriene B_4 synthetic capacity, suggesting that induction of 5-lipoxygenase and leukotriene B_4 synthesis occurs independently of the enzymes required for NBT reduction (Table 1).

It should be noted that treatment of HL-60 cells with 1,25-dihydroxyvitamin D_3 consistently resulted in a 2-fold greater induction of 5-lipoxygenase enzyme activity than observed with Me_2SO or Bt_2 -cAMP, but in approx. one-half the leukotriene B_4 synthesis. One explanation for the discrepancy between 5-lipoxygenase enzyme activity and leukotriene B_4 production may be that differentiation of the HL-60 cells with 1,25-dihydroxyvitamin D_3 results in cells which predominantly synthesize

Table 1 Induction of 5-lipoxygenase in differentiated HL-60 cells

HL-60 cells were treated for 5 days with the indicated agent. Cells were assayed for leukotriene B_4 released in response to 10 μM A23187 ($n = 2$), 5-lipoxygenase enzyme activity ($n = 4$) and NBT reduction ($n = 2$) as described in the Materials and methods section. Similar results were obtained in two separate experiments. n.d. not detectable.

Agent	LTB ₄ production (pg/10 ⁶ cells)	5-Lipoxygenase activity (pmol/15 min per mg)	NBT reduction (% positive)
None	53	190 ± 65	0.5
1.3% Me_2SO	200	518 ± 21	22.4
500 μM Bt_2 -cAMP	190	510 ± 37	83.6
1 μM Retinoic acid	74	419 ± 50	37.8
0.1 μM 1,25-Dihydroxy- vitamin D_3	115	1112 ± 344	16.8
10 nM PMA	nd	220 ± 29	15.0

Figure 1 Induction of 5-lipoxygenase and FLAP mRNAs by different differentiation agents

HL-60 cells were treated with 1.3% Me₂SO, 0.1 μ M 1,25-dihydroxyvitamin D₃ (Vit. D₃), 1 μ M retinoic acid (RA), 500 μ M Bt₂-cAMP (cAMP) or 10 nM PMA for 5 days. Total cellular RNA was isolated and analysed by Northern blot analysis using 5-lipoxygenase (5-LO)- and FLAP-specific cDNA probes as described in the Materials and methods section.

Figure 2 Kinetics of 5-lipoxygenase and leukotriene B₄ induction

HL-60 cells were treated with 1.3% Me₂SO (a) or 0.1 μ M 1,25-dihydroxyvitamin D₃ (b) for the indicated periods of time. Cells were analysed for either 5-lipoxygenase enzyme activity ($\square$ — $\square$) or A23187-induced leukotriene B₄ production ($\square$ --- $\square$) as described in the Materials and methods section. Data represent the means $\pm$ S.E.M. ($n = 3$).

peptidyl leukotrienes, which would not be detected with the leukotriene B₄ radioimmunoassay. To address this issue, cells were analysed by reverse-phase h.p.l.c. for lipoxygenase products following A23187 challenge. Basal and 1,25-dihydroxyvitamin-D₃-differentiated HL-60 cells challenged with A23187 produced

small quantities of leukotriene C₄ and leukotriene B₄, while Me₂SO-differentiated HL-60 cells markedly increased production of di-HETEs, leukotriene B₄, leukotriene D₄ and leukotriene C₄ (results not shown). Thus the discrepancy in 5-lipoxygenase enzyme activity and leukotriene B₄ synthesis cannot be attributed to an increase in peptidyl leukotriene biosynthesis (leukotrienes C₄, D₄ or E₄), as h.p.l.c. analysis of the extracts failed to detect significant amounts of these products in 1,25-dihydroxyvitamin-D₃-differentiated cells.

Another explanation for the discrepancy is that 1,25-dihydroxyvitamin D₃ induces 5-lipoxygenase, but fails to induce FLAP, which is also required for leukotriene biosynthesis [10]. To test this hypothesis, Northern blot analyses were performed using total cellular RNA isolated from HL-60 cells differentiated with various agents (Figure 1). Treatment of HL-60 cells with 1.3% Me₂SO, 500 μ M Bt₂-cAMP, and 1 μ M retinoic acid for 4 days resulted in 4–5-fold increases in 5-lipoxygenase mRNA (Figure 1). Consistent with enzyme activity measurement, treatment of the HL-60 cells with 10 nM PMA failed to induce 5-lipoxygenase mRNA (Figure 1), while treatment of the cells with 0.1 μ M 1,25-dihydroxyvitamin D₃ resulted in a 6-fold increase in 5-lipoxygenase mRNA. Similar to 5-lipoxygenase, treatment with Me₂SO, Bt₂-cAMP and retinoic acid resulted in 3–5-fold increases in FLAP mRNA (Figure 1). However, treatment of the cells with 1,25-dihydroxyvitamin D₃ induced FLAP mRNA only 1.3-fold, while PMA treatment induced FLAP mRNA 2-fold (Figure 1). These data suggest that differentiation of HL-60 cells into granulocytic cells resulted in co-ordinate expression of 5-lipoxygenase and FLAP. In contrast, differentiation of HL-60 cells into monocytic cells resulted in the differential expression of 5-lipoxygenase and FLAP, in that PMA induced FLAP mRNA but not 5-lipoxygenase, while differentiation of HL-60 cells with 1,25-dihydroxyvitamin D₃ induced 5-lipoxygenase mRNA but only modestly induced FLAP mRNA. Thus the discrepancy in 5-lipoxygenase enzyme activity and leukotriene production observed upon 1,25-dihydroxyvitamin D₃ treatment may be explained by a lack of induction of FLAP, which is required for translocation of 5-lipoxygenase to cellular membranes.

