Up-regulation of steroid sulphatase activity in HL60 promyelocytic cells by retinoids and 1 α ,25-dihydroxyvitamin D₃

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HL60 promyeloid cells express both classes of oestrogen receptor (ER α and ER β). We show that hydrolysis of oestrone sulphate by steroid sulphatase is a major source of oestrone in HL60 cells, and that most of the released oestrone is not metabolized further to 17 β -oestradiol. Treatment of HL60 cells with retinoids or 1 α ,25-dihydroxyvitamin D₃ increased steroid sulphatase mRNA and activity in parallel with the induction of CD11b, an early marker of myeloid differentiation that is expressed before the differentiating cells stop proliferating. Use of agonists and antagonists against retinoid receptor- α and retinoid receptor-X revealed that both classes of retinoid receptor can drive steroid sulphatase up-regulation. Steroid sulphatase activity fluctuates during the cell cycle, being highest around the transition from G1 to S phase. During the differentiation of HL60 cells induced by

all-*trans*-retinoic acid or 1 α ,25-dihydroxyvitamin D₃, there is increased conversion of 17 β -oestradiol into oestrone by an oxidative 17 β -hydroxysteroid dehydrogenase. Treatment of Caco-2 colon adenocarcinoma cells with all-*trans*-retinoic acid or 1 α ,25-dihydroxyvitamin D₃ also increases 17 β -oestradiol oxidation to oestrone. An increase in local oestrone production therefore occurs in multiple cell types following treatment with retinoids and 1 α ,25-dihydroxyvitamin D₃. The possible involvement of locally produced oestrogenic steroids in regulating the proliferation and differentiation of myeloid cells is discussed.

Key words: differentiation, myeloid cell, oestrone, steroid sulphatase.

INTRODUCTION

Nuclear hormone receptor agonists, such as 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] and all-*trans*-retinoic acid (ATRA), have recently been shown to regulate the expression of several enzymes involved in the synthesis of oestrogenic steroids and may, therefore, modulate oestrogen function in hormone-sensitive tissues. Most published studies have concentrated on the effects of these ligands on aromatase activity or expression. Aromatase, encoded by the *CYP19* gene, converts C₁₉ androgens into C₁₈ oestrogens [e.g. testosterone into 17 β -oestradiol (E₂)] [1], and 1 α ,25(OH)₂D₃ up-regulates expression of this enzyme in several human cell lines [2,3]. The physiological relevance of this effect has been confirmed *in vivo*, since aromatase expression is reduced in the gonads of mice lacking the 1 α ,25(OH)₂D₃ receptor. This leads to uterine hypoplasia and impaired folliculogenesis in females and incomplete spermatogenesis in males; these defects are reversed by exogenous oestrogens [4]. Furthermore, the combination of a retinoid receptor- α (RAR) agonist [(E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB)] with a retinoid receptor-X (RXR) agonist (LG100268) enhanced aromatase expression in MCF-7 breast cancer cells [5]. A retinoid response element (RARE) occurs in exon 1a of the *CYP19* gene in human choriocarcinoma cells [6]. However, LG100268 inhibited aromatase expression in human ovarian granulosa [7]. The mechanism underlying this effect is not understood.

Steroid sulphatase (STS; EC 3.1.6.2) [8] lies on an alternative pathway for the generation of biologically active oestrogenic steroids. STS is a widely distributed enzyme which converts biologically 'inert' steroid sulphates, such as oestrone 3-sulphate (E₁S), dehydroepiandrosterone sulphate (DHEAS) and pregnenolone sulphate, into their respective pro-hormones, which can then be metabolized further into biologically active steroids. For example, oestrone (E₁) can be converted into E₂ by type 1 17 β -hydroxysteroid dehydrogenase (17 β -HSD) [9]. Since E₁S and E₂S are present in blood at much higher concentrations than either unconjugated E₁ or E₂ and have much longer half-lives, the desulphation of oestrogen sulphates *in situ* may make an important contribution to the local supply of E₁ and E₂ [10]. Similarly, adult plasma levels of the inert adrenal-derived steroid DHEAS (~5 μ M) are 100–500 times higher than those of testosterone and 1000–10000 times higher than those of E₂, providing a large reservoir for the generation of androgens or oestrogens in peripheral target tissues. It has been estimated that inert adrenal steroids contribute ~30–50% of the androgens in men and ~75% or ~100% of peripheral oestrogens in pre- and post-menopausal women respectively (reviewed in [11]). STS converts DHEAS into dehydroepiandrosterone (DHEA) [8], which can be metabolized further into 5-androstene-3 β ,17 β -diol or 4-androstene-3 β ,17 β -dione by 17 β -HSD or 3 β -HSD respectively [9], both of which are moderately potent oestrogen receptor agonists [12]. Pharmacological inhibition of STS blocks oestrogen-receptor-mediated proliferation of human breast

Abbreviations used: ATRA, all-*trans*-retinoic acid; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; E₁, oestrone; E₁S, oestrone 3-sulphate; E₂, 17 β -oestradiol; ER α and ER β , oestrogen receptor isoforms; HSD, hydroxysteroid dehydrogenase; NBT, Nitro Blue Tetrazolium; 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; RAR, retinoid receptor- α ; RARE, retinoid response element; RT-PCR, reverse transcriptase-PCR; RXR, retinoid receptor-X; STS, steroid sulphatase; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid; VDR, vitamin D receptor.

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cancer and granulosa cells induced by physiological concentrations of DHEAS [13,14].

Deregulation of STS activity may contribute to the initiation of some hormone-dependent cancers [8]. For example, STS is 40–500 times more active than aromatase in breast tumours and hence generates most of the biologically active oestrogens [15]. Furthermore, STS expression is elevated in breast cancers [16], and there is an inverse correlation between intratumoural STS mRNA expression and relapse-free survival in breast cancer patients [17]. STS expression can be modified by growth factors and cytokines (reviewed in [8]), but the effects of nuclear receptor agonists on STS have not been examined in detail. A recent publication reported that, at high concentrations, ATRA stimulated STS expression in the hormone-responsive MCF-7 breast cancer cell line, but not in hormone-unresponsive MDA-MB-231 breast cancer cells [18].