Kinetics of 5-lipoxygenase induction

The kinetics of Me₂SO- and 1,25-dihydroxyvitamin-D₃-induced 5-lipoxygenase enzyme activity and leukotriene B₄ production were compared. Me₂SO-treated HL-60 cells maximally expressed 5-lipoxygenase enzyme activity and leukotriene B₄ production 4 days after addition of the differentiation agent (Figure 2a). Induction of 5-lipoxygenase enzyme activity was first detectable 24 h after treatment with 1,25-dihydroxyvitamin D₃ (Figure 2b) and 42 h after Me₂SO treatment (Figure 2a). In contrast to Me₂SO-differentiated HL-60 cells, the induction of 5-lipoxygenase and A23187-induced leukotriene synthesis failed to plateau within the 5-day treatment period in 1,25-dihydroxyvitamin-D₃-treated cells (Figure 2b). Induction of the mRNA encoding 5-lipoxygenase preceded the induction of 5-lipoxygenase enzyme activity, with maximal induction occurring 2 days after addition of Me₂SO and 3 days after 1,25-dihydroxyvitamin D₃ (Figure 3). Increases in mRNA were first detectable 20 h after the addition of the differentiation agents (Figure 3). The induction kinetics of FLAP mRNA in Me₂SO-differentiated HL-60 cells were similar to those of 5-lipoxygenase (Figure 3). However, the induction kinetics for FLAP mRNA in 1,25-dihydroxyvitamin-D₃-treated HL-60 cells differed from those of 5-lipoxygenase mRNA induction, in that significant increases in FLAP mRNA were not detectable until 2 days after addition of 1,25-dihydroxyvitamin D₃ (Figure 3).

Figure 3 Kinetics of 5-lipoxygenase and FLAP mRNA induction

HL-60 cells were treated with either 1.3% Me_2SO (a) or 0.1 μM 1,25-dihydroxyvitamin D_3 (b) for the indicated periods of time. Total cellular RNA was isolated and analysed by Northern blotting for 5-lipoxygenase (5-LO) and FLAP expression as described in the Materials and methods section.

Induction of 5-lipoxygenase and FLAP is dependent upon new protein synthesis

Induction of leukotriene production in Me_2SO -differentiated HL-60 cells was inhibited by treatment with 10 μM cycloheximide (a concentration of cycloheximide which inhibits 95 % of protein synthesis during the course of the experiment) up to 30 h after addition of Me_2SO (results not shown). These data suggest that induction of leukotriene B_4 synthetic capability required new protein synthesis. To determine if induction of 5-lipoxygenase and FLAP mRNAs required new protein synthesis, HL-60 cells were treated with 10 μM cycloheximide at 0, 2, 5 and 28 h after addition of Me_2SO . Cellular RNA was isolated and analysed by Northern blot analysis 48 h after treatment with Me_2SO . The induction of both 5-lipoxygenase and FLAP mRNA was inhibited by treatment of the cells with cycloheximide up to 5 h after the addition of Me_2SO (Figure 4). Addition of cycloheximide to the cultures 28 h after Me_2SO addition did not inhibit the induction of 5-lipoxygenase mRNA and enhanced the induction of FLAP mRNA (Figure 4). Similar results were obtained for 1,25-dihydroxyvitamin- D_3 -differentiated cells (results not shown). These data suggest that new protein synthesis was required for the induction of 5-lipoxygenase and FLAP mRNAs.

Figure 4 Inhibition of 5-lipoxygenase and FLAP mRNA induction by cycloheximide

At time zero, 1.3% Me_2SO was added to the HL-60 cultures. At the indicated times 10 μM cycloheximide was added to the cells to inhibit new protein synthesis. Total cellular RNA was isolated 48 h after the addition of 1.3% Me_2SO and analysed for 5-lipoxygenase (5-LO) and FLAP transcripts by Northern blot analysis as described in the Materials and methods section. Similar results were obtained for 1,25-dihydroxyvitamin- D_3 -differentiated HL-60 cells.