In the present study, the effects of retinoids and $1\alpha,25(\text{OH})_2\text{D}_3$ on STS expression and activity are examined in the HL60 human promyelocytic cell line. The HL60 cell line was used for four main reasons: (i) $1\alpha,25(\text{OH})_2\text{D}_3$ and retinoids induce HL60 cells to differentiate into monocytes and neutrophils respectively [19,20]; (ii) myeloid cells have the potential to generate oestrogenic steroids via both the aromatase and the STS/17 β -HSD pathways [3,21–24]; (iii) oestrogen binding sites are present in myeloid cells [21,25–27]; and (iv) circumstantial evidence suggests that immature myeloid cells may proliferate (or in some cases differentiate) in response to physiological concentrations of oestrogenic steroids [21,27–33]. We report that STS activity increases in parallel with known markers of neutrophilic and monocytic differentiation during retinoid- and $1\alpha,25(\text{OH})_2\text{D}_3$ -induced differentiation of HL60 cells.

EXPERIMENTAL

Chemicals

ATRA and 9-*cis*-retinoic acid were purchased from Sigma (Poole, Dorset, U.K.). The RXR agonist AGN194204, the RXR antagonist AGN195393, the pan-RAR agonist TTNPB and the pan-RAR antagonist AGN194310 were supplied by Allergan Inc. (Irvine, CA, U.S.A.). $1\alpha,25(\text{OH})_2\text{D}_3$ was obtained from Dr Lise Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). Radiolabelled steroids were from NEN Life Science Products-UK Ltd. (Hounslow, Middx., U.K.). All other chemicals were from Sigma.

Cell culture

HL60 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) in 95% air/5% CO_2 at 37 °C. Cells were seeded at 2.5×10^5 cells/ml as 4 ml cultures in 25 cm² flasks, and differentiation was induced with 100 nM ATRA (neutrophilic) or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (monocytic) for 5 days, unless otherwise stated. In some experiments, cells were exposed to increasing concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ (3.125–100 nM final concentration) in the presence of a fixed concentration of E_1S (5–40 μM) for 5 days. Differentiation was assessed by measuring the proportions of cells that reduced Nitro Blue Tetrazolium (NBT) or phagocytosed complement-coated yeast [19,20]. Cell surface expression of the early differentiation marker CD11b was detected by flow cytometry with an FITC-conjugated antibody (IOtest[®]; Immunotech, Marseilles, France) [34]. Samples were analysed using a Becton Dickinson FACScan flow cytometer equipped with the Cellquest software.

Asynchronously growing HL60 cells were separated by size into populations enriched in particular phases of the cell cycle by counter-current elutriation [35]. To determine cell cycle distributions, 10^6 cells from each elutriated fraction were washed twice in PBS, fixed overnight in ice-cold ethanol, stained with propidium iodide (50 $\mu\text{g}/\text{ml}$) and incubated with 100 $\mu\text{g}/\text{ml}$ ribonuclease for 2 h at room temperature. DNA content was analysed by flow cytometry. The remainder of the cells were assayed for STS activity.

The human MCF-7 hormone-dependent breast cancer and MG-63 osteosarcoma cell lines were from A. T. C. C. (Manassas, VA, U.S.A.). The human colon adenocarcinoma cell line Caco-2 was a gift from Dr M. Eggo (Division of Medicine, University of Birmingham, U.K.). These cell lines were grown routinely in Phenol Red-free RPMI 1640 supplemented with 10% (v/v) foetal calf serum, glutamine, non-essential amino acids and antibiotics in 95% air/5% CO_2 at 37 °C.

Western blotting of oestrogen receptors

HL60 cells were lysed by resuspending cell pellets in lysis buffer [2% (w/v) SDS, 60 mM Tris/HCl (pH 6.8), 5 mM EDTA, 10 mM dithiothreitol and 1 mM PMSF] and boiling for 10 min. Samples (100 μg) were separated by SDS/PAGE (10% gels), electroblotted on to Hybond-ECL membranes (Amersham Life Science) and probed overnight using polyclonal antibodies to human oestrogen receptor isoforms ER α and ER β , both diluted 1:1000 (G-20 and L-20 respectively; Santa Cruz Biotechnology Inc., Wembley, Middx., U.K.). Blots were visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated secondary antibodies (Amersham Life Sciences).

Reverse transcriptase-PCR (RT-PCR) for STS and oestrogen receptor mRNA expression

HL60 cells were grown as described above. PBS-washed cells (5×10^6) were suspended in 0.7 ml of hypertonic homogenization buffer (50 mM Tris/HCl and 0.1 mM EDTA, pH 7.4) and disrupted using a QIAshredder (Qiagen, Crawley, W. Sussex, U.K.). RNA was extracted using an RNeasy mini kit (Qiagen). A sample of 3–5 μg of RNA was used for first-strand cDNA synthesis using the SUPERScript[®] preamplification system (Gibco Life Sciences, Paisley, Scotland, U.K.), and PCR was carried out using a HotStart Taq Mastermix kit (Qiagen). The sense and antisense primers for human STS were as described by Fujikawa et al. [36]. In order to achieve linear amplifying conditions, the PCR reactions were performed for 30 cycles (denaturation at 95 °C for 30 s annealing at 59 °C for 30 s and extension at 72 °C for 2 min). Using these conditions, the expected PCR product would be 275 bp in size [36].

We also used RT-PCR to determine whether HL60 cells expressed ER α and/or ER β . The following sense and antisense primers were used to detect the expression of full-length ER β [37]: ER β sense, 5'-AGTATGTACCCTCTGGTCACAGCG-3'; ER β antisense, 5'-CCAAATTGAGGGACCACAGCAG-3'. These primers were designed to give a 408 bp product [37]. The conditions for PCR were 94 °C for 1 min, then 30 cycles of 94 °C for 30 s, 64 °C for 40 s and 72 °C for 45 s [37]. We also designed additional primers to detect ER β expression, based on exon 5 of the published ER β sequence, which would be expected to yield a PCR product of 150 bp: ER β sense, 5'-TGCTTTGGTTTGGTGTGATTGC-3'; ER β antisense, 5'-GTGGTGGACCTCATGGCCACATG-3'. To probe for the expression of ER α in HL60 cells, we designed primers based on the exon 5 region of the

published full-length ER α sequence: ER α sense, 5'-CAGGGT-GAAGTGGGGCTGCTG-3'; ER α antisense, 5'-ATGCGGAA-CCGAGATGATGTAGC-3'. In order to achieve linear amplifying conditions, the PCR reactions for both ER α and ER β were performed for 30 cycles (denaturation at 95 °C for 9 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min). In all cases the PCR products were electrophoresed on a 2 % (w/v) agarose gel and stained with ethidium bromide.

Measurement of STS and 17 β -HSD activity in intact HL60 cells

HL60 cells (1×10^6) were incubated in 1 ml of Phenol Red-free RPMI 1640 containing 1 % (v/v) charcoal-stripped foetal calf serum with increasing concentrations of E $_1$ S, E $_1$ or E $_2$ for up to 24 h (final concentrations in each assay ranged from 0.1 to 250 μ M, and contained 1 μ Ci of the relevant 3 H-labelled steroid). Culture supernatants were centrifuged at 15000 g for 15 min and passed through a 0.2 μ m filter. The extracts were applied to a 25 cm $\times$ 0.46 cm LUNA 5 μ M C18 column (Phenomenex, Macclesfield, Cheshire, U.K.) and steroids were eluted using 48 % (v/v) acetonitrile in HPLC-grade water at 1 ml/min. The identities of metabolites were determined by comparison of the retention times with those of authentic standards determined in parallel runs.

Measurement of STS activity in cell fractions

Cells were collected by centrifugation and resuspended in 1 ml of hypotonic homogenization buffer (50 mM Tris/HCl and 0.1 mM EDTA, pH 7.4). After 30 min on ice, the cells were disrupted by repeated sonication and centrifuged at 100000 g at 4 °C for 60 min. The pellet was resuspended in 0.5 ml of the same buffer by sonication and its protein concentration was measured (kit from Bio-Rad, Hemel Hempstead, Herts., U.K.). STS was assayed at 37 °C in 0.02 M Tris/HCl (pH 6.5) containing 20 μ M E $_1$ S (spiked with ~ 100000 d.p.m. of [3 H]E $_1$ S) in a final volume of 100 μ l. Reactions were started with 30–80 μ g of protein and were quenched after 60 min with 900 μ l of ice-cold 0.1 M sodium bicarbonate containing 5000 d.p.m. of [14 C]E $_1$ to determine the recovery of the extraction process. A volume of 950 μ l of the resulting mixture was extracted with 3 ml of toluene, and the organic and aqueous layers were separated. Then 8 ml of Emulsifier Safe scintillant (Canberra Packard, Pangborne, Berks., U.K.) was added to each fraction and radioactivity was determined in a Packard 2000CA TriCarb liquid scintillation counter. The results were corrected for carry-over of radiolabelled substrate into the organic layer determined in blank incubations without cell protein. In experiments where the substrate concentration was varied, reaction times were chosen (usually 15–60 min) so that less than 10 % of the substrate was consumed. Kinetic parameters were calculated using either the Enzfitter[™] software package or the enzyme kinetics module in the Sigmaplot[™] graphics software package.

Statistical analysis

All results were obtained from 3–6 experiments performed in quadruplicate. Results are expressed as means $\pm$ S.E.M. Where appropriate, results were analysed by the Mann–Whitney non-parametric test of significance using the Instat statistical software package (version 1.15; Graphpad Software, San Diego, CA, U.S.A.).

Figure 1 STS activity in HL60 cells is increased following treatment with 100 nM ATRA or 100 nM 1 α ,25(OH) $_2$ D $_3$

(A) HL60 cells were treated with 100 nM ATRA (Δ) or 100 nM 1 α ,25(OH) $_2$ D $_3$ (∇) or left untreated ($\square$) for 5 days. Cells were harvested and incubated for 24 h with [3 H]E $_1$ S (100 nM; 0.5 μ Ci), and metabolites were separated by reverse-phase HPLC as described in the Experimental section. Also shown is the HPLC profile obtained with cell-free controls ($\circ$). The traces are typical of those obtained in five experiments of similar design. (B) HL60 cells were treated with 100 nM ATRA (Δ) or 100 nM 1 α ,25(OH) $_2$ D $_3$ (∇) or left untreated ($\square$) for 5 days. Cells were harvested and STS activity was determined as described in the Experimental section. The data are means $\pm$ S.E.M. from two experiments, performed in quadruplicate. The rate of E $_1$ formation was significantly higher in both the ATRA- and 1 α ,25(OH) $_2$ D $_3$ -treated cells when compared with undifferentiated cells at all E $_1$ S concentrations ($P < 0.05$).

RESULTS

STS activity in intact HL60 cells increases following treatment with ATRA or 1 α ,25(OH) $_2$ D $_3$

Preliminary experiments showed that E $_1$ S was metabolized only into E $_1$ by intact HL60 cells, and that metabolism was linear for more than 24 h at all concentrations between 0.001 and 250 μ M. Figure 1 shows typical HPLC profiles of the metabolites formed during a 24 h incubation with [3 H]E $_1$ S (100 nM) by undifferentiated HL60 cells and by cells that had differentiated for 120 h along the neutrophilic (100 nM ATRA) or monocytic [100 nM 1 α ,25(OH) $_2$ D $_3$] lineages. In all three cell populations, E $_1$ S was converted into a single metabolite with the retention time of E $_1$. There was no significant further metabolism of E $_1$ to E $_2$: < 0.1 % of 100 nM [14 C]E $_1$ was converted into [14 C]E $_2$ in 24 h (results not shown). At each E $_1$ S concentration tested, the rate of E $_1$ formation was higher in the ATRA- or 1 α ,25(OH) $_2$ D $_3$ -treated cells than in the undifferentiated cells (Figure 1B). For example, at a 'physiological' substrate concentration of 100 nM E $_1$ S, the rate of E $_1$ formation in undifferentiated cells was 750 ± 35 fmol/h per 10^6 cells, compared with 3450 ± 90 and 2800 ± 75 fmol/h per 10^6 cells in ATRA- and 1 α ,25(OH) $_2$ D $_3$ -treated cells respectively.