Figure 5 Transcriptional rate of the 3'-portion of the 5-lipoxygenase gene

The transcriptional activity through 5-lipoxygenase introns E and M, as well as β -actin, *c-myc* and FLAP, were determined in Me_2SO - and 1,25-dihydroxyvitamin- D_3 -differentiated HL-60 cells. Cells were treated for the indicated periods of time, then nuclei were isolated and analysed for transcriptional activity using 5-lipoxygenase intron E- and intron M-specific probes as described in the Materials and methods section.

shown). These experiments were repeated numerous times, and in no case was an increase in 5-lipoxygenase or FLAP transcription observed.

It was possible that the use of full-length cDNA probes in the nuclear run-off reactions would not detect changes in completion of transcription, i.e. full-length cDNA probes would not detect transcriptional arrest within the 5-lipoxygenase gene, as was the case for *c-myc* [28,29]. To evaluate whether transcriptional arrest accounts for the changes in 5-lipoxygenase mRNA in differentiated HL-60 cells, probes to 5-lipoxygenase intron E and intron M [14] were used to determine if transcription was uniform on the 5-lipoxygenase gene. The last intron in the 5-lipoxygenase gene is intron M [14]. Differentiation of HL-60 cells with either Me_2SO or 1,25-dihydroxyvitamin D_3 did not result in detectable changes in the transcriptional rate of 5-lipoxygenase introns E or M. In addition, 1,25-dihydroxyvitamin D_3 treatment of HL-60 cells did not change the transcriptional rate of the FLAP or β -actin genes (Figure 5). In both cases the signal detected using 5-lipoxygenase probes was not significantly greater than back-

Induction of 5-lipoxygenase and FLAP is not due to transcriptional activation

The results above and others [19] suggest that the increase in 5-lipoxygenase enzyme activity and leukotriene production detected upon differentiation of HL-60 cells was due to an increase in 5-lipoxygenase and FLAP transcripts. To determine if the increases in 5-lipoxygenase and FLAP mRNAs were due to transcriptional activation of their respective genes, nuclear run-off reactions were performed using a full-length human 5-lipoxygenase cDNA probe and a partial human FLAP cDNA (a 500 bp fragment covering the coding region of the protein). As controls, a genomic probe containing the last exon of human *c-myc* and a full-length human β -actin probe were also included in the study. Using cDNA probes, low levels of transcription of both 5-lipoxygenase and FLAP genes were detected in basal HL-60 cells (results not shown). Treatment of HL-60 cells with Me_2SO for 18, 24 or 50 h did not result in a significant increase in transcription of the 5-lipoxygenase or FLAP genes (results not

Figure 6 Half-life of 5-lipoxygenase, FLAP and *c-myc* mRNAs

Actinomycin D (5 µg/ml) was added to basal HL-60 cells (■), or cells differentiated with Me₂SO for 48 h (●), or with 1,25 dihydroxyvitamin D₃ for 48 hours (▲). Total cellular RNA was isolated and analysed for 5-lipoxygenase transcripts (a), FLAP transcripts (b) or *c-myc* transcripts (c). Results represent the means ± S.E.M. of at least three separate determinations.

ground detected with the pGEM vector (Figure 5). These data suggest that 5-lipoxygenase is not regulated by either transcriptional initiation or transcriptional elongation in Me₂SO- or 1,25-dihydroxyvitamin-D₃-differentiated HL-60 cells. As previously reported [30], treatment with Me₂SO resulted in a decrease in transcription of the *c-myc* gene, as detected using a 3'-specific probe to human *c-myc* (Figure 5). Differentiation of HL-60 cells with Me₂SO results in a 2–3-fold increase in β-actin mRNA (results not shown). In agreement with the induction of β-actin mRNA there was a 2-fold increase in β-actin transcription.

mRNA half-life

Because the data from the nuclear run-off reactions suggested that the increase in 5-lipoxygenase and FLAP transcripts was not due to transcriptional activation of their respective genes, the half-lives of 5-lipoxygenase and FLAP mRNAs were examined in basal and differentiated HL-60 cells, as described in the Materials and methods section. The half-life for 5-lipoxygenase mRNA was not significantly different in basal, Me₂SO-differentiated or 1,25-dihydroxyvitamin-D₃-differentiated HL-60 cells (Figure 6). In all three cases the mRNA half-life was approx. 3 h. FLAP mRNA exhibited a half-life of approx. 3.5 h in basal HL-60 cells, which was extended to approx. 4.5 h in Me₂SO- and 1,25-dihydroxyvitamin-D₃-differentiated HL-60 cells (Figure 6b). There was a reproducible 2 h lag in the decay of FLAP RNA in all samples examined (Figure 6b). This lag was not observed in 5-lipoxygenase samples. To verify that the technique accurately predicted mRNA half-life, the stability of *c-myc* was determined. As previously reported [31], we determined that the half-life of *c-myc* RNA was less than 30 min in basal and 1,25-dihydroxyvitamin-D₃-treated cells, and increased to approx. 1 h in Me₂SO-differentiated cells (Figure 6c).