Peripheral blood mononuclear cells and myeloid-derived cell lines express two enzymes that might hydrolyse E $_1$ S and other 3-

Table 1 Treatment of HL60 cells for 120 h with ATRA, 9-*cis*-retinoic acid or 1 α ,25(OH) $_2$ D $_3$ increases the $V_{\max}$ of STS, but has no effect on the K_m

Enzyme activities were determined in particulate fractions from 2–7 separate HL60 cultures. K_m and $V_{\max}$ values were calculated using the EnzFitter software program, and are means $\pm$ S.E.M. of quadruplicate determinations. Significance of differences compared with control: * $P < 0.05$; ** $P < 0.002$; *** $P < 0.001$

Treatment	K_m (μ M)	$V_{\max}$ (nmol/h per mg)
Control	4.9 $\pm$ 0.5 ($n = 7$)	1.45 $\pm$ 0.06 ($n = 4$)
100 nM ATRA	5.7 $\pm$ 0.8 ($n = 4$)	21.6 $\pm$ 0.9*** ($n = 4$)
100 nM 9- <i>cis</i> -retinoic acid	8.7 $\pm$ 3.3 ($n = 6$)	19.7 $\pm$ 0.4** ($n = 6$)
100 nM 1 α ,25(OH) $_2$ D $_3$	5.6 $\pm$ 0.9 ($n = 4$)	20.1 $\pm$ 1.1** ($n = 4$)
1.25% DMSO	6.3 $\pm$ 0.6 ($n = 2$)	4.7 $\pm$ 0.4* ($n = 2$)

sulphated steroids: microsomal STS has a low capacity and high affinity ($K_m \leq 25 \mu$ M) [24], and a non-specific arylsulphatase has a high capacity but low affinity ($K_m > 500 \mu$ M) [38]. Kinetic parameters were measured in particulate fractions from undifferentiated, ATRA- or 1 α ,25(OH) $_2$ D $_3$ -treated HL60 cells, incubated with 0.1–200 μ M E $_1$ S. In all cell populations, Eadie–Hofstee and Lineweaver–Burk transformations of the kinetic data were best fitted by a single line, suggesting that E $_1$ S was hydrolysed by a single enzyme (results not shown). The K_m for E $_1$ S was 4.9 $\pm$ 0.5 μ M ($n = 7$) in undifferentiated HL60 cells. This value is similar to the K_m for STS activity against E $_1$ S in other human blood cells, including thrombocytes (3.2 μ M) [39], platelets, neutrophils and monocytes (~ 1 –2 μ M; D. M. Wood, C. J. Kirk and P. J. Hughes, unpublished work), as well as in human placenta (25 μ M), liver (14 μ M), breast tumour tissue (20 μ M), ovary (14.3 μ M) and endometrium (5.7 μ M) [39]. E $_1$ S hydrolysis in undifferentiated HL60 cells was irreversibly inhibited by sub-micromolar concentrations of the specific STS inhibitor oestrone sulphamate (results not shown). Several 3-hydroxysteroid sulphates have been reported to be STS substrates in leucocytes [24]. STS activity against [3 H]E $_1$ S in HL60 particulate fractions was competitively inhibited by DHEAS (IC $_{50} \sim 10 \mu$ M), pregnenolone sulphate (IC $_{50} \sim 5 \mu$ M), E $_2$ S (IC $_{50} \sim 30 \mu$ M) and cholesterol sulphate (IC $_{50} \sim 15 \mu$ M). These IC $_{50}$ values are close to the reported affinities of leucocyte STS for the respective substrates [24]. Conversely, warfarin, an inhibitor of non-specific arylsulphatases [40], did not significantly inhibit hydrolysis of E $_1$ S in HL60 cell particulate fractions, even at concentrations as high as 100 μ M (results not shown). These results suggest that desulphation of E $_1$ S in HL60 cells is catalysed by a high-affinity STS.

Kinetic parameters for STS in particulate fractions from undifferentiated cells and from populations that had been differentiated for 120 h by 100 nM ATRA, 100 nM 1 α ,25(OH) $_2$ D $_3$, 100 nM 9-*cis*-retinoic acid or 1.25% DMSO are shown in Table 1. Induction of neutrophilic or monocytic differentiation in HL60 cells did not change the K_m for STS against E $_1$ S. The K_m of the enzyme in the broken-cell preparations was ~ 10 -fold lower than the E $_1$ S concentration at which conversion into E $_1$ in intact cells was half-maximal (Figure 1B). This may reflect saturation of the E $_1$ S uptake transporter or the presence in these cells of an endogenous sulphatase inhibitor [41]. However, the $V_{\max}$ of the enzyme was increased significantly by treatment with 1 α ,25(OH) $_2$ D $_3$, retinoids or DMSO.

The increases in total STS activity induced by these differentiating agents seem likely, therefore, to reflect increased enzyme expression. We therefore measured STS expression in control and differentiated cell populations by PCR, using primers

Figure 2 PCR analysis of STS expression in myeloid cells

Total RNA was reverse-transcribed and amplified by PCR using primers specific for human STS [33] (275 bp product). The expression of STS was measured relative to the expression of β -actin (not shown) in HL60 cells that were treated for 120 h with 100 nM ATRA, 100 nM 9-*cis*-retinoic acid (9-*cis*-RA) or 100 nM 1 α ,25(OH) $_2$ D $_3$ (and also in MCF-7 breast cancer cells as a positive control; not shown).

Table 2 Effects of ATRA and 1 α ,25(OH) $_2$ D $_3$ on STS activity in Caco-2 human colon adenocarcinoma cells, MCF-7 human breast cancer cells and MG-63 human osteosarcoma cells

Cell monolayers at 60–70% confluence were treated with 100 nM ATRA or 100 nM 1 α ,25(OH) $_2$ D $_3$ for 72 h. At the end of the incubation period, the cells were harvested by trypsin treatment. STS activity was determined in the particulate fraction as described in the Experimental section at a substrate concentration of 20 μ M E $_1$ S. Results are expressed as the amount of E $_1$ formed and are means $\pm$ S.E.M. from three experiments performed in quadruplicate. Significance of differences compared with control: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

Treatment	E $_1$ formed (nmol/h per mg of protein)		
	Caco-2	MCF-7	MG-63
Untreated	10.2 $\pm$ 2.3	18.8 $\pm$ 1.5	21.5 $\pm$ 3.8
+ 100 nM ATRA	16.8 $\pm$ 1.1*	31.2 $\pm$ 2.1***	37.3 $\pm$ 1.9**
+ 100 nM 1 α ,25(OH) $_2$ D $_3$	18.6 $\pm$ 2.4*	36.3 $\pm$ 3.2**	40.2 $\pm$ 1.3***

based on the human STS sequence [36], which yielded a single 275 bp amplified product. MCF-7 hormone-dependent breast cancer cells served as a positive control [41], and an identical product was observed in the control and differentiated HL60 cells. After normalizing STS expression to that of the housekeeping gene β -actin, there was an 8–10-fold increase in the expression of the 275 bp transcript in HL60 cells following treatment for 120 h with 100 nM ATRA, 9-*cis*-retinoic acid or 1 α ,25(OH) $_2$ D $_3$ (Figure 2).

A smaller, but statistically significant, increase in STS activity against 20 μ M E $_1$ S was also seen in particulate fractions from Caco-2 human colon adenocarcinoma, MCF-7 human breast cancer and MG-63 human osteosarcoma cells following treatment with 100 nM ATRA or 100 nM 1 α ,25(OH) $_2$ D $_3$ for 72 h (Table 2).

Oxidative 17 β -HSD activity increases in HL60 and Caco-2 cells treated with ATRA or 1 α ,25(OH) $_2$ D $_3$

E $_2$ was readily converted into E $_1$ in undifferentiated HL60 cells, but the reverse reaction did not occur at a significant rate (Figure 3), confirming recent observations by Mountford et al. [21].

Figure 3 Oxidative 17β -HSD activity is increased in HL60 cells following treatment with 100 nM ATRA or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$

HL60 cells were treated with 100 nM ATRA ($\blacktriangle$) or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ ($\blacktriangledown$) for 5 days. The cells were harvested as in Figure 1 and incubated for 24 h with [^3H]E $_2$ (100 nM; 0.5 μCi). Metabolites were separated as in Figure 1. Also shown are the HPLC profiles obtained from cells that had been left untreated ($\blacksquare$) and from cell-free controls ($\bullet$). The traces are typical of those obtained in five experiments of similar design.

Using a supraphysiological concentration of E $_2$ (100 nM), conversion of E $_2$ into E $_1$ was $\sim 1500 \pm 125$ fmol/h per 10^6 cells in undifferentiated HL60 cells, doubling to $\sim 3600 \pm 210$ ($n = 4$; $P < 0.05$) and $\sim 3100 \pm 280$ ($n = 4$; $P < 0.03$) fmol/h per 10^6 cells in cells that had been exposed for 120 h to 100 nM ATRA or $1\alpha,25(\text{OH})_2\text{D}_3$ respectively. Again, this phenomenon was not restricted to myeloid cells. Most of the E $_1$ S added to untreated Caco-2 cells was converted into E $_2$ within 24 h, showing that these cells have efficient STS and reductive 17β -HSD activities (Figure 4A). Untreated Caco-2 cells converted $\sim 95\%$ of added E $_1$ into E $_2$ (Figure 4B), but did not convert E $_2$ into E $_1$ (Figure 4C). This is the converse of the pattern seen in HL60 cells, but similar to that in hormone-dependent breast cancer cell lines [41]. The pattern of oestrogen metabolism in Caco-2 cells changed after 12 days of treatment with ATRA or $1\alpha,25(\text{OH})_2\text{D}_3$: E $_1$ S was still metabolized extensively, but the major product was E $_1$ rather than E $_2$ (Figure 4A). The rate of conversion of E $_1$ into E $_2$ was lower in these treated cells (Figure 4B) and there was substantial conversion of E $_2$ into E $_1$ (Figure 4B).

The retinoid receptors RAR and RXR are involved in ATRA- and 9-*cis*-retinoic acid-mediated differentiation and the induction of STS in HL60 cells

Treatment of HL60 cells with 100 nM ATRA (an RAR agonist) or 9-*cis*-retinoic acid (a dual RAR/RXR agonist) for 120 h provoked 70–80 % of the cells to differentiate into neutrophils, as did the specific RXR and RAR agonists AGN194204 and TTNPB respectively (100 nM) (Figure 5A). A pan-specific RAR antagonist (AGN194310; 100 nM) greatly inhibited the differentiation induced by either ATRA ($P < 0.03$) or 9-*cis*-retinoic acid ($P < 0.001$; Figure 5C), supporting previous evidence that RAR plays an essential role in HL60 neutrophil differentiation [42]. The RXR-specific antagonist AGN195393 (100 nM) slightly reduced ATRA-mediated neutrophil differentiation ($P \sim 0.05$), but inhibited neutrophil differentiation in-

Figure 4 Effects on oestrogen metabolism in Caco-2 colon adenocarcinoma cells of treatment with 100 nM ATRA or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$