DISCUSSION

HL-60 cells provide a convenient model to study the mechanisms regulating leukotriene production. Reid et al. [19] reported that 5-lipoxygenase and FLAP were induced by Me₂SO treatment of HL-60 cells, which is in agreement with our findings that 5-lipoxygenase and FLAP were co-ordinately regulated in HL-60 cells when the cells were differentiated into granulocytic-like cells by Me₂SO, Bt₂-cAMP or retinoic acid. In contrast, differentiation of HL-60 cells into monocytic-like cells resulted in marked differences in 5-lipoxygenase and FLAP expression. Treatment

of the cells with PMA induced FLAP mRNA 2-fold and did not induce 5-lipoxygenase mRNA, while treatment of the cells with 1,25-dihydroxyvitamin D₃ induced 5-lipoxygenase mRNA 6-fold and FLAP mRNA only 1.3-fold. The lack of leukotriene products in 1,25-dihydroxyvitamin-D₃-differentiated HL-60 cells supports previous observations that both 5-lipoxygenase and FLAP are required for leukotriene synthesis [9,10].

The regulation of 5-lipoxygenase and FLAP expression was found to be very similar when the HL-60 cells were induced to differentiate into granulocytic cells. The kinetics of their mRNA induction and protein induction [19] were almost identical. In both cases, induction of mRNA preceded appearance of protein. The induction of both mRNAs was inhibited by treatment with cycloheximide, suggesting that synthesis of a protein or proteins was required for the induction of 5-lipoxygenase and FLAP mRNAs. The induction of neither gene product appears to be due to transcriptional activation of their respective genes. Thus, it is likely that a post-transcriptional mechanism is responsible for the increase in mRNA in HL-60 cells. However, we did not detect significant differences in mRNA half-life in basal and induced cells.

Both 5-lipoxygenase and FLAP are encoded by relatively large genes. The gene for 5-lipoxygenase is at least 80 kb in length, comprising 14 exons and 13 introns which are processed to a 2.5 kb mRNA [14], while the gene for FLAP is 30 kb in length and is processed to a 0.9 kb mRNA [16]. Sequences 5' to the 5-lipoxygenase transcription start site contain consensus sequences typical of housekeeping enzymes, suggesting that transcription would be constitutive. Our data support this finding, in that we did not observe any changes in the transcription of the lipoxygenase gene upon induction of the mRNA. Hoshiko et al. [15] utilized a reporter gene to identify both positive and negative regulatory elements which control expression of the 5-lipoxygenase gene. However, the elements function in HeLa cells, which do not normally express 5-lipoxygenase; therefore it is unlikely that these sequences are responsible for the tissue-specific expression of 5-lipoxygenase. In contrast, sequences 5' to the FLAP gene were identified which result in enhanced expression in a macrophage-derived cell line compared with a hepatoma cell line [16]. The first 600 bp in front of the FLAP transcription initiation site contains a TATA box, a putative AP-2 binding site and a glucocorticoid receptor binding site [16]. Our data also suggest that the FLAP gene is not regulated by transcriptional activation in either Me₂SO- or 1,25-dihydroxyvitamin-D₃-differentiated HL-60 cells. It is unknown if other

mechanisms exist for transcriptional activation of the 5-lipoxygenase or FLAP genes.

We have been unable to identify the molecular mechanism which controls 5-lipoxygenase and FLAP expression in HL-60 cells. Recently, Kiledjian and Kadesch [32] described that a liver/bone/kidney form of alkaline phosphatase (LBK AP) was regulated by a post-transcriptional mechanism other than mRNA stability or translational efficiency. Like 5-lipoxygenase and FLAP, LBK AP is encoded by a relatively large gene (greater than 50 kb), consisting of 12 exons. The authors concluded from their study that the tissue-specific expression of LBK AP was regulated by a post-transcriptional mechanism involving destabilization of the nascent LBK AP RNA in the nucleus of low-expressing cells. Dihydrofolate reductase expression in mouse cell lines was also thought to be due to regulation of RNA stability in the nucleus [33]. It is possible that 5-lipoxygenase and FLAP are both regulated by similar mechanisms. Alternatively, both proteins may be regulated by an RNA-processing step or transport out of the nucleus. Further studies on the molecular mechanisms which regulate 5-lipoxygenase and FLAP expression could provide important insights into possible molecular approaches to the control of the production of leukotrienes in diseased states.

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