Caco-2 cells at 50 % confluence were treated with 100 nM ATRA or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$. The cells were passaged every third day and re-seeded in 'differentiating' medium at $\sim 50\%$ of their confluent cell density to prevent spontaneous differentiation. At 10 days after the addition of differentiating agents, the cells were passaged at 1×10^6 cells per well into 6-well culture plates in the continued presence of differentiating agents and, 2 days later, the differentiating medium was removed by three washes with PBS. A 1 ml portion of serum-free RPMI 1640 containing 50 nM of the oestrogen under test (containing 0.5 μCi of the relevant ^3H -labelled steroid) was added. After a 24 h incubation, the metabolites in the tissue-culture supernatant were analysed by HPLC as described in the Experimental section. The results are expressed as the percentage of eluted radioactivity recovered as E $_1$ S (grey), E $_1$ (white) or E $_2$ (black). The results are from a single experiment, assayed in quadruplicate, and are typical of three similar experiments (error bars are omitted for clarity).

duced by 100 nM 9-*cis*-retinoic acid by $\sim 50\%$ ($P < 0.02$; Figure 5C). Neither antagonist caused significant differentiation when added alone. This confirms previous observations that both RAR- and RXR-stimulated pathways can play roles in the differentiation of HL60 cells. $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated monocytic differentiation was also significantly inhibited by the RXR antagonist, perhaps reflecting a role for vitamin D receptor (VDR)/RXR heterodimer formation (results not shown).

Similar results were obtained when the effects of the retinoid analogues on STS activity were examined (Figure 5B). Activity was increased ~ 12 -fold by ATRA, ~ 6 -fold by TTNPB, ~ 20 -fold by 9-*cis*-retinoic acid and ~ 13 -fold by the RXR agonist AGN194202, suggesting that both RAR and RXR participate in the up-regulation of STS activity. This was confirmed by the use

Figure 5 RAR and RXR agonists mimic, and specific RAR and RXR antagonists inhibit, the differentiation of HL60 cells and induction of STS activity provoked by ATRA or 9-*cis*-retinoic acid

HL60 cells were treated with 100 nM of retinoid agonists alone or in combination with antagonists as shown. TTPB and AGN194204 are specific agonists of RAR and RXR respectively. AGN194310 and AGN195393 are pan-specific antagonists of RAR and RXR respectively. 9-*cis* RA and 9-*cis*, 9-*cis*-retinoic acid. After 5 days, some cells were removed and assessed for their ability to reduce NBT (**A** and **C**), and STS activity was assayed in particulate fractions (**B** and **D**). The results are means $\pm$ S.E.M. from 3–6 experiments.

of specific RAR and RXR antagonists. The RXR antagonist AGN195393 slightly attenuated the increase in STS activity produced by ATRA ($P = 0.05$), but greatly reduced that provoked by 9-*cis*-retinoic acid ($P = 0.02$; Figure 5D). Similarly, the pan-specific RAR antagonist AGN194310 almost completely inhibited the increases in STS activity mediated by ATRA ($P = 0.002$) or by 9-*cis*-retinoic acid ($P = 0.001$; Figure 5D). The increase in STS activity provoked by $1\alpha,25(\text{OH})_2\text{D}_3$ was also abrogated by the pan-specific RXR antagonist (results not shown), suggesting that RXR/VDR heterodimers may be involved in the up-regulation of STS expression.

ATRA and $1\alpha,25(\text{OH})_2\text{D}_3$ increase STS activity in parallel with expression of an early marker of differentiation

We compared the time courses for the induction of STS activity and for the appearance of early-expressed (CD11b) [34] and late-expressed (NBT reduction and phagocytosis) [19,20] markers of differentiation in $1\alpha,25(\text{OH})_2\text{D}_3$ - and ATRA-treated cells. Cell numbers and cell cycle status were monitored in parallel. The specific activity of STS had increased ~ 6 -fold and ~ 3 -fold respectively by 24 h after addition of $1\alpha,25(\text{OH})_2\text{D}_3$ or ATRA (Figure 6A). As with other markers of differentiation,

ATRA induced STS activity more slowly than did $1\alpha,25(\text{OH})_2\text{D}_3$. The increase in STS activity preceded the appearance of phagocytic cells and cells that reduced NBT in both cases, suggesting that STS activity increases while the cells are still multiplying (Figures 6B and 6C).

Expression of CD11b is one of the earliest indications that cells have responded to differentiation stimuli [34]. CD11b expression and STS activity started to rise within 12–16 h of exposure to $1\alpha,25(\text{OH})_2\text{D}_3$ and increased in an approximately linear fashion for the next 10–18 h. Again, the acquisition of CD11b was somewhat slower during exposure to ATRA. In both cases, STS induction and CD11b expression had similar time courses (Figure 7).

We also investigated whether STS activity varies during the cell cycle. Asynchronously growing HL60 cell populations were fractionated by centrifugal elutriation, and their DNA content was analysed by flow cytometry to determine the cell cycle distribution of each fraction. STS activity varied widely between fractions, with activity being highest in those fractions containing cells that were predominately in late G1 and early S phase of the cell cycle (Figure 8). Activity was much lower in small, early G1 cells and in fractions containing larger cells, in which $> 50\%$ were in S phase and/or at the G2/M boundary.

Figure 6 Time course of the increase in STS activity in HL60 cells challenged with ATRA or $1\alpha,25(\text{OH})_2\text{D}_3$

HL60 cells were treated with 100 nM ATRA (●) or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (■) or left untreated (▲) for up to 5 days, harvested and assayed for: (A) particulate STS activity; (B) ability to phagocytose yeast; (C) ability to reduce NBT; (D) cell number; and (E) cell cycle distribution. Results are means $\pm$ S.E.M. from three experiments.

HL60 cells express ER α and ER β

The presence of oestrogen-binding sites has been demonstrated in HL60 and other myeloid cell lines [21,25–27], but these have not been characterized in molecular terms. To date, two oestrogen receptor isoforms (ER α and ER β) have been characterized from several species. The human ER α comprises 595 amino acids and has a molecular mass of $\sim$ 66–70 kDa. By Western blotting with the G-20 polyclonal antibody (against a peptide derived from the hinge region of ER α) a band at $\sim$ 68 kDa was clearly seen in both undifferentiated and differentiated HL60 cells and in the MCF-7 positive control (Figure 9A). The human ER β protein is composed of 530 amino acids and migrates in SDS/PAGE gels

as a protein of 58–62 kDa. We detected a band at $\sim$ 59 kDa using the L-20 polyclonal antibody (against a peptide from the C-terminal region of human ER β) in undifferentiated and differentiated HL60 cells, and in both MCF-7 breast carcinoma cells and LNCaP prostate adenocarcinoma cells (Figure 9B). There were no obvious changes in the expression of either receptor during neutrophilic or monocytic differentiation (Figures 9A and 9B). Since some doubts have been raised regarding the ability of commercial anti-ER β antibodies to reliably detect the expression of ER β at the low levels found in many tissues [43], we also used RT-PCR to confirm that HL60 cells express the two oestrogen receptor isoforms. Using oligonucleotide primers

Figure 7 Comparison of the early time courses for the increases in STS activity and CD11b expression provoked by ATRA or $1\alpha,25(\text{OH})_2\text{D}_3$

HL60 cells were treated with 100 nM ATRA (●) or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (■). Cells were harvested every 2 or 4 h for 36 h. STS activity was assayed in particulate fractions, and the percentage of cells expressing CD11b was assessed by flow cytometry. The results are means $\pm$ S.E.M. from three experiments. Untreated cells showed no change in STS activity or CD11b expression (results not shown).

Figure 8 STS activity at different stages of the cell cycle

Asynchronously growing HL60 cells were subjected to counter-current elutriation, flow cytometry and propidium iodide labelling to determine the proportion of cells in each fraction in G1 (○), in S phase (□) and in G2/M (▽). STS activity (■) was measured in particulate fractions prepared from these cells and expressed per mg of protein. The results are means $\pm$ S.E.M. from four experiments.

specific for $\text{ER}\alpha$ and $\text{ER}\beta$, we detected the expression of both receptor types in undifferentiated and differentiated HL60 cells (Figures 9C and 9D) and there were no overt changes in expression during differentiation.

E_1S stimulates proliferation in HL60 cells

When bulk cultures of HL60 cells that had been adapted to grow in the absence of serum were exposed to relatively high concentrations of E_1S (0.1–10 μM), there was a modest increase in their growth rate. The proliferative effect was seen most clearly in HL60 cells grown singly in 96-well plates. For example, over a 96 h period, untreated singly seeded cells divided just over four times, as indicated by an average recovery of 22.7 ± 1.7 cells per well ($n = 73$). Over the same time period, the cells treated with 10 μM E_1S divided just over five times, and an average of 36.9 ± 2.2 cells were recovered per well ($n = 73$; $P < 0.001$).

DISCUSSION

Several novel sites of peripheral oestrogen biosynthesis have been identified in recent years, including adipose tissue, skin, bone, testes, endometrium and brain [1]. While the total amount of oestrogen synthesized at these sites may be small, significant local tissue concentrations can be achieved, thereby influencing normal cell physiology [1]. Our results, and those from other recent papers, add human myeloid cells to the list of peripheral tissues and cells that can metabolize steroid precursors to bioactive oestrogens.

Primitive myeloid cells (THP-1 promonocytoid and HL60 promyelocytic cells), normal human $\text{CD}34^+$ primary blast cells and their mature counterparts all express aromatase activity [3,21–23]. Aromatase expression was increased slightly in THP-1 cells [3], but not in HL60 cells or cord blood samples, during vitamin D_3 -induced differentiation [21]. Similarly, ATRA and vitamin D_3 increased the activity of several $17\beta\text{-HSD}$ isoforms which catalyse the oxidation of E_2 to E_1 in myeloid cell lines and normal human blast cells [3,21,44]. Hence HL60 and other primitive blast cells can convert circulating testosterone and androstenedione directly into E_1 using aromatase and/or $17\beta\text{-HSD}$ respectively, and these pathways may be regulated during differentiation. However, aromatase activity in these cells is very

Figure 9 Immunoblots and RT-PCR showing the presence of (A, C) ER α and (B, D) ER β in HL60 cells treated with ATRA or 1 α ,25(OH) $_2$ D $_3$

In the experiments shown in (A) and (B), HL60 cells were treated with 100 nM ATRA or 100 nM 1 α ,25(OH) $_2$ D $_3$ for 24 h. Cells were then collected and cell lysates were prepared as described in the Experimental section. Cell lysates were fractionated by electrophoresis in a 10% (w/v) polyacrylamide gel. After electroblotting, nitrocellulose filters were probed with antibodies against ER α or ER β and visualized by enzyme-linked chemiluminescence. Similar results were obtained in three other experiments. Similarly, in the experiments shown in (C) and (D), HL60 cells were treated with 100 nM ATRA or 1 α ,25(OH) $_2$ D $_3$ for 120 h. Total RNA was extracted from the HL60 cells as described in the Experimental section and was reverse-transcribed to cDNA. The cDNA was probed by PCR with primers designed to detect ER α or ER β , as described in the Experimental section. Similar results were obtained in three other experiments.

low [21] when compared with the activities of STS and 17 β -HSD, so aromatization of C $_{19}$ androgens may not contribute significantly to the generation of oestrogenic steroids.

We have demonstrated that HL60 cells can make E $_1$ by an alternative route, the STS-catalysed hydrolysis of E $_1$, and also that they express both ER α and ER β . HL60 cells therefore have both the enzymic machinery to generate oestrogens and also the receptors with which to respond to these locally generated steroids. Treatment of HL60 cells with retinoids or 1 α ,25(OH) $_2$ D $_3$ produced a rapid increase in STS activity, coincident with the start of monocytic or granulocytic differentiation. Retinoids and 1 α ,25(OH) $_2$ D $_3$ mediate their transcriptional effects via specific nuclear receptors. There are two types of retinoid receptor (RAR and RXR), both of which have at least three isoforms [45], and a single VDR [46]. HL60 cells express both classes of retinoid receptor [42]. The retinoid receptors differ in their ligand specificities: RARs bind ATRA and 9-*cis*-retinoic acid, while RXRs bind only 9-*cis*-retinoic acid [45]. STS activity in HL60 cells was up-regulated by ATRA, by 9-*cis*-retinoic acid, by a pan-specific RAR agonist (TTNPB) and also by a specific RXR agonist (AGN194202), suggesting roles for both RAR and RXR.

It is thought that retinoid-induced neutrophilic differentiation of HL60 cells is driven by RXR/RAR heterodimers [42], and that VDR/RXR heterodimers mediate 1 α ,25(OH) $_2$ D $_3$ -induced monocytic differentiation [19,42]. A selective RAR antagonist prevented ATRA- and 9-*cis*-retinoic acid-mediated differentiation and STS induction, and a specific RXR antagonist blocked STS induction in response to 9-*cis*-retinoic acid or 1 α ,25(OH) $_2$ D $_3$, but not to ATRA. It is therefore likely that the formation of the same nuclear receptor heterodimers drives both STS expression and cell differentiation. These receptor dimers bind

to well defined transcriptional control motifs [RAREs and 1 α ,25(OH) $_2$ D $_3$ response elements] [45,46] which are not obviously present in a published partial promoter sequence for the human STS gene [47]. However, such elements may exist in the full STS promoter region, which remains to be sequenced.

We confirm that HL60 cells differentiating along the monocytic or neutrophilic lineage increase the oxidation of E $_2$ to E $_1$, presumably via a 17 β -HSD. HL60 cells express type 1 and type 4 17 β -HSDs [21]. It is generally thought that the type 1 17 β -HSD preferentially catalyses the reduction of E $_1$ to E $_2$ *in vivo*; however, under some circumstances the reverse reaction is favoured [9]. The type 4 isoform is a multifunctional enzyme which oxidizes 3-hydroxyacyl-CoA, 5-androstene-3 β ,17 β -diol and E $_2$ [9]. A RARE upstream of the coding region of the type 1 17 β -HSD gene may mediate enzyme induction by ATRA in the T-47D breast cancer cell line [48], but this appears to be silent in HL60 cells where ATRA does not influence type 1 17 β -HSD expression [21]. Little is known about the control of type 4 17 β -HSD expression, but its activity has been reported to decrease as HL60 cells differentiate in response to both ATRA and 1 α ,25(OH) $_2$ D $_3$ [25] and when U937 cells are treated with 1 α ,25(OH) $_2$ D $_3$ [44]. Differentiation-related changes in the balance of E $_2$ /E $_1$ synthesis are not exclusive to myeloid cells. In the present paper we show that ATRA and 1 α ,25(OH) $_2$ D $_3$ provoke a switch in favour of oxidative 17 β -HSD activity and increased E $_1$ synthesis in Caco-2 cells. Interestingly, the ability to convert E $_2$ into E $_1$ is compromised in some human colon tumours and colon cancer cell lines [49]. ATRA and 1 α ,25(OH) $_2$ D $_3$ induce growth arrest and differentiation of some colon epithelial carcinoma cell lines [50], and their use has been proposed for differentiation therapy of colon cancers [51]. Similarly, conversion of E $_2$ into E $_1$ is also enhanced during the

terminal differentiation phase of $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated keratinocytes [52] and during terminal differentiation of SV-HFO osteoblasts in long-term culture [53].

The increased capacity to generate biologically active steroids from local inert precursors may reflect the acquisition of a differentiated phenotype by myeloid cells, and may suggest that mature cells need to produce, and respond to, oestrogens in order to fulfil their physiological functions. However, data presented in this paper and elsewhere [21] show that the increased expression of oestrogenic enzymes coincides with the acquisition of early markers of myeloid differentiation. There is some circumstantial evidence that oestrogenic steroids may influence the maturation of monocytes and neutrophils. For example, sub-micromolar concentrations of E_1 induce neutrophilic differentiation of murine 32D myeloblasts [30], and higher doses ($> 1 \mu\text{M}$) potentiate $1\alpha,25(\text{OH})_2\text{D}_3$ -induced monocytic differentiation of HL60 cells and human $\text{CD}34^+$ blasts [21,29]. However, we have seen no consistent effects of E_1S on $1\alpha,25(\text{OH})_2\text{D}_3$ -induced monocytic differentiation in HL60 cells, even at concentrations as high as $40 \mu\text{M}$ (results not shown).

In contrast, we observed a modest increase in the growth of bulk cultures of HL60 cells that had been adapted to grow in the absence of serum following their exposure to relatively high concentrations of E_1S (0.1 – $10 \mu\text{M}$). Other workers have shown that physiological concentrations of E_1 and E_2 provoke proliferation rather than differentiation in HL60 cells and cord blood-derived myelomonocytic cells [27,29]. This may be of physiological relevance, since the number of circulating monocytes fluctuates during the menstrual cycle, peaking around ovulation when the plasma E_2 concentration is at its highest [28]. Furthermore, the STS product DHEA and its 17β -HSD product 5-androstene- $3\beta,17\beta$ -diol can stimulate the proliferation of a number of classes of haematopoietic cells in culture and *in vivo* [32,33]. Hence it is possible that oestrogens and related steroids are mitogens rather than differentiating agents in myeloid cells.

Oestrogens are known to be potent mitogens in mammary epithelial cells, where oestrogen-induced mitogenesis is associated with the recruitment of non-cycling, G0, cells into the cell cycle, a rapid progression through G1 and an increased rate of entry of cells into S phase (reviewed in [54]). As far as we know, ours is the first observation that the activity of an enzyme involved in the formation of biologically active oestrogens fluctuates during the cell cycle. Our data clearly show that STS activity was low in early G1 phase cell populations, peaked in late G1/early S phase and declined to a minimum in G2/M phase. The biochemical mechanisms underlying these oscillations in STS activity and their physiological significance are currently undergoing further investigation.

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